

ORIGINAL ARTICLE

Gut microbiome composition and function in experimental colitis during active disease and treatment-induced remission

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Dysregulated immune responses to gut microbes are central to inflammatory bowel disease (IBD), and gut microbial activity can fuel chronic inflammation. Examining how IBD-directed therapies influence gut microbiomes may identify microbial community features integral to mitigating disease and maintaining health. However, IBD patients often receive multiple treatments during disease flares, confounding such analyses. Preclinical models of IBD with well-defined disease courses and opportunities for controlled treatment exposures provide a valuable solution. Here, we surveyed the gut microbiome of the *T-bet*^{-/-} *Rag2*^{-/-} mouse model of colitis during active disease and treatment-induced remission. Microbial features modified among these conditions included altered potential for carbohydrate and energy metabolism and bacterial pathogenesis, specifically cell motility and signal transduction pathways. We also observed an increased capacity for xenobiotics metabolism, including benzoate degradation, a pathway linking host adrenergic stress with enhanced bacterial virulence, and found decreased levels of fecal dopamine in active colitis. When transferred to gnotobiotic mice, gut microbiomes from mice with active disease versus treatment-induced remission elicited varying degrees of colitis. Thus, our study provides insight into specific microbial clades and pathways associated with health, active disease and treatment interventions in a mouse model of colitis.

The ISME Journal (2014) 8, 1403–1417; doi:10.1038/ismej.2014.3; published online 6 February 2014

Subject Category: Microbe-microbe and microbe-host interactions

Keywords: colitis; community structure; gnotobiotic mice; gut microbiome; gut microbiota; treatment

Introduction

Inflammatory bowel disease (IBD) is linked to alterations in gut microbial communities and dysregulated mucosal immune responses (Ott *et al.*, 2004; Frank *et al.*, 2007; Sokol *et al.*, 2008; Packey and Sartor, 2009; Frank *et al.*, 2011; Erickson *et al.*, 2012; Morgan *et al.*, 2012; Presley *et al.*, 2012). Management of IBD has relied on nonspecific immunosuppressive therapies, agents targeting pro-inflammatory pathways, antibiotics and more recently, probiotics (Dethlefsen *et al.*, 2008; Engel and Neurath, 2010; Hill *et al.*, 2010; Jakobsson *et al.*, 2010; Manichanh *et al.*,

2010; Pineton de Chambrun and Sandborn, 2012; Ubeda and Pamer, 2012). However, many IBD-directed therapies are not effective in all patients and some carry a high risk of complications and side effects. How these therapies perturb the aggregate ecology and biomolecular environment of the gut microbiome is poorly understood. Thus, determining what aspects of the gut microbiome structurally and functionally change in active colitis and treatment-induced remission may provide improved therapeutic targets.

Mouse models of IBD provide an opportunity to identify microbes and microbial pathways involved in IBD and host-microbiota responses to therapies, which can be difficult to discern in humans given their genetic diversity and variability in environmental and treatment exposures. Deciphering which gut microbial community members and functions are similarly or differentially modulated by therapeutic interventions has important implications for IBD management and may facilitate customization of existing and future therapies.

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Received 7 August 2013; revised 20 November 2013; accepted 6 January 2014; published online 6 February 2014

TRUC (*T-bet*^{-/-} *Rag2*^{-/-} ulcerative colitis) mice develop an early onset, spontaneous ulcerative colitis (UC) due to genetic defects in innate and adaptive immunity (Garrett *et al.*, 2007). TRUC pathogenesis is driven, in part, by tumor necrosis factor- α (TNF- α) (Garrett *et al.*, 2007) and is dependent on the gut microbiome, as germ-free (GF) TRUC mice do not develop disease (Garrett *et al.*, 2010). In the presence of an endogenous microbiota, specific gut microbes have been shown to trigger TRUC colitis, including *Klebsiella pneumoniae* and *Proteus mirabilis* (Garrett *et al.*, 2010) – and more recently, *Helicobacter typhlonius* (Powell *et al.*, 2012). Like human IBD, TRUC colitis is responsive to immunomodulators that dampen pro-inflammatory responses to gut microbes (Garrett *et al.*, 2007) and to oral antibiotics (Garrett *et al.*, 2010). Daily consumption of a fermented milk product (FMP) has also been shown to mitigate TRUC colitis (Veiga *et al.*, 2010). Provided that antibiotics, immunomodulators and dietary interventions mechanistically act on different aspects of the host–microbiota interface to ameliorate TRUC colitis, they likely differentially modify the gut microbiome. Thus, TRUC mice offer a tractable model for evaluating gut microbiome contributions to colonic inflammatory pathogenesis and for characterizing gut microbiome responses to therapeutic interventions.

Here, we investigated the effects of diverse treatment interventions on host disease status and on gut microbiome structure and function in TRUC mice. Using 16S ribosomal RNA (rRNA) gene surveys, we analyzed gut microbial communities following treatment with: oral antibiotics (gentamicin, metronidazole or vancomycin), immunomodulators (TNF- α neutralizing antibodies (anti-TNF- α) or infusion of regulatory T cells (T_{Reg} s)) and dietary interventions (FMP or non-fermented milk control (MC)). In addition to examining taxonomic shifts associated with treatment exposure, we used gnotobiotic TRUC mice to test the inflammatory phenotypes of gut microbiomes from treatment groups *in vivo*. To functionally interrogate the metabolic potential of gut microbiomes associated with active colitis versus treatment-induced remission, we performed *in silico* analysis of 16S rRNA gene sequences coupled with reference genomes to infer microbial community function. We found that TRUC gut microbiomes with active colitis had a reduced potential for both carbohydrate and energy metabolism and an enhanced potential for flagellar assembly, tetrathionate respiration and benzoate degradation. Collectively, our study identified microbes and microbial functions underlying colitis-associated dysbiosis that were similarly or differentially modulated by host- and microbiota-targeted therapies, illustrating the potential for therapeutic manipulation of the gut microbiome in colitis.

Materials and methods

Animal husbandry

Specified-pathogen-free (SPF) BALB/c *T-bet*^{-/-} *Rag2*^{-/-} mice have been described (Garrett *et al.*, 2007), see Supplementary Methods.

Treatment interventions

Antibiotics. Mice were treated with antibiotics dissolved in their autoclaved drinking water: gentamicin (2 g l⁻¹; Cellgro, Manassas, VA, USA), metronidazole (1 g l⁻¹; Sigma-Aldrich, St Louis, MO, USA), vancomycin (500 mg l⁻¹; Sigma-Aldrich) (Garrett *et al.*, 2010).

Anti-TNF- α injections. Mice were injected (15 mg kg⁻¹) with a hamster anti-mouse TNF- α neutralizing antibody (clone TN4-19.12) (Bio X Cell, West Lebanon, NH, USA) weekly for 4 weeks starting at 4 weeks of age (Garrett *et al.*, 2007).

T_{Reg} cell infusion. FACS-sorted peripheral lymph node CD4⁺CD62L^{hi}CD25⁺ cells (75 000 cells per mouse) were intravenously injected at 4 weeks of age (Garrett *et al.*, 2007).

Dietary interventions. For details on the FMP and MC, see Supplementary Methods.

Histology

Upon sacrifice, colons were resected, fixed in 4% paraformaldehyde (Sigma-Aldrich) and embedded in paraffin. Five-micron sections were H&E-stained and evaluated in a blinded fashion for epithelial hyperplasia (0–3), epithelial injury (0–3), polymorphonuclear infiltration (0–3) and mononuclear infiltration (0–3), these indices were summed to generate the histologic colitis score (Garrett *et al.*, 2007).

Microbial DNA preparation from stool and mesenteric lymph nodes (MLNs)

Samples were collected and homogenized in RNA-later (Ambion, Grand Island, NY, USA), held at 4 °C overnight and stored at –80 °C before processing. For details on DNA extraction, see Supplementary Methods.

16S rRNA gene survey analysis of gut microbial communities

For details on 16S rRNA gene amplification, 454 pyrosequencing, sequence processing, operational taxonomic unit (OTU) selection, microbial composition and community structure analyses, metagenome inference and metabolic pathway reconstruction, and microbial biomarker discovery, see Supplementary Methods.

