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RESEARCH PAPER

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Commensal-pathogen interactions in the intestinal tract

Lactobacilli promote infection with, and are promoted by, helminth parasites

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Keywords: commensal, microbiota, duodenum, *Lactobacillus*, nematode, Th17, Treg

Abbreviations: cfu, colony forming units; GALT, gut-associated lymphoid tissue; GF, germ-free; HES, *H. polygyrus* excretory-secretory antigen; L3, third stage larvae; LP, lamina propria; MLN, mesenteric lymph node; OD, optical density; PP, Peyer's patch; qPCR, quantitative RT-PCR; SPF, specific pathogen-free; Th, T helper; Treg, T regulatory cell

The intestinal microbiota are pivotal in determining the developmental, metabolic and immunological status of the mammalian host. However, the intestinal tract may also accommodate pathogenic organisms, including helminth parasites which are highly prevalent in most tropical countries. Both microbes and helminths must evade or manipulate the host immune system to reside in the intestinal environment, yet whether they influence each other's persistence in the host remains unknown. We now show that abundance of *Lactobacillus* bacteria correlates positively with infection with the mouse intestinal nematode parasite, *Heligmosomoides polygyrus*, as well as with heightened regulatory T cell (Treg) and Th17 responses. Moreover, *H. polygyrus* raises *Lactobacillus* species abundance in the duodenum of C57BL/6 mice, which are highly susceptible to *H. polygyrus* infection, but not in BALB/c mice, which are relatively resistant. Sequencing of samples at the bacterial *gyrB* locus identified the principal *Lactobacillus* species as *L. taiwanensis*, a previously characterized rodent commensal. Experimental administration of *L. taiwanensis* to BALB/c mice elevates regulatory T cell frequencies and results in greater helminth establishment, demonstrating a causal relationship in which commensal bacteria promote infection with an intestinal parasite and implicating a bacterially-induced expansion of Tregs as a mechanism of greater helminth susceptibility. The discovery of this tripartite interaction between host, bacteria and parasite has important implications for both antibiotic and anthelmintic use in endemic human populations.

Introduction

In both humans and mice, the intestinal microbiota are essential for development of a mature immune system, and for maintaining immunological homeostasis in the intestinal tract.[1-4] Although many hundreds of bacterial species colonize the mammalian gut, the balance between these species varies widely between individuals in a population.[5] Importantly, the presence or absence of specific species of bacteria within the microbiota can be instrumental in driving immunological differentiation in the intestinal tract. For example, segmented filamentous bacteria stimulate naïve T cells toward an inflammatory T helper (Th)17 phenotype,[6-8] while a number of bacterial species promote immunosuppressive T regulatory cell (Treg) differentiation.[9-13] More broadly, germ-free (GF) mice raised in the absence of commensal microbes show impaired immunological development,[14] and a systemic skewing toward Th2 responses.[15,16]

While GF mice are generally more susceptible to infections with bacterial or viral agents,[14] persistence of the gastrointestinal helminth parasite *Heligmosomoides polygyrus* is markedly reduced in these animals compared with conventionally raised mice.[17-19] *H. polygyrus* establishes in the anterior small intestine of mice, alongside a substantial microbial community. As the outcome of *H. polygyrus* infection is likely dependent on the immediate cytokine environment,[20-22] we postulated that the composition of the microbiota may alter the priming of the murine immune system to alter susceptibility to helminth infection.

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Whether the composition of the intestinal microbiota is sufficient to drive differential susceptibility to infectious pathogens is an area which remains poorly understood. Here, we show that it is the composition of the gastrointestinal microbiota, rather than the total load of bacteria, which impacts the survival of *H. polygyrus* within the murine host. Specifically, Lactobacillaceae species abundance in the duodenum positively correlates with susceptibility to *H. polygyrus*. Furthermore, administration of a single species of this bacterial family, *Lactobacillus taiwanensis*, is sufficient to render a naturally *H. polygyrus*-resistant mouse stain more susceptible to helminth infection. To our knowledge, this is the first demonstration of a causal link between commensal bacteria and susceptibility to helminth infection.

Results

Intestinal microbiota composition alters *H. polygyrus* persistence

The role of the intestinal microbiota in susceptibility to pathogen infection can be studied in GF mice; however it is then difficult to ascertain whether differences in the ability to clear infections are due to an abnormally-developed immune system, differences in intestinal physiology, or to the absence of bacteria that may alter the immune response or physiology of the host at the time of infection. We therefore chose to examine helminth-microbiota interactions in mice maintained in a conventional, but specific pathogen-free (SPF) BALB/c colony. We hypothesized that that composition of the microbiota, rather than the overall level of colonization may be important in controlling helminth infection. Treating BALB/c mice with a low level of the antibiotic vancomycin prior to *H. polygyrus* infection (**Fig. 1A**) did not significantly reduce total fecal bacteria numbers (**Fig. 1B**), yet resulted in increased *H. polygyrus* persistence in the host (**Fig. 1C**). Examining the composition of the fecal microbiota following vancomycin treatment revealed an elevated abundance of members of the Lactobacillaceae and Enterobacteriaceae families and reduced abundance of *Eubacterium*/*Clostridium* species (**Fig. 1D–F**), leading us to hypothesize that altered levels of these bacterial groups affected *H. polygyrus* survival.

Lactobacillaceae species abundance in the duodenum positively correlates with susceptibility to *H polygyrus*

We next investigated whether the composition of the intestinal microbiota in the duodenum—the primary site of *H. polygyrus* colonization—was a factor contributing to the marked differences between mouse strains in susceptibility to *H. polygyrus* infection.[23] We first used quantitative PCR (qPCR) to compare the relative levels of broad phyletic groups in duodenal samples from naïve and infected animals of two major mouse strains, BALB/c and C57BL/6. Whereas the majority of BALB/c mice had begun to expel *H. polygyrus* by 28 d post-infection, C57BL/6 mice maintained high worm burdens at this time point (**Fig. 2A**). Additionally, infected C57BL/6 mice showed elevated levels both of duodenal Lactobacillaceae (**Fig. 2B**) and Enterobacteriaceae (**Fig. 2C**) family members compared with naïve mice. This result, extending from earlier reports,[24,25] indicates that the presence of helminths positively promotes establishment of certain bacterial groups. A previous report suggests that the increase in abundance of these bacterial groups following *H. polygyrus* infection in C57BL/6 mice is not limited to the site of *H. polygyrus* colonization alone, but extends to the large intestine.[25] In contrast to C57BL/6 mice in the more *H. polygyrus*-resistant BALB/c mice, the abundance of these bacterial groups is reduced, rather than elevated, following *H. polygyrus* infection (**Fig. 2D and E**).

