Microbiome composition in both wild-type and disease model mice is heavily influenced by mouse facility

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Author contribution statement

JG, AG, and NW acquired funding, designed the approaches used for the HSCR studies, and contributed to manuscript writing. KP designed the approaches for the C57BL/6J study, performed all Laramie-based experiments, coded all bioinformatic and statistical analyses, visualized and interpreted the data, and wrote the manuscript. SA was sensei for initial coding work, provided direction for statistical analysis, and contributed to manuscript writing.. JG provided guidance for initial mouse husbandry in Laramie, and contributed to manuscript writing.. All authors read, reviewed, and approved the manuscript prior to submission.

Keywords

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Abstract

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Gut microbiomes are associated with a broad range of human health problems. Murine models have become essential tools for understanding the complex interactions between gut microbes, their hosts, and disease. Multiple factors are known to influence the structure of mouse microbiomes and confound cross-study comparison. However, the contribution of inter-facility variation to mouse microbiome composition, especially in the context of disease, remains under-investigated. We show that when microbiome experiments are conducted in identical mouse lines housed in two separate facilities, facility effects are the primary driver of microbiome structure. Microbiomes differed in composition and diversity not only in the context of a disease model (the Ednrb-/- Hirschsprung Disease mouse), but also in wild-type C57BL/6J mice, which provide the genetic background for mouse models used in many microbiome studies. The facility differences between C57BL/6J mice were independent of cage or sex effects. In addition, we investigated the reproducibility of microbiome dysbiosis previously associated with Ednrb-/- mice. Notably, in 20-day old (P20) mice we observed strong genotype-based differences in microbiome diversity and composition that were conserved between facilities. In seven-day old (P07) mice and 24-day old (P24) mice, conserved genotype-based differences were not observed. Through differential abundance testing using data from two different facilities, we identified conserved candidate taxa and candidate OTUs that may be responsible for contributing to, or promoting, colitis, as well as those that may serve a protective role. Our findings raise the possibility that previously reported microbiome-disease associations from murine studies conducted in a single facility may be heavily influenced by facility-specific effects. More generally, these results provide a strong rationale for replication of mouse microbiome studies at multiple facilities, and for the meticulous collection of metadata that will allow the confounding effects of facility to be more specifically identified.

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The Institutional Animal Care and Use Committees (IACUC) of the Massachusetts General Hospital for Children, and the University of Wyoming, approved all mouse experiments conducted in Boston, MA (C57BL/6J study and Hirschsprung Disease Boston study) and Laramie, WY (C57BL/6J study and Hirschsprung Disease Laramie study), respectively.

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ABSTRACT

Gut microbiomes are associated with a broad range of human health problems. Murine models have become essential tools for understanding the complex interactions between gut microbes, their hosts, and disease. Multiple factors are known to influence the structure of mouse microbiomes and confound cross-study comparison. However, the contribution of inter-facility variation to mouse microbiome composition, especially in the context of disease, remains under-investigated. We show that when microbiome experiments are conducted in identical mouse lines housed in two separate facilities, facility effects are the primary driver of microbiome structure. Microbiomes differed in composition and diversity not only in the context of a disease model (the Ednrb$^{-/-}$ Hirschsprung Disease mouse), but also in wild-type C57BL/6J mice, which provide the genetic background for mouse models used in many microbiome studies. The facility differences between C57BL/6J mice were independent of cage or sex effects. In addition, we investigated the reproducibility of microbiome dysbiosis previously associated with Ednrb$^{-/-}$ mice. Notably, in 20-day old (P20) mice we observed strong genotype-based differences in microbiome diversity and composition that were conserved between facilities. In seven-day old (P07) mice and 24-day old (P24) mice, conserved genotype-based differences were not observed. Through differential abundance testing using data from two different facilities, we identified conserved candidate taxa and candidate OTUs that may be responsible for contributing to, or promoting, colitis, as well as those that may serve a protective role. Our findings raise the possibility that previously reported microbiome-disease associations from murine studies conducted in a single facility may be heavily influenced by facility-specific effects. More generally, these results provide a strong rationale for replication of mouse microbiome studies at multiple facilities, and for the meticulous collection of metadata that will allow the confounding effects of facility to be more specifically identified.

1 INTRODUCTION

A growing body of evidence has revealed the substantial impact of gut microbes on maintaining health of the human host. Alterations or disruptions to healthy gut microbiomes have been associated with a wide variety of diseases, including metabolic (Ley et al. 2006), inflammatory (Swidsinski et al. 2002), and autoimmune diseases (Vaahtovuo et al. 2008), as well as cancer (Moore and Moore 1995), mental illness (Cryan and Dinan 2012), and developmental disorders (Kang et al. 2013; Ward et al. 2012).

Murine models dominate gut microbiome research due to their low cost, high reproductive rates, and ease of experimental manipulation. These manipulations are fundamental to investigating the potential causality in associations between disrupted gut microbiomes and disease. Various factors that influence mouse gut microbiome structure and composition have been well documented, including comparisons of gut microbiome variability within a single facility. Among these factors are mouse vendor and inter-individual variation (Alexander et al. 2006; Hildebrand et al. 2013; Ericsson et al. 2015), cage and intra-facility effects (Hildebrand et al. 2013; Alexander et al. 2006), sex and genetic backgrounds (Alexander et al. 2006; Kovacs et al. 2011; Hufeldt et al. 2010), maternal effects and diet (Hildebrandt et al. 2009; Grönlund et al. 2011), and a wide range of stress responses and other environmental factors (Alexander et al. 2006; Bangsgaard Bendtsen et al. 2012). However, infrastructure, technology, housing and husbandry practices, diet, and other variables likely differ from one animal facility to another. The first systematic study highlighting facility-specific effects on mouse microbiomes found a high-degree of facility-level individuality in fecal microbiomes of normal C57BL/6J mice across 21 animal facilities in Germany (Rausch et al. 2016), suggesting that differences in facility standards and practices may confound cross-study comparisons of mouse microbiome structure and composition. However, to the best of our knowledge, there have been no reports describing how facility-specific effects shape mouse microbiomes in the context of disease.

Employing next-generation sequencing (NGS) approaches, we compared gut microbiome structure and composition in a mouse model of Hirschsprung disease ($Ednrb^{-/-}$, Endothelin receptor Type B knockout mouse) at two separate animal facilities, one of which had been the location of a previous Hirschsprung study (Ward et al. 2012). Hirschsprung Disease (HSCR) is a congenital colorectal aganglionosis caused by failure of neural crest-derived cells to migrate into the distal portion of the colon. While the most obvious manifestation of HSCR is colonic dysmotility in the aganglionic section, the most serious complication is the development of Hirschsprung-associated enterocolitis (HAEC), an inflammatory colitis arising in up to 50% of HSCR patients (Burkardt et al. 2014). Patients with HAEC exhibit distension, diarrhea, and fever, while those most severely affected suffer from bacterial translocation, sepsis, and death (Bill and Chapman 1962). The etiology of HAEC remains unknown, hindering the ability to generate effective therapies for its prevention. Previous work using murine models of HSCR and HAEC revealed intestinal dysbiosis associated with aganglionosis, indicating a potential role for intestinal microbiomes in the promotion of HAEC (Ward et al. 2012; Pierre et al. 2014). We also investigated the contribution of facility-specific effects to normal mouse microbiomes, reproducing the findings of Rausch et al. 2016 on a smaller scale. In summary, our analyses highlight the significant impact of facility-based differences in microbiomes at multiple levels of ecological classification, including alpha and beta diversity, core microbiome composition, and taxon abundances, both in normal mice and in the context of a disease model.

