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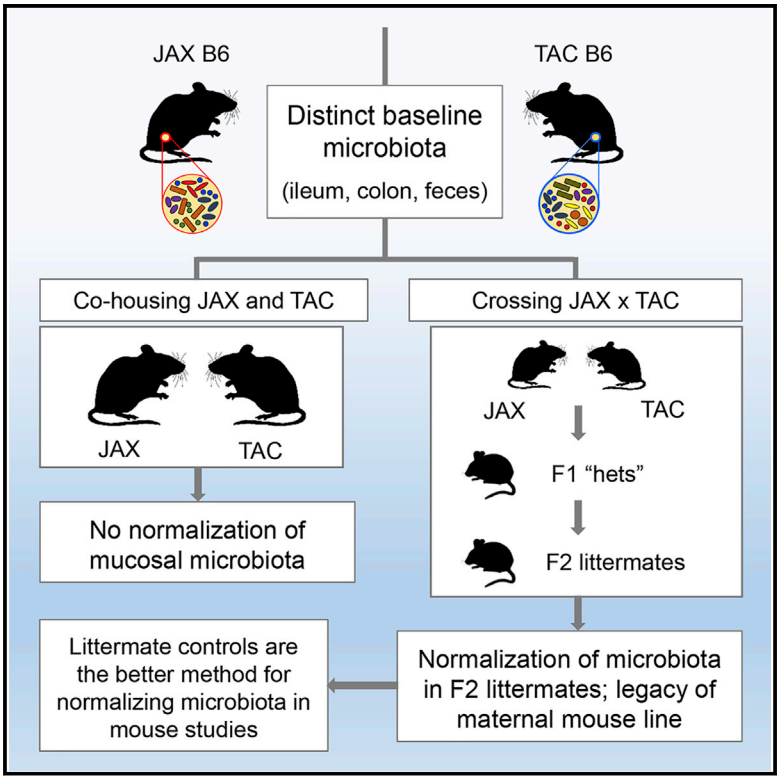
Comparison of Co-housing and Littermate Methods for Microbiota Standardization in Mouse Models

Graphical Abstract



Highlights

- Composition of the gut microbiota is a variable that can influence mouse phenotypes
- Techniques to standardize the microbiota include using littermate mice or co-housing
- The use of littermates from a heterozygous cross is optimal for standardization

Authors

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In Brief

Standardization of the microbiota in mouse models ensures reproducibility of findings and avoids erroneous conclusions. Here, Robertson et al. demonstrate that the use of F2-generation littermates is the superior method for standardization of the gut microbiota between experimental groups to minimize the influence of the microbiota in genotype-phenotype studies.

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Comparison of Co-housing and Littermate Methods for Microbiota Standardization in Mouse Models

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SUMMARY

The intestinal microbiota is a fundamental factor that broadly influences physiology. Thus, studies using transgenic animals should be designed to limit the confounding effects of microbiota variation between strains. Here, we report the impact on intestinal microbiota of co-housed versus F2-generation littermates, two commonly used techniques to standardize microbiota in animal models. Our results establish that while fecal microbiota is partially normalized by extended co-housing, mucosal communities associated with the proximal colon and terminal ileum remain stable and distinct. In contrast, strain inter-crossing to generate F2 littermates allows robust microbiota standardization in fecal, colon, and ileum sampling locations. Using reciprocal inter-crosses of P1 parents, we identify dissymmetry in F2 community structures caused by maternal transmission, in particular of the Verrucomicrobiaceae. Thus, F2 littermate animals from a unidirectional P1 cross should be used as a standard method to minimize the influence of the microbiota in genotype-phenotype studies.

INTRODUCTION

It is widely recognized that the intestinal microbiota plays an essential role in health and disease. Not surprisingly, some of the most fundamental functions of the intestinal microbiota relate to the regulation of intestinal functions, such as digestion of food and generation of various metabolites. In addition, gut microbes are important for the maturation and activity of the enteric immune, endocrine, and nervous systems (Cani and Knauf, 2016; Collins et al., 2012; McCoy et al., 2017; Yoo and Mazmanian, 2017). Shifts in bacterial community structure (i.e., composition and relative abundance of taxa), or dysbiosis, are associated with multiple metabolic and digestive diseases, such as inflammatory bowel disease, obesity, type 2 diabetes, and colorectal cancer (Bouter et al., 2017; Park et al., 2018; Sartor and Wu, 2017; Schroeder and Bäckhed, 2016). In addition, there is an increasing understanding that the intestinal microbiota influences physiology and health at sites distant from the intestine, as demonstrated by studies linking microbiota and cardiovascular diseases, neurodegeneration, or autism (Battson et al., 2018; Hsiao et al., 2013; Sharon et al., 2016).

The fundamental importance of the intestinal microbiota in normal physiological functions needs to be considered for the design and interpretation of experiments using animal models. Many *in vivo* studies use knockout (KO) mice to investigate the role of a specific gene in a defined experimental setting, which therefore requires comparison of two or more groups of mice (typically wild type [WT] and KO). It is widely recognized that independent lineages of animals, whether bred in the same facility or purchased from a vendor, display differences in community structure of the gut microbiota (Franklin and Ericsson, 2017); thus, differences in phenotypes observed in the KO mouse line compared to the WT line could be caused by differences in the microbiota rather than by gene mutation. To circumvent this problem, several techniques aiming to normalize microbiota between mouse groups have been developed.

The gold-standard method for standardizing the gut microbiota in experimental mice is use of F2-generation littermates from heterozygous crosses (Stappenbeck and Virgin, 2016), in which litters contain all possible genotypes and experience the same microbial and environmental exposures up to and following birth (Gomez de Agüero et al., 2016). Disadvantages of this method include excess production of heterozygotes, necessity for genotyping, and difficulty with generating adequate control mice in the case of the mutation of more than one gene (e.g., for double-KO mice). Another approach involves embryo transfers from the WT and KO strains into a foster dam, which can be effective if the microbiota is normalized across foster dams (e.g., using littermates), but involves coordinating timing and a potentially cost-limiting surgery. Cross-fostering of WT and KO pups at birth has also been proposed but requires complex logistics to achieve synchronous birth from both mouse lines, as well as the foster dam. Finally, an increasing number of studies use transient co-housing of mice from separate lines in the same cage to take advantage

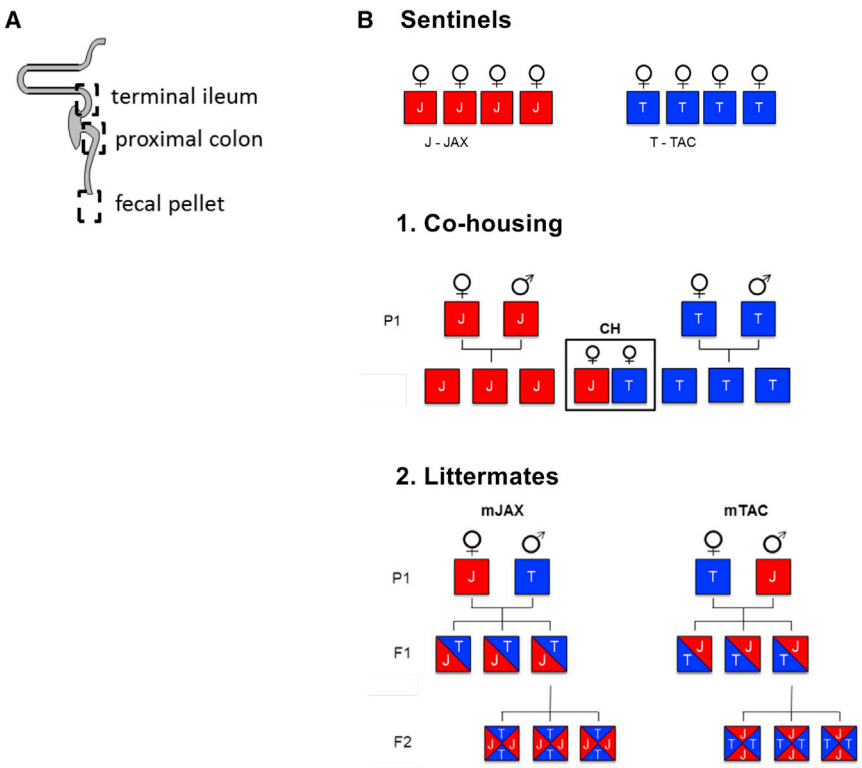

Figure 1. Gut Sampling Locations and Experimental Design

Mice were purchased from Jackson Laboratory (JAX) or Taconic Farms (TAC) and housed in our SPF facility for one week.

