

Alteration of the Murine Gut Microbiota During Infection with the Parasitic Helminth *Heligmosomoides polygyrus*

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Background: In a murine model of inflammatory bowel disease (IBD), treatment of colitis in IL-10 gene-deficient mice with the parasitic helminth *Heligmosomoides polygyrus* ameliorates colonic inflammation. The cellular and molecular mechanisms driving this therapeutic host response are being studied vigorously. One proposed mechanism is that *H. polygyrus* infection favors the outgrowth or suppression of certain bacteria, which in turn help modulate host immunity.

Methods: To quantify the effect of *H. polygyrus* infection on the composition of the gastrointestinal (GI) tract microbiota, we conducted two independent microbial ecology analyses of C57BL/6 mice. We obtained and analyzed 3,353 bacterial 16S rRNA encoding gene sequences from the ileum and cecum of infected and uninfected mice as well as inceptive *H. polygyrus* larvae at the outset of the second experiment and adult worms taken directly from the mouse duodenum at the end of the second experiment.

Results: We found that a significant shift in the abundance and relative distribution of bacterial species in the ileum of mice is associated with *H. polygyrus* infection. Members of the bacterial family *Lactobacillaceae* significantly increased in abundance in

the ileum of infected mice reproducibly in two independent experiments despite having different microbiotas present at the outset of each experiment.

Conclusions: These data support the concept that helminth infection shifts the composition of intestinal bacteria. The clinical consequences of these shifts in intestinal flora are yet to be explored.

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Key Words: inflammatory bowel diseases, microbial ecology, *Heligmosomoides polygyrus*, microbiota, microbiome

The etiology of inflammatory bowel disease (IBD) is complex and influenced by genetic, microbiologic, and environmental factors. Lifestyle changes accompanying the modernization of developing countries have been epidemiologically linked to an increase in IBD incidence.^{1–6} These observations have led to the IBD hygiene hypothesis, which states that raising children in extremely hygienic environments negatively affects immune development and predisposes them to immunological diseases later in life.⁶

Empirical support for the IBD hygiene hypothesis has been developed in mouse and rat models.^{7–9} These studies revealed that intestinal parasites and parasite products modulate host immunity and decrease IBD-like inflammation.^{6,10} Likewise, *Trichuris suis* (pig whipworm) treatment was effective in decreasing symptoms of IBD in human clinical trials.^{11,12}

It is not entirely understood how helminthic parasites modulate the host mucosal immune response and alter susceptibility to immunological diseases.^{10,13} One unexplored hypothesis is that helminth infection changes the abundance and/or distribution of gastrointestinal (GI) tract bacteria (the microbiota), resulting in an overall decrease in the proinflammatory cytokine milieu associated with the chronically inflamed state.⁶ To begin to address this hypothesis, we studied the effect of helminthic infection on the composition of the GI tract microbiota of their host.

We found that the murine roundworm, *Heligmosomoides polygyrus*, changes the microbiota of the GI tract of otherwise healthy wildtype mice. We used clone library analysis of the bacterial 16S rRNA encoding gene (16S) to

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quantify changes that occur in the microbiota associated with the distal small intestine (ileum) and the tip of the cecum. In two independent experiments, we found that the ileal microbiota from *H. polygyrus*-infected mice was significantly different from uninfected controls. Also, the total bacterial load was greater in infected mice versus controls, and the dominant bacteria associated with the ileum of treated mice were also associated with adult worms that had been removed at the time of necropsy. These data support the hypothesis that parasites alter the abundance and relative distribution of GI tract bacteria and provide the basis for future experiments that investigate the overall importance that this alteration has on host immunity.

MATERIALS AND METHODS

Mouse Experiments and Sample Collection

Two independent mouse experiments were completed as outlined in Figure 1. In each experiment, 5 of 10 C57BL/6 mice were infected with L3 stage *H. polygyrus* for 2 weeks. Upon necropsy, ≈ 2 cm of the distal ileum and 1–1.5 cm of the cecal tip were collected from all animals, rinsed with phosphate-buffered saline (PBS) to remove luminal contents, added to Dry Bead Tubes (MoBio Laboratories, Carlsbad, CA), snap-frozen in liquid nitrogen, and cryopreserved at -80°C for DNA extraction. During the second experiment, infective L3 stage *H. polygyrus* and adult worms from the duodenum of infected mice were also obtained for analysis.