Notably, BALB/c mice show substantial within-strain variation in their response ability to clear *H. polygyrus* infection (**Fig. 2A**). We therefore investigated whether the abundance of Lactobacillaceae family members correlated with infection intensity within a cohort of BALB/c mice, as well as between mouse strains. Revealingly, a positive correlation between these parameters was found: those BALB/c mice which maintained the highest duodenal levels of *Lactobacillus*/*Lactococcus* species following *H. polygyrus* infection had the highest parasite burdens 28 d post-infection (**Fig. 2F**). In contrast, the within-strain variation in worm burdens in BALB/c mice did not correlate with levels of Enterobacteriaceae, *Bacteroides, Eubacterium*, or *Clostridium* species, and no changes in the abundance of duodenal *Bacteroides*, *Eubacterium*, or *Clostridium* species were seen following infection in either inbred strain (data not shown). Segmented filamentous bacteria were not detectable in our BALB/c colony, in naïve or infected mice (data not shown).

Lactobacillaceae species abundance positively correlates with HES-specific IL-17A and Foxp3$^+$ cell levels

We therefore focused on the relationship between duodenal *Lactobacillus/Lactococcus* abundance and *H. polygyrus* persistence in BALB/c mice, and explored how levels of these bacteria were related to the cytokine response to *H. polygyrus*. IL-17A production by mesenteric lymph node (MLN) cells in response to *H. polygyrus* excretory-secretory antigen (HES) positively correlated with *H. polygyrus* burden, and with duodenal *Lactobacillus/Lactococcus* abundance at day 28 of infection (**Fig. 3A and B**). Some, but not all, species of this group have previously been reported to evoke IL-17A responses in other settings.[26] HES-specific IL-17A was the only cytokine to correlate positively with bacterial load; no association with *Lactobacillus/Lactococcus* levels was seen with HES-specific IL-4, IL-5, IL-9, IL-10, IL-13, or IFN-γ production (data not shown). No evidence exists to suggest that IL-17 is an effective cytokine in promoting immunity toward helminths,[27] despite an induction of IL-17A production following infection in BALB/c mice (**Fig. S1A**; ref. 27), thus it is possible that IL-17A responses are induced by the microbiota rather than the parasite during helminth invasion of the submucosa. Moreover, *Lactobacillus/Lactococcus* species abundance also positively correlated with total numbers of Tregs within the MLN at day 28 of *H. polygyrus* infection (**Fig. 3C**), as may be expected from the known propensity of these bacteria to stimulate regulatory properties of T cells.[13,28] Treg number also positively correlated with *H. polygyrus* burden at this time point (**Fig. 3D**), although at this late time point considerable worm expulsion had already occurred. Importantly, production of HES-specific IL-4, a cytokine key to expulsion of this helminth,[20,21] did not show a positive correlation with *H. polygyrus* burden (**Fig. S1B**).