(A) Four female JAX mice and four female TAC mice were sacrificed, and samples were taken from the terminal ileum, proximal colon, and fecal pellet, as described in the STAR Methods. These mice served as sentinels for assessing microbial community structure in parental JAX and TAC mice at baseline (P1). For the co-housing experiment, female mice from JAX or TAC lines were age matched and co-housed (1:1) at weaning until eight weeks of age and then sampled. Non-co-housed male and female siblings served as comparators.

(B) For the littermate experiment, reciprocal crosses of the parental JAX and TAC mice generated separate maternal JAX (mJAX) and maternal TAC (mTAC) lineages. The F1 and F2 generations were analyzed compared to the parental lines.

of coprophagic and grooming behaviors for sharing microbes across co-caged individuals. This technique may be the simplest and most convenient method, because independent mouse lines can be bred separately and then the females can be co-caged once weaned. However, because there is no standardized method for co-housing in the literature, studies vary widely in terms of timing post-weaning or duration, which could influence the success of the normalization technique. Moreover, precise experimental characterization of the impact of co-housing on the mucosa-associated versus fecal microbiota is lacking.

Here, we performed a thorough analysis of microbiota standardization in co-housed mice compared to F2-generation littermates through analysis of bacterial community structure in the fecal pellet and mucosa of the proximal colon and terminal ileum. The analysis was performed on two lines of C57BL/6 mice, acquired from separate vendors, Jackson Laboratory and Taconic Farms, which had significantly distinct microbiotas. Our results establish that F2 littermates have greater homogeneity in microbiota than co-housed mice, even when co-housing is under the best possible conditions in terms of timing and duration of co-caging. Our analysis also identified varying patterns of inter-generational (vertical) transmission of taxa and specifically highlighted the importance of maternal transmission for *Akkermansia*, an important component of mucosal communities in healthy humans. Altogether, our results highlight the importance of using littermate controls to avoid confounding microbial influences on gene-driven phenotypes and misinterpretation of findings.

RESULTS

Baseline Microbiota Varies in the JAX and TAC P1 Sentinels

We initially purchased two groups of 7-week-old C57BL/6 mice from two vendors, Jackson Laboratory (JAX) and Taconic Farms (TAC), which were immediately transferred to our specific pathogen-free (SPF) facility and housed in the same room. After one week of acclimatization, four female sentinels from JAX and TAC (co-caged by vendor) were sacrificed for analysis of baseline bacterial community structure in the parental generation (P1) of JAX and TAC mice (Figure 1). Permutational analysis of variance (PERMANOVA) of the principal coordinates analysis (PCoA) plots (Figure 2A) generated from Bray-Curtis distance matrices demonstrated that the JAX and TAC lineages had significantly distinct microbiotas in all three sampled gut compartments: fecal pellet ($p = 0.029$, $R^2 = 0.800$), proximal colon mucosa ($p = 0.024$, $R^2 = 0.633$), and terminal ileum mucosa ($p = 0.022$, $R^2 = 0.617$).

The diversity of JAX and TAC communities in the P1 sentinel mice was relatively low, consisting of only ~28 bacterial families. The JAX and TAC lineages did not vary in terms of alpha (within community) diversity, as determined by number of operational taxonomic units (OTU richness), Shannon index (measure of OTU richness and evenness), and Simpson index (measure of OTU richness and abundance weighted toward the more dominant OTUs) on data rarified to 10,000 OTU (Figure 2B). Relative abundance plots (Figure 2C) show distinctive community structure associated with gut compartment, with the fecal pellet dominated by the S24-7 group of Bacteroidetes and the mucosal compartments dominated by Clostridales. Greater abundance of certain taxa tended to be more closely associated with the JAX lineage, including Verrucomicrobiaceae (*Akkermansia muciniphila*), Lactobacillaceae, Bifidobacteriaceae, and Rikenellaceae, while Deferribacteraceae and the Bacteroidetes families,

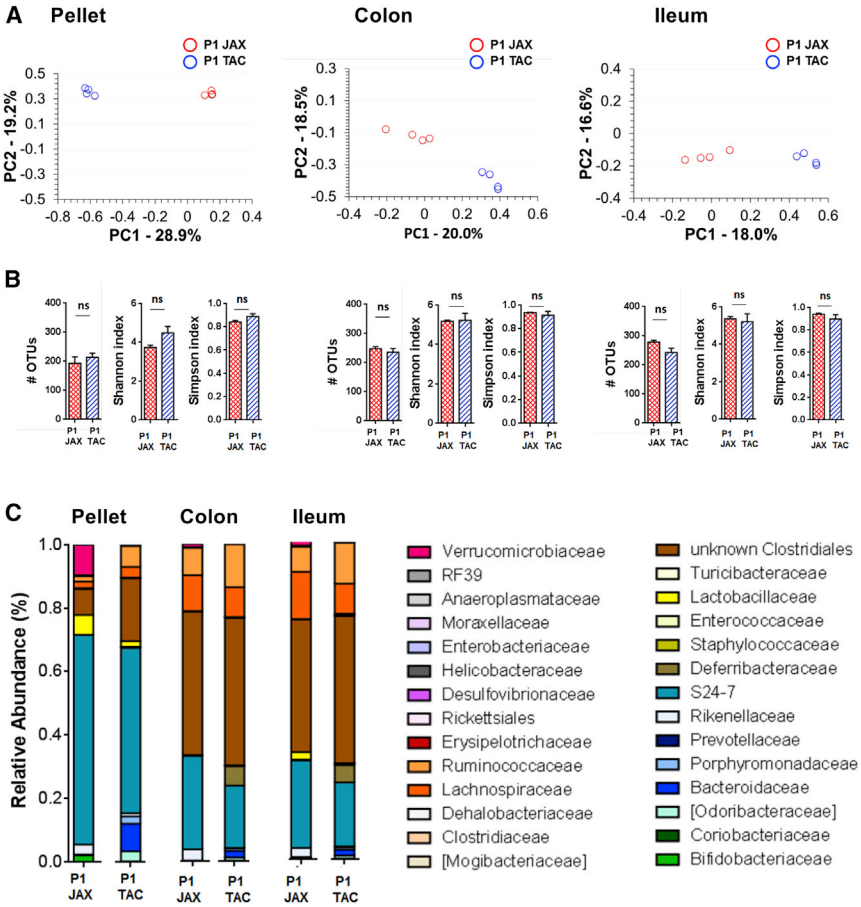

Figure 2. Distinct Baseline Microbiota in the JAX and TAC P1 Sentinels

(A) PCoA plots show significant differences in community structure of P1 sentinels in three gut locations.

(B) Alpha diversity measures (number of OTUs and Shannon and Simpson indices) did not vary between P1 sentinels.

(C) Relative abundance (family level) varied between P1 sentinels and along the length of the gut. Statistical significance and variance of Bray-Curtis dissimilarity data were assessed using PERMANOVA; alpha diversity data are represented as mean ± SEM, and statistical significance was assessed using t tests. n = 4 mice per group.

including Bacteroidaceae, Porphyromonadaceae, Prevotellaceae, and Odoribacteriaceae, tended to be more closely associated with the TAC lineage.

Co-housing Does Not Normalize Mucosal Bacterial Community Structure

Because there is no standard method for co-housing experimental mice, we chose a method that was expected to result in the greatest likelihood of success in terms of reducing variation in bacterial communities between co-housed groups. Specifically, we produced JAX and TAC mice from JAX x JAX and TAC x TAC crosses and co-housed age-matched females (1:1) in individual cages from weaning (3 weeks old) until sampling (8 weeks old); non-co-housed male and female siblings were analyzed as controls (Figure 1).