16S Clone Library Construction and Analyses

Total bulk DNA was extracted from tissue samples obtained during each mouse experiment. In the first experiment we used a developed protocol that combined bead-beating with the Qiagen (Chatsworth, CA) DNeasy Blood & Tissue kit.¹⁴ In the second experiment we again used bead-beating, but combined this with the MagNA Pure Compact System (Roche Diagnostics, Indianapolis, IN) to isolate the DNA. Briefly, a mixture of MagNA Pure Bacterial Lysis Buffer (BLB, Roche Diagnostics) and sterile PBS was added to frozen samples at a ratio of 9 parts BLB to 10 parts PBS. Samples were homogenized using a Mini-Beadbeater (BioSpec Products, Bartlesville, OK) for 1 minute. Samples were centrifuged at $\approx 13,000$ RPM in a microcentrifuge for 1 minute after which 40 μL of Proteinase K (>600 mAU/mL solution; Qiagen) was added. The solution was incubated at 65°C for 10 minutes and homogenized a second time on the Mini-Beadbeater for 1 minute. The remaining liquid was decanted after a 1-minute spin in a microcentrifuge ($\approx 13,000$ RPM) and added to a fresh sample tube. DNA was extracted using the MagNA Pure Compact System per the manufacturer's instructions (default tissue program, 50 μL final elution volume).

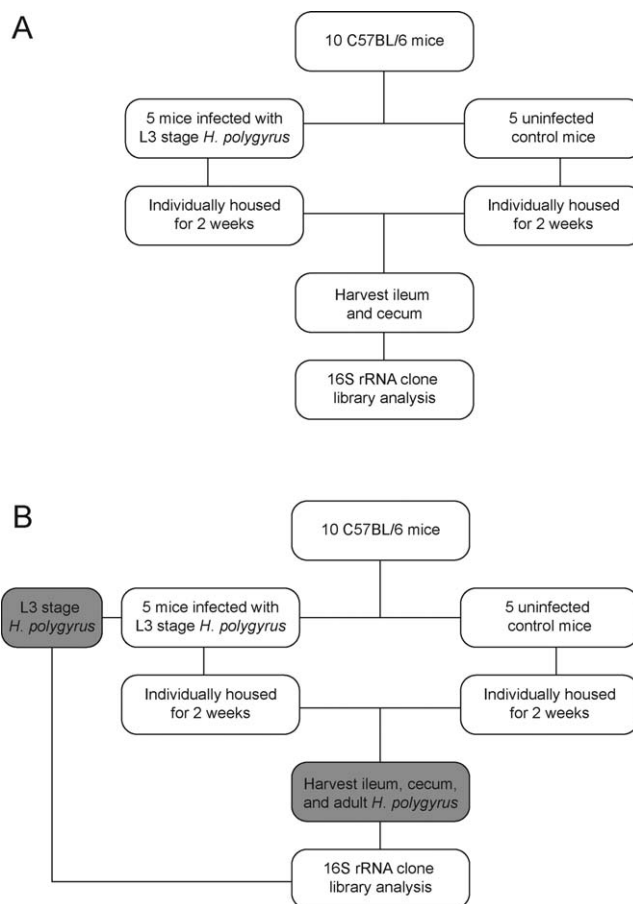


FIGURE 1. Overview of two independent murine infection experiments. The difference between the first experiment (A) and the second (B) was the inclusion of the L3 larval stage and adult *H. polygyrus* worms.

Clone libraries of the 16S rRNA-encoding gene were generated as previously described.¹⁵ Briefly, broad range primers (8F and 1492R) were used to polymerase chain reaction (PCR) amplify ≈ 1484 basepairs of the 16S rRNA-encoding gene. Amplicons were purified and ligated into plasmids, which were transformed into competent *E. coli* cells and grown overnight on LB agar (Topo TA cloning, Invitrogen, Carlsbad, CA). Single *E. coli* colonies were randomly sampled and screened for inserts of the correct size using plasmid specific PCR primers. Approximately 96 inserts were selected, purified, and submitted for Sanger-style sequencing for each sample (cecum, ileum, and/or adult worm). Partial sequences (≈ 800 bp) were obtained using the 8F primer for the 5-prime initiation of sequencing reads.

