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Infection with a small intestinal helminth, *Heligmosomoides polygyrus bakeri*, consistently alters microbial communities throughout the murine small and large intestine ☆

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ABSTRACT

Increasing evidence suggests that intestinal helminth infection can alter intestinal microbial communities with important impacts on the mammalian host. However, all of the studies to date utilize different techniques to study the microbiome and access different sites of the intestine with little consistency noted between studies. In the present study, we set out to perform a comprehensive analysis of the impact of intestinal helminth infection on the mammalian intestinal bacterial microbiome. For this purpose, we investigated the impact of experimental infection using the natural murine small intestinal helminth, *Heligmosomoides polygyrus bakeri* and examined possible alterations in both the mucous and luminal bacterial communities along the entire small and large intestine. We also explored the impact of common experimental variables including the parasite batch and pre-infection microbiome, on the outcome of helminth-bacterial interactions. This work provides evidence that helminth infection reproducibly alters intestinal microbial communities, with an impact of infection noted along the entire length of the intestine. Although the exact nature of helminth-induced alterations to the intestinal microbiome differed depending on the microbiome community structure present prior to infection, changes extended well beyond the introduction of new bacterial species by the infecting larvae. Moreover, striking similarities between different experiments were noted, including the consistent outgrowth of a bacterium belonging to the Peptostreptococcaceae family throughout the intestine.

1. Introduction

The eradication of helminths from developed regions constitutes a major achievement towards the improvement of human health, and continued efforts to lower the burden of infection in endemic areas remain essential. Yet, intestinal helminths have inhabited the intestines of mammals throughout evolution and are highly likely to have interacted with, and impacted, the complex bacterial communities present at these sites (Rapin and Harris, 2018). The eradication of helminths is also thought to contribute to the increased incidence of immune disorders including allergy and autoimmunity, observed in developed societies (Maizels, 2005; Cooper, 2009). The mammalian host and its common intestinal inhabitants – including helminths and bacteria – form a complex ecosystem, and the disruption of one of these partners might have important implications for other players in the ecosystem and ultimately for human health. Indeed, evidence is increasingly showing that interactions between helminths and bacteria do occur and that these interactions can impact the mammalian host and shape its immune response (Zaiss et al., 2015; Ramanan et al., 2016). Although the hypothesis that intestinal helminth infection impacts the mammalian intestinal microbiome is generally accepted, the impact of intestinal helminth infection on the human intestinal microbiome has only been investigated in a small number of studies, and studies using veterinary animals or experimental models have yielded contrasting results. These differences are to be expected as the field has been hampered by difficulties in characterizing the intestinal microbiome as a result

☆ Nucleotide sequence data reported in this paper are available in the GenBank, EMBL and DDBJ databases under the accession number PRJEB32288.

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of strong inter-individual variations, not only in humans but also in inbred mice (Mamantopoulos et al., 2017). Past studies have also employed a wide array of helminth parasites, host species and sampling sites, and utilized diverse technologies to characterize the microbiome (Rapin and Harris, 2018), making cross-study comparisons difficult.

Many of the experimental studies have utilized the natural murine parasite, *Heligmosomoides polygyrus bakeri*, which has a strictly enteric life cycle that is similar to that of trichostrongyloid parasites affecting humans and livestock (Bartlett and Ball, 1972; Pritchard et al., 1983; Gause et al., 2003). Experimental infection with this parasite occurs via oral gavage of infective larvae from laboratory cultures. Following gavage, the larvae penetrate the intestinal epithelium in the upper part of the small intestine and reside in the outer muscle layer of the intestinal wall (Sukhdeo et al., 1984). Here, the worm matures into its sexually mature form in a process that includes two successive moults at days 3–4 and 8–9 p.i. It finally exits the host tissue and establishes itself as an adult worm in the lumen of the small intestine, where it can survive for months (Camberis et al., 2003; Reynolds et al., 2012). *Heligmosomoides polygyrus bakeri* infection triggers a highly polarized type 2 immune response characterized by the production of IL-4, IL-5 and IL-13 cytokines, proliferation of IgE and IgG1 producing B cells and mucous secretion (Hasnain et al., 2011, 2013). Increased smooth muscle contractility together with increased epithelial cells secretions favor the eventual expulsion of adult worms from the intestinal lumen in a combined mechanism that is commonly referred to as the "weep and sweep" response (Shea-Donohue et al., 2001).

To date, seven studies have investigated the possible interaction between *H. polygyrus bakeri* and the intestinal microbiome (Walk et al., 2010; Rausch et al., 2013, 2018; Reynolds et al., 2014; Zaiss et al., 2015; Ramanan et al., 2016; Su et al., 2018). The first of these studies used a method based on the generation of 16S rRNA clone libraries and Sanger sequencing to investigate the impact of infection on the bacterial communities, and reported higher proportions of Lactobacillaceae in the ileum of infected mice (Walk et al., 2010). A later study, using real-time PCR and culture-based methods, reported differences between infected and non-infected mice in the bacterial communities residing in the ileum, the cecum and the colon at 2 weeks p.i. (Rausch et al., 2013). Another study using quantitative PCR and Sanger sequencing reported the association of *H. polygyrus bakeri* infection with the abundance of the bacterium *Lactobacillus taiwanensis* in the duodenum of susceptible C57BL/6 mice, but not in resistant Balb/c mice. These authors also reported that administration of *Lactobacillus taiwanensis* to resistant Balb/c mice resulted in higher worm burdens, suggesting that *Lactobacillus taiwanensis* promotes helminth infection (Reynolds et al., 2014). More recently, using high throughput sequencing of the bacterial 16S rRNA gene, our own group reported that *H. polygyrus bakeri* infection leads to long-lasting impacts on cecal bacterial communities which are maintained following fecal transfer (Zaiss et al., 2015). These findings were confirmed by a later study using a similar method but focusing on the bacterial communities in the colon (Su et al., 2018). Lastly, *H. polygyrus bakeri* infection has been shown to provide resistance against Bacteroides vulgatus outgrowth in Nod2-deficient mice, through a mechanisms involving type 2 cytokine production (Ramanan et al., 2016). Of note, *H. polygyrus bakeri* excretory/secretory products were recently described to exert anti-microbial activity, raising the possibility that direct helminth-bacterial interactions may take place (Rausch et al., 2018). Taken together, these observations endorse the view that intestinal helminths can and do impact the microbiome, but indicate that the outcome of helminth-microbial-host interactions may be variable.

Numerous factors might be expected to influence the impact of helminth infection on the intestinal microbiome, including the genetic variability of the parasites used for infection, the pre-infection or 'starting' microbiome of the host, and the possible carry-over of bacteria by the infective parasite larvae. Moreover, *H. polygyrus bakeri*-associated changes in the bacterial community composition might occur only within distinct sites of the intestine, and the infection might impact communities in the intestinal mucous layer differently from the communities present in the intestinal lumen. We considered it particularly important to address the latter question, given the hypothesis that the worm might impact the intestinal microbiome indirectly through the host immune response (Ramanan et al., 2016), and would thus be expected to have a major impact on those bacteria living in close association with the host intestinal epithelium. We therefore set out to determine the full extent of the impact of *H. polygyrus bakeri* infection on bacterial communities by analyzing microbiomes from the intestinal lumen and epithelium-attached mucous at multiple points along the entire length of the small and large intestines. We also investigated the impact of common variables, including the use of different parasite batches or mice with distinct pre-infection microbiomes, on the ability of *H. polygyrus bakeri* to alter murine intestinal microbial communities.

2. Materials and methods

2.1. Ethics statement

All animal experiments were approved by the Service de la consommation et des affaires vétérinaires (1066 Epalinges, Switzerland), authorization number 2238.