1 RESULTS

1.1 Facility-specific differences are major contributors to mouse microbiome composition in a disease model

We performed permutational multivariate analysis of variance (PERMANOVA) of UniFrac distances from age- and genotype-matched mice from the HSCR-dataset, using the R-vegan function adonis. This revealed significant differences associated with facility for both colon and fecal samples (Table S1) with the exception of three groupings: weighted P24-KO (24-day old mice, knock-out; Ednrb$^{-/-}$) colon samples, and weighted P24-KO and P24-WT (wild-type; Ednrb$^{+/+}$) fecal samples. PERMANOVA using the R-vegan function anosim supported these differences and R-values near 1.0 indicated that the groups from different facilities were highly dissimilar, with almost no overlap (Table S1). Hierarchical clustering visually demonstrated the facility bifurcation of P20 (20-day old mice) Boston and P20 Laramie colon samples from the HSCR-dataset (Fig 1). The same topology was seen in P07 (seven-day old mice) and P24 colon samples, with some inter-digitation at both ages (Figure S1, S2). These observations indicated a strong facility-specific effect on the composition of Ednrb$^{+/+}$ and Ednrb$^{-/-}$ mouse microbiomes at all ages.

To identify potential confounding factors underlying the composition differences attributed to facility, we tested the HSCR-Laramie dataset to determine whether microbiomes from mice housed as littermates clustered together by cage. Metadata necessary to examine cage effects were not available for the HSCR-Boston dataset. Given the variability in litter sizes, littermates at each age ranged from single pairs to multiple pairs of mice. While several groups of mice at P07 appeared to cluster by cage, only one of these clusters contained only mice from the same cage (Figure S3B, highlighted in orange). For P20 and P24 colon and fecal samples, no clear clustering by cage was observed (Figure S4, S5).

Given the facility differences reported above, we were interested in whether the core microbiomes from each facility were also different. These analyses were performed for all groups on each facility individually (“Boston core”, “Laramie core”) and for both facilities together (“Conserved core”). Conserved core Operational Taxonomic Units (OTUs) represented less than 40% of the total number of OTUs for all groups at the 50% threshold (Table S2). The number of conserved core OTUs dropped off considerably at the 75% and 100% thresholds, representing 4% or less of the total observed OTUs for all groups.

We next asked the question: when we consider identical OTUs present in each facility, were the abundances of those OTUs different between Boston and Laramie? To assess this, we calculated weighted UniFrac distances for the conserved core OTU tables and performed PERMANOVA to test for facility differences in relative abundance of identical OTUs. Given minimal core conservation at the 75% and 100% thresholds, we calculated weighted UniFrac distances at only the 50% threshold. No difference between facilities was observed for P24-KO colon samples or P24-WT fecal samples, while significant differences were observed for all other groups (Table S1). Weighted UniFrac distances for core 50% OTUs were ordinated using principal coordinates analysis (PCoA) with 95% confidence ellipses around sample points for each facility (Figure 2, Figure S6, S7). Ordination mostly corroborated the results of statistical analyses with three exceptions: P24-WT fecal and P24-KO colon samples exhibited no overlap of ellipses (Figure S7B and S7C), despite statistical analysis revealing no support for facility-based differences within these groups (Table S1). In addition, P24-WT colon samples showed

some overlap (Figure S7A), despite statistical support for facility-based differences between these groups (Table S1). This observation highlights the general need for statistical analysis of group- or treatment-based differences in microbiome composition, rather than reliance on ordination or clustering approaches, even when 95% confidence intervals are used.

We also assessed mice for facility-based differences in diversity independent of taxonomy, using two alpha diversity metrics: Chao1 and observed OTUs. For P07-WT colon samples, there was some statistical support for facility effects, although not consistently for both diversity metrics and both statistical tests (Table S3). For the remaining groups, facility differences were not supported for either alpha diversity metric. These results suggest minimal contribution of facility-specific differences to the shaping of mouse microbiome diversity, as opposed to composition.

1.1 Facility-specific differences drive microbiome differences at all taxonomic levels

The compositional and abundance disparities between facilities in the HSCR-dataset were also evident when taxonomic information was considered. Mean relative abundances of dominant phyla showed marked and statistically supported differences in pattern when age- and genotype-matched mice were compared by facility (Figure 3A-D, Table S4). Firmicutes dominated P07-WT and -KO Boston mice, while P07-WT and -KO Laramie mice exhibited a more even composition of Firmicutes, Proteobacteria, and, as a smaller component, Actinobacteria. At P20, an enrichment of Bacteroidetes was observed in colon and fecal microbiomes from both facilities, although Laramie KO mice displayed statistically supported higher relative abundance of this phylum (Table S4). In P24-WT fecal samples (Figure 3C), the phylum-level taxonomic composition of samples from the two facilities appeared to converge, showing similar patterns of abundance for Firmicutes and Bacteroidetes, while other sample groups retained a P24 composition similar to their P20 composition. At this age, there was no support for differences between facilities for any phyla (Table S4).

We also tested for significant differences in relative abundance between sets of age- and genotype-matched mice from the two facilities, and report here only those taxa exhibiting a mean relative abundance of at least 6%. Taxonomy was assigned to the lowest rank possible. In both colon and fecal samples, significant facility differences within a given mouse genotype were supported at all ages and every taxonomic level from order to genus (Table S4). Taken together, the results of taxonomic assignment demonstrate facility-driven differences in taxon abundance at all classification levels, with mice from each facility demonstrating drastic differences in dominant taxa.

1.1 Genotype-based differences in diversity and composition are present within facilities

We next asked whether previously reported genotype-based differences in beta diversity and in taxonomic composition between WT and KO HSCR mouse microbiomes (Ward et al. 2012) could be detected within each facility, in spite of the inter-facility variation reported above. We first re-assessed the HSCR-Boston study, where the differences between WT and KO mouse microbiomes (genotype-based differences) were originally observed.

Analysis using Wilcoxon Rank Sum Tests, but not Kruskal-Wallis, supported genotype-based differences in observed OTUs in P07 fecal and P20 colon samples (Table S3). Comparisons of beta diversity between age-matched mice using PERMANOVA showed support for genotype-based differences for colon and fecal samples from P20 mice in both unweighted and weighted UniFrac (Table S5). A genotype difference in unweighted UniFrac for P07 colon samples was

revealed by only the anosim function. For P07 fecal samples, both PERMANOVA tests supported differences for unweighted UniFrac, while a difference in weighted UniFrac was observed only with the anosim function. No differences were observed in P24 mice for either sample type or either beta diversity metric. Statistical analysis of mean relative abundances for taxa with greater than 6% abundance supported differences between genotypes in the abundance of phylum Bacteroidetes and family S24-7 in P20 fecal samples and family Enterobacteriaceae in P20 colon samples (Table S6). These taxa were associated with P20-KO mice. Taken together, these results indicate a strong genotype-based difference in microbiome composition between P20 WT and KO mice, while a difference in P07 mice is suggested, but not as well supported.

We applied the same comparative approach to the HSCR-Laramie study. Statistical analysis of alpha diversity supported no differences between genotypes at any age (Table S3). Differences in microbiome composition based on unweighted (but not weighted) UniFrac distances were supported for P24 mice in both sample types using both PERMANOVA tests (Table S5). Differences between genotypes at other ages were not supported. These results suggest that Laramie WT and KO P24 microbial communities differ in composition, but comparable patterns of taxon abundance drive these groups towards similarity. Comparisons of mean relative abundances for taxa above 6%, revealed genotype-based differences only in fecal samples (Table S6). Phylum Proteobacteria, family Enterobacteriaceae, and genera *Bacteroides* and *Parabacteroides* were associated with P24-KO mice. Family Enterobacteriaceae was also observed in higher abundance in P20-KO mice. Family S24-7 was associated with P20- and P24-WT mice, the inverse of what was observed in HSCR-Boston mice at P20.