For operational taxonomic unit (OTU)-based analyses, a matrix of all pairwise sequence identities (p-distance, or the proportion of nucleotide differences between sequences) was generated and downloaded using the Ribosomal

Database Project (RDP) Website^{16,17} and the RDP Download tool. This matrix was loaded into the DOTUR program¹⁸ to identify and bin sequences according to an idealized bacterial group, called an OTU. The OTU definition used here considered all sequences $\geq 97\%$ identical to be the same OTU. This definition is based on numerous microbial population and community analyses that suggest that this level of sequence similarity ($\geq 97\%$) among 16S rRNA encoding genes defines a bacterial species.^{19,20} An OTU-count matrix (a square matrix, where communities are rows, OTUs are columns, and each element of the matrix is a count of the number of times the OTU was observed in a particular community) was generated from the binned sequences and loaded into the EstimateS²¹ program for the generation of a Bray–Curtis similarity matrix. The Bray–Curtis metric is a pairwise numerical representation of community similarity based on the abundance and relative distribution of OTUs in the communities being compared.²² To visualize Bray–Curtis similarities, a principal coordinates analysis was generated using the Numerical Taxonomy System, NTSYSpc v. 2.2e (Exeter Software, Setauket, NY). For taxonomic classification, sequences were uploaded and aligned using the RDP Classifier tool.²³ Taxonomic classifications in each sample were made at the 80% confidence level.

Quantitative PCR

Quantitative PCRs were used to measure the amount of 16S rRNA gene operons in each sample relative to the single-copy mouse gene, TNF- α (tumor necrosis factor alpha) as previously described.^{14,24,25} Individual PCR assays were done for each target using the LightCycler 480 Probes Master reaction mixture (Roche Diagnostics) and the LightCycler 480 instrument (Roche Diagnostics). The comparative C_T method was used to compare the bacterial load in each sample.²⁶

Statistical Analyses

Differences in delta C_T (relative difference in the copy numbers of bacterial 16S rRNA and murine TNF α operons) were tested using Student's *t*-test and SAS statistical software (SAS Institute, Cary, NC). An analysis of variance (ANOVA) was conducted on the family-level taxonomic classification of samples in each experiment using the Generalized Linear Model (GLM) procedure and SAS statistical software. The Bonferroni correction was used to adjust *P*-values for multiple comparisons.

Ethical Considerations

All animal experiments were completed in accordance with protocols approved by use and care of animals committees at both the University of Michigan and Tufts University.

RESULTS

Two independent experiments were conducted. In the first experiment, no data were collected from the *H. polygyrus* L3 infective larvae or adult worms. In the second experiment, the microbiota associated with both L3 larvae and adult worms were analyzed. All mice treated with *H. polygyrus* L3 infective larvae became colonized, and adult worms were observed upon necropsy in both experiments. Adult worms were only present in the proximal small bowel.

H. polygyrus Increases Bacterial Abundance in the Ileum

Significantly more bacterial 16S rRNA operons were detected by quantitative PCR in cecal samples compared to ileal samples (Experiment 1 = 9.5-fold greater; Experiment 2 = 20.2-fold greater), indicating an overall difference in the total bacterial load (overall abundance of bacteria) of these two intestinal locations. *H. polygyrus* infection did not statistically affect bacterial load in the cecum of mice ($t = -1.74$, $df = 17$, $P = 0.099$). However, 1.8-fold more 16S rRNA operons were detected in the ileum of infected mice compared to uninfected mice ($t = -2.33$, $df = 18$, $P = 0.031$), indicating that parasitic infection increased the abundance of bacteria in this part of the GI tract.

Differences in Mouse Gut Microbiota

A total of 1647 and 1706 16S sequences were generated from samples in each experiment and an average of 87 and 89 sequences were obtained per sample (GI location) per animal, respectively. A cecum sample from an uninfected mouse in Experiment 1 was lost before clone library construction was completed and an ileum sample from an uninfected mouse in Experiment 2 yielded insufficient bacterial DNA to allow amplification of 16S. For all other samples, two approaches were used to characterize differences within and between the microbiota. The first approach was OTU-based, meaning that the analysis was based on the abundance and distribution of groups of nucleotide sequences that were $\geq 97\%$ identical (i.e., a theoretically based sequence similarity cutoff for bacterial species). The second approach was also based on nucleotide sequence data, but instead of binning all similar sequences together, we determined the taxonomic classification for each sequence by making comparisons to an archived and curated 16S sequence database (see Materials and Methods).