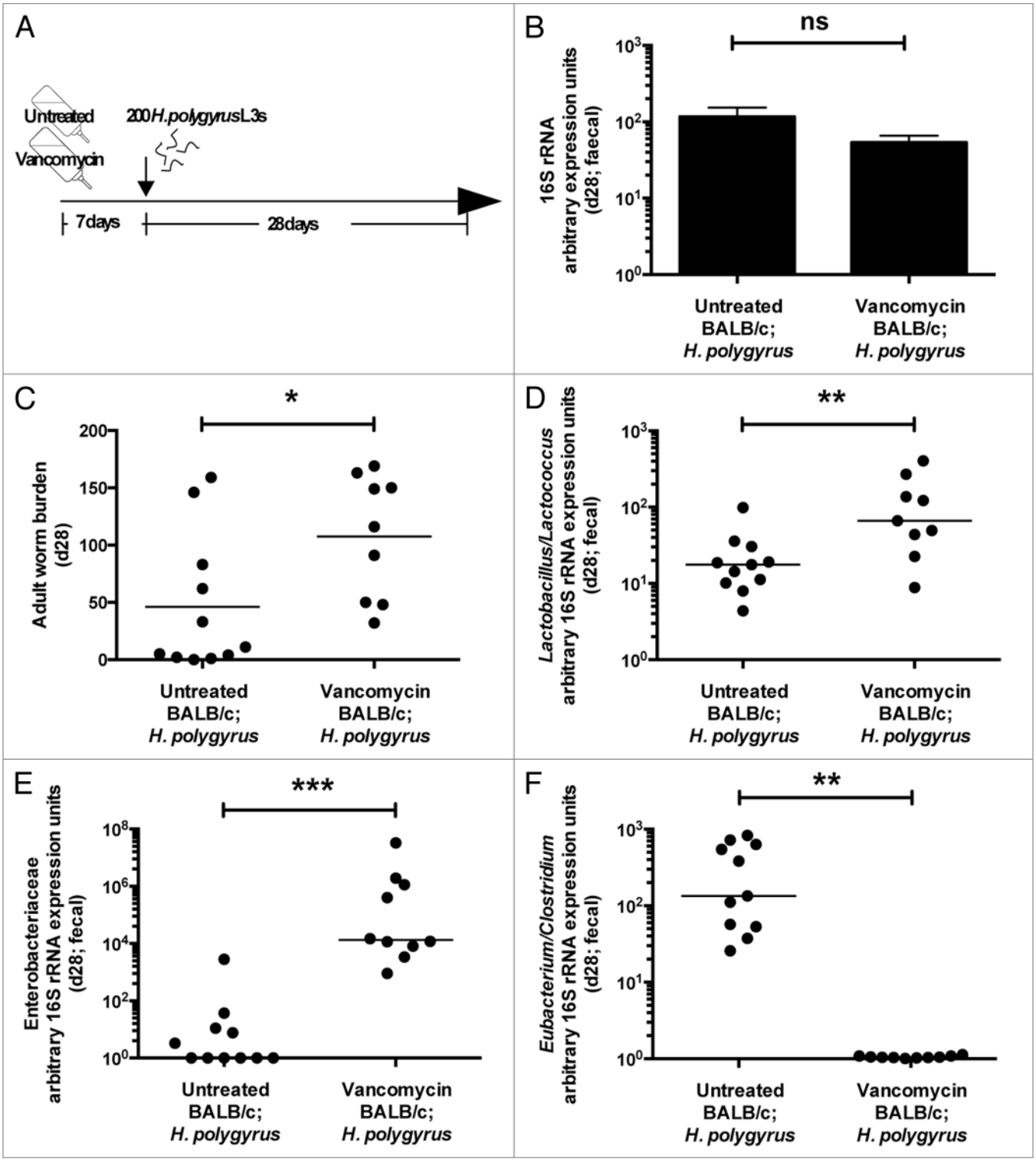

Figure 1. Intestinal microbiota composition alters *H. polygyrus* persistence. (**A–F**) BALB/c mice were administered untreated water, or water containing 0.5 g/L vancomycin for one week prior to infection with 200 *H. polygyrus* L3s, and throughout the experiment. Data pooled from two experiments. (**A**) Experimental protocol. (**B**) qPCR analysis of fecal 16S rRNA gene expression 28 d post-infection. (**C**) Intestinal *H. polygyrus* burden 28 d post-infection. (**D**) qPCR analysis of fecal *Lactobacillus/Lactococcus*-specific 16S rRNA gene expression 28 d post-infection. (**E**) qPCR analysis of fecal Enterobacteriaceae-specific 16S rRNA gene expression 28 d post-infection. (**F**) qPCR analysis of fecal *Eubacterium/Clostridium*-specific 16S rRNA gene expression 28 d post-infection. NS denotes no statistical differences; * indicates $P = \leq 0.05$; ** indicates $P = \leq 0.01$; *** indicates $P = \leq 0.001$.

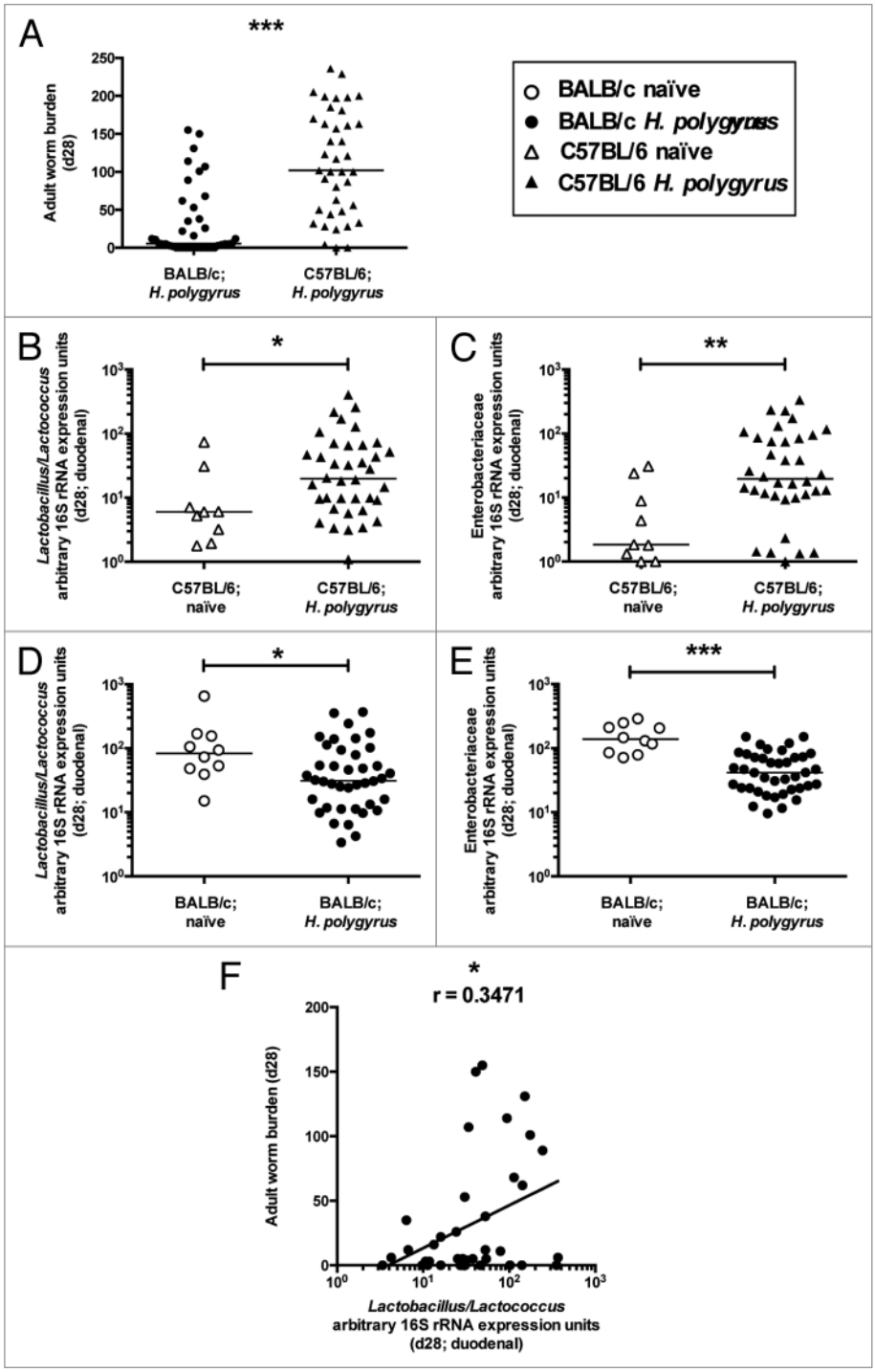

Figure 2. Lactobacillaceae species abundance positively correlates with susceptibility to *H polygyrus*. Six-week old female mice were left naïve or infected with 200 *H. polygyrus* L3s. Ten naïve mice and 40 *H. polygyrus*-infected mice, either C57BL/6 or BALB/c were used in each experiment. Experiments in each strain were performed separately and so data are not intended to be directly comparable between strains. A direct comparison of differential strain resistances to *H. polygyrus* can be found in ref. 23. (**A**) Intestinal *H. polygyrus* burden 28 d post-infection. (**B**) qPCR analysis of duodenal *Lactobacillus/Lactococcus*-specific 16S rRNA gene expression 28 d post-infection in C57BL/6 mice. (**C**) qPCR analysis of duodenal Enterobacteriaceae-specific 16S rRNA gene expression 28 d post-infection in C57BL/6 mice. (**D**) qPCR analysis of duodenal *Lactobacillus/Lactococcus*-specific 16S rRNA gene expression 28 d post-infection in BALB/c mice. (**E**) qPCR analysis of duodenal Enterobacteriaceae-specific 16S rRNA gene expression 28 d post-infection in BALB/c mice. (**F**) Correlation between intestinal *H. polygyrus* burden and duodenal *Lactobacillus/Lactococcus*-specific 16S rRNA gene expression measured by qPCR at day 28 post-infection in BALB/c mice. Statistics shown indicate analysis by Spearman correlation test. * indicates $P \leq 0.05$; ** indicates $P \leq 0.01$; *** indicates $P \leq 0.001$ and r indicates the correlation co-efficient.