Whole metagenome shotgun (WMS) sequence analysis
For details on Illumina shotgun sequencing, sequence processing and microbial gene and pathway abundance analyses, see Supplementary Methods.

Dopamine ELISA

To measure dopamine levels, the DOP Research ELISA (Labor Diagnostika Nord, Nordhorn, Germany) was employed following the manufacturer's instructions. For details, see Supplementary Methods.

Gnotobiotic mouse experiments

GF Balb/c *T-bet*^{-/-} *Rag2*^{-/-} mice were maintained on autoclavable Mouse Breeder Diet 5021 (LabDiet, St Louis, MO, USA) and housed in gnotobiotic isolators in the Harvard Digestive Disease Center Facility. For details, see Supplementary Methods.

RT-qPCR for FMP strains

For detailed RT-qPCR (real-time quantitative PCR) methods and primers, see Supplementary Methods.

Statistical analysis

Significant *P*-values associated with microbial clades and functions identified by LEfSe (Linear Discriminant Analysis with Effect Size) were corrected for multiple hypothesis testing using the Benjamini and Hochberg false discovery rate correction (Benjamini and Hochberg, 1995). All *P*-values and *q*-values are in Supplementary Table S3. Other statistical tests for significance were performed in GraphPad Prism version 5.0b for Mac OS X (GraphPad Software, La Jolla, CA, USA). All averages are mean ± SEM.

Accession number

Sequences have been deposited on MG-RAST under project ID 6698.

Results

Assessing gut microbiome structure during active disease and treatment-induced remission with 16S rRNA gene surveys

TRUC mice manifest signs of colitis prior to 3 weeks of age, which increase in severity over time (Garrett *et al.*, 2007). At 3 weeks of age, mice were randomized to the following groups: antibiotics (gentamicin, metronidazole or vancomycin), immunomodulators (anti-TNF- α or T_{Regs}), dietary interventions (FMP or MC), or untreated (sham) control, and began treatment 1 week later. Stool samples were collected from mice at 4 weeks of age (baseline/pre-intervention) and directly upon completion of the intervention at 8 weeks of age (post-intervention). The study design and histology-based colitis

scores to relate gut microbiome changes to host disease status are shown (Figures 1a and b).

In total, 1 014 181 quality-filtered 16S rRNA gene sequences were obtained from 152 stool samples (6672 ± 335 reads per sample; Supplementary Table S1). Reads were binned *de novo* into approximately species-level OTUs at ≥97% sequence similarity (586 ± 30 OTUs per sample; Supplementary Table S1). Microbiome analysis tools included: QIIME (Quantitative Insights Into Microbial Ecology) for sequence processing (Caporaso *et al.*, 2010), PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) for metagenome inference (Langille *et al.*, 2013), HUMAnN (The HMP Unified Metabolic Analysis Network) for functional profiling (Abubucker *et al.*, 2012), and LEfSe for univariate contrasts (Segata *et al.*, 2011) (Supplementary Figure S1).

Effects of treatment interventions on gut microbiome composition

Principal coordinate analysis (PCoA) of the unweighted UniFrac distances (Lozupone *et al.*, 2011) revealed that baseline (pre-intervention) communities clustered together (Figure 1c, far left panel). Comparing post-treatment stool, samples tended to separate by type of intervention, with distinct clusters observed for gut microbiomes exposed to antibiotics, immunomodulators and dietary interventions (Figure 1c, middle right panel). PCoA of the weighted UniFrac distances demonstrated similar trends (Supplementary Figure S2), as did a single-dimensional UPGMA (unweighted pair group method with arithmetic mean) hierarchical clustering of these samples (Figure 1d).

The 16S rRNA gene survey data revealed the greatest variation in gut microbial community composition with antibiotic exposure, particularly for gentamicin and vancomycin (Figure 1c, far right panel). In contrast, immunomodulators and dietary interventions (Figure 1c, right panels) induced changes of smaller effect size. Shifts in phylum-level relative abundances of samples collected at baseline and upon intervention completion also confirmed these high taxonomic level gut microbial response patterns (Figure 1e). Both in terms of microbial presence/absence and in overall clade abundances, immunomodulators, dietary interventions and individual antibiotics each perturbed the gut microbiome in distinctive ways and to varying extents, with antibiotics having the most substantial effect on microbial community structure.

Antibiotic-driven microbial community shifts may be influenced by early-life exposures and are associated with specific clade responses

Despite mitigating some IBD flares (Wang *et al.*, 2012), antibiotics disrupt gut microbial community