OTU-based Characterization

To visualize the variation in bacterial communities, we generated Bray–Curtis similarity values from an OTU-count matrix. A principal coordinates analysis (PCoA) was used to visualize these data by positioning each sample in

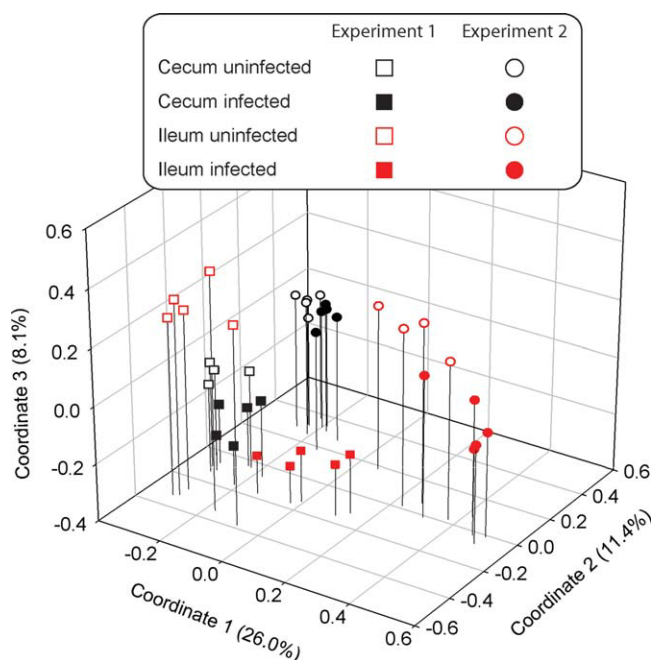


FIGURE 2. Principal coordinates analysis of the structure of the GI tract microbiotas of *H. polygyrus*-infected and uninfected mice. Cecal and ileal microbiotas are represented by symbols of different color (cecum = black, ileum = red); Experiments 1 and 2 are represented by symbols of different shape (square = Experiment 1, circle = Experiment 2); and microbiotas from infected and uninfected mice are represented by filled and unfilled symbols (uninfected = unfilled, infected = filled). Cecal microbiotas grouped according to the experiments. Ileal microbiotas from infected mice were different from uninfected mice in both experiments.

3D space (Fig. 2). The more similar two communities are, the closer they appear in the figure. The microbiotas of uninfected mice grouped together in 3D coordinate space according to experiment, suggesting that the baseline composition at the outset of each experiment was different. Within each experiment, cecal samples were similar to each other, and there was only a modest difference between the communities from *H. polygyrus* (*Hp*) infected and uninfected mice. In contrast, ileal bacterial communities tended to be more variable, and there was a clearer distinction between the intestinal microbiotas of *Hp* infected and uninfected animals.

Taxonomic Differences

The PCoA in Figure 2 is useful for visualizing how similar the samples were to each other based solely on sequence similarity. To understand how samples were different based on bacterial taxonomy, we analyzed the variation in the abundance of bacterial families in each sample (Table 1). Within each experiment there were significant differences between cecal and ileal microbiotas, regardless

of whether the mice were infected with *Hp*. For example, members of the *Lachnospiraceae* and *Ruminococcaceae* families were more abundant in the cecum of mice in both experiments (Fig. 3). After correcting for multiple comparisons, no differences were found between the cecal microbiotas of *Hp*-infected and uninfected mice, supporting the observation from the PCoA that these communities are highly similar (Fig. 2).

In contrast, the abundance of multiple bacterial families in the ileum of *Hp*-infected and uninfected animals was significantly different in each experiment (Fig. 3). In the first experiment, *Hp*-infected mice had a greater abundance of *Porphyromonadaceae*, *Lactobacillaceae*, and *Clostridiaceae* in their ileum compared to uninfected mice ($P = 0.0007$, $P = 0.0167$, and $P = 0.0442$, respectively). A similar difference in *Lactobacillaceae* abundance was seen in the ileum of *Hp*-infected mice in the second experiment ($P < 0.0022$). Compared to *Hp*-infected animals, uninfected mice in the first experiment had more *Clostridiales* ($P = 0.0001$) in their ileum, while uninfected mice from the second experiment had more *Erysipelotrichaceae* ($P < 0.0001$).