1.1 Boston and Laramie facilities share patterns of differential abundance

We next attempted to identify organisms that may be responsible for contributing to or promoting colitis (associated with KO mice) or those that may serve a protective role (associated with WT mice). To assess this, we determined which OTUs and higher taxa were differentially abundant in microbiomes of WT or KO mice. This analysis was performed for each facility separately, and the results were combined to produce a list of conserved OTUs and taxa, the counts and relative abundances of which were consistently associated with a given genotype in both facilities. When a conserved OTU or taxon shared an identical genotype association in both facilities, it was classified as a conserved candidate. A total of five conserved OTUs were discovered, of which all five were considered candidates. One candidate was associated with P20- and P24-WT mice, two candidates were associated with P24-WT mice, and two candidates were associated with P24-KO mice (Table 1). Candidate OTU-549756, representing *Lactobacillus* (Table 1), was also observed in P07 fecal samples; however, it was associated with WT mice in Boston and KO mice in Laramie (Table S7). We also identified six conserved taxa representing several taxonomic levels. Of those six, three shared identical genotype association across facilities: family S24-7 associated with P07-KO mice, and family Enterobacteriaceae and genus *Coprobacillus*, both associated with P24-KO mice (Table 1). Family S24-7 was also conserved in P20 fecal samples, although it shared an inverse relationship between genotypes (Table S7).

1.1 Facility-specific differences are major contributors to microbiome composition in C57BL/6J mice

Our observations of the HSCR-dataset, together with a previous study showing inter-facility differences in normal mouse microbiomes (Rausch et al. 2016), prompted us to determine

whether facility effects also drive microbiome structure of normal mice housed in our mouse facilities. Using the same in-bred mouse strain (C57BL/6J), we compared fecal microbiomes of mice raised in Boston or Laramie.

PERMANOVA (using adonis) of UniFrac distances from the C57BL/6J dataset revealed significant differences in fecal microbiome composition associated with facility (Table S1). The anosim function corroborated these differences and R-values near 0.5 indicated that the groups from each facility were dissimilar, with some overlap. Hierarchical clustering of UniFrac values provided a clear visualization of the facility effect (Figure 4A and B), although clusters derived from weighted analysis (Figure 4B) displayed minor inter-digitation of each facility group. Given reported sex and cage effects on microbiome composition (Hildebrand et al. 2013; Alexander et al. 2006), we looked at whether mice within a facility clustered according to these factors. There was some evidence for clustering by sex in both unweighted and weighted analysis (Figure S8A and S8B). In both cases, the groups that clustered by sex also clustered by cage. One cage in the Laramie facility clustered together in both unweighted and weighted UniFrac, while one Boston cage exhibited clustering only in unweighted UniFrac (Figure S8C and S8D). These observations indicated a strong effect of facility on normal mouse microbiomes that was independent of sex and cage effects. Conserved core OTUs represented 53% and 25% of the total number of OTUs at the 50% and 75% thresholds, respectively (Table S2). The percentage of conserved core OTUs was higher at all thresholds for C57BL/6J mice than for any group in the HSCR-dataset (Table S2), although at the 100% threshold, the conserved core accounted for only 7% of the total observed OTUs. This observation suggests higher similarity of normal mouse microbiomes as compared to microbiomes from diseased mice. The facility effect was present at all percentage thresholds, with $P < 0.001$ for both PERMANOVA functions (Table S1). Principal coordinates analysis of weighted UniFrac distances for 50% core OTUs visually demonstrated the facility difference, while revealing overlap shared between Boston and Laramie ellipses (Figure 4C). No statistical support for differences in alpha diversity between facilities was found for C57BL/6J mice, using either Chao1 or observed OTUs metrics (Table S3). Mean relative abundances of dominant phyla showed statistically supported differences between facilities (Table S4). Boston mice were dominated by Bacteroidetes while a higher abundance of Firmicutes was observed in Laramie mice (Figure 4D).

1 DISCUSSION

Murine models are crucial for microbiome research. They have led to the identification of multiple microbiome-disease associations, but have also allowed the field to transition from association-based observations to causality-based conclusions that definitively link disrupted microbiomes to promotion of disease. While factors that influence mouse microbiomes within a single animal facility have been identified, less is known about the broader influence of these factors between animal facilities. Additionally, the contribution of these inter-facility factors in the context of a disease model, where host- and disease-specific factors influence the composition and structure of microbiomes, remains unclear. Practices and standards differ between animal facilities, which can confound cross-study comparisons.

In this study, we sampled colon and fecal microbiomes from two animal facilities, using identical host genetic backgrounds. These backgrounds included both an intestinal disease model ($Ednrb^{-/-}$) used for the HSCR studies and one of the most widely used in-bred mouse strains (C57BL/6J) used for the C57BL/6J study. The sampling time points chosen for the HSCR

studies featured both weaning and disease progression to capture the environment prior to disease. At P07 and P20, mice are suckling, with P20 mice having additional access to solid food. P24 mice are completely weaned and near the usual onset time of HAEC (P28-P30). We identified a strong facility-specific effect on the composition of both Ednrb$^{-/-}$ mouse microbiomes at all ages tested, and on C57BL/6J mouse microbiomes. These effects were independent of cage or sex influences. A previous study identified similar facility-specific effects on normal mouse microbiomes across 21 animal facilities at 13 institutions (Rausch et al. 2016). Our findings are consistent with this report.

In the originally published mouse study (HSCR-Boston) (Ward et al. 2012), statistical support for a significant differences in microbiome composition and structure between Ednrb$^{-/-}$ (KO) and Ednrb$^{+/+}$ (WT) mice were observed using n = 5/genotype at P07 and P20, and n = 3/genotype at P24. Our replication study (HSCR-Laramie) used similar numbers: n = 5/genotype at P07 and P24, and n = 6/genotype at P20. Two mice per genotype from the original Laramie P24 cohort sequenced poorly, and an additional two mice per genotype were sampled to maintain n = 5/genotype. The C57BL/6J dataset used n = 10 mice/facility. In a methodologically similar study comparing fecal microbiomes of C57BL/6J mice from several animal facilities, Rausch et al. 2016 were able to make statistically meaningful and biologically relevant conclusions based upon n = 5 mice/facility (Rausch et al. 2016). Other studies using smaller numbers of animals (n = 4/group) (Kovacs et al. 2011) or similar numbers (n = 7-10/group) were able to demonstrate statistical support for group-based differences (Shogan et al. 2014; Ryan et al. 2017; Allen et al. 2015). Based upon these published studies, it was reasonable to expect that the work performed here was adequately powered.

For both datasets (HSCR and C57BL/6J), mouse facility practices and standards differed between Boston and Laramie in a number of ways, including diet, housing, and husbandry. In both humans and mice, the effects of diet on gut microbiomes have been well established (Brown et al. 2012); however, little is known about the effects of different brands of standard mouse chow on the composition of gut microbiomes (Laukens et al. 2016). Associations between mouse chow fat content and microbiome composition have been reported (Hildebrandt et al. 2009; Turnbaugh et al. 2008; Ma et al. 2012). Chow fed to Boston mice contained 12.3% fat, while Laramie mice were fed chow containing 11.4% fat. For age- and genotype-matched mice in the HSCR-dataset, inter-facility comparisons showed a higher abundance of Firmicutes and a lower abundance of Bacteroidetes for Boston mice relative to Laramie mice, in eleven out of twelve groups. This relationship is a hallmark observation in mice fed high-fat diets (Hildebrandt et al. 2009; Brown et al. 2012). In contrast, the Boston C57BL/6J mice possessed lower Firmicutes and higher Bacteroidetes compared to Laramie mice. These observations suggest that the 0.9% difference in chow fat content may play a small role separating microbial communities from the two facilities, but is not the sole contributor to variation.