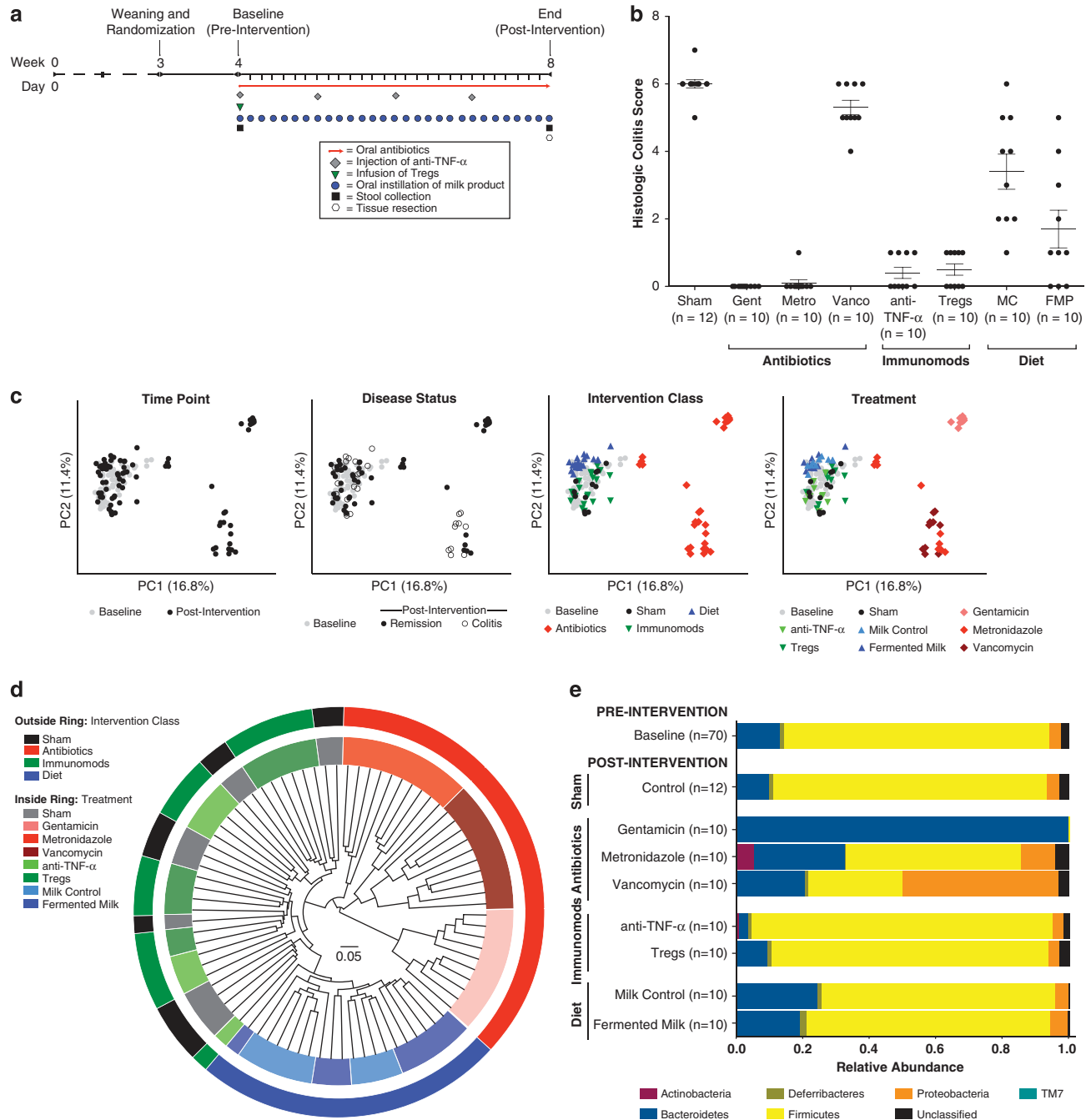


Figure 1 Experimental design and influence of interventions on the gut microbiome. **(a)** Study experimental schema. **(b)** Histologic colitis scores. Symbols represent individual mice. Error bars indicate mean \pm SEM. Colitis scores > 2 indicate active colitis and scores ≤ 2 remission. Sham, untreated, handling control; Gent, gentamicin; Metro, metronidazole; Vanco, vancomycin; Immunomodulators, anti-TNF- α or T_{Regs}; MC, non-fermented milk control; FMP, fermented milk product; Diet, dietary intervention with FMP or MC in addition to *ad libitum* chow. **(c)** PCoA using unweighted UniFrac distances of gut microbial communities obtained from stool samples collected at baseline (pre-intervention) and upon treatment completion (post-intervention). The first two principal coordinates (PC) from the PCoA are plotted. Symbols represent data from individual mice, color-coded by the indicated metadata. **(d)** Gut microbiomes were clustered by similarity using the UPGMA clustering algorithm on the unweighted UniFrac distances. Samples from individual mice were clustered by the indicated intervention class (outside ring) or by the specific treatment (inside ring). **(e)** Phylum-level phylogenetic classification of 16S rRNA gene sequences. Bars represent mean relative abundances for each pre- or post-intervention group.

structure and diversity, pushing the microbiome to an alternate state and potentially prolonging susceptibility to pathogens (Dethlefsen *et al.*, 2008; Hill *et al.*, 2010; Jakobsson *et al.*, 2010; Manichanh *et al.*,

2010; Ubeda and Pamer, 2012). We investigated the extent to which three oral antibiotics perturb the gut microbiomes of TRUC mice: metronidazole, gentamicin and vancomycin. Gentamicin and

metronidazole (the latter used clinically to treat IBD and *Clostridium difficile* colitis) both ameliorated TRUC colitis (Figure 1b). However, vancomycin, also employed clinically to treat *C. difficile* colitis, did not attenuate TRUC colitis (Figure 1b). Given that gentamicin, metronidazole and vancomycin have disparate modes of action and effects on TRUC colitis, we characterized shifts in community composition and diversity following treatment exposure and identified which members of the gut microbiome were similarly or differentially modulated by each antibiotic.

In contrast to baseline microbial communities, which consisted mostly of Firmicutes followed in decreasing order of relative abundance by Bacteroidetes, Proteobacteria, Actinobacteria, Deferribacteres and TM7, each antibiotic had marked effects on gut microbiome composition (Figure 2a). Gentamicin exposure led to a dominance of Bacteroidetes ($99.6 \pm 0.1\%$, relative abundance), particularly *Bacteroidaceae*, whereas vancomycin promoted an expansion of Proteobacteria – levels increased from $3.4 \pm 1.8\%$ to $47.0 \pm 10.8\%$. Responses within the metronidazole and vancomycin treatment groups were distinct in terms of overall community composition (Figure 1c, far right panel) and by relative abundance analysis (Figure 2a). To address if host or environmental factors affect gut microbiome responses to these antibiotics, we evaluated whether treatment, caging or legacy effects (influence of parental transmission on microbial composition) corresponded to the greatest degree of microbial variation. The greatest source of variation was not cage effects – observed in some mouse gut microbiome studies and attributed to cohabitation and coprophagia (Hildebrand *et al.*, 2013). Rather, microbial communities segregated first by parental origin and second by the antibiotic administered (metronidazole versus vancomycin) (Figures 2b and c; Supplementary Figure S3a), suggesting that community structure may be influenced by early-life exposures and by post-weaning interactions with other environmental exposures, respectively.

To identify gut microbiome responses associated with antibiotics and legacy effects, we determined pre- and post-treatment microbial clade differences in progeny of breeding pairs using LEfSe. Mice enriched in Actinobacteria and *Bacteroides* (BP-II) (Supplementary Figure S3b) at baseline became further enriched in these microbial clades with metronidazole treatment (Figure 2a, Metro-6-8,10). Because metronidazole selectively targets anaerobic bacteria, it may enable aerotolerant members of the Actinobacteria and Bacteroidetes phyla to bloom. Mice enriched in δ -proteobacteria (BP-III) (Supplementary Figure S3c) at baseline became further enriched in Proteobacteria with vancomycin treatment (Figure 2a, Vanco-1-5). This vancomycin-induced expansion of Proteobacteria has been observed previously (Carvalho *et al.*, 2012; Ubeda and Pamer, 2012). Thus, identifying microbial clades enriched at baseline may predict gut

microbial responses to antibiotics and may be useful for tailoring antibiotic treatment.

Alterations of gut microbial communities following gentamicin, metronidazole and vancomycin treatment coupled with their disparate effects on colitis provided an opportunity to identify which gut microbiome members are preferentially targeted by a given antibiotic and probe for members that are consistently associated with disease pathogenesis. Using LEfSe, we performed an all-against-all multi-class comparison of gut microbiome samples from antibiotic-treated mice to identify clades specifically modulated by each antibiotic. Although gentamicin-treated communities were completely dominated by Bacteroidetes, the remaining Firmicutes were enriched in *Erysipelotrichi* (Supplementary Figure S4a). Metronidazole treatment was associated with enrichments in Firmicutes and unclassified bacteria (Supplementary Figure S4a). In contrast with gentamicin and metronidazole, vancomycin treatment led to a significant expansion of γ -proteobacteria and ϵ -proteobacteria, including *Escherichia* and *Helicobacter* (Supplementary Figure S4a), which have been associated with intestinal inflammatory pathogenesis (Mukhopadhyaya *et al.*, 2012).

To explore microbial biomarkers of active colitis, we looked for clades consistently reduced in gentamicin- and metronidazole-treated communities but augmented in vancomycin and sham communities. There were significant enrichments across three microbial lineages in colitogenic gut microbiomes: *Mucispirillum*, *Desulfovibrio* and *Helicobacteraceae* (Figure 2d; Supplementary Figure S4b), all of which discriminated between active colitis versus remission. Other studies have pointed to their opportunistic nature given their putative capacity to degrade mucin (*Mucispirillum*) (Robertson *et al.*, 2005; Berry *et al.*, 2012) and during active inflammation to produce high levels of hydrogen sulfide (*Desulfovibrio*) (Carbonero *et al.*, 2012) and ammonia (*Helicobacteraceae*) (Hansen *et al.*, 2011), which may further fuel inflammation.

Immunomodulators alter low abundance communities of the gut microbiome and drive distinct clade responses

Use of immunomodulators in IBD has increased given their longer disease-remission periods and fewer side effects as compared with corticosteroids (Yanai and Hanauer, 2011; Pineton de Chambrun and Sandborn, 2012). However, knowledge of the effects of specific immunomodulators on gut microbiomes is limited (Engel and Neurath, 2010). Because TRUC colitis is driven, in part, by dysregulated colonic TNF- α production, which can be ameliorated with TNF- α neutralizing antibodies or infusion of immunosuppressive T_{Regs} (Garrett *et al.*, 2007), we utilized TRUC mice to unravel the effects of TNF- α -directed therapies on a colitogenic gut microbiome.