Hp-associated Microbiota

In the second experiment, L3 larvae and adult worms were sampled to determine the microbiota carried by (or associated with) *Hp*. The level of bacterial DNA extracted from L3 stage larvae was low, and only 28 16S sequences were obtained even after multiple PCR reactions were pooled. The amount of bacterial DNA in these samples was also extremely low by quantitative PCR (cycle threshold of 30.8 cycles). In contrast, bacterial DNA in the adult worms harvested from the proximal small bowel was abundant, and we had little problem cloning and sequencing 16S amplicons from these samples.

The microbiota associated with L3 stage larvae was completely unique (Fig. 4). None of the sequences from L3 stage larvae were found in mice or adult worms, suggesting that *Hp* worms develop a unique microbiota as they mature into adults. Although only 28 sequences were obtained from the L3 stage larvae, six bacterial families were detected, suggesting that these worms had a complex microbiota at the time of infection. In contrast to the uniqueness of the L3 microbiota, the microbiota associated with adult worms looked remarkably similar to the microbiota of the ileum of their murine hosts, suggesting that the microbiotas of *Hp*-infected animals and adult *Hp* worms are highly related.

DISCUSSION

H. polygyrus L3 stage larvae invade the mucosa of the mouse jejunum, where they develop into adults and emerge into the intestinal lumen 8 days after ingestion.

TABLE 1. Statistically Significant Differences in the Abundance of Bacterial Families for Each Mouse Experiment

| Bacterial Family | F-value | df | Pr > F | Significant Difference |
|-----------------------------------|---------|----|---------|--------------------------------------|
| Experiment 1 | | | | |
| <i>Porphyromonadaceae</i> | 13.1 | 3 | 0.0002 | Abundant in ileum of infected mice |
| <i>Lachnospiraceae</i> | 6.71 | 3 | 0.0043 | Abundant in cecum of mice |
| <i>Ruminococcaceae</i> | 5.31 | 3 | 0.0108 | Abundant in cecum of mice |
| <i>Clostridiaceae</i> | 4.94 | 3 | 0.0139 | Abundant in ileum of infected mice |
| Unclassified <i>Clostridiales</i> | 19.94 | 3 | <0.0001 | Abundant in ileum of uninfected mice |
| <i>Lactobacillaceae</i> | 7.66 | 3 | 0.0025 | Abundant in ileum of infected mice |
| Experiment 2 | | | | |
| <i>Anaeroplasmataceae</i> | 7.37 | 3 | 0.0029 | Abundant in cecum of mice |
| <i>Lachnospiraceae</i> | 51.99 | 3 | <0.0001 | Abundant in cecum of mice |
| <i>Ruminococcaceae</i> | 16.25 | 3 | <0.0001 | Abundant in cecum of mice |
| Unclassified <i>Clostridiales</i> | 11.74 | 3 | 0.0003 | Abundant in ileum of mice |
| <i>Lactobacillaceae</i> | 17.26 | 3 | <0.0001 | Abundant in ileum of infected mice |
| <i>Erysipelotrichaceae</i> | 23.67 | 3 | <0.0001 | Abundant in ileum of uninfected mice |

Infection induces a polarizing T-helper cell type 2 (Th2) host immune response characterized by the production of IL-4 and IL-13, as well as activation of the STAT-6 pathway.²⁷ These events activate mast cells, induce the alternatively activated phenotype in tissue-resident macrophages, increase mucus secretion by goblet cells, and enhance intestinal smooth muscle contraction.^{28,29} During a primary *H. polygyrus* infection, the host Th2 response does not result in rapid clearance of the adult worms; rather, it has a significant deleterious effect on *H. polygyrus* fecundity, as measured by the number of eggs shed per gram of feces.^{28,30}

H. polygyrus infection has a profound influence on a number of immunoregulatory signals that decrease intestinal inflammation. *H. polygyrus* infection increases the production of the global transcription factor, FoxP3, in T cells of the lamina propria, and these cells decrease intestinal inflammation upon transfer to mice with established colitis.³¹ In the terminal ileum of mice, *H. polygyrus* induces mucosal T cells to express Toll-like receptor 4 (TLR4).³² TLR4 signaling by bacterial lipopolysaccharide (LPS) normally stimulates cells to produce proinflammatory cytokines, like TNF α and IL-12. In contrast to what happens normally, mucosal T cells from *H. polygyrus*-infected mice do not produce TNF α or IL-12, but instead produce the regulatory cytokine TGF β .³² In the absence of this TGF β signaling, there is insufficient control of interferon gamma (IFN- γ) production by IL-10 and colitis is maintained.³³