Boston mouse chow contained 55.6% total carbohydrates, of which 51.6% and 4.0% were simple sugars and crude fiber, respectively. Laramie mouse chow contained 52.8% total carbohydrates, with 47.5% simple sugars and 5.3% crude fiber. An increase in Bacteroidetes is associated with lower carbohydrate diets in mice (Walker, Ince, et al. 2011). For age- and genotype-matched mice in the HSCR-dataset, inter-facility comparisons revealed statistical support for higher abundance of Bacteroidetes for only Laramie P20-KO mice, although the remaining Laramie mice displayed a higher abundance of this phylum. This suggests minimal contribution of carbohydrate content in driving the differences between the facilities.

Treatment of mouse chow via autoclaving or irradiation is known to influence the

physical properties and nutrient availability of chow (Caulfield, Cassidy, and Kelly 2008). The use of irradiated chow has been associated with a decrease in microbiome diversity (Rausch et al. 2016); however, the factors underlying that association remain unclear. Our results showed minimal differences in alpha diversity between Boston mice, fed irradiated chow, and Laramie mice, fed autoclaved chow. Although not statistically supported, Boston C57BL/6J mice had a lower average diversity for both alpha diversity metrics compared to Laramie mice. Further studies that control for other confounding factors, including housing and husbandry discrepancies and mouse chow brand, could substantiate the possible link between irradiated chow and decreased alpha diversity.

Our results indicate some contribution of cage effects to shaping distinct, facility-specific microbiomes. Intrinsic environmental factors, such as the interaction of mice with their cage mates, are known to influence the composition of gut microbiomes (Hildebrand et al. 2013; Alexander et al. 2006; Kovacs et al. 2011). Mice are known to ingest feces excreted by their co-housed mates either through direct consumption or through grooming (Soave and Brand 1991). This practice is called coprophagy and is likely the primary driver of gut microbiota convergence or synchronization in co-housed mice (Hildebrand et al. 2013; Alexander et al. 2006; Nguyen et al. 2015). Transference of susceptibility to chemically induced colitis has been shown when wild-type mice are co-housed with mice deficient in immune system components, and is likely a result of transmission of colitogenic microbiota through coprophagy (Elinav et al. 2011; Zenewicz et al. 2013). For these reasons, our mice were housed as littermates where applicable. While metadata for the HSCR-Boston dataset were limited, precluding a cage effect analysis, the HSCR-Laramie mice showed minimal evidence for cage effects. This was evident for only one group of P07 mice. Mice in the C57BL/6J dataset showed some evidence for cage contribution to the shaping of microbiome composition. In both mouse lines analyzed in this study, cage effects are likely not the primary driver of facility differences reported here, although in combination with other facility-specific factors may help drive microbial communities apart.

Our observation of strong facility effects on microbiome composition in both the HSCR and C57BL/6J datasets naturally raises the question of whether the genotype-based differences we previously reported for HSCR mice in Boston (Ward et al. 2012) would also be detected in Laramie. We analyzed our replication study (HSCR-Laramie) alongside the original study (HSCR-Boston) to ensure consistency across all parameters used for data processing and analysis. The genotype-based differences observed between WT and KO mouse microbiomes when the HSCR-Boston data were re-assessed were not as clear as compared to the original published conclusions. This is likely attributable to the increased stringency of our re-assessment, and the addition of rarefaction. Results from our replication study showed partial reproducibility of genotype-based differences in colon and fecal microbiomes between WT and KO mice; however, these genotype-based differences and the overall microbiome composition and structure were not identical in both animal facilities.

Re-assessment of phylum-level relative abundances corroborated the original observation of increased Bacteroidetes in the feces of Boston P20-KO mice, relative to wild-type mice of the same age. This relationship was not seen in Laramie mice at this age; feces from those mice showed enrichment of family Enterobacteriaceae from phylum Proteobacteria. Observations for these taxa are consistent with previously published work in another HSCR/HAEC mouse model (Pierre et al. 2014) and shifts in Bacteroidetes (Walker, Sanderson, et al. 2011; Frank et al. 2007) and Proteobacteria (Frank et al. 2007) have been reported in other inflammatory bowel diseases (IBD) related to HAEC.

Members of the genus *Bacteroides* have been previously suggested to play a role in IBD (Swidsinski et al. 2005). In the context of HAEC, Frkyman et al. 2015 observed higher abundance of *Bacteroides* in fecal samples from patients with a history of HAEC compared to patients with no history of enterocolitis, although this relationship was not statistically supported (Frykman et al. 2015). In contrast, Li et al. 2016 observed statistically supported higher abundance of *Bacteroides* in the intestinal contents of patients with no history of enterocolitis compared to patients with active enterocolitis or patients in HAEC remission (Li et al. 2016). This genus was previously observed in higher abundance in the cecal contents of knockout-mice in a similar HSCR/HAEC model (Pierre et al. 2014). Our original analysis revealed statistical support for higher abundance of *Bacteroides* in the colons of Boston P24-KO mice compared to WT mice of the same age; however, this association was not statistically supported after re-assessment. Interestingly, Laramie P24-KO mice displayed a higher abundance of this genus, which was statistically supported in fecal samples. Further investigation of *Bacteroides* in the context of HAEC is warranted, to determine whether this genus possesses proinflammatory ability or contributes to development of enterocolitis.

The striking observation of depleted *Lactobacillus* and enriched *Staphylococcus* in P07-KO mouse colons, relative to P07 WT mice, was not observed to be statistically significant after re-assessment. Furthermore, the original observation of enriched *Tannerella* in P20-KO fecal samples was also not observed after re-assessment. This is likely due to the use of a different database for the taxonomic assignment reported here. In the original publication (Ward et al. 2012), a genus-level label of *Tannerella* was used to refer to a genus-level taxon that could not be unambiguously assigned to one genus, but was most closely related to *Tannerella* and *Barnesiella*. Previous reports show database-dependent classification for closely related sequences as either family S24-7 or family Porphyromonadaceae, to which *Tannerella* and *Barnesiella* belong (Lagkouvardos et al. 2016). Re-assessment likely classified these sequences to family S24-7, resulting in the loss of *Tannerella*. Family S24-7 has been shown to be a dominant member of mouse gut microbiomes (Lagkouvardos et al. 2016) and repeatedly appeared in our data. This family was associated in higher abundance with Boston P20-KO fecal samples relative to P20-WT, while the inverse was observed for Laramie P20- and P24-KO mice.

A powerful advantage of replicating the original work (Boston) in a different location (Laramie) was the ability to construct a conserved candidate list of differentially abundant organisms (OTUs) and taxa that share the same relationship in both facilities. We identified five candidate OTUs and three candidate taxa. Here, we want to emphasize caution when interpreting the results, as further empirical study of each candidate will be needed.

One candidate taxon corresponding to family S24-7 was over-represented in P07-KO mice compared to WT mice of the same age. This taxon did not maintain candidacy at the later ages as it was associated with Boston KO and Laramie WT mice at P20, and was not conserved for either genotype at P24. Members of family S24-7 appear to be abundant in laboratory mice, but have also been found in other mammals, including humans (Ormerod et al. 2016; Lagkouvardos et al. 2016, 24). Increased abundance of family S24-7 was associated with treatment-induced remission in a mouse model of colitis (Rooks et al. 2014), suggesting a possible role for members of this family in protection from colitis. The inconsistent genotype associations for this candidate and the relative abundance of this family (see above) likely preclude this candidate from influencing the pathogenesis of HAEC, although repeat studies are needed.