2.2. Mice

Four weeks old female C57BL/6 wild-type mice were purchased from Charles River Laboratories (France), and housed under specific pathogen-free (SPF) conditions at the Faculty of Life Sciences facility for animal housing, Ecole polytechnique Fédérale de Lausanne (EPFL), Switzerland. Mice were infected with 300 infective *H. polygyrus bakeri* L3 larvae, administered by oral gavage in 200 µl of saline (Gibco, USA, 10010-015). Control mice received 200 µl of saline by the same route.

2.3. Parasites

Heligmosomoides polygyrus bakeri infective L3s were generated in the laboratory by Manuel Kulagin and Luc Lebon based on previously described methods (Camberis et al., 2003). Briefly, faeces from infected mice were mixed with charcoal and water, and incubated at 26 °C for 1 week. The larvae were recovered using a modified Baermann apparatus and washed with saline. Larvae were then incubated for 4–6 h in an antibiotic saline solution containing 5 mg/ml of enrofloaxin (injectable Baytril, Bayer, Germany), 2 mg/ml of amoxicilin and 0.2 mg/ml of clavulanic acid (injectable Co-amoxi-Mepha 2200, Mepha Pharma, Switzerland). Larvae were finally washed in saline and stored at 4 °C until infection.

2.4. Analysis of intestinal bacterial communities

Bacterial communities were assessed by high-throughput sequencing of the v1-v2 hyper-variable regions of the bacterial 16S rRNA gene as previously described ((Rapin et al., 2017), following the basic protocol "Bacterial 16S rRNA sequencing for bacterial communities present in intestinal contents of mice, or from fecal samples collected from mice or humans"). Samples were processed

Table 1
Number of samples obtained per intestinal site per experimental mouse group.

	Duo.^{m}	Duo.^{l}	Jej.^{m}	Jej.^{l}	Ile.^{m}	Ile.^{l}	Cec.^{m}	Cec.^{l}	Col.^{m}	Col.^{l}	Fec.
Hpb-1	4	10	–	10	8	8	10	10	10	10	9
Hpb + a	6	8	–	10	10	6	10	10	9	10	10
Hpb + b	5	10	–	10	10	10	9	10	10	10	9
Hpb-2	8	5	10	12	14	15	15	14	15	15	–
Hpb + c	12	10	14	13	15	15	14	15	15	14	–

Duo., duodenum; Jej., jejunum; Ile., ileum; Cec., cecum; Col., colon; Fec., faeces; ^{m}, mucous; ^{l}, lumen, *Hpb*, *Heligmosomoides polygyrus bakeri*; *Hpb*-1, non-infected mice from experiment 1; *Hpb* + a, mice infected with infective larvae from batch 'a'; *Hpb* + b, mice infected with infective larvae from batch 'b'; *Hpb*-2, non-infected mice from experiment 2; *Hpb* + c, mice infected with infective larvae from batch 'c'.

Table 2
Minimal number of bacterial 16S rRNA gene amplicon sequences obtained per sample for each murine intestinal site.

	Duo.^{m}	Duo.^{l}	Jej.^{m}	Jej.^{l}	Ile.^{m}	Ile.^{l}	Cec.^{m}	Cec.^{l}	Col.^{m}	Col.^{l}	Fec.
Exp. 1	6,213	23,528	–	17,217	26,165	42,587	53,071	9,367	20,404	49,050	17,019
Exp. 2	5,318	12,306	5,058	21,777	5,168	58,390	7,204	9,955	5,873	5,442	–

Duo., duodenum; Jej., jejunum; Ile., ileum; Cec., cecum; Col., colon; Fec., faeces; ^{m}, mucous; ^{l}, lumen; Exp. 1, experiment 1; Exp. 2, experiment 2.

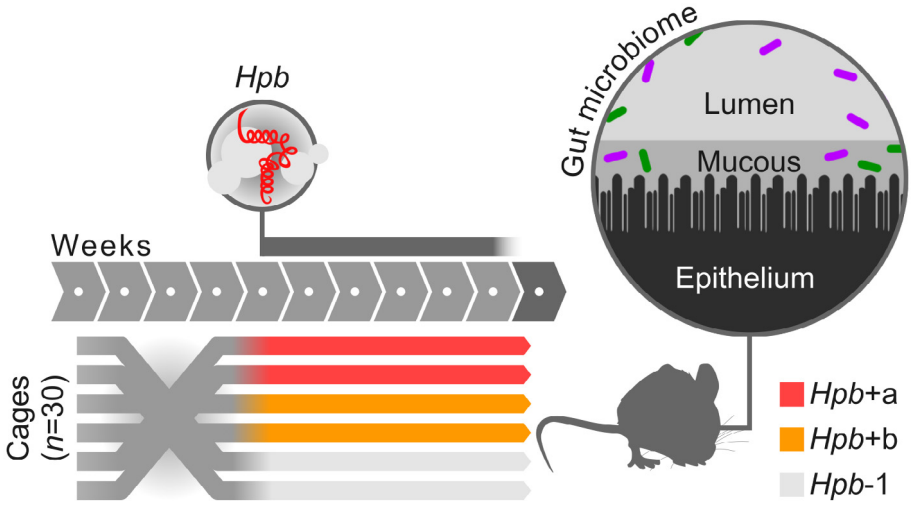

Fig. 1. Experimental design of experiment 1. Analysis of the bacterial communities in the murine intestinal lumen and intestinal mucous layer 40 days p.i. with two distinct batches of *Heligmosomoides polygyrus bakeri* (*Hpb*) larvae (batches 'a' and 'b'). *Hpb* + a, mice infected with infective larvae from batch 'a'; *Hpb* + b, mice infected with infective larvae from batch 'b'; *Hpb*-1, non-infected mice used in experiment 1.

in nine sequencing runs on an Illumina MiSeq platform using Paired End (PE) v2 2x250 chemistry. Raw nucleotide sequences were deposited in the European Nucleotide Archive database and are accessible in GenBank, EMBL and DDBJ databases under accession number PRJEB32288. The sequences were processed using scripts from the Quantitative Insight Into Microbial Ecology (Qiime) v1.9.0 pipeline (Caporaso et al., 2010). Briefly, sequences were trimmed to remove bases showing a Phred quality score lower than 20 using Seqtk software. Forward and reverse reads were then merged using the join_paired_ends.py script from Qiime (which implements the fastq-join algorithm), setting a minimal overlap of 100 bp and allowing a maximal alignment mismatch of 10%. Sample demultiplexing was done using the split_libarries_fastq.py script from Qiime. In addition, reads were truncated at the first three consecutive bases showing a Phred quality score below 20, and the resulting sequences were discarded if truncated by more than 25% of their length. Reads containing more than 2 N (unknown) nucleotides were discarded. Reads were finally clustered at 97% similarity and mapped to the GreenGene database v13_8 (DeSantis et al., 2006) as described previously (Caporaso et al., 2012). The computation was performed using the cluster of the Vital-it center for high-performance computing of the Swiss Institute of Bioinformatics. Sequences that were not assigned to any taxonomy were discarded, representing 26,688,913 out of the 48,531,909 (54%) sequences obtained in total, from intestinal samples in experiment 1 and experiment 2. Samples showing less than 5000 observations were also discarded. The final distribution of samples among experimental groups is described in Table 1.