To our knowledge, it has never been examined if *H. polygyrus* infection alters the composition of the GI tract microbiota. A number of observations from experiments with germfree mice suggest that *H. polygyrus* needs GI tract bacteria to establish a robust infection.³⁴ Compared to mice with a conventional microbiota, total worm burden

was lower in germfree mice 13 days postinoculation. Moreover, germfree mice rapidly cleared these worms between days 13–30 postinoculation, while worm burden remained high in conventional mice. Also, a robust intestinal mucosal eosinophilia developed in germfree mice, which was absent in conventional mice, suggesting that the host immune response to *H. polygyrus* differs in the absence of intestinal bacteria. Interestingly, mortality in mice given a “heavy” infection (1600 L3 infective larvae) was only observed in conventional mice. These observations suggest that the GI microbiota is necessary for the normal host response to *H. polygyrus*.

Infection of mice with *H. polygyrus* resulted in an increased abundance of various bacteria, suggesting that some species find conditions in the ileum of *H. polygyrus*-infected mice favorable. This observation is what one would expect from a microbial-helminth mutualism, where both bacteria and worm benefit from symbiosis. Interestingly, members of the *Lactobacillaceae* family increased in both experiments. The *Lactobacillaceae* family is composed of the genera *Lactobacillus*, *Paralactobacillus*, *Pediococcus*, and other bacteria that are not yet classified by microbial taxonomists. Most of the known *Lactobacillaceae* species belong to the genus *Lactobacillus*, which is a diverse group of Gram-positive, facultative anaerobic species commonly known as lactic acid bacteria because most convert sugars into lactic acid. Certain *Lactobacillus* species, like *L. rhamnosus*³⁵ and *L. casei*,³⁶ decrease intestinal inflammation in murine models of IBD, and it is tempting to speculate that *Lactobacillus* species play a role in the antiinflammatory/immune modulatory effects of *H. polygyrus* infection. More studies are needed to elucidate the role of this potentially important mutualism.