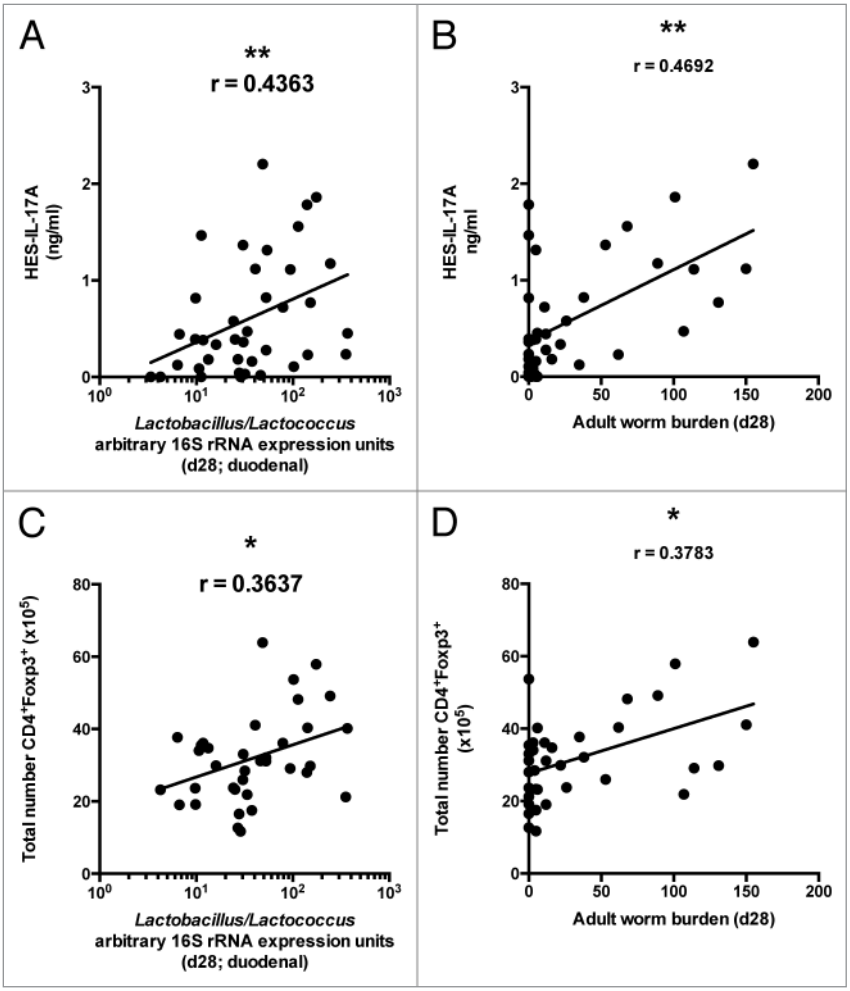

Figure 3. Lactobacillaceae species and *H. polygyrus* abundance both positively correlate with Th17 and Treg phenotypes. Six-week old BALB/c female mice were infected with 200 *H. polygyrus* L3s. Twenty-eight days post-infection MLN cells were stained for Foxp3 expression, or MLN cells were restimulated with 1 μg HES for 72 h after which IL-17A production was determined by ELISA. (**A, B**) Correlation between MLN cell HES-specific IL-17A production and (**A**) duodenal *Lactobacillus/Lactococcus*-specific 16S rRNA gene expression measured by qPCR, and (**B**) intestinal *H. polygyrus* burden 28 d post-infection. (**C, D**) Correlation between total number of MLN $CD4^+Foxp3^+$ cells 28 d post-infection and (**C**) duodenal *Lactobacillus/Lactococcus*-specific 16S rRNA gene expression measured by qPCR, and (**D**) intestinal *H. polygyrus* burden 28 d post-infection. Statistics shown indicate analysis by Spearman correlation test. * indicates $P \leq 0.05$; ** indicates $P \leq 0.01$; and r indicates the correlation co-efficient.

***Lactobacillus taiwanensis* correlates with *Lactobacillus/Lactococcus* effects in *H. polygyrus* infection**

To experimentally test whether increased *Lactobacillus/Lactococcus* levels were causal in increasing susceptibility to *H. polygyrus*, we chose to identify the most prominent species from this bacterial group residing in the small intestine of infected mice. DNA was extracted from the duodenum of BALB/c mice showing the highest worm burdens 28 d post-infection. Primers specific for the 16S rRNA gene of the *Lactobacillus/Lactococcus* family members[29] were used to amplify that region of the DNA, after which individual amplicons were sequenced and the results searched against a BLAST database. Of 13 amplicons screened, 12 were a 100% nucleotide match to the sequence of the Gram-positive, facultatively anaerobic *Lactobacillus taiwanensis* first identified from silage cattle feed[30] and since isolated from the intestinal contents of rats.[31] The remaining sample sequenced showed one nucleotide change from the sequence described for *L. taiwanensis*, which was still the closest match. Notably, *L. taiwanensis* is most closely related to *L. johnsonii* (**Fig. 4A**), isolates of which were reported to favor Th17 differentiation.[26]

It has previously been reported that *L. taiwanensis* is relatively highly divergent from closely related species at the *gyrB* gene locus,[30] therefore this region of the genome was chosen to design primers which would amplify DNA from *L. taiwanensis*, but not its close family members (**Fig. S2**). Levels of *L. taiwanensis* present in the duodenum of 28 d-*H. polygyrus* infected mice positively correlated not only with the total *Lactobacillus/Lactococcus* abundance readout from the same samples (**Fig. 4B**), but also with the number of worms remaining in the host at the same time point (**Fig. 4C**), suggesting that levels of this single species may play a role in controlling susceptibility to *H. polygyrus*.

***Lactobacillus taiwanensis* administration enhances Treg frequencies and susceptibility to *H. polygyrus* infection**

To establish if the correlation between *L. taiwanensis* and *H. polygyrus* abundance had a causal basis, we then tested the effects of feeding *L. taiwanensis* to BALB/c mice. Animals were given untreated drinking water or water containing 2×10^8 colony forming units (cfu)/ml *L. taiwanensis* for one week prior to *H. polygyrus* infection, and throughout the course of infection (**Fig. 5A**). Egg output from *H. polygyrus* was measured each week; at day 14 post-infection, the bacteria-fed and control mice showed similar levels of egg production, however by day 21 post-infection egg output was significantly higher from those mice that had received *L. taiwanensis* (**Fig. 5B**). *L. taiwanensis*-fed mice also bore an increased *H. polygyrus* burden 28 d post-infection compared with untreated mice in each of four independent experiments (**Fig. 5C and D**), suggesting that the presence of *L. taiwanensis* prolongs *H. polygyrus* infection in BALB/c mice. Co-infection of mice with the bacterial pathogen *Bordetella bronchiseptica* has previously been shown to promote *H. polygyrus* survival in mice,[32] but this is the first report to our knowledge that a component of the commensal microbiota can promote helminth parasite persistence.