2.5. Data analyses

Data analyses were performed using Genocrunch (https://genocrunch.epfl.ch). Differences between bacteria communities at each sampling site in Fig. 2 and Fig. 8A were assessed at the species level using the Adonis method based on the Jaccard index. Venn diagrams representing significant changes ($P < 0.05$) in individual Operational Taxonomic Units (OTUs) across sampling sites were generated in R (https://www.r-project.org). Rarefaction at the depth of 5141 observations per sample was applied prior to all analyses with the exception of Figs. 4, 5 and 9, where rarefaction depth was adapted individually for each sampling site to maximize discovery of bacteria affected by the worm infection (Table 2). Differences in individual OTU proportions and diversity between infected and non-infected mice were assessed by ANOVA. OTUs

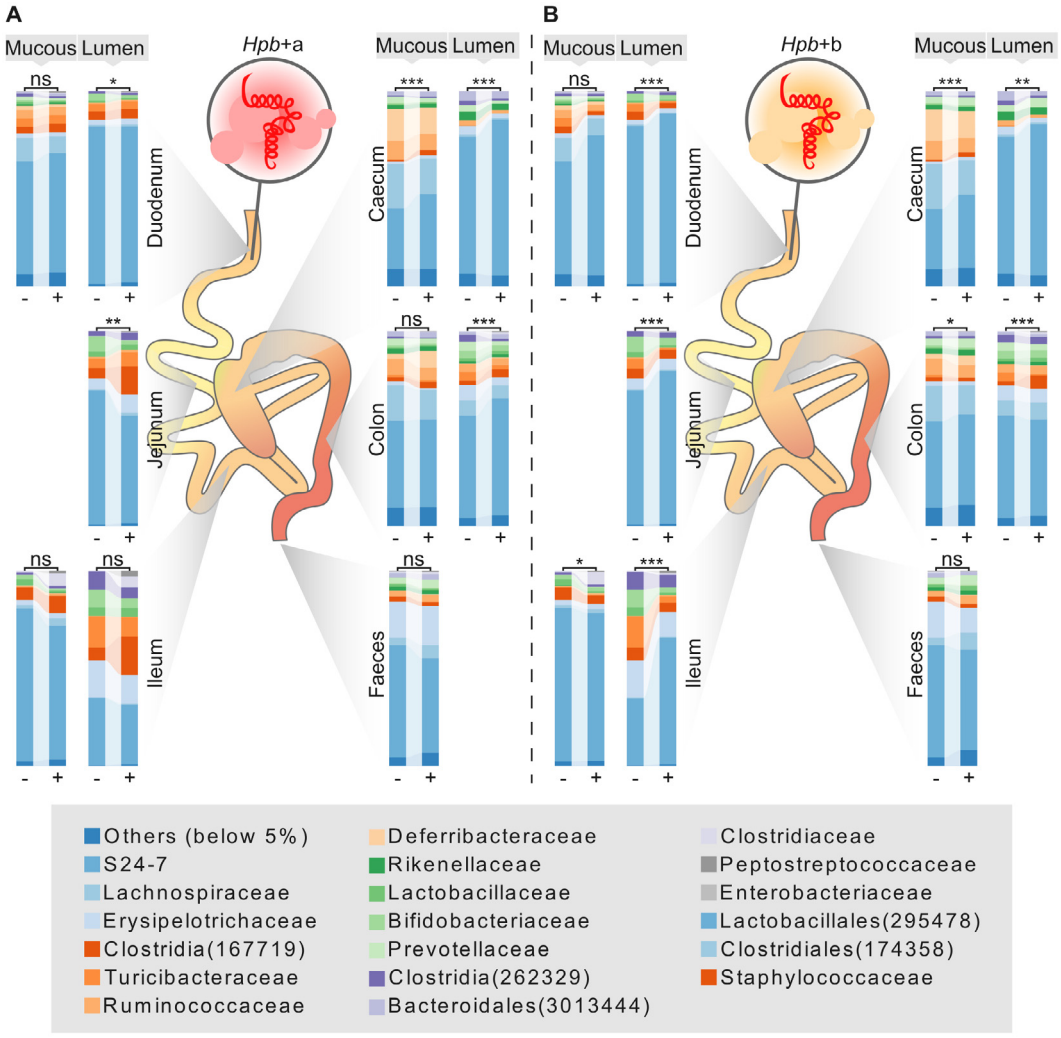

Fig. 2. Bacterial communities are affected by *Heligmosomoides polygyrus bakeri* (*Hpb*) infection at multiple sites along the mouse gut. Average proportions of bacterial families in non-infected (−) and infected (+) mice from experiment 1 are shown. Operational Taxonomic Units (OTUs) for which the family is unknown are labeled at the last resolved taxonomic level. The OTU ID is shown for families represented by a single OTU. Non-infected mice (−) are compared with mice infected with two distinct batches of invective *H. polygyrus bakeri* larvae (batches 'a' and 'b'). (A) Non-infected mice (*Hpb*-1) are compared with mice infected with infective larvae from batch 'a' (*Hpb* + a). (B) Non-infected mice (Hpb-1) are compared with mice infected with infective larvae from batch 'b' (*Hpb* + b). Groups were compared using the Adonis method based on the Jaccard distance (*P < 0.05; **P < 0.01; ***P < 0.001; ns, P > 0.05).

named in Figs. 4 and 9A were selected based on the following criteria: OTUs were first scored based on statistical differences between infected and non-infected groups across sampling sites. Briefly, for each sampling site, a score was assigned (P < 0.001 = 3, 0.001 < P < 0.01 = 2, 0.01 < P < 0.05 = 1, P > 0.05 = 0) and the overall score was calculated as the sum across all sampling sites. OTUs showing consistency in fold-change sign across at least five sampling sites, together with an overall score higher than 5, where selected.

3. Results

3.1. Small intestine helminth infection impacts on the microbiome present at multiple sites along the small and large intestine, with distinct parasite batches contributing to variability in the exact bacterial community composition found at each site

Many parasitic helminths, including *H. polygyrus bakeri*, exhibit a sexual reproductive cycle resulting in large genetic heterogeneity amongst individual worms. Even parasites maintained in laboratories for use in experimental work are produced in batches that can each be considered different communities and might show significant differences, including in term of virulence (Camberis et al., 2003; Bouchery et al., 2017). To test the impact of distinct parasite batches on helminth-induced microbial changes, we assessed the impact of two batches of worms (batches 'a' and 'b'), derived from distinct *H. polygyrus bakeri*-infected mice and produced as described in Section 2.3, on the same microbiome, by performing a large experiment in which all mice were subjected to 'normalization' of the 'starting intestinal microbiome' by mixing beddings between cages and randomizing mice once each week for 4 weeks prior to infection (Fig. 1). Normalization of the microbiome is necessary as mice bred in the same facility demonstrate inter-cage variations (Mamantopoulos et al., 2017). Mice were then sacrificed at day 40 p.i. and the bacterial communities in the duodenum, jejunum, ileum, caecum, colon, and faeces evaluated by high-throughput sequencing of the v1–v2 hyper-variable regions of the bacterial 16S rRNA gene as described in Section 2.4. Statistical evidence that infection with *H. polygyrus bakeri* affected the composition of the bacterial communities was found at multiple intestinal sites, both in the mucous and in the luminal content