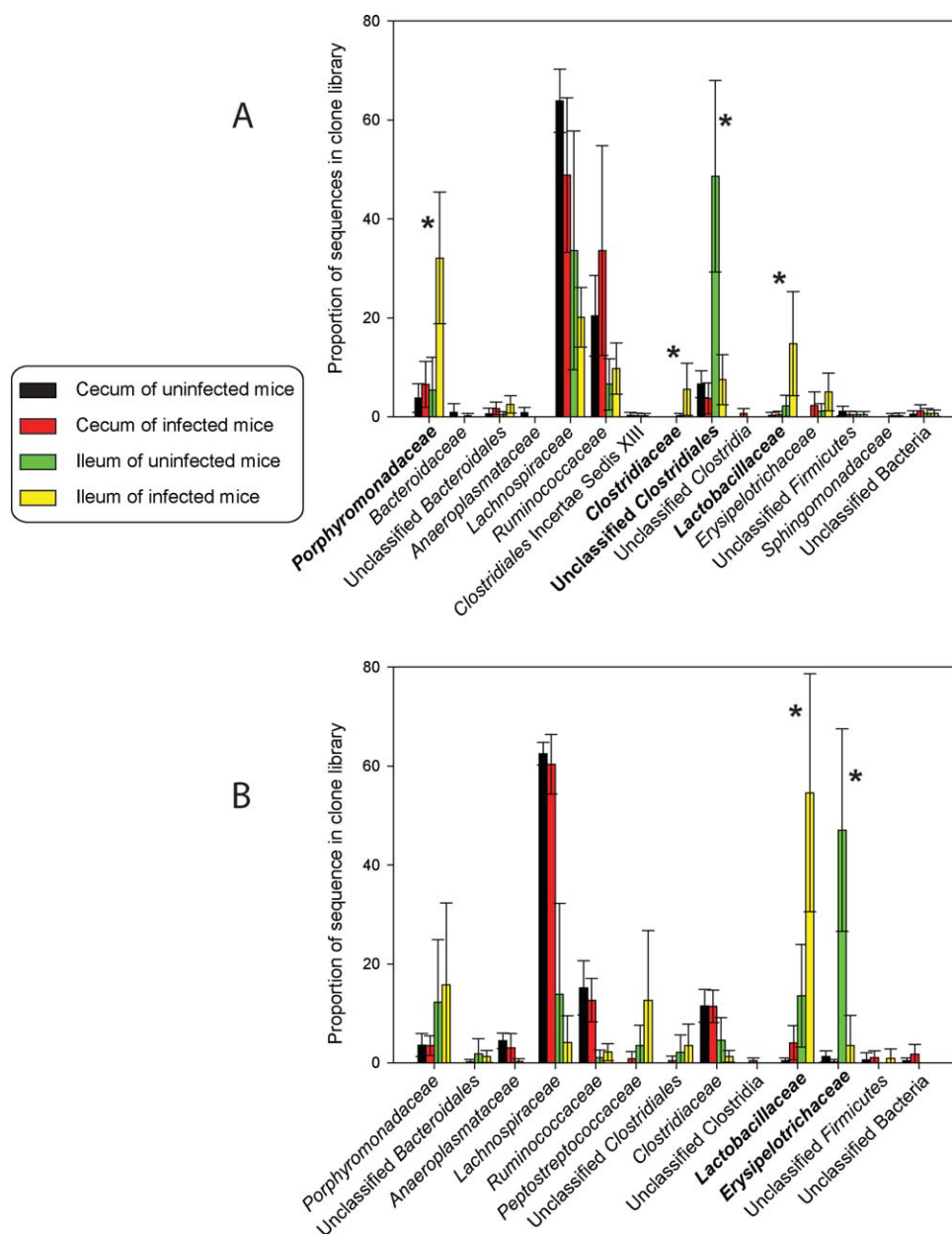


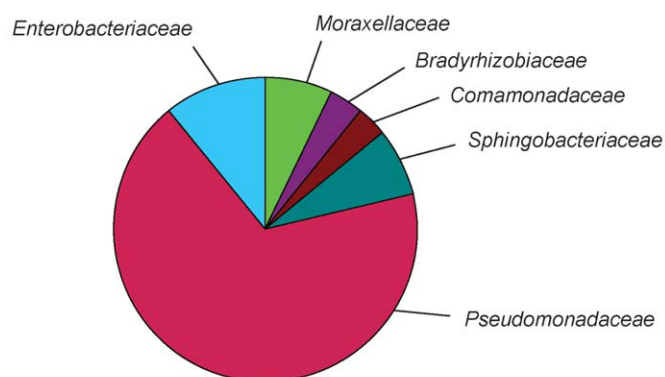
FIGURE 3. Abundance of bacterial families in microbiotas from the first (A) and second (B) experiments. Samples are represented by different-colored bars (see legend). Error bars represent the standard deviation in abundance among the mice. No differences were detected between infected and uninfected cecal samples. Significant differences between the infected and uninfected ileal samples are indicated with asterisks and bolded bacterial family name.

Identifying members of the GI tract microbiota that help to control gut inflammation is an active area of research (see Ref. 37 for a list of bacteria). Such organisms span much of the genetic breadth of the GI tract bacterial community, and it is likely that no single species is responsible for all of these microbially mediated effects. Even within a genus, different species have different ways of modulating the host immune system. For example, *L. rhamnosus* inhibits NF- κ B activation by producing reac-

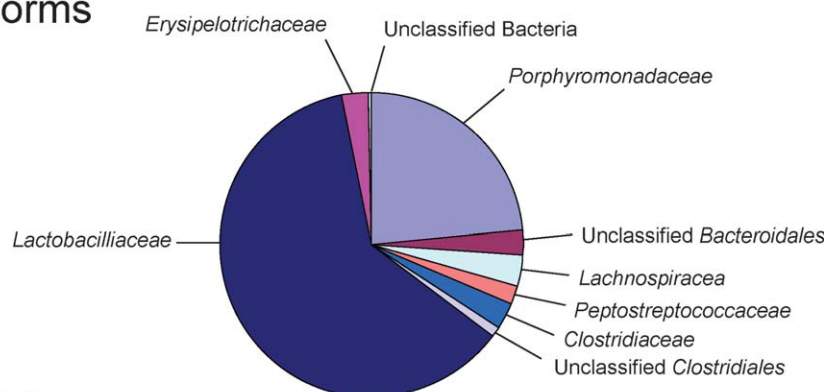
tive oxygen species,³⁵ while cell wall components of *L. casei* inhibit IL-6 production.³⁶ These observations suggest that the particular influence of the microbiota on host immunity depends on the structure of the microbiota (i.e., their abundance and relative distribution) present at the outset of helminth infection.