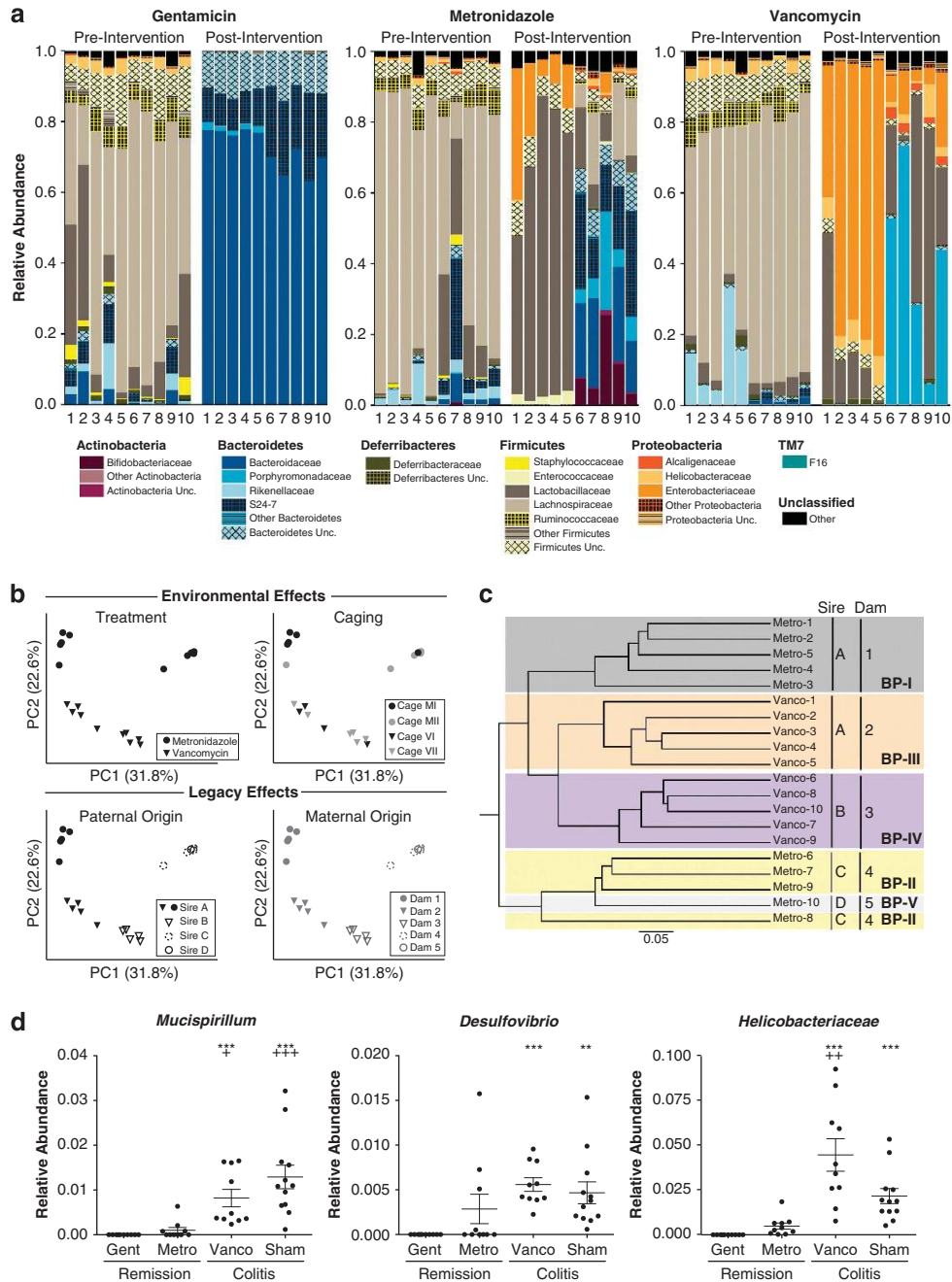


Figure 2 Antibiotic-driven microbial community shifts may be influenced by early-life exposures and are associated with specific clade responses. (a) Family-level phylogenetic classification of 16S rRNA gene sequences from stool samples collected pre- and post-intervention. Bars represent relative abundances of samples from individual mice. Labels indicate families with average relative abundances $\geq 1\%$ in at least one pre- or post-intervention group. Remaining families and reads assigned to higher level taxonomies were binned together in their associated phylum as 'other' or 'unclassified' (uncl.), respectively. (b) PCoA plots of the unweighted UniFrac distances of post-intervention stool samples from metronidazole- ($n = 10$) and vancomycin ($n = 10$)-treated mice. The first two PCs from the PCoA are plotted. Symbols represent data from individual mice, color-coded by the indicated metadata. For caging, M, metronidazole-treated; V, vancomycin-treated. (c) UPGMA clustering algorithm on the unweighted UniFrac distances of samples, color-coded by breeding pair. The letter and number assigned to the sire and dam, respectively, correspond to the PCoA plots in b. (d) Differentially abundant microbial clades in stool from mice with active colitis versus remission. Symbols represent data from individual mice from three independent experiments. Error bars indicate \pm SEM. Kruskal–Wallis test with Dunn's multiple comparison test: * or + $P < 0.05$; ** or ++ $P < 0.01$; and *** or +++ $P < 0.001$.

To determine the extent to which gut microbiomes are affected by immunomodulators, we inspected exclusive and shared species-level phylotypes (OTUs) among anti-TNF- α injected, T_{Reg} infused

and untreated mice. In all, 1 611 out of 4 420 total OTUs (36.5%) identified in immunomodulatory-treated and untreated mice were shared (Figure 3a, left panel). Shared OTUs were mostly more

abundant species – 240 306 out of 265 344 (90.6%) of all the sequences present across samples – whereas OTUs unique to each group were mostly low abundance species (Figure 3a, right panel). T_{Reg} -infused mice had the largest number of unique species (17.3%) compared to anti-TNF- α treated (10.3%) and untreated (10.4%) mice (Figure 3a, left panel), suggesting that immunomodulators, particularly T_{Reg} s, modulate low abundance gut microbiome members as compared to antibiotics, in agreement with the smaller effect size of shifts in community structure (Figure 1c, right panels).

As this argued for a focus on clade abundances rather than microbial presence/absence, we identified which microbial clades were consistently modified by immunomodulators using LEfSe. With immunomodulators, gut microbiomes were most significantly enriched in Actinobacteria, including *Corynebacterium*, as well as, *Bacillales* – specifically

the genus *Staphylococcus* (Figure 3b; Supplementary Figure S5a). The gut microbiomes of anti-TNF- α -treated mice were enriched in *Staphylococcus*, in contrast with that of T_{Reg} -infused mice, which were most significantly enriched in *Aerococcaceae* (Figure 3c; Supplementary Figure S5b). Examining shared and differential taxonomic responses illustrated that *Staphylococcus* expanded with both immunomodulators, however, to a greater extent following treatment with anti-TNF- α . *Helicobacter* spp. were also enriched in immunomodulatory-treated versus sham mice and reflect the presence of *Helicobacter ganmani* – a species found in mice and humans but not associated with colitis (Robertson *et al.*, 2001; Tolia *et al.*, 2004). Although treatment with anti-TNF- α and T_{Reg} s resulted in colitis remission, these immunomodulators drove specific microbial responses. These findings suggest that gut microbiomes may