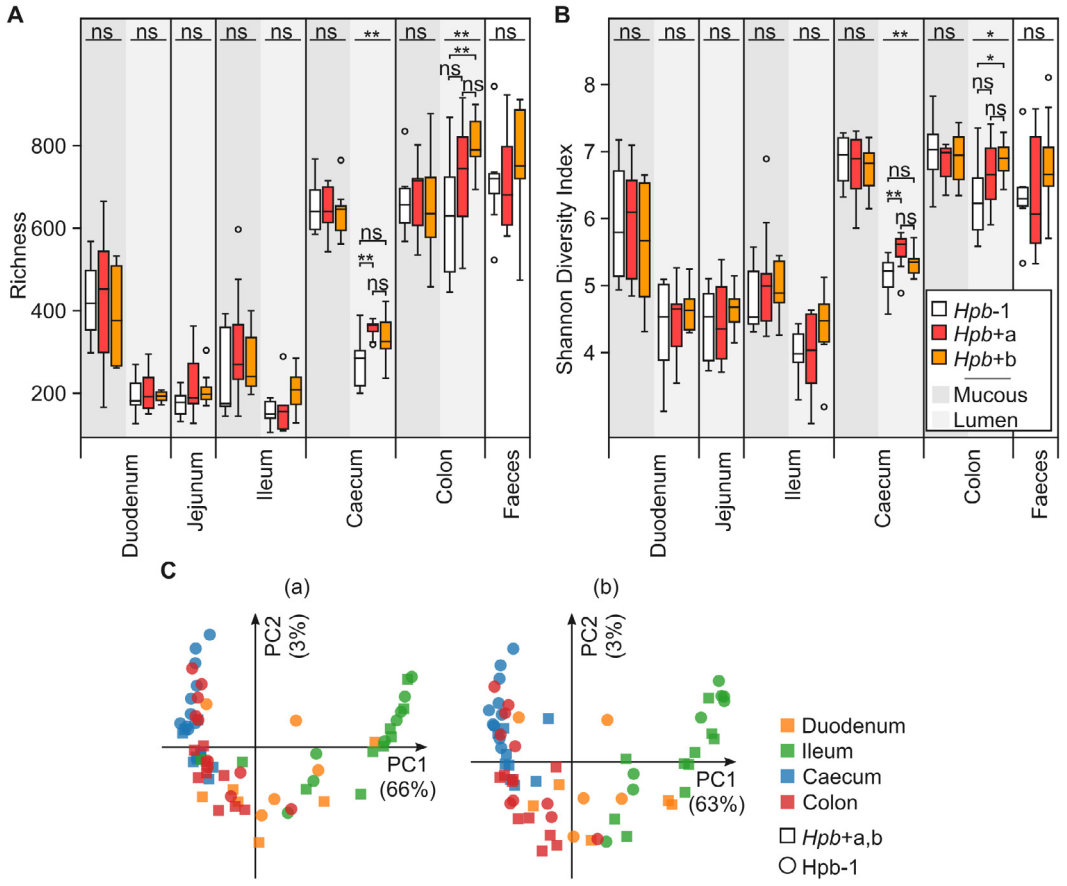

Fig. 3. *Heligmosomoides polygyrus bakeri* (*Hpb*) infection affects murine microbiome bacterial community diversity. Bacterial community diversity in the mucous layer and lumen of the intestinal tract and faeces in terms of richness (A) and Shannon diversity index (B) for non-infected mice from experiment 1 (*Hpb*-1), mice infected with infective larvae from batch 'a' (*Hpb* + a) and mice infected with infective larvae from batch 'b' (*Hpb* + b) are shown. Groups were compared by ANOVA (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns, $P > 0.05$). (C) Principal coordinates analysis based on the Jaccard distance of bacterial communities in the mucous layer along the intestinal tract for non-infected mice from experiment 1 and mice infected with infective larvae from batch 'a' (a) or batch 'b' (b).

(Fig. 2). This evidence was reproducibly observed using both batches of *H. polygyrus bakeri*, either in the mucous or the lumen of the duodenum, the jejunum, the caecum and the colon, but not in the ileum (Fig. 2). Interestingly, no statistical evidence for differences in bacterial community composition was observed in the faeces with either of the two batches of worms (Fig. 2). In addition, infection with batch 'a' larvae led to a higher diversity in the caecum lumen while infection with batch 'b' larvae led to a higher diversity in the colonic lumen, as shown in term of species richness (Fig. 3A) and Shannon diversity index (Fig. 3B).

Beyond these differences, a principal coordinates analysis (PCoA) highlighted that bacterial communities tend to cluster according to sampling site rather than infection, with the ileum clearly separating from the rest of the samples (Fig. 3C). This indicates that, although helminth infection can alter bacterial communities, the niche provided by distinct intestinal sites plays a more important role in determining community structure, with the ileum forming a particularly unique environment compared with the rest of the intestine. Differences in the proportions of individual bacterial species between *H. polygyrus bakeri* infected and non-infected mice at each sampling site along the intestine notably involved members of the families Lachnospiraceae, Clostridiaceae, S24-7, Lactobacillaceae, Ruminococcaceae and Peptostreptococcaceae, a member of the class Clostridia as well as members of the *Allobaculum*, *Bifidobacterium*, *Ruminococcus*, *Sutterella* and *Turicibacter* genera (Fig. 4). Finally, the relative abundances of some bacteria were found to be significantly ($P < 0.05$) altered across all studies in response to infection (Fig. 5). Most strikingly, a member of the family Peptostreptococcaceae was found in higher proportions in the intestinal mucous layer of infected mice at all sampling sites along the intestine independently of the larval batch used to infect the mice (Fig. 5A and C). By contrast, a member of the *Bifidobacterium* genus was found in lower proportions in both intestinal mucous and lumen at all sampling sites in mice infected with larvae from batch 'a', but was not detected in mice infected with batch 'b' larvae (Fig. 5A and B). Members of the *Allobaculum* and *Turicibacter* genera were found in higher proportions in the intestinal lumen of all infected mice, regardless of larval batch used (Fig. 5B and D).

Taken together, these data demonstrate that infection with a small intestinal helminth, *H. polygyrus bakeri*, leads to significant changes in bacterial community composition throughout the small and large intestine, but that parasitic variability (resulting from batch variability) can influence the exact nature of these changes.

3.2. The 'starting point' of the host intestinal microbiome impacts the outcome of helminth-bacterial interactions

Beyond inter-individual or inter-cage variations within a given experiment, the microbial communities of mice bred in laboratories are well known to differ between facilities and to change over time (Rausch et al., 2016; Franklin and Ericsson, 2017). For evident