It is well established that the GI tract microbiotas of mammals are highly complex and variable among individuals.³⁸ In fact, mice from different commercial vendors (e.g.,

L3 larvae



Adult worms



Infected ileum

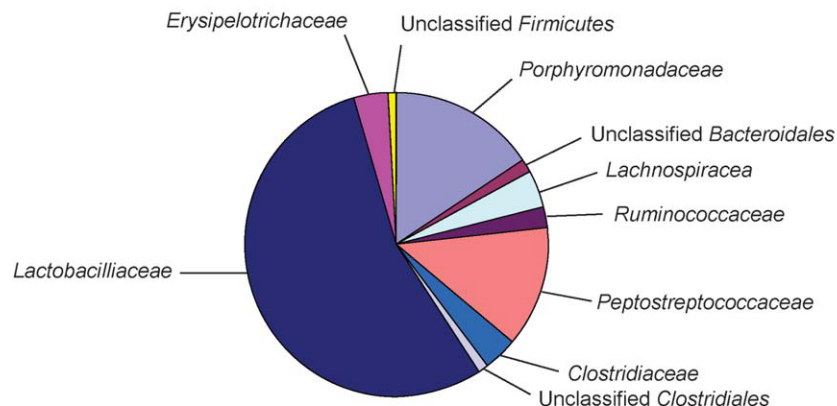


FIGURE 4. Worm-associated microbiotas. Pie charts display the proportion of bacterial families in sequences from the L3 larvae (28 sequences), adult worms from infected mice (473 sequences), and samples from the ileum of mice (422 sequences). On average, the microbiota associated with adult worms is highly similar to the microbiota from ileal tissue samples.

Taconic Farms, Germantown, NY, and Jackson Laboratory, Bar Harbor, ME) have different GI tract microbiotas despite having the same genetic (wildtype C57BL/6) background.³⁹ These observations support the notion that the GI tract microbiota within an individual or group of individuals is constantly evolving. Not surprisingly, the intestinal microbiotas of the uninfected mice in the experiments presented here were significantly different at the outset of each experi-

ment. This result says little about the evolutionary dynamics of the microbiota of C57BL/6 mice, but since the two experiments were separated by ≈ 2 years, it does suggest that changes evolve rapidly. Whether the initial microbiota structure influences the host immune response to *H. polygyrus* infection remains to be investigated. However, the observation that organisms of the *Lactobacillaceae* family increased in abundance in both experiments, regardless of different

initial microbiotas, suggests that there may be compositional similarities among *H. polygyrus* infections that ultimately influence host immunomodulation.

Relevance of This Study to Helminth Treatment of IBD

Normally, Th1/Th17 driven inflammation characterized by the production of proinflammatory cytokines (IL-12, IL-23, IL-17, and IFN- γ) is held in check by the production of immunoregulatory cytokines and transcription factors (e.g., TGF- β , IL-10, and FoxP3). In patients with IBD, homeostatic regulatory signals are impaired, and a chronic inflammation develops that is perpetuated by antigens from the commensal GI tract microbiota.⁴⁰ A number of therapies commonly used in IBD like azathioprine, anti-TNF monoclonal antibodies, and corticosteroids suppress this inflammatory cascade.⁴¹ Some studies suggest that helminths may prove useful for the treatment of both Crohn's disease and ulcerative colitis.^{11,12} The data presented here demonstrate that *H. polygyrus* infection is associated with a significant shift in the abundance and relative distribution of GI tract bacteria. The relatively large effect size of the shift among ileal-associated microbiotas compared to cecal-associated microbiotas suggests that either helminths directly influenced the microbiota or that the host immune response to infection mediated the observed alterations. The dynamic interactions among *H. polygyrus*, the host mucosal immune response, and the intestinal microbiota require more study.

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