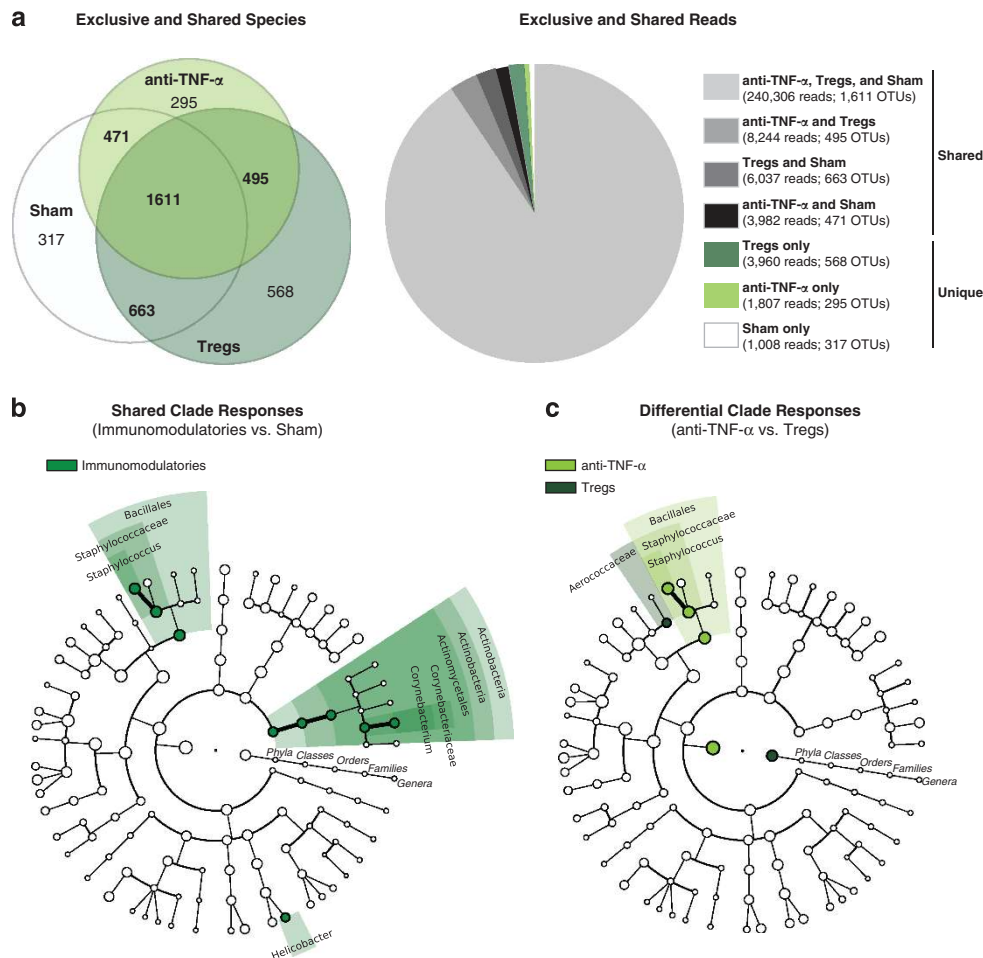


Figure 3 Immunomodulators influence low abundance gut microbial community members and drive distinct microbial responses. (a) Left panel: Venn diagram of exclusive and shared species-level phylotypes (non-singleton OTUs, at $\geq 97\%$ sequence identity) in anti-TNF- α injected ($n = 10$), T_{Reg} infused ($n = 10$) or sham (untreated; $n = 12$) mice. In total, 4420 OTUs were present across samples. Right panel: number of 16S rRNA gene sequences in each of the indicated segments of the Venn diagram. In total, 265 344 sequences were present across samples. (b) Differentially abundant microbial clades in stool from immunomodulatory-treated (anti-TNF- α or T_{Reg} s; $n = 20$) versus sham ($n = 12$) mice. (c) Differentially abundant microbial clades in stool from anti-TNF- α -($n = 10$) versus T_{Reg} -treated mice. For cladograms, white circles represent non-significant microbial clades.

distinguish and differentially respond to distinct host-directed therapies.

FMP consumption has subtle but distinct effects on the gut microbiome

Lactic acid-producing bacteria (LAB) within FMPs can improve gut homeostasis by providing microbes with beneficial functions to the host (Nanau and Neuman, 2012; Veerappan *et al.*, 2012). LAB have shown promise in IBD management, given their ability to promote anti-inflammatory immune responses without inducing severe side effects in IBD patients (Veerappan *et al.*, 2012). However, little is known of how LAB modify the gut microbiome in IBD in relation to other therapies (Rijkers *et al.*, 2010). Previous studies in TRUC mice have shown that colitis can be ameliorated with daily consumption of a LAB-containing FMP consisting of *Bifidobacterium animalis* subsp. *lactis* (CNCM I-2494), *Streptococcus thermophilus* (CNCM I-1630), two strains of *Lactobacillus delbrueckii* subsp. *bulgaricus* (CNCM I-1632 and CNCM I-1519) and *Lactococcus lactis* subsp. *cremoris* (CNCM I-1631) (Veiga *et al.*, 2010) (Figure 1b). In contrast, MC administration was less effective in attenuating colitis (Veiga *et al.*, 2010) (Figure 1b). To determine the differential effects of the FMP and MC on the gut microbiome, we orally instilled either product daily for 4 weeks.

Bifidobacteriaceae and *Coriobacteriaceae* have been associated with human colonic health and IBD remission (Morgan *et al.*, 2012; Papa *et al.*, 2012) and were enriched with FMP treatment (Figure 4a; Supplementary Figure S6a). The observed increase in *Bifidobacteriaceae* may reflect its presence in the FMP or an FMP-mediated expansion of endogenous bifidobacteria. Similar to earlier studies, we found an increase in lactate-consuming bacteria with FMP, such as *Desulfovibrionaceae*, that may be associated with elevated levels of the electron donor lactate produced by the dietary LAB in the FMP (Veiga *et al.*, 2010). Differential clade responses between FMP- and MC-fed mice included enrichments of *Lactobacillus* and *Streptococcus* in the MC group, which may have been secondary to the presence of lactose and absence of LAB in the MC product (Figure 4b; Supplementary Figures S6b and c).

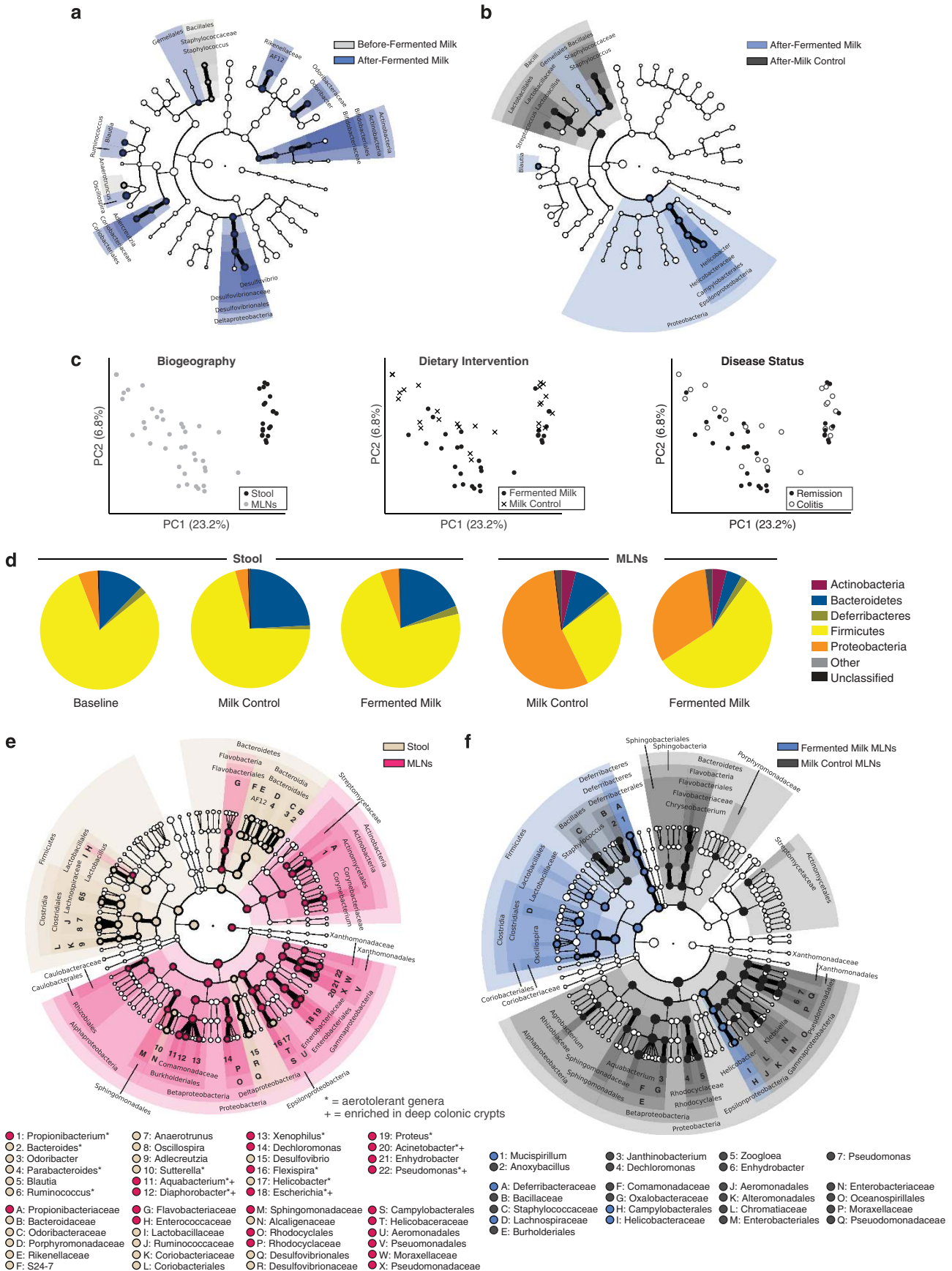
Proteobacteria were enriched with FMP – this finding reflects the presence of *Helicobacter* spp., specifically *H. ganmani*, as was observed with immunomodulators (Figures 3b and 4b, Supplementary Figure S6c). These results point to administration of FMP having subtle but distinctive effects on the gut microbiome, which facilitate gut microbial community changes that directly or indirectly ameliorate colitis.