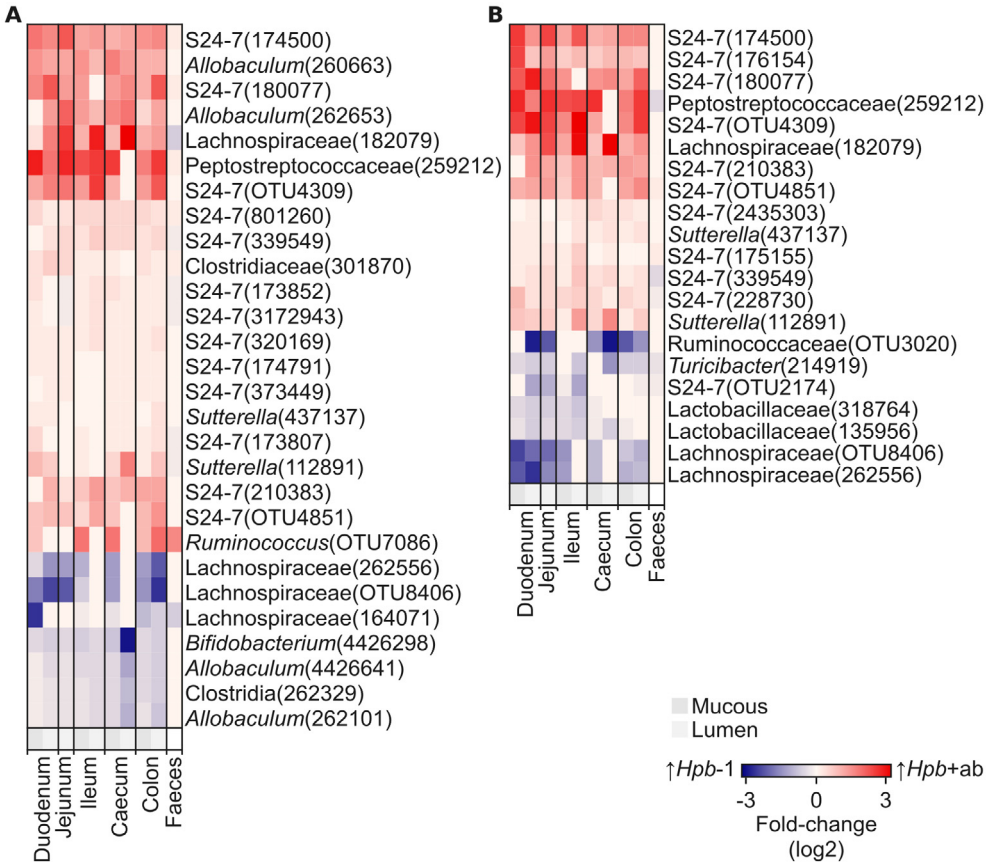

Fig. 4. Mouse microbiome bacteria are altered by *Heligmosomoides polygyrus bakeri* (*Hpb*) infection. Differences in proportions of selected bacterial species in the mucous layer and lumen of the intestine and faeces between non-infected mice from experiment 1 (*Hpb*-1) and mice infected with larvae from batch 'a' (*Hpb* + a) (A) or batch 'b' (*Hpb* + b) (B). Differences are represented by the log value of the fold-change. Species were selected based on statistical significance and consistency across multiple sampling sites as described in Section 2.5. Higher proportions in samples from *Hpb*-1 are represented by a negative (blue) scale while higher proportions in infected samples are represented by a positive (red) scale. Operational Taxonomic Units (OTUs) for which the specie is unknown are labeled at the last resolved taxonomic level. OTU IDs are shown in parenthesis.

reasons, any impact of helminth infection on the microbiome would first depend on its initial state. For instance, outgrowth of particular species following helminth infection could only be observed if these species were present in the community prior to the infection. To gain further insight into the robustness of the observed impact of *H. polygyrus bakeri* infection on intestinal bacterial communities, we conducted a second experiment (experiment 2) employing a "starting point" distinct from our first experiment (experiment 1, reported in Figs. 1–5). These different "starting points" were obtained by simply conducting experiments at different time periods (approximately 12 months) using mice obtained from the same provider. As for experiment 1, normalization of the starting microbiome within mice entered into experiment 2 was achieved by employing a period where mice were randomized and bedding was mixed between cages once each week for 4 weeks prior to infection (Fig. 6). By necessity, the experiment also employed a new batch of parasite larvae (batch 'c') as *H. polygyrus bakeri* larvae only maintain viability for a period of 8–12 weeks. As expected, major differences were found between bacterial communities from the mice employed in experiment 1 (*Hpb*-1) and experiment 2 (*Hpb*-2) (Fig. 7A). Notably, 510 identified OTUs were not shared between these two groups (Fig. 7B).

Statistical evidences for an alteration of the microbiome were found in the lumen of the duodenum and in both the mucous layer and the lumen of the ileum, the caecum and the colon (Fig. 8A). As seen in experiment 1, a PCoA analysis revealed that the intestinal site, rather than the infection status, has the largest impact on bacterial community structure, with the trend for a separation of ileal samples being repeated (Fig. 8B). Similarly to infection with larvae batch 'a' in experiment 1, a higher bacterial diversity (measured by the Shannon diversity index) was observed in the lumen of the cecum in infected mice (Fig. 8D). However, in contrast to experiment 1, *H. polygyrus bakeri* infection resulted in a lower diversity in the ileum mucous, both in terms of richness (Fig. 8C) and Shannon diversity index (Fig. 8D). Differences between *H. polygyrus bakeri*-infected and non-infected mice at each sampling site along the intestine notably involved members of the families Lachnospiraceae, S24-7, Rikenellaceae, Ruminococcaceae and Peptostreptococcaceae, two OTUs. Respectively, assigned to orders Clostridiales and Coriobacteriales, an OTU assigned to class Clostridia, members of the *Ruminococcus*, *Turicibacter*, *Escherichia* and *Sutterella* genera as well as *Ruminocuccus gnavus* (Fig. 9A). As for experiment 1, some bacteria were found significantly ($P < 0.05$) and consistently increased or decreased by the infection across all sampling sites (Fig. 9B and C). Strikingly, the same bacterium belonging to the family Peptostreptococcaceae that was consistently seen in higher relative abundance in the intestinal mucous layer of infected mice from experiment 1 (Fig. 5A and C), was also seen consistently higher in both the mucous layer (Fig. 9B) and the lumen (Fig. 9C) of infected mice in experiment 2, with the exception of the ileal mucous layer, where its relative abundance was lower (Fig. 9A). In addition, a member of the family Clostridiaceae was found in higher proportions in both the intestinal mucous and lumen of infected mice at all sampling sites except the ileal

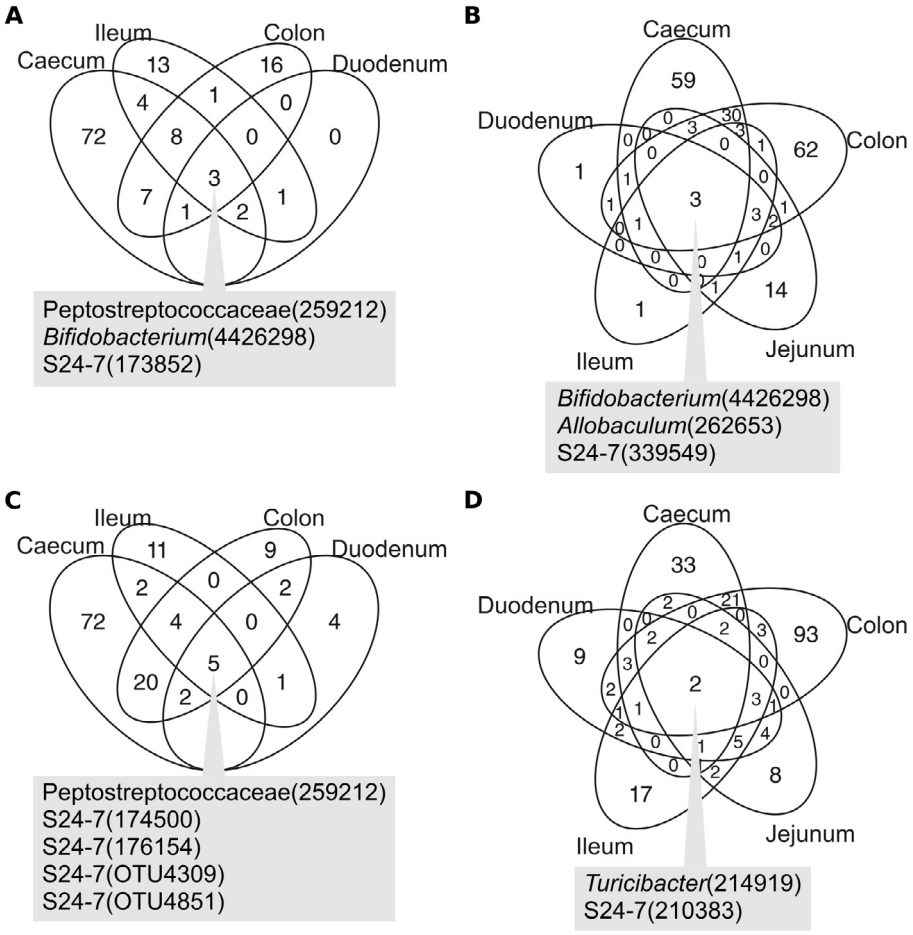

Fig. 5. A subset of bacteria are consistently associated with *Heligmosomoides polygyrus bakeri* (*Hpb*) infection across all murine intestinal sites. Venn diagrams highlighting overlaps between sampling sites along the intestine for all significant ($P < 0.05$) differences in proportions of observed species between non-infected mice from experiment 1 (*Hpb*-1) and mice infected with larvae from batch 'a' (*Hpb* + a) (A, B) or batch 'b' (*Hpb* + b) (C, D) in the mucous layer (A, C) and the lumen (B, D). Operational Taxonomic Units (OTUs) for which the specie is unknown are labeled at the last resolved taxonomic level. OTU IDs are shown in parenthesis.