The PCoA plots from all gut locations show separate community clusters of the unrelated JAX and TAC lineages, with the co-housed mice forming mostly non-overlapping clusters between them (Figures 3A, 3F, and 3K). Differences in community structure of all clusters were significant (one-way PERMANOVA) in the fecal pellet ($p = 0.001$, $R^2 = 0.088$), proximal colon mucosa ($p = 0.001$, $R^2 = 0.146$), and terminal ileum mucosa ($p = 0.001$, $R^2 = 0.142$). There were no substantial cage or litter effects influencing clustering patterns of co-caged or control communities on the PCoA plots (Figure S1). Broad differences in relative abundance between co-housed JAX (JAX-CH) and co-housed TAC (TAC-CH) mice were most evident in pellet compared to mucosal communities (Figures 3C, 3H, and 3M), even though pellet communities appear to cluster less markedly (i.e., are more homogeneous) on PCoA plots. In general, co-housing increased OTU richness in the mouse lines (significant in all gut locations of TAC mice and in the pellet of JAX mice) compared to sibling controls, but there were few other differences in alpha diversity comparisons (Figure S2A). In the pellet, the Bray-Curtis distances between the JAX-CH and the JAX siblings and between the TAC-CH and the TAC siblings were significantly lower (indicating higher similarity) than comparison of the JAX-CH and TAC-CH groups (Figure S2B). In the colon and ileum, the Bray-Curtis distances were about equal between the co-housed groups and their separately caged sibling controls. There were no differences in beta dispersion (group variance) for any sampling location (data not shown). These data suggest that co-housing provides opportunities for some microbial mixing and community shifts toward co-housed partners, particularly in the fecal pellet compared to the mucosal sampling sites, but that early life exposure and parental influences on community structure are preserved.

Linear discriminant analysis (LDA) for effect size (LEfSe) was used for high-dimensional class comparisons between experimental groups. We first investigated which bacterial families or genera discriminated between JAX-CH and TAC-CH mice that were co-housed for five weeks from weaning (Figures 3B, 3G, and 3L; Table S1). Taxa that are more dominant in one group over the other may suggest an inability to transmit horizontally (via coprophagy or grooming) due to environmental constraints (e.g., O_2 exposure) or that the pre-weaning microbiota is somehow resistant to colonization by these taxa. In all three gut compartments, the JAX-CH mice had significantly more Verrucomicrobiaceae, Turicobacteriaceae, and Mogibacteriaceae (except ileum), than the TAC-CH mice. However, these were the only differentiating taxa in the fecal pellet

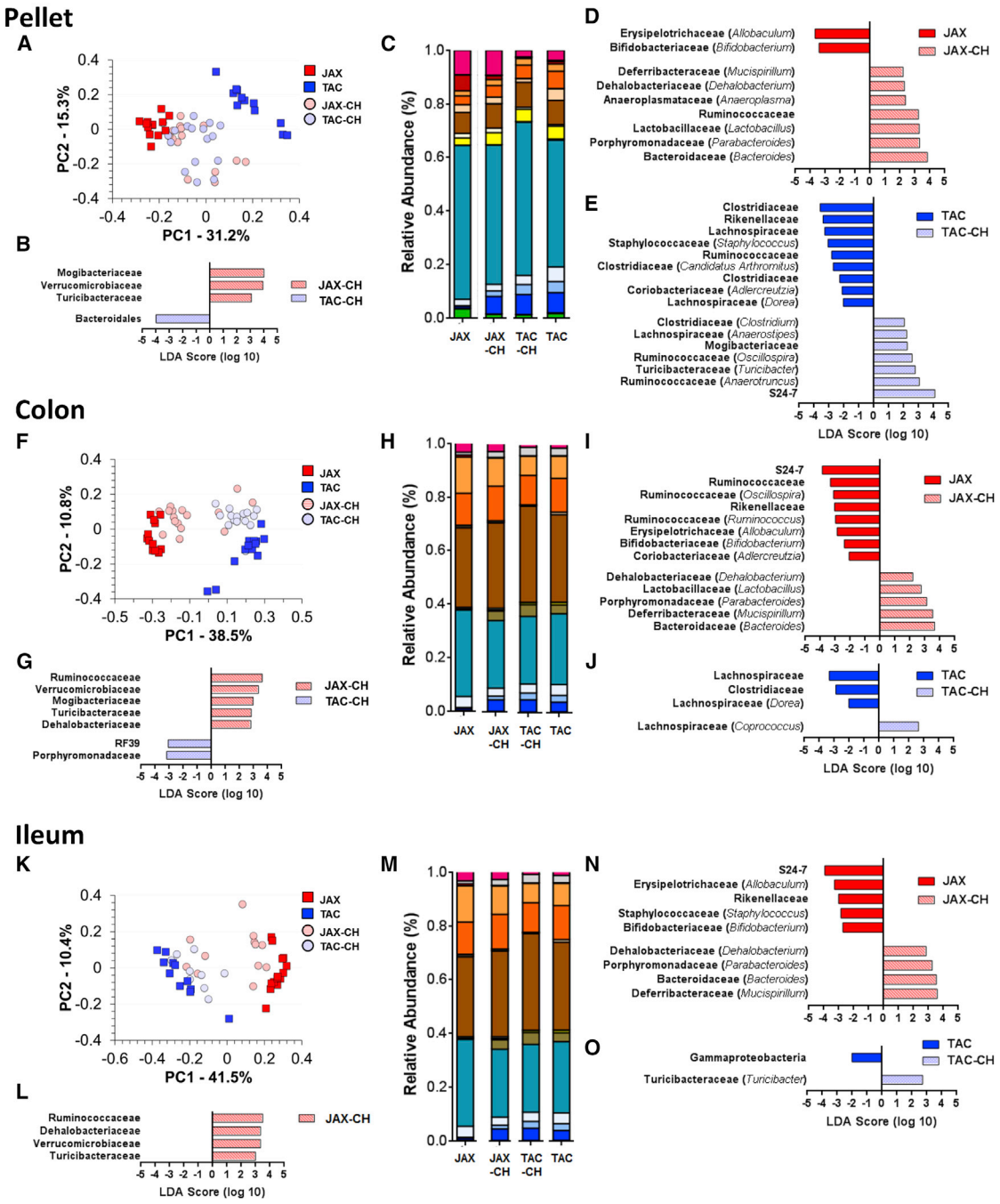

Figure 3. Co-housing Does Not Normalize Mucosal Bacterial Community Structure

(A–O) For each fecal pellet (A–E), proximal colon (F–J), and terminal ileum (K–O) gut location, Bray-Curtis PCoA (A, F, and K) and relative abundance (C, H, and M) plots show community variation in JAX-CH and TAC-CH mice compared to their non-co-housed siblings. LEfSe plots show differentiating taxa (family level) in JAX-CH and TAC-CH mice (B, G, and L). LEfSe plots also show discriminating taxa (genus level) in JAX-CH (D, I, and N) and TAC-CH (E, J, and O) mice and sibling controls. Fecal pellets showed more microbiota homogenization than either of the mucosal communities. Statistical significance and variance of Bray-Curtis dissimilarity data were assessed using one-way PERMANOVA. n = 16 (co-housed groups); n = 12 (control groups). See also Figures S1–S3 and Table S1.

(in addition to more Bacteroidaceae in the TAC-CH mice), suggesting a more homogeneous microbiota in JAX-CH and TAC-CH pellets compared to mucosal communities of the proximal colon and terminal ileum. The JAX-CH mice also had more Ruminococcaceae and Dehalobacteriaceae in the colonic and ileal communities, while Porphyromonadaceae and RF39 were more dominant in the colonic microbiota of the TAC-CH mice.