We next examined whether *L. taiwanensis* administration alone for one week (**Fig. 5E**) skewed cytokine production from α-CD3-stimulated MLN cells. No differences were seen in

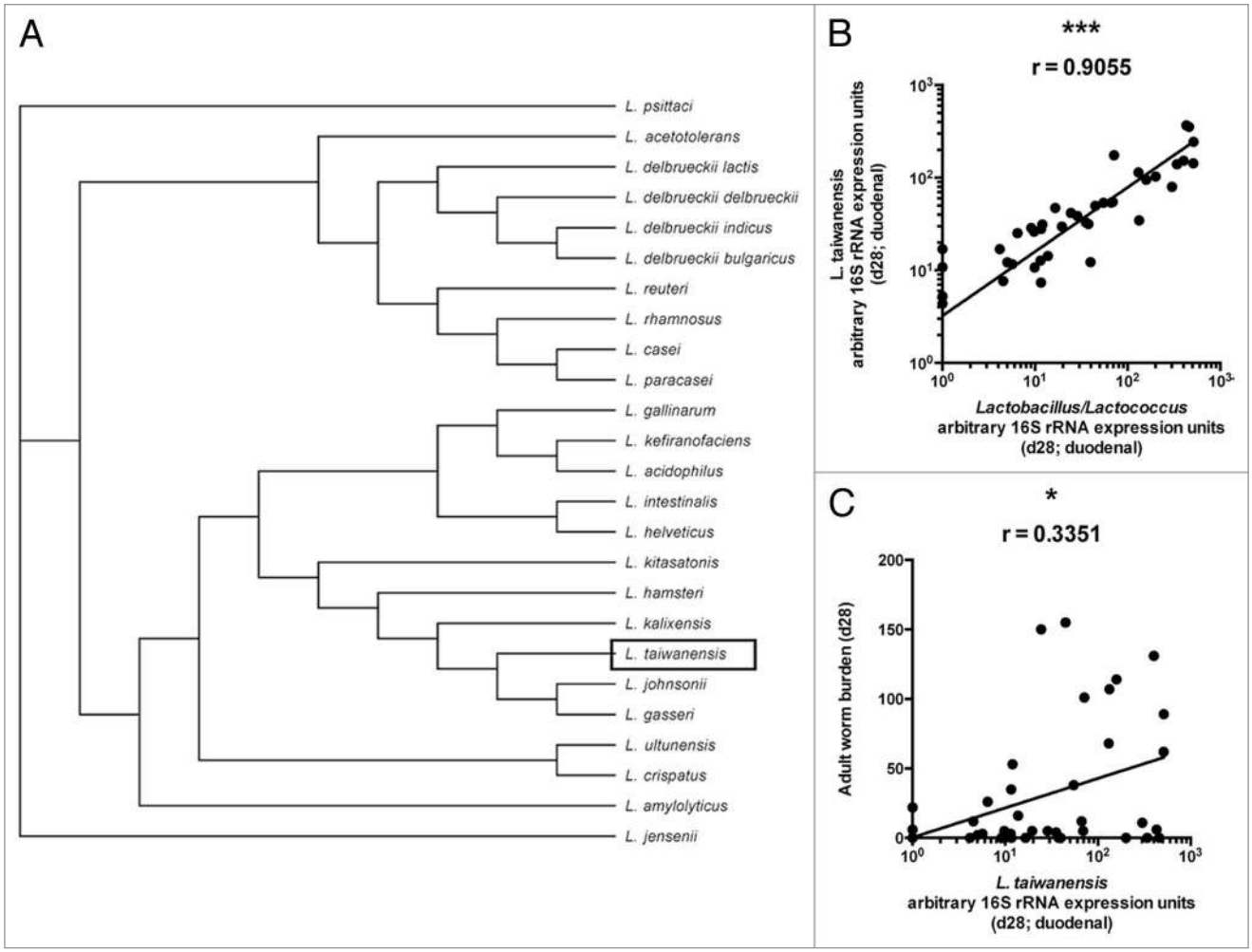

Figure 4. *Lactobacillus taiwanensis* correlates with *Lactobacillus/Lactococcus* effects in *H. polygyrus* infection. (**A**) Neighbor-joining tree showing relatedness of *Lactobacillus* species based on the similarity at the region of the *gyrB* gene shown in **Fig. S2**. (**B**) Correlation between duodenal *Lactobacillus/Lactococcus*-specific 16S rRNA gene expression and duodenal *L. taiwanensis*-specific *gyrB* gene expression measured by qPCR 28 d post-infection. (**C**) Correlation between intestinal *H. polygyrus* burden 28 d post-infection and duodenal *L. taiwanensis*-specific *gyrB* gene expression measured by qPCR at the same time point. (**B, C**) Statistics shown indicate analysis by Spearman correlation test. * indicates $P \leq 0.05$; *** indicates $P \leq 0.001$; and r indicates the correlation co-efficient.

IFN-γ, IL-4, IL-5, IL-10, IL-13, or IL-17A production between cells from untreated and *L. taiwanensis*-treated BALB/c mice (**Fig. S3A–F**).

Treg expansion has previously been reported in mice which have been exposed to *Lactobacillus* bacteria,[33-37] and thus the presence of Foxp3$^+$ cells in the gut-associated lymphoid tissue (GALT) was examined after one week of *L. taiwanensis* exposure, prior to *H. polygyrus* infection (**Fig. 5E**). The frequency of Foxp3$^+$ cells among CD4$^+$ T cells was significantly elevated in both the MLN and Peyer's patch (PP) cells of *L. taiwanensis*-treated mice compared with untreated BALB/c mice (**Fig. 5F and G**), confirming that *L. taiwanensis* exposure alone is sufficient to elevate Treg frequencies. Foxp3$^+$CD4$^+$ cell levels were next examined at day 7 and 28 of *H. polygyrus*-infection, in mice which had been exposed to *L. taiwanensis* for one week prior to and throughout the course of *H. polygyrus*-infection. At these time points, no differences in Treg frequencies were seen in the MLN or small intestinal lamina propria (LP) between untreated and *L. taiwanensis*-treated mice (**Fig. S3G–I**), suggesting that it is the GALT Treg frequency at the time of infection which determines the outcome of *H. polygyrus* infection.