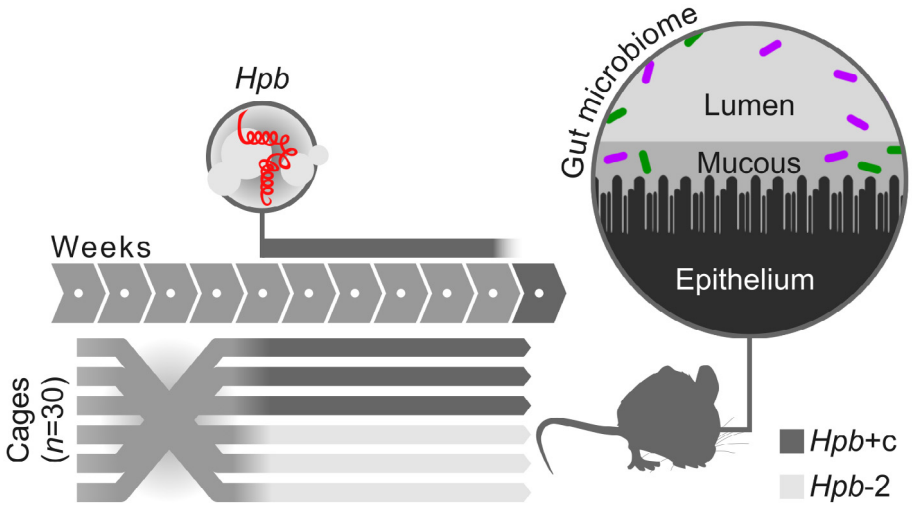

Fig. 6. Experimental design of experiment 2. Analysis of the bacterial communities in the murine intestinal lumen and intestinal mucous layer 40 days p.i. with *Heligmosomoides polygyrus bakeri* (*Hpb*) larvae. *Hpb* + c, mice infected with infective larvae from batch 'c'; *Hpb*-2, non-infected mice used in experiment 2.

mucous layer, where its relative abundance was lower (Fig. 9). As seen in mice infected with larvae from batch 'b' in experiment 1, an OTU assigned to genus *Turicibacter* was observed to be consistently less abundant along the intestinal lumen in infected mice from experiment 2 (Fig. 9). Also, a member of the family Lachnospiraceae, together with a member of the genus *Sutterella*, were seen in higher proportions in the lumen at all sampling sites except in the ileum (Fig. 9).

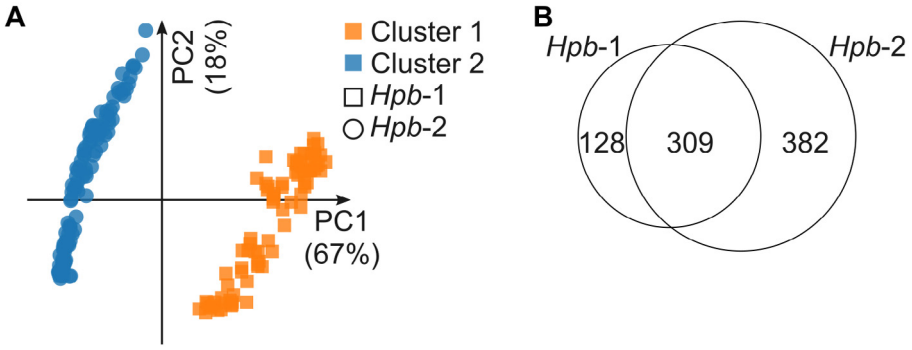

Fig. 7. Mice from experiment 1 and experiment 2 harbor distinct bacterial communities. (A) Principal coordinates analysis based on the Bray-Curtis dissimilarity for intestinal and fecal bacterial communities (from both mucous layer and lumen samples) in non-infected mice from experiments 1 (*Hpb*-1) and 2 (*Hpb*-2). Colors represent clusters of bacterial communities as defined by similarity network clustering based on the Bray-Curtis dissimilarity, as implemented on the Genocrunch web application. (B) Overlaps between non-infected mice from experiments 1 (*Hpb*-1) and 2 (*Hpb*-2) for observed bacterial species in intestinal and fecal sample (from the mucous layer and the lumen).

3.3. Helminth-induced alterations to the bacterial microbiome extend well beyond the introduction of new bacterial species by parasitic larvae

Nematodes are known to carry their own microbiome (Hsu et al., 1986; Zhang et al., 2017) and *H. polygyrus bakeri* is hatched from laboratory cultures of faeces harvested from infected mice and containing parasite eggs (Camberis et al., 2003). We therefore assessed the possibility that bacteria observed in higher proportions within the intestine of infected mice were simply carried there from infecting larvae. To address this question, we characterized the microbiome of the same batch of *H. polygyrus bakeri* larvae (batch 'c') used to perform the infections detailed in experiment 2. Amongst all the bacteria significantly affected by the infection at any sampling site and either in the intestinal mucous layer or in the intestinal lumen, only 26 were detected in *H. polygyrus bakeri* batch 'c', including *Turicibacter* and Clostridiaceae (Fig. 10A and B). Of note, Peptostreptococcaceae 259212 was not present in the *H. polygyrus bakeri* larvae batch 'c', showing that the effect of *H. polygyrus bakeri* infection on the abundance of this bacterium – which was noted across all experiments performed – was not due to its introduction into the host by the parasite.

4. Discussion

The results of this study showed that infection with a small intestinal helminth, *H. polygyrus bakeri*, can consistently and significantly alter bacterial microbial communities throughout the mucous layer and intestinal lumen of the small and large intestine. The use of a large set of mice with a 'normalized' microbiome and infected with two distinct batches of helminth larvae provides unequivocal proof that helminth infection can alter the intestinal microbiome. The finding that both the microbiome starting point and the parasite batch employed leads to important differences in the exact nature and site of microbiome alterations is likely to explain, at least in part, why previous studies of helminth-bacterial interactions have reported diverse outcomes. These data highlight the need to carefully control all experiments investigating helminth-microbiome interactions and the difficulty in comparing data generated across different laboratories, or even within the same laboratory, at different times.

The contribution of parasite batch to variability in helminth-induced microbial alterations likely arises from genetic variations resulting from the sexual life cycle of these complex organisms. However they may also arise from differences in the microbiome associated with the infective larvae. Major differences were also observed in the 'starting' microbiome of mice sourced at different times from the same provider, also introducing variability to the data obtained. However, in spite of these variables we found that *H. polygyrus bakeri* infection consistently altered the community structure of bacteria found in the mucous and luminal compartments of the cecum and colon. Alterations also occurred within the small intestine, yet the exact compartment affected varied between experimental conditions. In terms of diversity, infection resulted in differences across all experimental conditions, but these were smaller and less consistent than alterations to community structure. Of note, variations in bacterial communities along the intestinal tract were poorly reflected in the faeces, suggesting a greater stability of the microbial composition in the faeces compared with the intestinal tract. This observation emphasizes that particular attention should be given to sampling site in study of the microbiome. Based on this observation in mice, we further hypothesize that the changes in fecal bacterial composition observed in humans after helminth clearance might reflect an even more dramatic change occurring within more distal sites of the intestine.