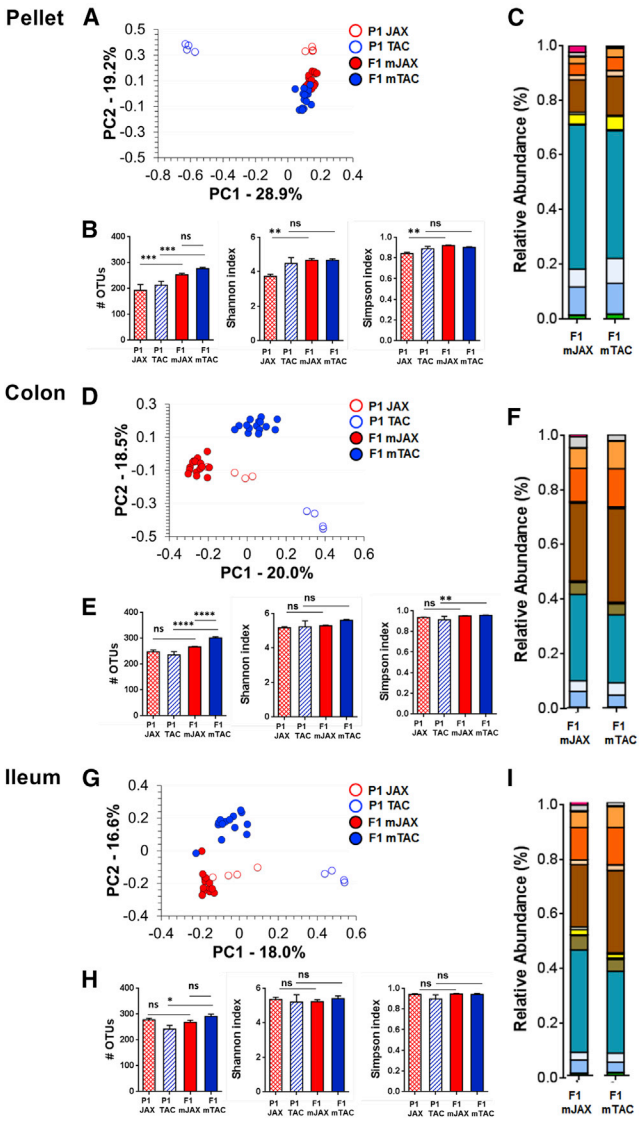

Figure 4. Microbiota of F1-Generation Mice from Reciprocal Crosses Shows Distinct Community Structure and Exhibits Little Variation within mJAX and mTAC Lines

(A–I) For each fecal pellet (A–C), proximal colon (D–F), and terminal ileum (G–I) gut location, Bray-Curtis PCoA plots show significant shifts in the microbiota from the P1 to F1 generation in each of the mJAX and mTAC lineages (A, D, and G). Alpha diversity based on OTU richness (number of OTUs) significantly increased from the P1 to the F1 generation in both the mJAX and the mTAC lineages, while Shannon and Simpson indices increased in the mJAX line only (B, E, and H). Family-level relative abundance (see legend in Figure 2) was similar in F1 mJAX and mTAC mice along the length of the gut (C, F, and I). Both sets of F1 microbiotas were more similar to the parental JAX microbiota. Statistical significance and variance of Bray-Curtis dissimilarity data were assessed using one-way PERMANOVA; alpha diversity data are represented as mean ± SEM, and statistical significance was assessed using one-way ANOVA with Sidak's multiple comparisons test. *$p < 0.05$, **$p < 0.01$. n = 4 (P1 groups); n = 15 (F1 groups). See also Figures S4 and S5.

We also investigated which taxa significantly contributed to microbiota differences between JAX-CH mice (Figures 3D, 3I, and 3N; Table S1) and TAC-CH mice (Figures 3E, 3J, and 3O; Table S1) and their non-co-housed sibling controls. Microbiota of JAX controls consistently had more *Allobaculum* and *Bifidobacterium* in all three gut compartments than their co-housed counterparts, while S24-7 and Rikenellaceae were more abundant in the mucosal communities of the colon and ileum. The JAX controls had more *Oscillospira*, *Ruminococcus*, and *Adlercreutzia* in the colon microbiota. The JAX-CH mice had comparatively more *Mucispirillum*, *Bacteroides*, *Parabacteroides*, *Dehalobacterium*, and *Lactobacillus* compared to their non-co-housed siblings, and this was consistent across sampling sites. Fewer taxa distinguished between the microbiota of co-housed and those of non-TAC-CH mice compared to the JAX groups. Most distinguishing taxa associated with both TAC-CH and TAC control groups were found in pellet communities and included genera within the Lachnospiraceae, Clostridiaceae, and Ruminococcaceae, as well as a few others. The S24-7 group had the greatest effect size in the TAC-CH mice. Taxa more abundant in the co-housed mice could have been acquired from the co-housing partner, or outgrowth may have been encouraged in its original host by other changes in the microbiome influenced by co-housing. Taxa more dominant in the control mice may have been outcompeted by horizontally acquired genera from the co-housing partner.

Finally, we used LEfSe analysis to compare the discriminating taxa of communities associated with the colonic mucosa (resident microbiota) to those associated with the pellet (more transient microbiota) in co-housed mice (Figure S3; Table S1). Many taxa were found in both JAX and TAC microbiota. There were more Clostridiaceae, Lachnospiraceae, Ruminococcaceae, Deferribacteraceae, and Anaeroplasmataceae in the microbiota of the colonic mucosa and more Coriobacteriaceae, Erysipelotrichaceae, Rikenellaceae, *Bifidobacterium*, *Lactobacillus*, *Akkermansia*, and S24-7 in pellet communities. Distinguishing taxa in the JAX colon included Dehalobacteriaceae and Enterococcaceae, while *Anaerostipes* and *Turicobacter* dominated in the pellet. The TAC pellet community had more RF39 and *Parabacteroides* than the mucosal community of the colon.

Microbiota of F1-Generation Littermates from Reciprocal Crosses Show Distinct Community Structure and Exhibit Little Variation within mJAX and mTAC Lineages

We performed reciprocal crosses of the parental JAX and TAC mice to produce two separate maternal JAX (mJAX) and maternal TAC (mTAC) lineages (Figure 1). These F1-generation mice showed significant shifts in microbiota from the P1 generation and clustered closely but distinctly on PCoA plots in each of the gut locations (Figures 4A, 4D, and 4G). Differences in community structure were significant (one-way PERMANOVA) for all groups in the fecal pellet ($p = 0.001$, $R^2 = 0.326$), proximal colon mucosa ($p = 0.001$, $R^2 = 0.426$), and terminal ileum mucosa ($p = 0.001$, $R^2 = 0.344$). Comparisons of Bray-Curtis distances showed that both F1-generation lineages clustered significantly closer to the P1 JAX (i.e., maternal lineage of mJAX and paternal lineage of mTAC) than the P1 TAC lineage (Figure S4), suggesting that the

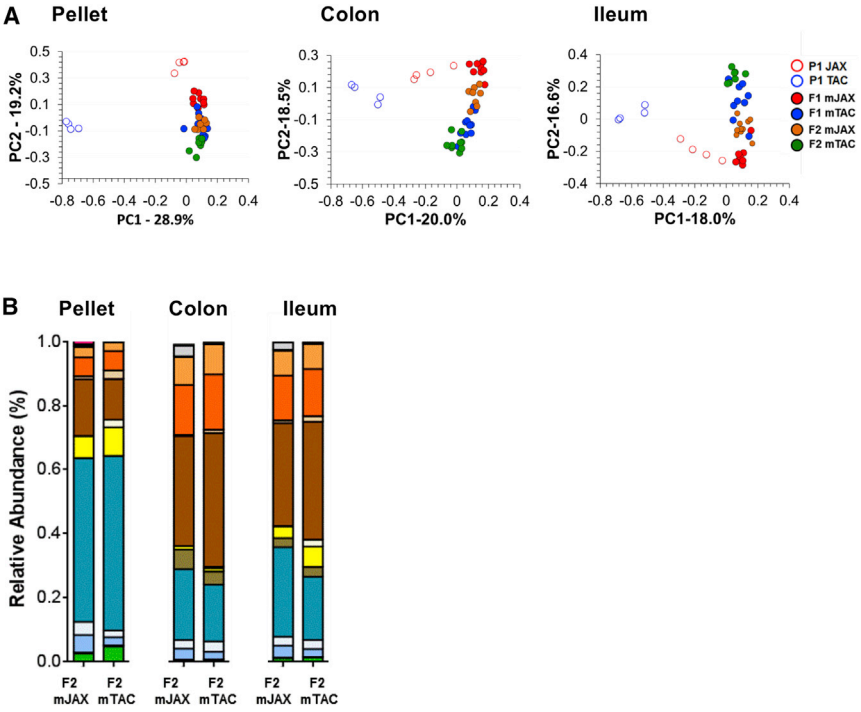

Figure 5. Microbiota of F2-Generation Littermates Does Not Vary from F1 Parents and Retains Legacy of the Original Maternal Cross

(A) For each gut compartment (pellet, colon, or ileum), PCoA plots show clustering of F2 littermates from both mJAX and mTAC with their F1 parental groups and with each other.