Three candidate OTUs were associated with older WT mice, two represented order Clostridiales and one represented genus *Lactobacillus*. Our inability to classify Clostridiales candidates below the Order level (for both these WT associations, and the KO associations described below) precludes even a tentative proposal of these candidates as colitogenic or colitis-protective, based on comparison with the results of other studies. A significant depletion of *Lactobacillus* was observed in stool obtained from patients with HAEC (Shen et al. 2009). Lower abundance of *Lactobacillus* was also observed in diseased mice compared to healthy mice in another HSCR/HAEC murine model (Pierre et al. 2014). In humans (Mattar, Coran, and Teitelbaum 2003; Thiagarajah et al. 2014) and in Ednrb$^{-/-}$ mice (Thiagarajah et al. 2014; Yildiz et al. 2015), a decrease in intestinal mucin production and alterations to the mucosal barrier have been associated with HAEC. A reduction in the availability of mucins may hinder the ability of lactobacilli to colonize the intestinal barrier and displace pathogens, a protective function normally performed by these organisms (Lee et al. 2003). Our discovery of a *Lactobacillus* candidate OTU in P24-WT colon samples further highlights the possible protective role of lactobacilli in prevention of HAEC.

We identified two candidate OTUs and two candidate taxa positively associated with P24-KO mice. The candidate OTUs corresponded to genus *Coprobacillus* and order Clostridiales, while the candidate taxa were identified as genus *Coprobacillus* and family Enterobacteriaceae. Enrichment of *Coprobacillus* has been observed in patients suffering primary sclerosing cholangitis (PSC) with concomitant IBD (Bajer et al. 2017). In contrast to these observations, *Coprobacillus* was found to be restricted to healthy patients rather than those with active Crohn's disease (CD) (Rausch et al. 2011). This genus has also been associated with irritable bowel syndrome (Kassinen et al. 2007). The contradictory associations for *Coprobacillus* may be attributed to the observation of this genus as a member of the core microbiome of the gut in humans (Tap et al. 2009). Our observation of *Coprobacillus* as both a candidate OTU and a candidate taxon found in colon and fecal samples of P24-KO mice, a time point just prior to onset of HAEC, suggests a role for this organism in promoting inflammation. Increased abundance of Enterobacteriaceae is considered a marker of intestinal inflammation and oxidative stress in human IBD and in murine colitis (Jia et al. 2017). In the context of existing literature, our observation of this taxon in the feces of P24-KO mice from both facilities suggests a potentially prominent role for members of this family in promoting HAEC. Although short, our conserved candidate list provides targets for investigation of the contribution of specific organisms to protection from, or promotion of, HAEC.

1 CONCLUSIONS

Our findings highlight the major effect that inter-facility variation has on microbiome composition both in a specific disease model and also in a mouse strain widely used in microbiome research. These results emphasize the need to exercise caution in the interpretation of microbiome-disease associations identified through single-facility murine studies. Our study was limited by inclusion of only two facilities, relatively low numbers of mice, and differences in available metadata from each facility. Identification of inter-facility differences through meticulous collection of metadata are essential to improve our understanding of the role that facility-specific factors play in shaping microbiomes and thereby diminish the variables that confound cross-study comparisons of murine microbiome work. Additionally, conducting microbiome studies in multiple facilities will strengthen the results obtained by permitting

identification of shared taxa that can be further investigated as disease-associated candidates.

1 MATERIALS AND METHODS

1.1 Ethics statement

The Institutional Animal Care and Use Committees (IACUC) of the Massachusetts General Hospital for Children, and the University of Wyoming, approved all mouse experiments conducted in Boston, MA (C57BL/6J study and Hirschsprung Disease Boston study) and Laramie, WY (C57BL/6J study and Hirschsprung Disease Laramie study), respectively.

1.1 Animal facility procedures

C57BL/6J or Ednrb^{tm1Ywa} mice on a hybrid C57BL/6J-129Sv background (JAX #003295) were housed under identical conditions within a given facility. A breeding pair from the Boston facility was used to establish the colony in Laramie. For both facilities, mice were maintained on a 12-h light-dark cycle at 25°C, supplied with autoclaved, hyper-acidified water (pH 3.0), and weaned at postnatal day 21 (P21). Homozygous knockout mice (Ednrb$^{-/-}$) were phenotypically identified by their piebald coat, and genotype was confirmed by polymerase chain reaction (PCR) as detailed in Supplementary Methods. Differences in mouse housing, diet, and husbandry between the Boston and Laramie facilities are listed below.

Boston housing, diet, and husbandry. Mice were housed in Allentown rectangular cages (Allentown Inc., Allentown, NJ, USA) and cage/bedding changes were performed weekly or as needed. Mice were fed a standard non-autoclaved rodent chow, Prolab Isopro RMH 3000 Irradiated (LabDiet, St. Louis, MO, USA). Mice were harem bred (1 male, 2 females per cage).

Laramie housing, diet, and husbandry. Mice were housed in Optimice polysulfone triangular cages (Animal Care Systems, Centennial, CO) and cage/bedding changes were performed every two weeks or as needed. Mice were fed a standard, autoclaved rodent chow, Laboratory Rodent Diet 5001 (LabDiet, St. Louis, MO, USA). Mice were monogamously bred (1 male, 1 female per cage), as this was the only breeding protocol approved by the University of Wyoming IACUC.

1.1 Sample collection and DNA extraction

Hirschsprung Disease studies (HSCR-Boston and HSCR-Laramie). Knockout (Ednrb$^{-/-}$) and wild-type (Ednrb$^{+/+}$) mice were euthanized by CO_2 asphyxiation on postnatal day 7, 20, or 24 (P07, P20, P24). The distal two-thirds of each mouse colon was removed and rinsed with sterile phosphate-buffered saline (PBS) (pH 7.4). Colons were collected for analysis of surface-associated microbial communities. Fecal material was collected from the colon washout. Colons and fecal material were immediately frozen at -80°C. The following numbers of mice were used in Boston: P07, n = 5 KO and WT; P20, n = 5 KO and WT; P24, n = 3 KO and WT. The following numbers of mice were used in Laramie: P07, n = 5 KO and WT; P20, n = 6 KO and WT; P24, n = 5 KO and WT. For Laramie P24 mice, re-samples of n = 2 KO and WT were collected as several of the originally submitted samples sequenced poorly or failed to sequence altogether.

C57BL/6J study (Boston and Laramie). Fecal samples were collected when mice were six weeks of age. Pellets were collected during defecation directly into sterile cryotubes pre-filled with 0.5mL 1X PBS (pH 7.4) supplemented with 10% glycerol. Material was collected from ten mice

at each facility, three females and seven males. Mice were housed by both litter and sex. DNA was extracted using the QIAmp DNA stool MiniKit (QIAGEN, Valencia, CA, USA) with the addition of a bead-beating step at the beginning. Full details are available in Supplementary Methods.

1.1 16S rRNA gene sequencing – Roche 454 and Illumina MiSeq

Roche 454 pyrosquencing utilized the 28F-588R primer pair targeting the V1-V3 hypervariable 16S rRNA regions and Illumina MiSeq sequencing utilized the primer pair 28F-388R targeting the V1-V2 hypervariable regions. Detailed sequencing methods are available in Supplementary Methods.