Although we highlight that different microbiome 'starting points' can lead to different responses, functional metagenomics has shown that a particular niche within the microbiome can be occupied by several species and that communities with different species compositions can display more similarity in terms of metabolic functions (Franzosa et al., 2014). Therefore, the taxonomy-focused metagenomic approach used in the present study may fail to capture a more robust impact of *H. polygyrus bakeri* infection at the functional level, a view supported by the previous finding that *H. polygyrus bakeri* consistently leads to higher levels of bacteria-derived short-chain-fatty-acids (SCFAs) within the caecum (Zaiss et al., 2015). We also consistently noted a higher proportion of a member of the family Peptostreptococcaceae following *H. polygyrus bakeri* infection. This was observed along the entire length of the intestine and across varying experimental conditions. The fact that *H. polygyrus bakeri* larvae did not harbor any Peptostreptococcaceae at the time of the infection rules out the possibility of co-inoculation and indicates that bacteria already present in the host intestine increased in abundance in response to infection. However this observation was only possible because the bacteria in question was already part of the microbial communities prior to infection and may not occur in other experiments, for instance using mice from other facilities. The family Peptostreptococcaceae (order Clostridiales) includes six described genera, of which members were isolated from various environments ranging from human and animal microbiomes to swine manure and deep-sea hydrothermal vents (Slobodkin, 2014). Of particular interest, this family of bacteria has been described as anaerobic and produces

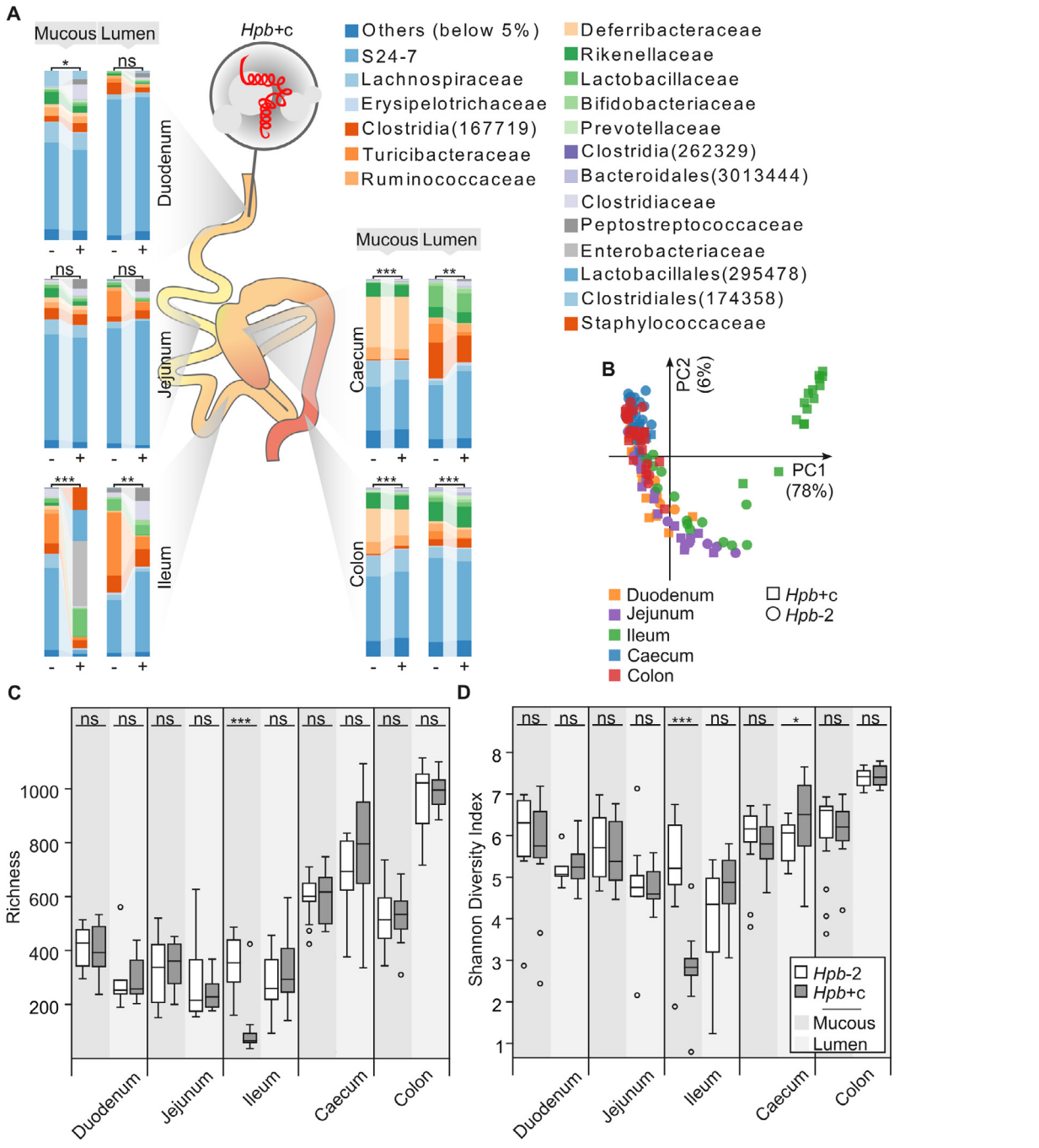

Fig. 8. The bacterial community is reproducibly impacted by *Heligmosomoides polygyrus bakeri* (*Hbp*) infection at multiple sites along the mouse gut, but the nature of the impact depends on the initial state of the microbiome. (A) Average proportions of bacterial families in non-infected (-, *Hpb*-2) and infected (+, *Hpb* + c) mice from experiment 2 are shown. Groups were compared using the Adonis method based on the Jaccard distance (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns, $P > 0.05$). (B) Principal coordinates analysis based on the Jaccard distance of bacterial communities in the mucous layer along the intestinal tract for non-infected (*Hpb*-2) and infected (*Hpb* + c) mice from experiment 2. (C, D) Bacterial community diversity in the mucous layer and lumen of the intestinal tract and faeces for experiment 2 in terms of richness (C) and Shannon diversity index (D) are shown. Groups were compared by ANOVA (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns, $P > 0.05$).

acetate as a product of fermentation. This raises the possibility that the promotion of outgrowth of species of the family Peptostreptococcaceae by *H. polygyrus bakeri* might contribute to the previously observed high SCFAs levels during *H. polygyrus bakeri* infection. The 16S rRNA based phylogeny of the family Peptostreptococcaceae has been subject to remodeling and several species of *Clostridium* have been included in this family in the database of the Ribosomal Database Project (RDP, https://rdp.cme.msu.edu). Based on the alignment of the 16S rRNA gene representative sequence for the Peptostreptococcaceae seen in the present study (OTU 259212) to the NCBI 16S database with BLAST, the phylogenetically closest identified match was *Romboutsia timonensis* strain DR1 (with only 91% similarity). Altogether, the exact taxonomy of this Peptostreptococcaceae remains unclear in the current state and further characterization (first requiring isolation from the mouse gut) would be necessary in order to understand the possible role of this bacteria within the helminth-associated microbiome and to identify possible mechanisms promoting its outgrowth during *H. polygyrus*