(B) Relative abundance was similar in the two lineages and retained similar family-level abundance to the original maternal cross.

Statistical significance and variance of Bray-Curtis dissimilarity data were assessed using one-way PERMANOVA. n = 4 (P1 groups); n = 15 (F1 groups); n = 8 (F2 groups). See also Figure S6.

microbiota of the JAX line exerted more influence on community structure than that of the TAC line. Significant effects of maternal lineage and litter number explained most variation in the microbiota of F1 mJAX and mTAC groups of mice, but sex of the mice and cage effect had no influence (Figure S5).

Alpha diversity, especially the number of OTUs, generally increased from the P1- to F1-generation microbiota in both the mJAX and the mTAC lineages (Figures 4B, 4E, and 4H). The similarities in relative abundance plots (Figures 4C, 4F, and 4I) for the F1-generation microbiota indicates that OTUs were acquired from both parents present in the cage and that competitive interactions between microbes shaped communities in similar ways. For example, both lineages of F1-generation mice had similar abundance of Lactobacillaceae, Bifidobacteriaceae, and Rikenellaceae (from the JAX line) and of Porphyromonadaceae and Deferribacteraceae (from the TAC line). In addition, both F1 lineages had greater abundance of Anaeroplasmataceae and Rikettsiales than either parental line. In contrast, the Bacteroidaceae and Prevotellaceae groups showed low abundance compared to the P1 TAC mice. The Verrucomicrobiaceae group was abundant only in the mJAX lineage of F1 mice, suggesting that it is primarily transmitted vertically from the dam.

Microbiota of F2-Generation Littermates Does Not Vary from F1 Parents and Retains Legacy of the Original Maternal Cross

The F2 littermates were produced from F1 x F1 crosses within each of the mJAX and mTAC lines. For each gut compartment, the PCoA plots show that F2-generation littermates from mJAX and mTAC lines cluster closely with their F1 parental groups and with each other (Figure 5A). Differences in community structure were significant (one-way PERMANOVA) between F2-generation mJAX and mTAC groups in the fecal pellet ($p = 0.001$, $R^2 = 0.166$), proximal colon mucosa ($p = 0.001$, $R^2 = 0.222$), and terminal ileum mucosa ($p = 0.001$, $R^2 = 0.197$). However, there were no differences in alpha diversity (data not shown) between groups, and family-level relative abundance was similar in both lineages (Figure 5B). The influence of maternal lineage on bacterial community structure was diminished compared to the F1-generation mice, but the legacy of the original cross was still visible (i.e., mJAX and mTAC communities still cluster distinctly from each other), particularly in the mucosal communities of the colon and ileum (Figure S6).

We explored various bacterial transmission patterns in the P1-F1 mice to gain some insights into the ecological niche of certain genera. Figure 6 shows examples of transmission patterns that were significant across all three gut compartments. *Akkermansia* was a more abundant component of the JAX microbiota compared to TAC that appeared to be primarily transmitted by the dam. *Akkermansia* was more abundant in the F1-generation mJAX (and F2 generation) than in the mTAC group, which only had exposure to this species via coprophagy or grooming (oral route), and these activities do not begin until several days after birth (Figure 6A). *Clostridium* was also more abundant in the P1 JAX mice but showed similar abundance in the F1 communities of both mJAX and mTAC lineages (Figure 6B). The P1 TAC mice had greater abundance of *Bacteroides* and *Parabacteroides* than the P1 JAX mice. However, while *Parabacteroides* established in relatively high abundance in the F1 progeny from both lineages (similar to the TAC parent), *Bacteroides* established in relatively low abundance (similar to the JAX parent), suggesting possible differences in competitive abilities of these taxa in this context (Figures 6C and 6D). Finally, segmented filamentous bacteria (SFB), the organism that first illuminated the issue of microbiota differences in JAX and TAC mice (Ivanov et al., 2009), was initially more abundant in TAC samples than in JAX samples and tended to retain this level of abundance in a greater proportion of F1 mice from the mTAC line compared to the mJAX line. However, high within-group variation clouds

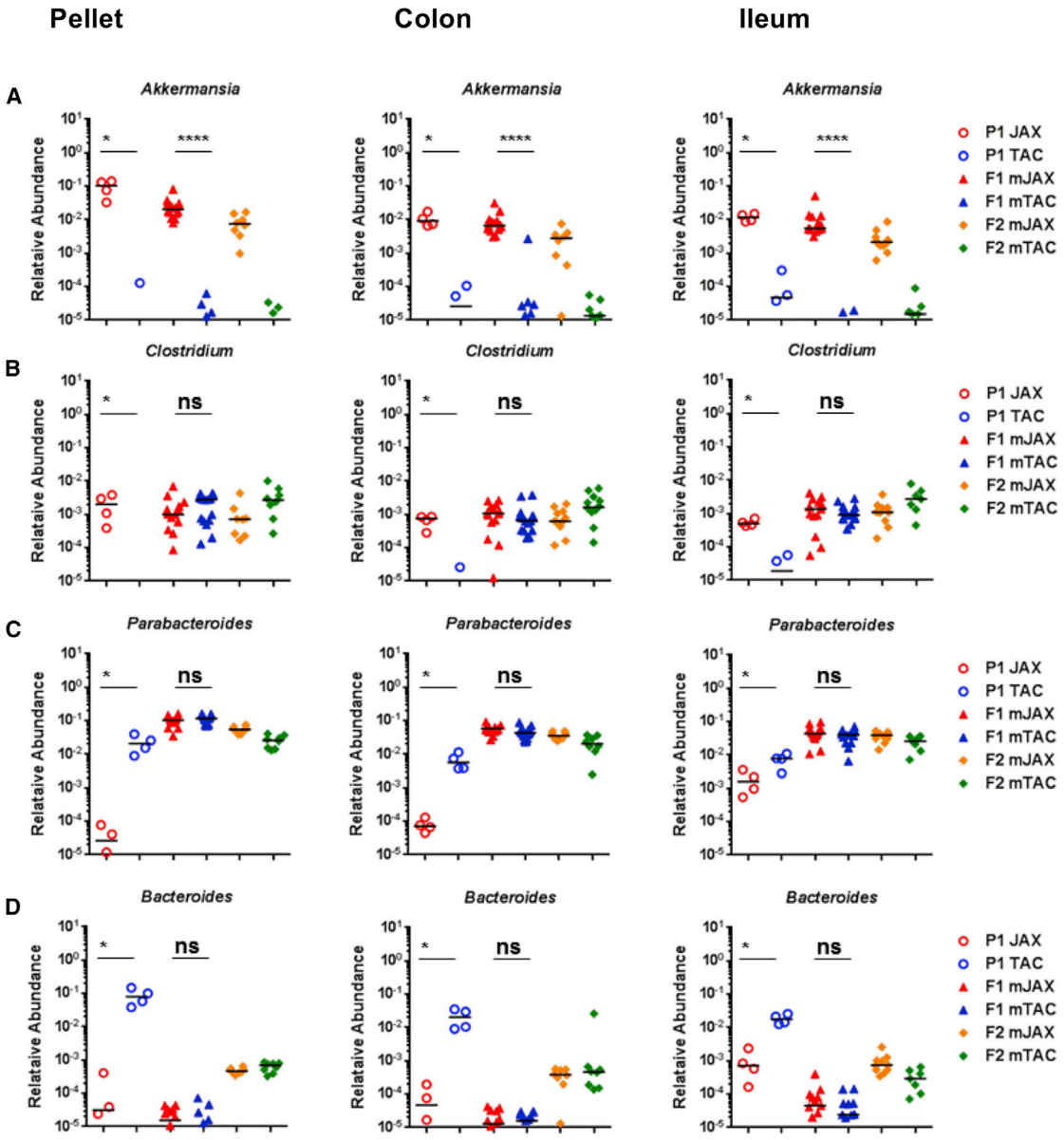

Figure 6. Variation in Bacterial Transmission Patterns from P1 to F1 and F2 Generations

(A) Different transmission patterns were observed among F1-generation mice across gut compartments. *Akkermansia* was abundant in the P1 JAX group and the F1 and F2 littermate offspring from mJAX compared to mTAC.