1.1 Sequence processing

Quality trimming, chimera checking, and denoising of raw datasets was performed by RTL (see: Supplementary Methods for parameters and details). Pre-processed sequencing files obtained from RTL were further processed and analyzed using the Quantitative Insights Into Microbial Ecology (QIIME) 1 pipeline (Caporaso, Kuczynski, et al. 2010). For all datasets, sequence reads were first demultiplexed and then quality filtered. Quality filtering removed any reads not matching the sample-specific barcode, reads shorter than 200bp or longer than 1000bp, reads with greater than 6 ambiguous bases, reads below the minimum quality score of 25, and reads with homopolymers in excess of 6bp. Individual sequence files for HSCR-Boston and HSCR-Laramie were merged following demultiplexing and quality filtering. Sequences were classified into Operational Taxonomic Units (OTUs), defined at 97% 16S rRNA gene sequence similarity, and selected using UCLUST open-reference clustering against Greengenes reference collection 13.8 (DeSantis et al. 2006; Edgar 2010). UCLUST was chosen over USEARCH as chimeric sequences were removed by RTL during the pre-processing steps. Reads that failed to cluster to a reference were subsequently clustered *de novo*. Representative sequences from each OTU were aligned to a core set of Greengenes 16S rRNA sequences using PyNAST and subsequently filtered (Caporaso, Bittinger, et al. 2010; DeSantis et al. 2006). Phylogenetic trees relating OTUs were constructed using FastTree (Price, Dehal, and Arkin 2010). Taxonomy for each representative OTU was assigned against Greengenes 13.8 using UCLUST consensus taxonomy (Edgar 2010; McDonald et al. 2012). Finally, chloroplast sequences were removed from each OTU table using QIIME 1. The above workflow produced an unrarified master OTU table with taxonomic assignments for each OTU and containing all samples within a given study. Samples from each unrarified master OTU table were filtered out as needed for subsequent analysis, yielding separate OTU tables. The fates of each of these tables are and their rarefaction depths are detailed in Supplementary Methods and Table S8, respectively.

1.1 OTU table analyses

Beta diversity was quantified using unweighted and weighted UniFrac distance metrics (Lozupone and Knight 2005). Unweighted UniFrac considers only presence/absence of an OTU, whereas weighted UniFrac takes into account the relative abundance of an OTU in addition to presence/absence. UniFrac distances were visualized with either hierarchical clustering using unweighted pair group with arithmetic mean (UPGMA) (Sokal and Michener 1958) or with ordination utilizing principal coordinates analysis (PCoA) (Borg and Groenen 2005). UPGMA clustering was multiscale bootstrap resampled in R using package pvclust (Suzuki and Shimodaira 2006). Bootstrapping was performed n = 10000 replications to calculate

approximately unbiased (AU) percentage values for each cluster and build a statistically supported consensus dendrogram. AU-values 95% and above indicate strong support for branches. Dendrograms were visualized in R using the base packages in combination with dendextend, dplyr, ggplot2, and gridExtra (R Core Team 2017; Tal Galili 2015; H Wickham et al. 2017; Hadley Wickham 2009; Baptiste Auguie 2016). Principal coordinates were calculated in QIIME 1 and illustrated in R using packages rKIN and ggplot2, respectively (S. Albeke 2017; Hadley Wickham 2009, 2). Package rKIN was also used to compute the percentage of overlap between 95% confidence ellipses in PCoA ordination. The species richness (alpha diversity) of samples was measured in QIIME 1 using both the Chao1 and observed OTUs metrics. The Chao1 index estimates the total number of distinct OTUs in a sample, while the observed OTUs metric measures the actual observed number of distinct OTUs per sample. Core microbiomes were computed using QIIME 1, and were defined as OTUs present in 50%, 75%, or 100% of samples within a grouping, allowing for comparisons at multiple percentage thresholds. Phylum- and genus-level relative abundances were extracted from OTU tables using QIIME 1. Phylum-level relative abundance plots were generated in R using a combination of dplyr, ggplot2, and gridExtra (H Wickham et al. 2017; Hadley Wickham 2009; Baptiste Auguie 2016).

1.1 Statistical analyses

Differences in UniFrac distances between sample groups were assessed using two permutational multivariate analysis of variance (PERMANOVA) approaches, adonis and anosim. These tests are incorporated into QIIME 1 from the vegan package in R (Jari Oksanen et al. 2017). Both tests exhibited similar results. Differential abundance testing of OTUs and taxa on age-matched WT and KO mice from each facility was carried out in QIIME 1 using the Kruskal-Wallis, nonparametric T, and g-tests on rarefied OTU tables. The outcome from each test was variable, therefore all results were reported for transparency. To assess differences in alpha diversity or relative abundance between sample groups, two non-parametric rank sums tests were employed, Kruskal-Wallis and Wilcoxon (Toutenburg 1975). Where applicable, Kruskal-Wallis was followed by Dunn's *post hoc* analysis for pairwise multiple comparisons using R packages stats and PMCMR, respectively (Pohlert T 2014; R Core Team 2017). R package reshape2 was used to construct Table S3 (Hadley Wickham 2007). Where applicable, the Wilcoxon test, also referred to as Mann-Whitney *U*, was followed by pairwise Wilcoxon comparisons between groups using R stats (R Core Team 2017). Both multiple comparisons tests used the FDR method to adjust *P*-values for false discovery rate. Pairwise comparisons were conducted for all possible pair combinations of all ages and all genotypes. These approaches yielded slightly different results. For transparency we chose to report the results from both statistical methods to ensure that conclusions were not made based upon a single statistical test.

1.1 Computational details

Computational analyses were performed using QIIME 1 version 1.9.1, R Version 2.4.3 "Kite-Eating Tree", R Studio version 1.1.383, conda version 4.5.0, Python version 3.5.5, running on macOS High Sierra version 10.13.4.

1.1 Availability of data and materials

The amplicon sequence datasets supporting the conclusions of this manuscript are available under the NCBI BioProject ID PRJNA418574. The QIIME 1 commands, OTU tables, accompanying metadata, R scripts and workspaces used for data processing, analysis, and

visualization are available in Data Sheet 2. File names, types, and descriptions for everything found in Data Sheet 2 are located on the last page of Data Sheet 1. All raw, filtered, and rarefied OTU tables, in addition to all commands, shell scripts, Jupyter notebooks, R scripts, and all other items generated and used during analysis are available in this project's GitHub repository located here: https://github.com/kdprkr/wards_wizard. Please contact the corresponding author if any item needed for analysis or generation of figures and tables cannot be found, or if any links are broken.

1 ABREVIATIONS

NGS: Next-generation sequencing. Ednrb: Endothelin receptor Type B. HSCR: Hirschsprung Disease. HAEC: Hirschsprung-associated enterocolitis. PERMANOVA: permutational multivariate analysis of variance. P07: postnatal day 7. P20: postnatal day 20. P24: postnatal day 24. KO: knock-out. WT: wild-type. OTUs: Operational Taxonomic Units. PCoA: principal coordinates analysis. PCR: polymerase chain reaction. IBD: Inflammatory bowel disease. PSC: primary sclerosing cholangitis. CD: Crohn's disease. QIIME 1: Quantitative Insights Into Microbial Ecology 1. UPGMA: unweighted pair group with arithmetic mean.

1 AUTHOR CONTRIBUTIONS

JG, AG, and NW acquired funding, designed the approaches used for the HSCR studies, and contributed to manuscript writing. KP designed the approaches for the C57BL/6J study, performed all Laramie-based experiments, coded all bioinformatic and statistical analyses, visualized and interpreted the data, and wrote the manuscript. SA was sensei for initial coding work, provided direction for statistical analysis, and contributed to manuscript writing.. JG provided guidance for initial mouse husbandry in Laramie, and contributed to manuscript writing. All authors read, reviewed, and approved the manuscript prior to submission.

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1 CONFLICTS OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships which could be construed as a potential conflict of interest.

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861 **FIGURE 1 | Colon samples from P20 HSCR-mice cluster by facility of origin.** Hierarchical
862 clustering of unweighted **(A, C)** and weighted **(B, D)** UniFrac distances for sequences obtained
863 from P20-WT and P20-KO colon samples. Shading emphasizes each facility's cluster.