Discussion

In recent years, the fundamental importance of the commensal microbiome to the developmental, immunological and metabolic well-being of the host organism has become clearly understood.[1,2,38] It has further been recognized that the immune system faces a profound challenge in discriminating between symbionts and pathogens, particularly when closely-related and competing organisms are present.[39] Hence, pathogens can overturn tolerance to commensals,[40] or otherwise dysregulate the steady-state floral constitution, giving rise to pathogenesis.[41,42] In contrast, we report

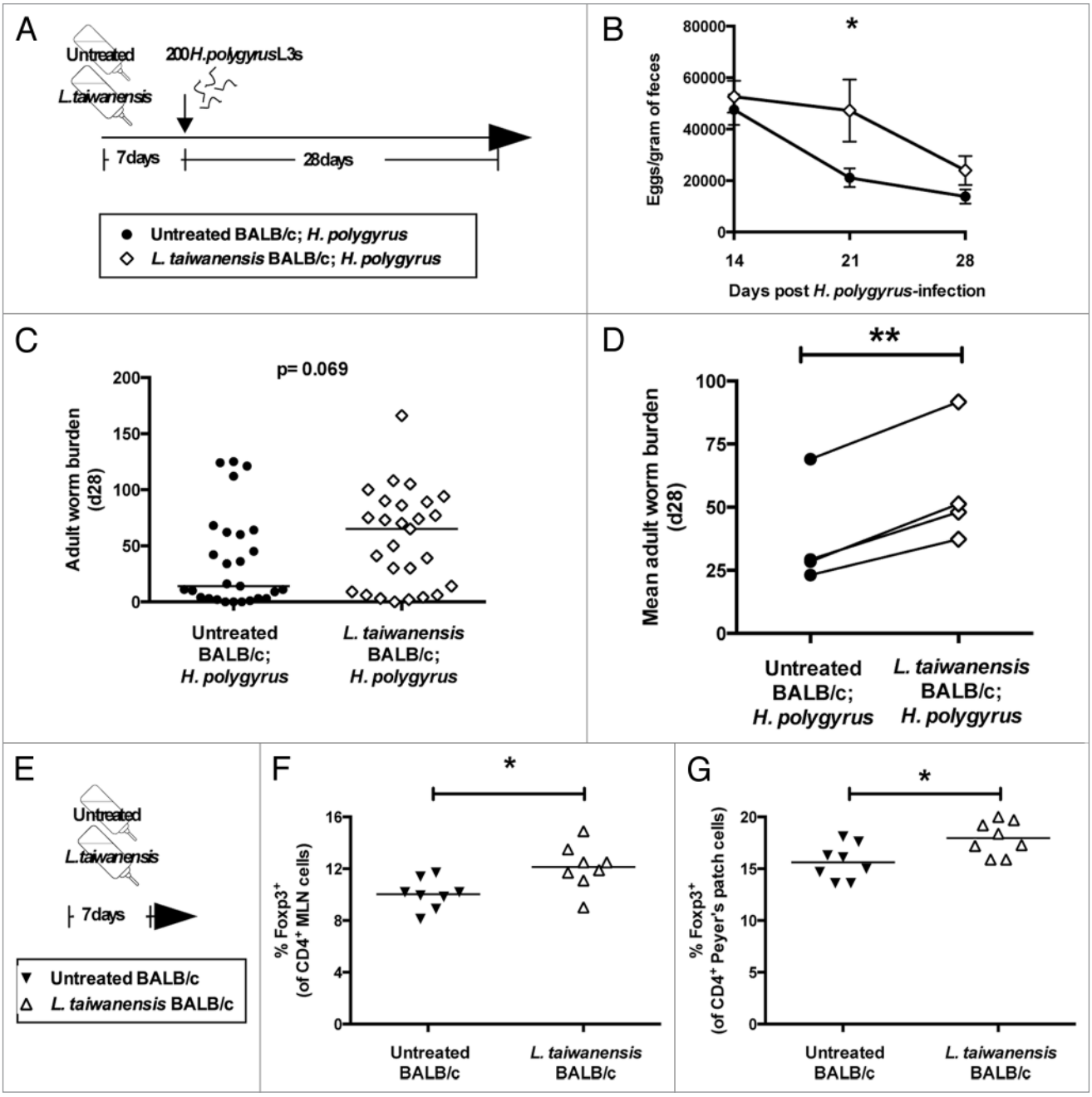

Figure 5. *Lactobacillus taiwanensis* administration enhances *H. polygyrus* infection. (**A**) Experimental protocol for (**B-D**). BALB/c mice were administered untreated drinking water, or water containing 2×10^8 colony forming units (cfu)/ml *L. taiwanensis* for one week prior to infection with 200 *H. polygyrus* L3s, and throughout the experiment. (**B**) Mean *H. polygyrus* egg output per gram of feces +/– SEM on days 14, 21 and 28 post-infection. (**C**) Intestinal *H. polygyrus* burden 28 d post-infection. Data shown are pooled from four independent experiments, each with 6–7 mice per group. (**D**) Mean intestinal *H. polygyrus* burden 28 d post-infection. Data shown are paired means from the four independent experiments shown in (**C**). (**E**) Experimental protocol for (**F-G**). BALB/c mice were administered untreated drinking water, or water containing 2 x 10^8 colony forming units (cfu)/ml *L. taiwanensis* for one week. (**F**) % Foxp3$^+$ cells among CD4$^+$ MLN cells. Data shown are pooled from two independent experiments each with 4 mice per group. (**G**) % Foxp3$^+$ cells among CD4$^+$ PP cells. Data shown are pooled from two independent experiments each with 4 mice per group. * indicates $P \leq 0.05$; ** indicates $P \leq 0.01$.

for the first time a more mutualistic relationship between a commensal microbe and a helminth parasite, which in the non-pathogenic setting of long-term infection, provides a new perspective on real-world interactions in the intestinal tract.

Our experiments have established two striking findings: that infection with the helminth *H. polygyrus* alters the microbial composition of the small intestine; and that the abundance of species present in the normal commensal microbiota can in turn alter *H. polygyrus* survival within the host. The expansion of Lactobacillaceae family members in the duodenum of C57BL/6 mice is in accordance with a previous report of greater abundance of Lactobacillaceae species in the ileum of 14-d *H.*

polygyrus-infected C57BL/6 mice.[24] However, as we show here, the opposite effect occurs in more resistant animals. An association between Lactobacillaceae species abundance and *H. polygyrus* survival was also noted in GF mice monocolonized with *Lactobacillus* spp., which showed increased susceptibility to *H. polygyrus*-infection.[17] Our data show that the administration of a single commensal species, *Lactobacillus taiwanesis*, is sufficient to elevate Treg frequencies in the GALT, yet the mechanism behind this interaction remains elusive. Previous work has attributed host immune modulation by specific commensal microbes to their metabolic products, such as short chain fatty acids.[43-46] Identifying the immunomodulatory molecules produced by commensal microbes will be crucial in developing tools to modulate host responses, without the need for administering live microbes.