(B) *Clostridium* was more abundant in the P1 JAX group but had similar abundance in the F1 and F2 littermates of both mJAX and mTAC.

(C and D) *Parabacteroides* (C) and *Bacteroides* (D) were more abundant in P1 TAC mice compared to P1 JAX mice. Whereas *Parabacteroides* established relatively high abundance in the F1 and F2 littermate mice, similar to the P1 TAC microbiota, *Bacteroides* established relatively low abundance in the F1 and F2 progeny, similar to the P1 JAX microbiota.

Statistical significance was assessed using Kruskal-Wallis with Dunn's multiple comparisons test. *$p < 0.05$, ****$p < 0.0001$. n = 4 (P1 groups); n = 15 (F1 groups); n = 8 (F2 groups). See also Figure S7.

these observations (Figure S7). All other significant patterns of genera transmission are shown in Figure S7.

DISCUSSION

In this proof-of-concept study, we have demonstrated that variation in microbiota is negligible in F2-generation littermate mice generated from two C57BL/6 (WT) parental lineages, each with distinct normal microbiota. Conversely, co-housing mouse lines was ineffective in normalizing the microbiota, especially with respect to the resident mucosal microbiota of the terminal ileum and proximal colon compared to the potentially more transient communities of the fecal pellet. These findings provide experimental support for promoting the use of littermate controls as

the gold-standard method of standardizing the gut microbiota for isolating the contribution of genetics to phenotype in KO mouse models of disease (Stappenbeck and Virgin, 2016).

Co-housing of mice is frequently used as a means of standardizing the microbiota between lines of WT and KO animals (Stappenbeck and Virgin, 2016). The KO mouse lines are regularly exchanged among researchers and thus carry the microbial legacy of traveling within or between mouse colonies and acquiring environmental bacteria, whereas WT animals tend to be either purchased from vendors like JAX or TAC, with strict barriers against environmental exposures, or bred within in-house colonies. It is generally assumed that co-housing normalizes the microbiota between strains due to coprophagy and grooming behaviors (Ericsson and Franklin, 2015). However, the effectiveness of the method may depend on the timing and duration of co-housing, which vary across studies, because there is a limited window of time (about one week) post-weaning during which some niche space exists for environmentally acquired microbes before the microbiota matures and gains colonization resistance functions (Pantoja-Feliciano et al., 2013). Moreover, it may be necessary for several inoculation events to occur for some microbes to colonize a new niche. Here, we analyzed the microbiota of co-housed mice from two vendor lines, JAX and TAC, in which community structure was shown to be different at baseline. Pairs of JAX and TAC mice were co-housed in individual cages at weaning (i.e., 3 weeks old) and analyzed after five more weeks to optimally allow for community mixing. We found that the pellet communities of the co-housed mice were distinct from their respective non-co-caged siblings but tended to cluster together on PCoA plots despite some differences in relative abundance of taxa. However, the mucosal communities sampled from the ileum and colon showed less homogenization between the two co-caged lines and remained more similar to their non-co-caged siblings. These findings demonstrate that co-caging does not standardize the gut microbiota between mouse groups and emphasizes that the fecal pellet microbiota is a poor reflection of mucosal communities. Thus, the reliance on horizontal transmission via coprophagy for microbiota normalization might select for microbes that are more tolerant of the oxygenated environment and capable of overcoming colonization resistance of the resident microbiota in the recipient mouse. In addition, a study of multigenerational bacterial transmission modes in 17 inbred mouse lines showed that only some bacterial taxa tended to be exchanged horizontally between mouse lines, and these tended to be genera that include pathogenic members (Moeller et al., 2018).

Analysis of the taxa that differentiate between co-housed mouse groups indicate bacteria whose horizontal transmission may be limited by the environment or their ability to colonize the new host. Compared to their co-housed partners, the JAX-CH mice had more Verrucomicrobiaceae, Mogibacteriaceae, and Turicobacteriaceae in all three gut compartments, as well as Ruminococcaceae and Dehalobacteriaceae in the mucosal communities, while the TAC-CH mice had more Bacteroidaceae in the pellet and Porphyromonadaceae and RF39 in the colonic mucosa. Further analysis of taxa that discriminate between co-housed mice and their non-co-housed siblings identified bacteria that were acquired from the co-housing partner versus those retained from early life exposures. Most bacterial families that differentiated JAX-CH and TAC-CH mice also varied between co-housed and control siblings, indicating their acquisition from the co-housing partner, although not to the same level of abundance in the new host. However, although abundance of *A. muciniphila*, the only member of the Verrucomicrobiaceae family inhabiting the gut mucosa, was greater in the JAX strain, it did not vary between JAX-CH and sibling controls, or TAC-CH and controls, indicating unaffected abundance in JAX and negligible movement through the environment. Another study exploring the discriminating taxa between two colonies of non-obese diabetic mice following co-housing (of pregnant dams for two weeks pre-birth) or oral gavage of gut contents similarly identified *A. muciniphila* as a non-transferable species (Hänninen et al., 2018). *A. muciniphila* has been identified as an important biomarker of human disease (Everard et al., 2013; Png et al., 2010; Schneeberger et al., 2015). In mice, colonization is linked to increased expression of genes associated with immune responses (Derrien et al., 2011) and strengthened gut barrier functions (Everard et al., 2013) but also shown to drive enhanced intestinal inflammation in the background of Nlrp6 and interleukin-10 (IL-10) deficiency (Seregin et al., 2017). The inability to standardize abundance of *A. muciniphila* and other bacterial groups (e.g., members of Bacteroidaceae and Porphyromonadaceae) known to influence physiology and disease phenotypes in experimental mice is a compelling reason to consider using an alternate effective method to control the microbiota.

As a comparison to co-housing, we performed reciprocal crosses of the two strains of mice to generate maternal lines of either JAX or TAC origin and analyzed bacterial community structure from these lines. This cross generated two sets of F1 mice that would correspond to heterozygous mice from a study of WT versus KO animals. Each F1-generation line was then crossed to obtain F2 littermate mice that would correspond to experimental animals for direct comparison of WT and KO genotypes. Communities from three gut locations of the two sets of F1 mice clustered closely to, yet still distinct from, their parental clusters, as well as from each other. Microbiota from the F2-generation littermates also formed tight clusters close to their parental groups and did not significantly differ from each other in terms of family-level relative abundance or alpha diversity. These findings confirm that F1 (or heterozygous) crosses standardize the microbiota among F2 littermates. While several studies have compared the influence of genotype on community structure of the microbiota in various KO mouse models and reported no differences among F2 littermates (Lemire et al., 2017; Mamantopoulos et al., 2017; Robertson et al., 2013; Shanahan et al., 2014; Ubeda et al., 2012), this work compares the microbiota of two lines of F2 littermates with their F1 parents and grandparents, all on a WT background.

An interesting and unexpected finding was that the microbiota of F1 littermates from reciprocal crosses was not necessarily more similar to their respective maternal microbiota, demonstrating that taxa from both parents contribute to community composition and structure in progeny. While inheritance of the maternal microbiota has been shown between mother and child (Ferretti et al., 2018), maternal transfer cannot be

assumed in animal studies, because it may depend on whether the sire remains in the cage during pup rearing (as was the case in this study) and whether taxa are capable of horizontal transmission via coprophagy. Comparisons of bacterial abundance in P1 and F1 mice of each line identified varying patterns of inter-generational (vertical) transmission for certain genera. For most bacterial groups that varied significantly in abundance between the parental JAX and the parental TAC strains, abundance was normalized in the F1-generation (or F2-generation) gut samples. The exception was *Akkermansia*, which was significantly more abundant in the mJAX line across generations, thus confirming the importance of maternal transmission for Verrucomicrobiaceae. Abundance of some genera, such as *Clostridium* and *Parabacteroides*, established within the F1 microbiota at similar abundance to the parental group with the higher abundance (i.e., JAX and TAC, respectively), while other genera, such as *Bacteroides*, established at similar levels to the lower-abundance parent (i.e., JAX); these observations reveal something about the competitive ecology of bacterial groups, but this is beyond current understanding of microbial ecology of the gut. These analyses suggest that the most robust experimental design for F2 littermate studies should include unidirectional P1 crosses (i.e., consistently using one strain as the maternal line and the other as the paternal line) to avoid a grandparent effect. Moreover, *a priori* knowledge of community structure in each strain in the original cross would be useful to rationally determine which strain should be the maternal line, because F2 littermates from reciprocal crosses may retain the legacy of maternal lineage.