FMP administration influences the mucosal immune system by modulating microbial communities trafficked to the MLNs

Given the subtle clade differences in stool observed with the FMP, we questioned whether there were greater changes in other gut-associated microbial communities, like the mesenteric lymph nodes (MLNs). MLNs function as a ‘firewall’ that prevents live gut microbes from reaching the systemic immune system (Macpherson and Smith, 2006; Eberl and Lochner, 2009). Microbial DNA was sequenced from pooled MLNs ($n = 5$ MLNs per mouse) of individual FMP- and MC-fed mice. In total, 288 500 quality-filtered 16S rRNA gene sequences were obtained with an average of 7797 ± 5728 reads per sample. Comparing stool and MLN microbial communities, we observed that the major variation is explained by sampling site (biogeography) (Figure 4c; Supplementary Figure S7).

To identify which microbes are preferentially sampled by the host mucosal immune system or otherwise trafficked through the lymphatics to the MLNs, we examined taxonomic differences between microbiomes from stool and MLNs. We observed greater differences between MLN microbial communities than between stool communities with administration of FMP versus MC, particularly for the ratio of Firmicutes to Bacteroidetes and the proportion of total Proteobacteria (Figure 4d). This suggests that FMP exposure might affect which gut microbes reach the MLNs and consequently shape host immune responses. Microbial communities of MLNs were highly enriched in aerobes and facultative anaerobes (Figure 4e). TRUC MLNs compositionally resembled deep colonic crypt communities (Pédrón *et al.*, 2012) with both being enriched in aerotolerant clades that are rare in stool (Figure 4e; Supplementary Figure S8).

Figure 4 A FMP influences the microbial communities of the gut and MLNs. (a) Differentially abundant microbial clades in stool collected before and after FMP ($n = 10$). (b) Differentially abundant microbial clades in stool after FMP ($n = 10$) versus MC ($n = 10$). (c) PCoA plots of the unweighted UniFrac distances of post-intervention stool samples (FMP, $n = 10$; MC, $n = 10$) and MLNs (FMP, $n = 21$; MC, $n = 16$; 5 MLNs/mouse). The first two PCs from the PCoA are plotted. Symbols represent data from individual mice, color-coded by the indicated metadata. (d) Phylum-level phylogenetic classification of 16S rRNA gene sequences from pre-intervention ($n = 20$) and post-intervention stool samples (FMP, $n = 10$; MC, $n = 10$) and post-intervention MLNs (FMP, $n = 21$; MC, $n = 16$; 5 MLNs/mouse). Pie charts represent the mean relative abundances of phyla across mice from each group. (e) Differentially abundant microbial clades in post-intervention samples from stool (FMP, $n = 10$; MC, $n = 10$) versus MLNs (FMP, $n = 21$; MC, $n = 16$; 5 MLNs/mouse). (f) Differentially abundant microbial clades in post-intervention MLNs of FMP- ($n = 21$) versus MC ($n = 16$)-fed mice. *, aerotolerant genera; +, genera shared between MLNs and deep colonic crypt communities (Pédrón *et al.*, 2012). For cladograms, white circles represent non-significant microbial clades.



Based on evidence for a dominant presence of aerotolerant genera at the oxygen-rich mucosal surfaces of intestinal epithelial cells (Engel and Neurath, 2010; Marteyn *et al.*, 2010; Pineton de Chambrun and Sandborn, 2012), our findings suggest that aerotolerant microbes may have greater access to the MLNs.

MLNs of MC-fed mice, which tended to have more severe colitis compared with FMP-fed mice (Figure 1b), were highly enriched in Proteobacteria, including an increased relative abundance of *Enterobacteriaceae*. Gut microbiome studies of IBD patients have demonstrated expansions of Proteobacteria, particularly *Enterobacteriaceae* (Garrett *et al.*, 2007; Mukhopadhyaya *et al.*, 2012). We observed increased levels of *Klebsiella* in the MLNs of MC-fed mice (Figure 4f; Supplementary Figure S9), which have been implicated as opportunistic drivers of inflammation in TRUC mice (Garrett *et al.*, 2007). In contrast, MLNs of FMP-fed mice were enriched in Firmicutes – including *Lactobacillales*, *Clostridiales* and *Coriobacteriales* (Figure 4f; Supplementary Figure S9). Gut microbiome status (homeostasis versus dysbiosis) can influence transport of commensal and pathogenic bacterial antigens from the lumen to the MLNs (Diehl *et al.*, 2013). Our analysis of stool and MLN microbial communities support these findings. Furthermore, increased Proteobacteria in stool and MLNs of mice with active colitis aligns with the association between Proteobacteria and IBD-associated dysbiosis.

Microbial community perturbations in active disease versus treatment-induced remission and their functional validation in gnotobiotic mice

Discriminatory microbial lineages for active colitis included the Deferribacteres, *Mucispirillum*; *Anaerotruncas*; and Proteobacteria, particularly *Enterobacteriaceae*, *Helicobacteraceae*, *Desulfovibrio* and *Sutterella* (Figure 5a; Supplementary Figure S10). In contrast, clades associated with remission included Actinobacteria; the Bacteroidetes, *S24-7*; and the Firmicutes, *Staphylococcaceae* and *Erysipelotrichales* (Figure 5a; Supplementary Figure S10).

Gnotobiotic mice represent a tractable system for testing the function or inflammatory capacity of gut microbiomes (Tremaroli and Bäckhed, 2012). Disease phenotypes, for example, diabetes and obesity, can be transferred to gnotobiotic mice by inoculating them with gut microbiomes of afflicted mice or human donors (Turnbaugh *et al.*, 2006; Wen *et al.*, 2008). To assess the inflammatory potential of gut microbiomes exposed to antibiotics, immunomodulators or FMP compared with an untreated (sham) control, we performed fecal transfers from treated or untreated SPF TRUC donors to GF TRUC recipients (Figure 5b experimental schema). Pro-inflammatory input communities from untreated donors, on average, induced colitis over the duration of an 8-week association, whereas

anti-inflammatory input communities from gentamicin and anti-TNF- α -treated donors did not induce colitis (Figure 5c). Despite FMP ameliorating colitis in SPF donors, gnotobiotic recipients tended to develop mild to moderate colitis (Figure 5c). To follow up on this finding, we measured levels of two FMP bacterial strains, *B. lactis* and *L. lactis*, in donor and recipient stool samples using RT-qPCR. FMP strains were detected in donor but not recipient stool (Figure 5d). These findings suggest inefficient intestinal colonization of FMP strains from the donor stool samples and support studies showing that maintaining the benefits of FMPs requires routine administration (Veiga *et al.*, 2010; Sanders, 2011). In contrast with antibiotics and immunomodulators, these experiments suggest a transient protective effect of FMP on the gut microbiome. Together, these experiments demonstrate that inflammatory phenotypes of gut microbiomes are capable of being transmitted and tested *in vivo*. Moreover, our results point to differences in the durability of gut microbiomes and their associated disease phenotypes with treatment.