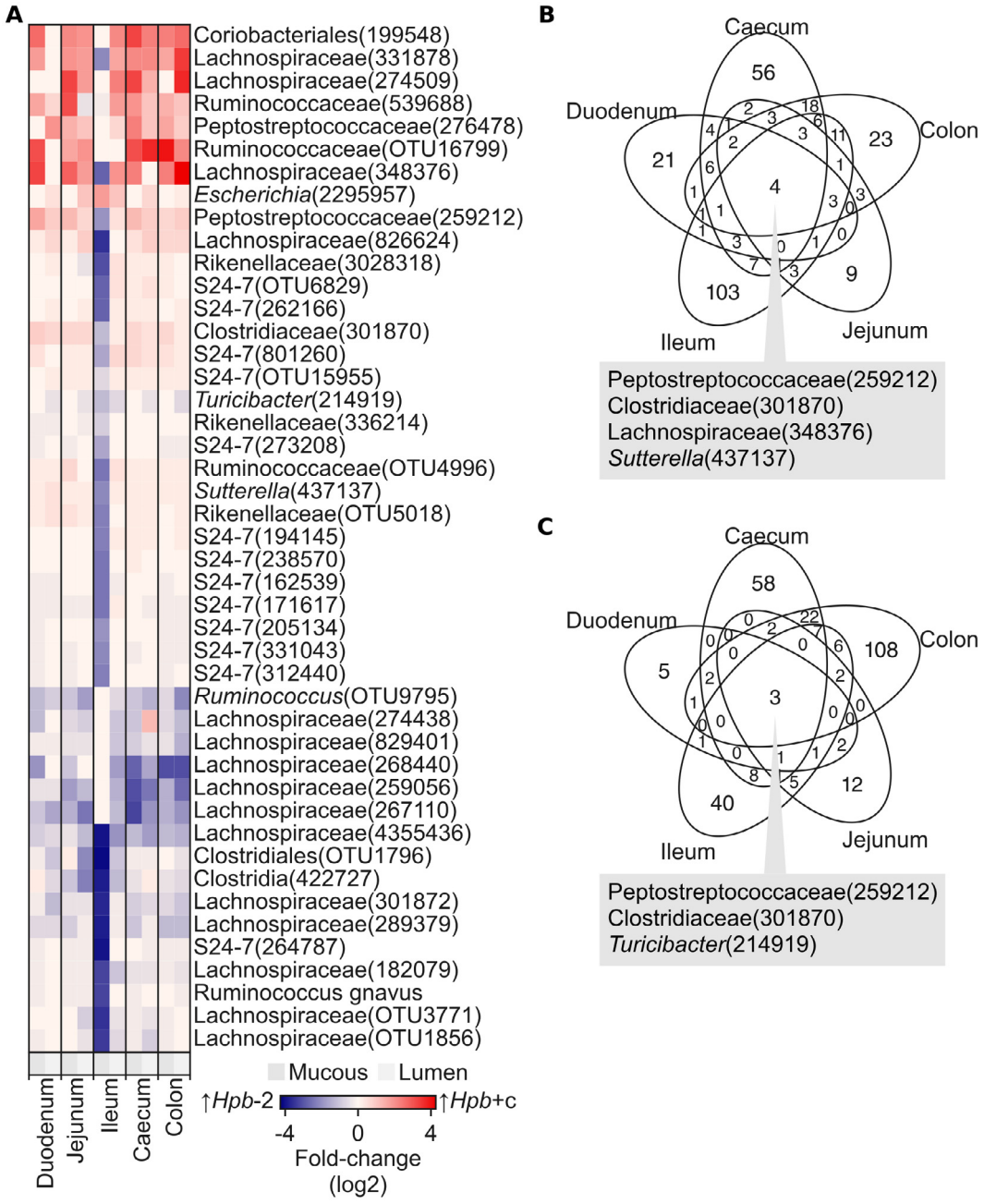

Fig. 9. A member of the Peptostreptococcaceae family is consistently associated with *Heligmosomoides polygyrus bakeri* (*Hpb*) infection across murine intestinal sites and experimental conditions. (A) As in Fig. 4, differences in proportions of selected bacterial species in the mucous layer and lumen of the intestine and faeces between non-infected (*Hpb*-2) and infected (*Hpb* + c) mice from experiment 2 are shown. (B, C) Overlaps between sampling sites along the intestine for all significant ($P < 0.05$) differences in proportions of observed species between non-infected (*Hpb*-2) and infected (*Hpb* + c) mice from experiment 2 in the mucous layer (B) and the lumen (C) are shown.

bakeri infection. Finally, as bioinformatics is a rapidly evolving field, the development of new analytical tools and the improvement of genetic database annotations might help to refine the present results in the future.

In conclusion, this work provides conclusive evidence confirming the hypothesis that intestinal helminths can impact the mammalian intestinal microbiome. Our work also indicates that helminth-induced changes can occur at regions distal to the parasite site of infection and extend well beyond the introduction of new bacterial species carried over by the infecting larvae. These findings provide impetus for further studies investigating the impact of helminth-microbial interactions on host health. Based on these findings, we believe that future exploratory studies should avoid being too reductionist and rather attempt to mimic the wide variety of conditions affecting host-microbiome-parasite interactions, starting with parasite genetics and microbiome composition. Although technically difficult, this could be achieved by performing experiments that introduce variability in a controlled manner. For instance, by carefully controlling microbiome composition whilst varying helminths and or helminth batches used. In the present study, this is achieved through microbiome normalization steps involving animal randomization and cage bedding mixes, as described in Sections 3.1 and 3.2. An example of an alternative, more tightly controlled approach would be to use gnotobiotic mice (mice that have been colonized by defined sets of bacteria), prior to infection. Regardless of the exact

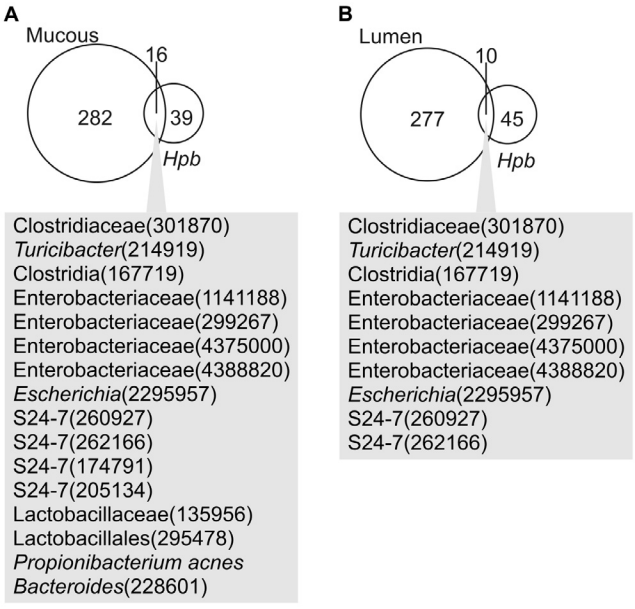

Fig. 10. Only a limited number of bacteria found affected by *Heligmosomoides polygyrus bakeri* (*Hpb*) infection in the gut were observed on *Hpb* infective larvae before the infection of mice. Venn diagrams highlighting overlaps between bacterial species included in Fig. 9B (A) or Fig. 9C (B) and bacterial species observed in the batch of infective *Hpb* larvae used in experiment 2 are shown.

approach, the underlying goal would be to obtain homogeneous groups of subjects allowing the critical use of statistics in the analysis. Applied on a larger scale, such a strategy not only could estimate the importance of each given variable being studied (e.g. helminth genetic variability) but could also allow the identification of conserved patterns not otherwise noted in less controlled studies (such as the association of helminth infection with a bacteria from the family Peptostreptococcaceae, as reported in the present study). We hypothesize that identification of such patterns would be very meaningful as they would likely give insight into an underlying conserved biology, and thus are more likely to translate into settings of human infection.

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