Two main hypotheses can be formulated to explain changes in the microbiota composition following *H. polygyrus* infection. First, *H. polygyrus* could be actively modifying the microbiota through secretory antimicrobial products such as the parasite-encoded lysozymes previously reported;[47] if it is possible that the helminth has evolved to select Th17- and Treg-generating microbes most favorable to its survival. As *L. taiwanensis* administration for 7 d expanded Treg frequencies, but did not raise Th17 responsiveness, we postulate that commensal promotion of Tregs, rather than of Th17-cells, may predispose mice to greater susceptibility to *H. polygyrus* infection. This hypothesis is also supported by other studies from our laboratory which show infection is unchanged in mice depleted with anti-IL-17 antibodies, but increased *H. polygyrus* susceptibility is seen when Tregs are expanded with IL-2:anti-IL-2 complexes.[27]

Second, either or both the inflammatory response elicited by helminth infection, or parasite disruption of the epithelial barrier may alter the intestinal niche in favor of certain commensals. Previously published data has reported that the composition of cultivatable microbes isolated from the luminal ileum differs between naïve and *H. polygyrus*-infected mice, and that similar compositional shifts occur following infection of IL-4Rα$^{-/-}$ mice,[25] suggesting that IL-4 or IL-13 signaling may not contribute to the modification of microbiota composition during infection. However, this does not rule out that other immune responses induced by the presence of *H. polygyrus*, such as Tregs,[48] may contribute to shifts in intestinal microbiota composition. Colonic microbiota populations are altered following *Trichuris suis* infection of the pig, and the authors suggest this is due to the availability of different nutritional sources for bacteria as a result of parasite damage to the intestinal epithelium.[49]

Overall, these data suggest that the microbiota composition in the duodenum can impact the survival of *H. polygyrus* within the murine host, and furthermore, *H. polygyrus* may be actively modifying the microbiota in order to promote its own survival. Following an earlier demonstration that establishment of another parasitic nematode, *Trichuris muris*, is dependent on the presence of colonic microbes,[50] our study suggests the existence of a richer and more interactive relationship between the microbiota and helminths in the gastrointestinal tract of mammals.[51]

An additional important dimension to the host-commensal-parasite trio is the genetic status of the host; in our studies we find that the modulatory effect of *L. taiwanensis* is most significant in the relatively resistant setting of the BALB/c mouse strain, rather than in the fully susceptible C57BL/6 mouse. This may reflect host genetic differences which regulate establishment of commensal species, or a more subtle role of the microbiome in modifying rather than dictating host immune responsiveness to infection.

It is notable that variation in *Lactobacillus* abundance significantly correlates with, but does not fully explain, variation in adult worm burdens (for example in **Figure 2F**). It is likely therefore that other commensal species may exert more subtle effects in restraining or promoting worm expulsion, and future work should explore this possibility. Indeed, identification of additional species that act in a similar manner to *L. taiwanensis* would open the possibility of defining a cocktail of microbes with complementary immunomodulatory functions that exert a more robust effect on host immunity.[52]

The immunomodulatory capacities of helminth parasites, including *H. polygyrus*, have been well described,[22] and helminth products are being tested for therapeutic use in autoimmune and allergic disease conditions.[53,54] Intriguingly, many of the perceived immunoregulatory effects of helminth infection have also been reported to be benefits of certain species and strains of *Lactobacillus*.[33,35-37,55,56] Mice that lack expression of MyD88, an adaptor protein which mediates signaling through Toll-like receptors and IL-1 family members[57] are rendered more resistant to *H. polygyrus* infection,[58] raising the possibility that functional MyD88 signaling is required for the recognition of specific microbiota components to maintain susceptibility to helminth infection. It will be important therefore for future work to tease apart the respective influences of parasite and microbe on the immune system, and to track the impact of ongoing antibiotic and anthelmintic treatments in human populations on the intestinal biota in the broadest sense.

Materials and Methods

Animals and parasites

BALB/c and C57BL/6 mice were bred in-house and housed in individually ventilated cages under SPF conditions. Littermates were randomly distributed between test groups for antibiotic treatment and *L. taiwanensis*-administration experiments. Mice were infected by oral gavage with 200 *H. polygyrus bakeri* third stage larvae (L3s) obtained from fecal cultures from *H. polygyrus*-infected mice, in 200 μl of dH_2O. To ensure microbiota population changes following infection were a result of parasite infection, and not due to the gavage procedure, control 'naïve' mice were orally gavaged with the same 200 μl dH_2O in which L3s had been stored in, after L3s had been filtered out using a 40 μm cell strainer (BD Biosciences). *H. polygyrus* egg counts were performed using a McMaster 2 cell counter (Hawksley). All animal studies were performed in accordance with UK Home Office guidelines.

Cell preparation and staining

MLN or PP were isolated and single cell suspensions prepared using a 70 μm cell strainer (BD Biosciences) in RPMI 1640

(Gibco) containing 10% FBS (HyClone), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Small intestinal LP cells were isolated 7 d post-*H. polygyrus* infection: first, PP were manually removed from the small intestine, which was longitudinally opened in ice cold RPMI 1640 (Gibco) containing 3% FBS (HyClone), and intestinal contents removed. The remaining tissue was diced into RPMI 1640 (Gibco) containing 2mM EDTA (Gibco) and 20 mM HEPES (Sigma-Aldrich), and manually shaken for 90 s, followed by a wash step in the same media. Tissue was then stirred for 15 min at 37 °C in RPMI 1640 (Gibco) containing 4% FBS (HyClone), 0.5 mM EDTA (Gibco) and 14.5 μg/ml DL-Dithiothreitol (Sigma-Aldrich), followed by further manual shaking for 90 s and washing in RPMI 1640 (Gibco) containing 2mM EDTA (Gibco) and 20 mM HEPES (Sigma-Aldrich). Next, tissue was placed into RPMI 1640 (Gibco) containing 0.5 mg/ml Deoxyribosnuclease I from bovine pancreas (Sigma-Aldrich) and 0.1 mg/ml Liberase TL (Roche) and digested for 24 min at 37 °C with gentle stirring. The digest reaction was stopped by addition of 3% FBS (HyClone), and single cell suspensions were made by passing tissue through 40 μm cell strainers (BD Biosciences). 2×10^6 MLN, PP, or LP cells were washed in PBS, and stained with CD4 (Clone RM4–5; BD Pharmingen) for 20 min at 4 °C. Cells were fixed for one hour in Fix/Perm (eBioscience) and stained with Foxp3 (Clone FJK-16s; eBioscience) in Permeabilization buffer (eBioscience) for 20 min at 4 °C. Isotype matched controls were also used. Marker expression was measured on a LSRII flow cytometer and analyzed using FlowJo software (Tree Star).