Microbial metabolic functions associated with active colitis versus treatment-induced remission

To investigate the gut microbiome functions associated with active colitis versus remission in TRUC mice following treatment, we used PICRUST to infer putative metagenomes from our 16S rRNA gene profiles (Langille *et al.*, 2013). Reads were binned into OTUs at $\geq 97\%$ sequence identity using a closed reference-based strategy that searches against the available collection of Greengenes reference OTUs. PICRUST transformed counts of reference-based OTUs into metagenome prediction counts of functional genes on a per-sample basis and evaluated prediction accuracy by calculating the extent to which microbes in a sample are related to sequenced reference genomes using the weighted Nearest Sequenced Taxon Index (Supplementary Table S2). Identified microbial gene families (specified by KEGG Orthology groups) were grouped into metabolic pathways and broader functional categories based on the BRITE hierarchy. We used LEfSe to identify significant, differentially abundant microbially relevant functions associated with active colitis versus remission following treatment (Supplementary Figure S11). Categories associated with remission included carbohydrate metabolism and energy metabolism, as well as the biosynthesis of secondary metabolites. In contrast, gut microbiomes with active colitis were enriched in categories associated with cell motility, signal transduction and xenobiotics biodegradation and metabolism, as well as lipid metabolism (Figure 6a). Thus, gut microbiomes associated with active colitis may have a reduced capacity for energy harvest and dysregulated microbial signaling and cellular processing pathways.

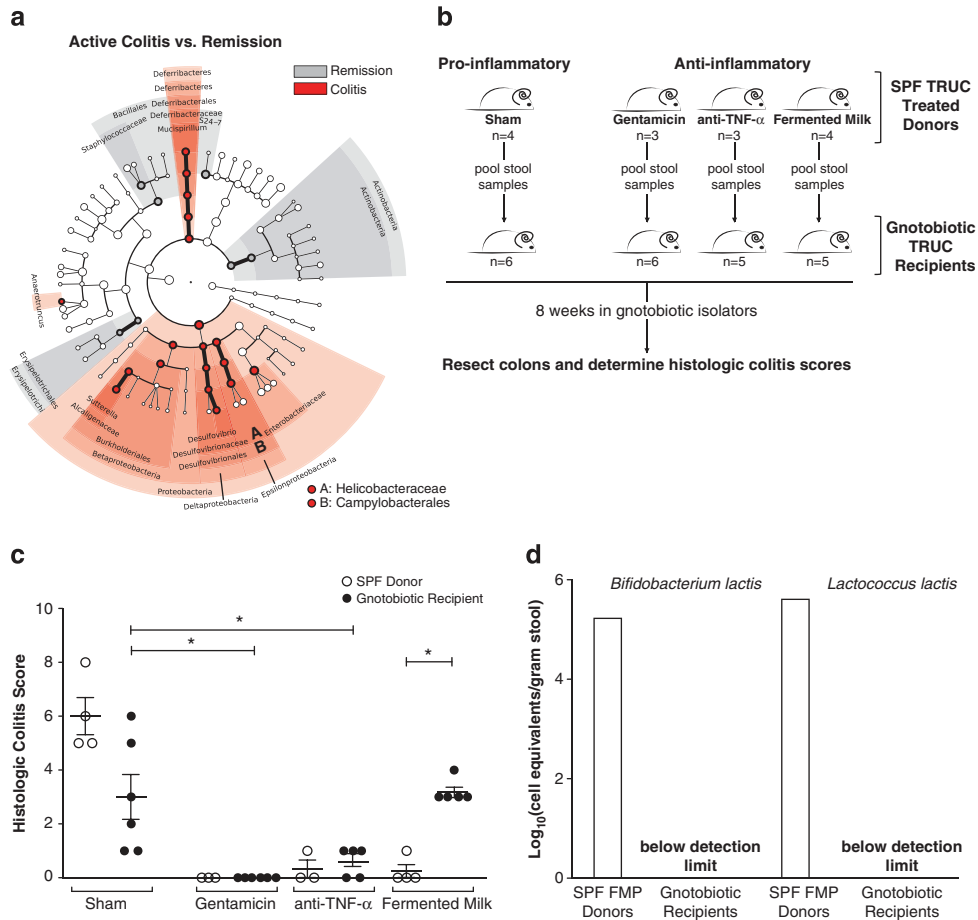


Figure 5 Gut microbiome composition in active colitis and treatment-induced remission with *in vivo* functional validation of pro-inflammatory activity in gnotobiotic TRUC mice. **(a)** Differentially abundant microbial clades in stool from mice with active colitis ($n = 31$) versus remission ($n = 51$) upon intervention completion. For cladogram, white circles represent non-significant microbial clades. **(b)** Experimental schema of 8-week gnotobiotic TRUC association with conventionally raised, SPF TRUC donor stool. SPF donors were treated for 4 weeks prior to stool collection. Stool from the indicated number of donors was pooled and transplanted into gnotobiotic TRUC recipients. **(c)** Histologic colitis scores of donors and recipients. Symbols represent data from individual mice. Error bars indicate \pm SEM. Mann–Whitney test: $*P < 0.05$. **(d)** *B. lactis* and *L. lactis* levels quantified by RT-qPCR in stool from fermented milk (FMP)-treated donors (pooled; $n = 4$) and their corresponding GF recipients ($n = 5$).

Within the cell motility category, we observed an increased capacity for bacterial motility proteins, including genes for flagellar assembly (Figure 6b). Flagellar bacterial antigens have been implicated as disease drivers in both mouse models of colitis and human IBD (Steiner, 2007). Within the signal transduction category, we detected the most significant gene abundances within two-component regulatory systems (Figure 6c). Data suggest that opportunistic microbes have an ability to utilize substrates generated under inflammatory conditions. We examined whether there were differential gene abundances for tetrathionate respiration, a metabolic pathway underlying the fitness advantage of *Salmonella enterica* subsp. Typhimurium in an inflamed gut (Winter et al., 2010), and observed enhanced potential for tetrathionate respiration with active disease in TRUC mice (KOs: 08357,08358,08539,13040,13041; Figure 6c,

inset). Within the xenobiotics biodegradation and metabolism category, we found an enhanced potential for benzoate degradation (Figure 6d). Catechols (1,2-dihydroxybenzene) – intermediaries of benzoate metabolism – have the ability to promote *Enterobacteriaceae* growth and virulence (Freestone et al., 2007; Lyte et al., 2011). Catecholamines, which are host-derived catechols with a side-chain amine, are detectable in stool (Asano et al., 2012). *K. pneumoniae* has been implicated in TRUC pathogenesis (Garrett et al., 2010) and members of the *Klebsiella* genus are one of the few *Enterobacteriaceae* with the genomic potential to fully metabolize catecholamines (see benzoate degradation pathway kpn00362). Thus, active colitis, with its *Enterobacteriaceae* enrichment, may result in decreased levels of fecal catecholamines. To test this hypothesis, we measured dopamine, the most abundant catecholamine in