In conclusion, our results establish that F2 littermates have greater homogeneity in microbiota than co-housed mice, even when co-housing is under the best possible conditions in terms of timing and duration of co-caging. These findings suggest that some components of the microbiota established in early life are difficult to displace by microbes introduced after weaning and some bacteria are limited in their ability to transfer via the external environment. Overall, these results highlight the importance of using littermate controls to avoid confounding microbial influences on gene-driven phenotypes and misinterpretation of findings in mouse models.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2019.04.023.

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AUTHOR CONTRIBUTIONS

Conceptualization, S.J.R., P.L., S.E.G., and D.J.P.; Methodology & Investigation, P.L. and S.J.R.; Formal Analysis, H.M., S.J.R., W.T., and L.B.; Writing – Original Draft, S.J.R., S.E.G., and D.J.P.; Writing – Review & Editing, S.J.R., P.L., H.M., A.G., S.E.G., and D.J.P.; Funding Acquisition, S.E.G. and D.J.P.; Supervision, D.S.G., K.C., S.E.G., and D.J.P.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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REFERENCES

Battson, M.L., Lee, D.M., Weir, T.L., and Gentile, C.L. (2018). The gut microbiota as a novel regulator of cardiovascular function and disease. J. Nutr. Biochem. *56*, 1–15.

Bokulich, N.A., Subramanian, S., Faith, J.J., Gevers, D., Gordon, J.I., Knight, R., Mills, D.A., and Caporaso, J.G. (2013). Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. Nat. Methods *10*, 57–59.

Bouter, K.E., van Raalte, D.H., Groen, A.K., and Nieuwdorp, M. (2017). Role of the Gut Microbiome in the Pathogenesis of Obesity and Obesity-Related Metabolic Dysfunction. Gastroenterology *152*, 1671–1678.

Cani, P.D., and Knauf, C. (2016). How gut microbes talk to organs: The role of endocrine and nervous routes. Mol. Metab. *5*, 743–752.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. Nat. Methods *7*, 335–336.

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Huntley, J., Fierer, N., Owens, S.M., Betley, J., Fraser, L., Bauer, M., et al. (2012). Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. ISME J. *6*, 1621–1624.

Collins, S.M., Surette, M., and Bercik, P. (2012). The interplay between the intestinal microbiota and the brain. Nat. Rev. Microbiol. *10*, 735–742.

Derrien, M., Van Baarlen, P., Hooiveld, G., Norin, E., Müller, M., and de Vos, W.M. (2011). Modulation of Mucosal Immune Response, Tolerance, and Proliferation in Mice Colonized by the Mucin-Degrader *Akkermansia muciniphila*. Front. Microbiol. *2*, 166.

Edgar, R.C. (2010). Search and clustering orders of magnitude faster than BLAST. Bioinformatics *26*, 2460–2461.

Edgar, R.C. (2013). UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat. Methods *10*, 996–998.

Ericsson, A.C., and Franklin, C.L. (2015). Manipulating the Gut Microbiota: Methods and Challenges. ILAR J. *56*, 205–217.

Everard, A., Belzer, C., Geurts, L., Ouwerkerk, J.P., Druart, C., Bindels, L.B., Guiot, Y., Derrien, M., Muccioli, G.G., Delzenne, N.M., et al. (2013). Cross-talk between *Akkermansia muciniphila* and intestinal epithelium controls diet-induced obesity. Proc. Natl. Acad. Sci. USA *110*, 9066–9071.

Ferretti, P., Pasolli, E., Tett, A., Asnicar, F., Gorfer, V., Fedi, S., Armanini, F., Truong, D.T., Manara, S., Zolfo, M., et al. (2018). Mother-to-infant microbial transmission from different body sites shapes the developing infant gut microbiome. Cell Host Microbe *24*, 133–145.

Franklin, C.L., and Ericsson, A.C. (2017). Microbiota and reproducibility of rodent models. Lab Anim. (NY) *46*, 114–122.

Gomez de Agüero, M., Ganal-Vonarburg, S.C., Fuhrer, T., Rupp, S., Uchimura, Y., Li, H., Steinert, A., Heikenwalder, M., Hapfelmeier, S., Sauer, U., et al. (2016). The maternal microbiota drives early postnatal innate immune development. Science *351*, 1296–1302.

Hänninen, A., Toivonen, R., Pöysti, S., Belzer, C., Plovier, H., Ouwerkerk, J.P., Emani, R., Cani, P.D., and De Vos, W.M. (2018). *Akkermansia muciniphila* induces gut microbiota remodelling and controls islet autoimmunity in NOD mice. Gut *67*, 1445–1453.

Hsiao, E.Y., McBride, S.W., Hsien, S., Sharon, G., Hyde, E.R., McCue, T., Codelli, J.A., Chow, J., Reisman, S.E., Petrosino, J.F., et al. (2013). Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. Cell *155*, 1451–1463.

Ivanov, I.I., Atarashi, K., Manel, N., Brodie, E.L., Shima, T., Karaoz, U., Wei, D., Goldfarb, K.C., Santee, C.A., Lynch, S.V., et al. (2009). Induction of intestinal Th17 cells by segmented filamentous bacteria. Cell *139*, 485–498.

Lemire, P., Robertson, S.J., Maughan, H., Tattoli, I., Streutker, C.J., Platnich, J.M., Muruve, D.A., Philpott, D.J., and Girardin, S.E. (2017). The NLR Protein NLRP6 Does Not Impact Gut Microbiota Composition. Cell Rep. *21*, 3653–3661.

Magurran, A.E. (2004). Measuring Biological Diversity (Wiley).

Mamantopoulos, M., Ronchi, F., Van Hauwermeiren, F., Vieira-Silva, S., Yilmaz, B., Martens, L., Saeys, Y., Drexler, S.K., Yazdi, A.S., Raes, J., et al. (2017). Nlrp6- and ASC-Dependent Inflammasomes Do Not Shape the Commensal Gut Microbiota Composition. Immunity *47*, 339–348.

McCoy, K.D., Ronchi, F., and Geuking, M.B. (2017). Host-microbiota interactions and adaptive immunity. Immunol. Rev. *279*, 63–69.

Moeller, A.H., Suzuki, T.A., Phifer-Rixey, M., and Nachman, M.W. (2018). Transmission modes of the mammalian gut microbiota. Science *362*, 453–457.

Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., et al. (2017). vegan: Community Ecology Package. R package version 2.4-2 (R Foundation for Statistical Computing).

Pantoja-Feliciano, I.G., Clemente, J.C., Costello, E.K., Perez, M.E., Blaser, M.J., Knight, R., and Dominguez-Bello, M.G. (2013). Biphasic assembly of the murine intestinal microbiota during early development. ISME J. *7*, 1112–1115.

Park, C.H., Eun, C.S., and Han, D.S. (2018). Intestinal microbiota, chronic inflammation, and colorectal cancer. Intest. Res. *16*, 338–345.

Png, C.W., Lindén, S.K., Gilshenan, K.S., Zoetendal, E.G., McSweeney, C.S., Sly, L.I., McGuckin, M.A., and Florin, T.H. (2010). Mucolytic bacteria with increased prevalence in IBD mucosa augment *in vitro* utilization of mucin by other bacteria. Am. J. Gastroenterol. *105*, 2420–2428.

Robertson, S.J., Zhou, J.Y., Geddes, K., Rubino, S.J., Cho, J.H., Girardin, S.E., and Philpott, D.J. (2013). Nod1 and Nod2 signaling does not alter the composition of intestinal bacterial communities at homeostasis. Gut Microbes *4*, 222–231.