Antigens and restimulation

For antigen-specific restimulation, HES was produced as described previously.[57] MLN cells were isolated as described above, and 1×10^6 cells were plated in duplicate in 96-well flat bottom plates with or without 1 μg/ml HES or 1 μg/ml α-CD3 (BD Pharmingen), at 37 °C with 5% CO_2 for 72 h. Supernatants were then collected, and analyzed for IL-17A or IL-4 presence by ELISA using IL-17A or IL-4 capture and detection antibodies (BD Pharmingen) or by a cytokine bead assay for IFN-γ, IL-4, IL-5, IL-10, IL-13, IL-17A (BD Biosciences) each by the manufacturer's protocol. Cytokine levels were quantified against a standard curve created using recombinant cytokines.

Antibiotic treatment

0.5 g/L vancomycin (Univeristy of Edinburgh Pharmacy) was dissolved in the drinking water of autoclaved, UV-sterilized drinking water, and replaced weekly.

qPCR for bacterial abundance

DNA from the duodenum or feces was extracted using the QIAamp DNA Stool Mini Kit (Qiagen) by the manufacturer's protocol, including the optional 95 °C incubation step, to lyse bacterial cell walls. DNA concentration was quantified using a Nanodrop 2000 (Thermo Scientific) and adjusted to 30 ng/μl. Four μl of DNA was mixed with 5 μl SYBR Green I (Roche), 0.4 μl DEPC-treated H_2O, and 0.2 μl each of 10 μM forward and reverse primers targeting the 16S rRNA or *gyrB* gene of all or specific bacterial groups. For detection of total bacteria, the 16S rRNA gene-specific 5′-3′ACTCCTACGG GAGGCAGCAG T and 5′-3′ ATTACCGCGG CTGCTGGC primers[59] were used, for detection of *Lactobacillus/Lactococcus* species, the *Lactobacillus/Lactococcus* 16S rRNA gene-specific 5′-3′ AGCAGTAGGG AATCTTCCA and 5′-3′ CACCGCTACA CATGGAG primers[29] were used, for detection of Enterobacteriaceae species, the Enterobacteriaceae 16S rRNA gene-specific 5′-3′ GTGCCAGCMG CCGCGGTAA and 5′-3′ GCCTCAAGGG CACAACCTCC AAG primers were used,[60] for detection of *Eubacterium/Clostridium* species, the *Eubacterium/Clostridium* 16S rRNA gene-specific 5′-3′ ACTCCTACGG GAGGCAGC and 5′-3′ GCTTCTTTAG TCAGGTACCG TCAT primers were used,[61] and for detection of *L. taiwanensis*, the *L. taiwanensis gyrB* gene-specific 5′-3′ CAACGGATAT AAGACAACAC TCATGACCTT C and 5′-3′ GGTAGACCGC GCATTTTCAG AAACC primers were used. The reactions were run on a LightCycler 480 II (Roche), with 45 cycles of 95 °C for 10 s, 60 °C for 10 s, 72 °C for 10 s. Reactions were set up in triplicate and run alongside a serially-diluted pool of all samples to be analyzed, to create a standard curve of Ct value vs gene expression level in arbitrary units. Data for each sample are presented as the mean expression level in arbitrary units.

***L. taiwanensis* identification, culture, and administration**

DNA from the duodenum of three highly *H. polygyrus*-infected BALB/c mice (each harboring <130 adult worms at 28 d post-infection) was extracted using the QIAamp DNA Stool Mini Kit (Qiagen) as described above. *Lactobacillus/Lactococcus* specific primers as described above were used to amplify a *Lactobacillus/Lactococcus* specific region of the 16S rRNA gene. The resulting amplicon was ligated into pGEM vectors (Promega) by the manufacturer's protocol, and JM109 cells (Promega) were transformed with the ligation reaction by the manufacturer's protocol. Colonies were left to grow overnight on agar plates containing 50 μg/ml ampicillin in a 37 °C incubator with 5% CO_2. Individual colonies were collected and grown overnight in LB broth containing 50 μg/ml ampicillin in a 37 °C shaking incubator, after which DNA was extracted using QIAprep Spin Miniprep Kits (Qiagen). DNA was sequenced in-house using pGEM-specific T7F (5′-3′: TAATACGACT CACTATAGGG) and SP6 (5′-3′: ATTTAGGTGA CACTATAGAA T) primers. Resulting sequences were analyzed on MacVector and BLAST-searched against known microbial sequences.

L. taiwanensis strain BL263 was obtained from the Lactic Acid Bacteria and Probiotics Laboratory (Instituto de Agroquímica y Tecnología de los Alimentos, IATA-CSIC). Static overnight cultures were set up at 37 °C with 5% CO_2 in *Lactobacillis* MRS broth (Difco). Optical density (OD) of the culture was measured at 600 nm and the number of cfu determined using the following equation, based on the standard curve for a closely related species, *L. casei*, calculated by the Lactic Acid Bacteria and Probiotics Laboratory (Instituto de Agroquímica y Tecnología de los Alimentos, IATA-CSIC): $\text{cfu/ml} = (8 \times 10^8 \times \text{OD}) - 6 \times 10^7$. Prior to administration in autoclaved, UV-sterilized drinking water, *L. taiwanensis* cells were washed twice in dH_2O, and resuspended in drinking water at a concentration of 2×10^8 cfu/ml. *L. taiwanensis*-containing drinking water was replaced every 48 h throughout the course of the experiment.

Statistical analysis

Data sets were first examined for normality. For comparisons between two groups, where data were parametrically distributed an unpaired *t* test was used, and if data were not parametrically distributed a Mann–Whitney test was used. A paired *t* test was used where indicated to examine differences between mean worm burdens in separate experiments, where each experiment measured worm burdens of control and treated mice in parallel. For correlation analyses on non parametric data, a Spearman correlation test was used. The correlation co-efficient r value was added to graphs where correlations reached significance. NS on graphs denotes no statistical differences; * indicates $P = \leq 0.05$; ** indicates $P = \leq 0.01$; *** indicates $P = \leq 0.001$.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/gutmicrobes/article/32155/

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