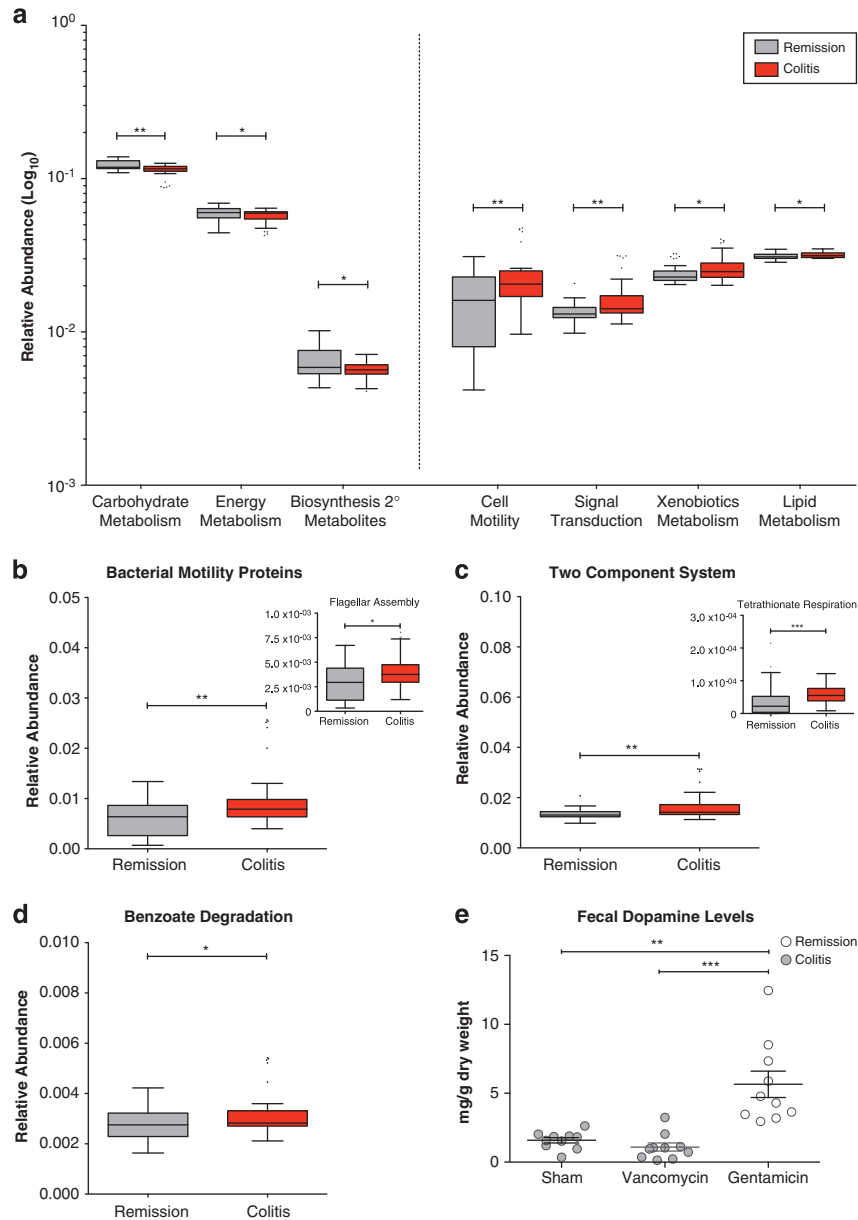


Figure 6 Inferred gut microbiome functions associated with active colitis and treatment-induced remission. Relative abundances of KO gene families grouped into BRITE functional hierarchies, as inferred by PICRUSt from 16S rRNA gene sequences. Differentially abundant microbial functions associated with active colitis ($n = 31$) versus remission ($n = 51$) upon intervention completion organized by KEGG BRITE categories (**a**) and pathways (**b–d**). Boxplots denote top quartile, median and bottom quartile. Whiskers and outliers are plotted by the Tukey method. (**e**) ELISA-based determinations of fecal dopamine levels. Symbols represent data from individual mice from three independent experiments. Mann–Whitney test: * $P < 0.05$; ** $P < 0.01$ and *** $P < 0.001$.

the colonic lumen (Asano *et al.*, 2012), in stool collected from mice with active colitis and treatment-induced remission. We examined samples from mice treated with vancomycin and gentamicin, which increases or decreases *Enterobacteriaceae*, respectively. Dopamine levels were significantly decreased in stool of sham- and vancomycin-treated mice with active colitis as compared with gentamicin-treated mice in remission. In addition, there was a trend, although not statistically significant, toward lower levels of dopamine in mice treated with vancomycin versus sham (Figure 6e).

To validate the inferred functions determined by PICRUSt, we performed WMS sequencing – the conventional means of assessing microbiome functional potential – on a subset of banked stool samples from anti-TNF- α -treated mice ($n = 6$). A total of 419 659 443 quality-filtered shotgun sequences were obtained with an average of $69\,943\,241 \pm 31\,774\,397$ reads per sample. Microbial gene abundances estimated from WMS and 16S rRNA gene sequence data were correlated (Spearman correlation, $r = 0.6835$). This was consistent with correlations in WMS and 16S functional data for human stool samples (Morgan *et al.*, 2012).

Discussion

A goal of microbiome research is defining the structure and function of the gut microbiome in health and active disease states (Lemon *et al.*, 2012). Our study identified shifts in microbial clades and inferred functions associated with active colitis and remission following treatment with antibiotics, immunomodulators or a fermented milk–dietary intervention in an experimental colitis mouse model. Using gnotobiotic fecal transplant experiments, we found that treatments' effect on the durability of gut microbiome inflammatory phenotypes varies. Collectively, we identified microbial biomarkers (both clades and functions) that may have clinical relevance for tracking disease when it is asymptomatic and utility as therapeutic targets for managing IBD.

An emerging concept in dysbiosis and bacterial pathogenesis is that certain bacteria have the ability to utilize host substrates to gain a fitness advantage during inflammation (Fleckenstein *et al.*, 2010; Buffie and Pamer, 2013; Winter *et al.*, 2013). The ability to respire tetrathionate and nitrate is central to the fitness of several *Enterobacteriaceae* (Winter *et al.*, 2013), as these metabolites are readily available in an inflamed gut and can be used as electron acceptors to generate ATP. We observed that tetrathionate utilization was associated with active colitis, which supports a link between enhanced oxidative stress and *Enterobacteriaceae*-mediated dysbiosis previously described in the TRUC model (Garrett *et al.*, 2010). Dampening the redox stress associated with intestinal inflammation may reduce the abundance of these electron acceptors and eliminate the fitness advantage of colitogenic bacteria, thus restoring intestinal homeostasis.

Enrichment of genes for microbial benzoate degradation in active colitis was unexpected. Catecholamines have garnered interest as communication molecules between host and microbes (Lyte *et al.*, 2011). *Enterobacteriaceae* can degrade catecholamines and catecholamines can promote *Enterobacteriaceae* growth and expression of bacterial virulence factors (Eloe-Fadrosh and Rasko, 2013). The histidine sensor kinase QseC is necessary for bacterial responses to host catecholamines and a compound that inhibits QseC, LED209, has been shown to inhibit pathogen virulence *in vivo* and *in vitro* (Rasko *et al.*, 2008). Given that LED209 selectively interferes with bacterial virulence and colonization without affecting bacterial growth, typical antibiotic resistance patterns that plague traditional antimicrobials are unlikely to develop (Rasko and Sperandio, 2010). Our observations on tetrathionate respiration and benzoate degradation highlight how gut microbiome studies in mouse models of disease are useful for identifying novel microbial therapeutic targets.

Fecal transplantation represents a long-standing treatment with the potential to address IBD

dysbioses and its practice is resurging. However, its use requires substantial consideration from a safety and regulatory perspective (Buffie and Pamer, 2013). We observed that the health status of a host and its gut microbiome could be transient in some cases. Despite a host being in a state of health, as confirmed by histology, transferring its gut microbiome to a GF recipient resulted in colonic inflammation. As applications for fecal transplantation develop for humans, gnotobiotic mouse models may prove useful for evaluating whether microbiomes selected for transplant will confer the intended health outcomes for the recipient.

In summary, our analyses point to features of microbiome dysbiosis and dysfunction in experimental colitis. Improvements in animal model systems and opportunities for translational medical research warrant future studies that incorporate 16S rRNA gene surveys with other 'omic' approaches to recognize the gut microbiome's full potential and ultimately guide therapeutic strategies for manipulating the microbiome to manage disease.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

We thank members of the Garrett and Huttenhower Labs for discussion. The following grants supported this study: to MGR, NIAID 5T32AI007638; to CH, NIH R01HG005969, NSF DBI-1053486 and ARO W911NF-11-1-0473; to WSG, NIH (R01CA154426, K08AI078942), Burroughs Wellcome CAMS, Searle Scholars, Cancer Research Institute and Danone Research Awards.

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