864 **FIGURE 2 | Core microbiomes of P20 HSCR-mice separate by facility of origin, with no**
865 **overlap.** Principal Coordinates Analysis of weighted UniFrac distances for core microbiome
866 OTUs at the 50% threshold obtained from P20-WT and P20-KO colon and fecal samples.
867 Percentage values along each axis indicate the amount of variability in the data explained by
868 each of the first two principal coordinates. Ellipses indicate 95% confidence intervals.

FIGURE 3 | Phylum-level taxonomic composition of microbiomes from HSCR-mice exhibit marked differences in pattern between facilities. Mean relative abundances of four dominant phyla for samples grouped by facility, age, genotype, and sample type. All taxonomic groups representing < 6% of Bacterial sequences were grouped into 'Other.' Any taxonomic group unable to be assigned to Kingdom Bacteria was grouped into 'Unassigned.'

FIGURE 4 | Microbiome composition of C57BL/6J mice differs by facility. (A-D) Analysis of sequence data from C57BL/6J mouse fecal samples from Boston and Laramie. **(A)** Hierarchical clustering of unweighted UniFrac distances. **(B)** Hierarchical clustering of weighted UniFrac distances. **(C)** Principal Coordinates Analysis of weighted UniFrac distances for core microbiome OTUs at the 50% threshold. Percentage values along each axis indicate the amount of variability in the data explained by each of the first two principal coordinates. Ellipses indicate 95% confidence intervals. Percentage of ellipse overlap is indicated. **(D)** Mean relative abundances of three dominant phyla for samples grouped by facility. All taxonomic groups representing < 6% of Bacterial sequences were grouped into 'Other.' Any taxonomic group unable to be assigned to Kingdom Bacteria was grouped into 'Unassigned.'

TABLE 1 | Differentially abundant candidate OTUs and taxa conserved between facilities. ^ indicates OTUs or taxa observed at multiple ages. O: represents Order. F: represents Family. G: represents Genus. Log_2 Fold Change (FC) was calculated KO/WT, therefore (+) indicates association with KO mice and (-) indicates association with WT mice.

OTU-ID	Taxonomic Identity	Statistical Test	Sample Type	Association	Facility	Log_2FC
376516^	O: Clostridiales	G-Test	Colon	P20-WT	Boston	-4.5
					Laramie	-10
376516^	O: Clostridiales	G-Test	Colon	P24-WT	Boston	-10
					Laramie	-2.9
354957	O: Clostridiales	Kruskal-Wallis	Colon	P24-WT	Boston	-3.2
					Laramie	-10
549756	G: *Lactobacillus*	G-Test	Colon	P24-WT	Boston	-1.8
					Laramie	-10
351309	O: Clostridiales	G-Test	Colon	P24-KO	Boston	+3.8
					Laramie	+3.0
4407703	G: *Coprobacillus*	Kruskal-Wallis	Fecal	P24-KO	Boston	+5.5
					Laramie	+10
4407703	G: *Coprobacillus*	Kruskal-Wallis	Colon	P24-KO	Boston	+10
					Laramie	+3.8
n/a	F: S24-7	Nonparametric-T	Colon	P07-KO	Boston	+7.1
					Laramie	+1.8
n/a	F: Enterobacteriaceae	Kruskal-Wallis	Fecal	P24-KO	Boston	+3.2
					Laramie	+9.6
n/a	G: *Coprobacillus*	Kruskal-Wallis	Fecal	P24-KO	Boston	+5.6
					Laramie	+10
n/a	G: *Coprobacillus*	Nonparametric-T	Colon	P24-KO	Boston	+5.4
					Laramie	+2.2

Figure 1.TIFF

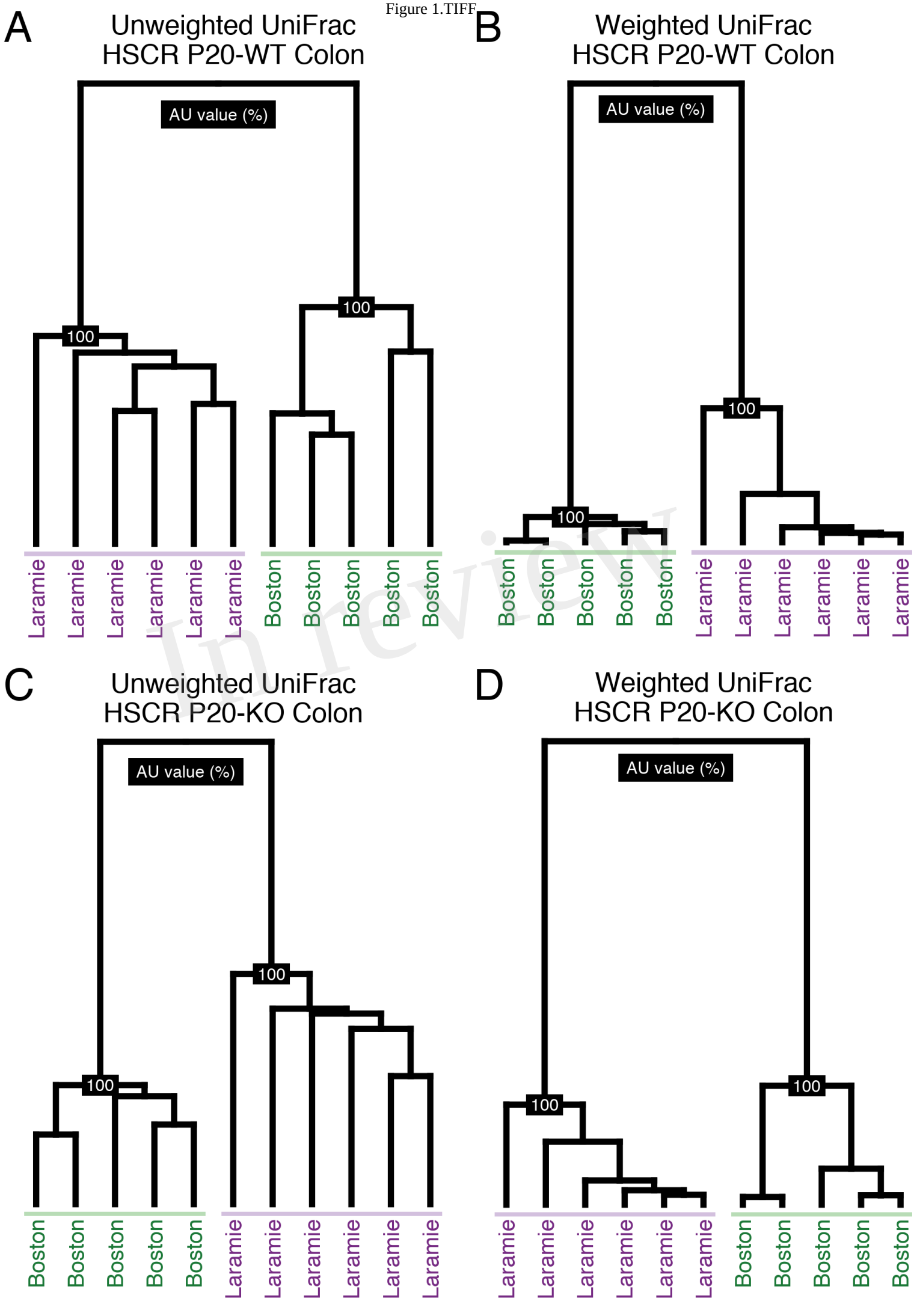

Figure 2.TIFF

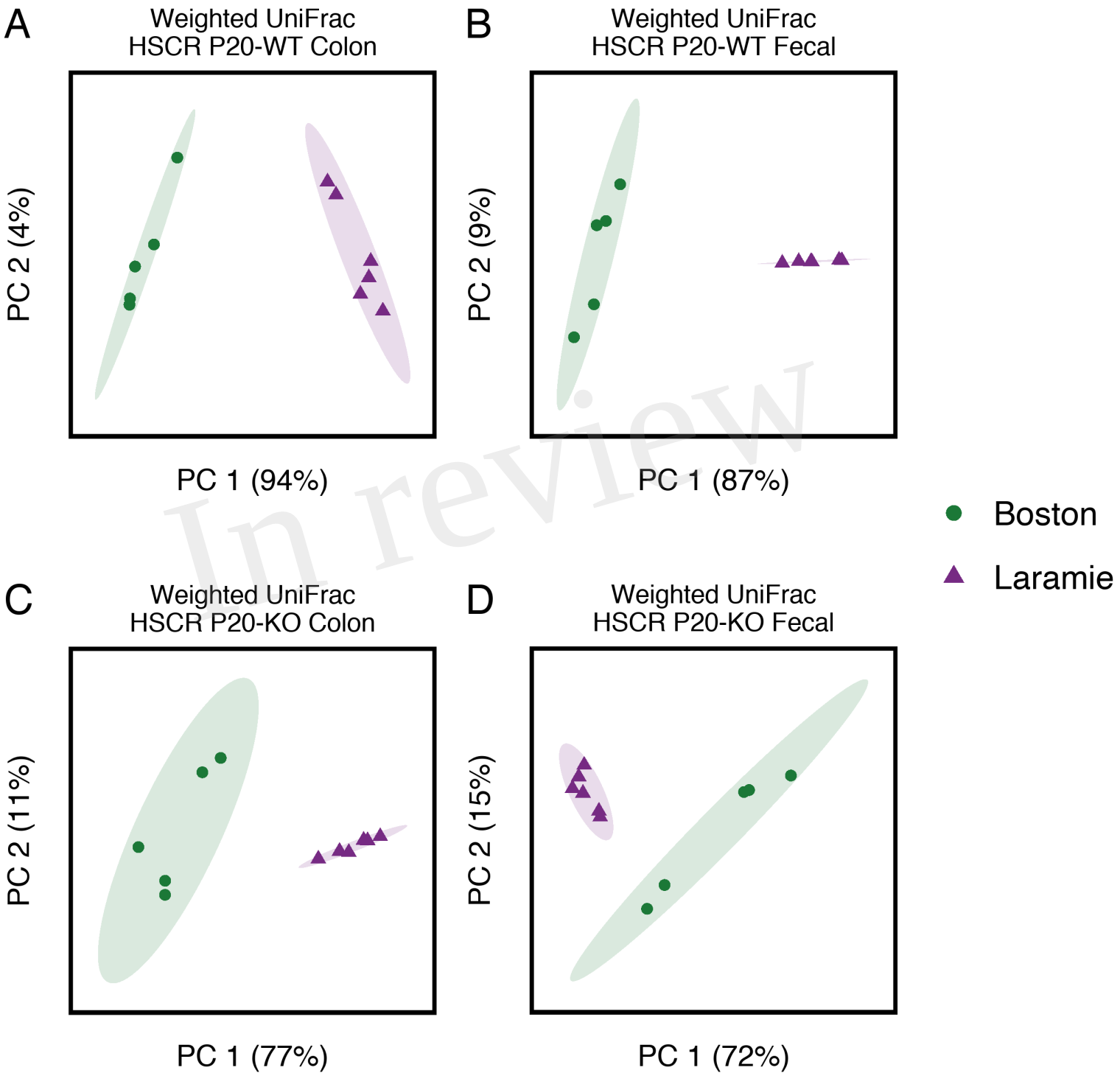

Figure 3.TIFF

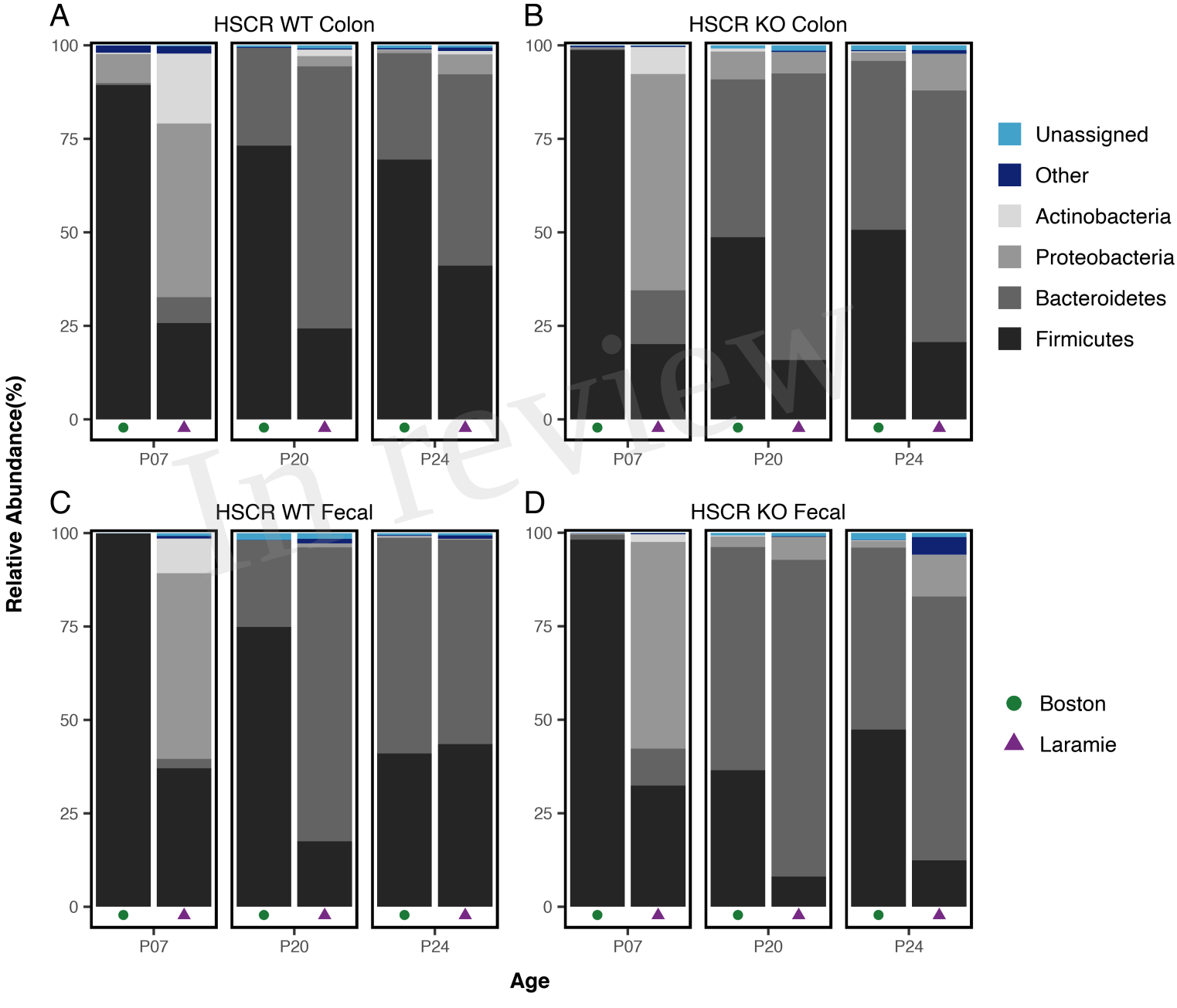

Figure 4.TIFF