Sartor, R.B., and Wu, G.D. (2017). Roles for intestinal bacteria, viruses, and fungi in pathogenesis of inflammatory bowel diseases and therapeutic approaches. Gastroenterology *152*, 327–339.

Schneeberger, M., Everard, A., Gómez-Valadés, A.G., Matamoros, S., Ramírez, S., Delzenne, N.M., Gomis, R., Claret, M., and Cani, P.D. (2015). *Akkermansia muciniphila* inversely correlates with the onset of inflammation, altered adipose tissue metabolism and metabolic disorders during obesity in mice. Sci. Rep. *5*, 16643.

Schroeder, B.O., and Bäckhed, F. (2016). Signals from the gut microbiota to distant organs in physiology and disease. Nat. Med. *22*, 1079–1089.

Segata, N., and Huttenhower, C. (2011). Toward an efficient method of identifying core genes for evolutionary and functional microbial phylogenies. PLoS ONE *6*, e24704.

Seregin, S.S., Golovchenko, N., Schaf, B., Chen, J., Pudlo, N.A., Mitchell, J., Baxter, N.T., Zhao, L., Schloss, P.D., Martens, E.C., et al. (2017). NLRP6 Protects $Il10^{-/-}$ Mice from Colitis by Limiting Colonization of *Akkermansia muciniphila*. Cell Rep. *19*, 2174.

Shanahan, M.T., Carroll, I.M., Grossniklaus, E., White, A., von Furstenberg, R.J., Barner, R., Fodor, A.A., Henning, S.J., Sartor, R.B., and Gulati, A.S. (2014). Mouse Paneth cell antimicrobial function is independent of Nod2. Gut *63*, 903–910.

Sharon, G., Sampson, T.R., Geschwind, D.H., and Mazmanian, S.K. (2016). The Central Nervous System and the Gut Microbiome. Cell *167*, 915–932.

Stappenbeck, T.S., and Virgin, H.W. (2016). Accounting for reciprocal host-microbiome interactions in experimental science. Nature *534*, 191–199.

Ubeda, C., Lipuma, L., Gobourne, A., Viale, A., Leiner, I., Equinda, M., Khanin, R., and Pamer, E.G. (2012). Familial transmission rather than defective innate immunity shapes the distinct intestinal microbiota of TLR-deficient mice. J. Exp. Med. *209*, 1445–1456.

Yoo, B.B., and Mazmanian, S.K. (2017). The Enteric Network: Interactions between the Immune and Nervous Systems of the Gut. Immunity *46*, 910–926.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical Commercial Assays		
MoBio PowerSoil kit	MoBio	Cat. #12888-100
Deposited Data		
Raw data	This paper	European Nucleotide Archive - study number PRJEB28381 (https://www.ebi.ac.uk/ena)
Experimental Models: Organisms/Strains		
Mouse: C57BL/6J	The Jackson Laboratory	Stock: 000664 JAXWEST:RB04
Mouse: C57BL/6N	The Taconic Farms	Lot: 20151005-IBU001502C-HC
Oligonucleotides		
Universal Forward: 5′-AGAGTTTGATCCTGGCTCAG-3′	Caporaso et al., 2012	N/A
Reverse: 5′-GACGGGCGGTGWGTRCA-3′	Caporaso et al., 2012	N/A
Software and Algorithms		
Quantitative Insights Into Microbial Ecology (QIIME) software (v1.9.1-20150604)	Caporaso et al., 2010	http://qiime.org/
UPARSE	Edgar, 2010, 2013	http://www.drive5.com/usearch/
GraphPad Prism 6	GraphPad Software Inc	http://www.graphpad.com
R software (v3.4.1) with R package vegan (v2.4-4)	R Foundation	https://www.r-project.org/https://cran.r-project.org/web/packages/vegan/index.html
Other		
Illumina MiSeq	Illumina	N/A

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for reagents should be directed to and will be fulfilled by the Lead Contact, Dana Philpott (dana.philpott@utoronto.ca).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Ethics statement

Experiments involving animals were performed according to guidelines and policies of the animal care program from the Division of Comparative Medicine (DCM) of the University of Toronto. The protocol was approved by the University of Toronto Committee on Use and Care of Animals (Protocol #20011441 to D.J.P.).

Mouse strains and husbandry

Wild-type mice were purchased (cage-mates) at seven weeks of age from Jackson Laboratory (C57/Bl6J) and Taconic Farms (C57/Bl6N) and were housed and bred under specific pathogen-free (SPF) conditions in at the Division of Comparative Medicine (DCM) of the University of Toronto. All mice were kept in filter-top cages that were changed bi-monthly by research personnel with free access to commercial pelleted food and water (non-acidified) *ad libitum*. Mice were housed under standardized conditions with regulated daylight (12 hour light-dark cycle), humidity, and temperature and were monitored daily for signs of any obvious physical stress and behavioral changes, and euthanized if found in distress. Both male and female mice were used in the study and all mice were sampled at eight weeks of age. Mice purchased from vendors were given at least one week to acclimate to the DCM prior to experimentation.

METHOD DETAILS

Study design and sampling

The parental (P1) mice included two breeding pairs plus an additional four ‘‘sentinel’’ females from each vendor (Jackson and Taconic). One breeding pair from each vendor was used to continue the JAX and TAC mouse lines (for the co-housing experiment)

while the other breeding pair was split to establish two reciprocal mouse lines, with dams from each line (for the littermate experiment). The sentinel mice (n = 4 co-caged female mice per group) were sampled at eight weeks of age to establish the baseline microbiota from each lineage. For the co-housing analysis, pairs of age-matched F1-generation JAX and TAC females were co-housed in 1:1 ratios in single cages (n = 16) for five weeks from weaning, with non-co-housed siblings (n = 12) serving as controls. Sibling controls were mostly males (JAX: 10M +2F; TAC: 9M+3F) and were co-caged with 1-3 of their siblings of the same sex. In all, mice from 6 litters and 3-4 breeding pairs from JAX or TAC parents were matched and used for the co-housing experiment. For the littermate analysis, reciprocal crosses of JAX and TAC parents (i.e., maternal (m)JAX and mTAC lineages) were established to produce ''heterozygous'' F1 mice (n = 15 per maternal group). F2-generation littermates (n = 8 per maternal group) were subsequently produced from F1 crosses. Fecal pellets were sampled along with mucosal communities (collected after three successive rounds of vigorous washing in PBS) from 1-cm lengths of terminal ileum and proximal colon (Lemire et al., 2017). All samples were stored at −80°C until processing. The littermate and co-housing experiments were performed concurrently; all samples were analyzed together so that data could be directly compared.

16S rRNA gene sequencing and OTU assembly

Total DNA was extracted from samples using the MoBio PowerSoil kit (MoBio) following manufacturer's instructions for increased yield. Samples from the littermate and co-housing experiments were submitted for sequence analysis together on the same plates. The V4 hypervariable region of the 16S rRNA gene was amplified using a universal forward sequencing primer and a uniquely barcoded reverse sequencing primer to allow for multiplexing (Caporaso et al., 2012) and sequenced at the Centre for Analysis of Genome Evolution and Function (CAGEF) at the University of Toronto. Triplicate amplifications were quality checked on a 1% agarose TBE gel and then pooled at approximately even concentrations to reduce amplification bias. The purified library was quantified and loaded on to the Illumina MiSeq for sequencing using the V2 (150 bp paired end) chemistry, according to manufacturer instructions (Illumina, San Diego, CA).

Fastq sequence reads were prepared using UPARSE (Edgar, 2010, 2013). Paired reads were joined and then trimmed for quality using –fastq_mergepairs and –fastq_filter, with a –fastq_maxee set at 0.5, respectively. Merged sequences shorter than 225 base pairs were not retained. Qiime (v1.9.1-20150604) (Caporaso et al., 2010) was then used to cluster fasta sequences into operational taxonomic units using open-reference OTU picking. OTUs with relative abundances below 0.005% were excluded from the analysis (Bokulich et al., 2013). OTU tables were rarefied to 10,674 sequences, the lowest number of sequences obtained for a sample.

QUANTIFICATION AND STATISTICAL ANALYSIS

Bacterial community analysis

Rarefied OTU tables were used to determine relative abundances of taxa at multiple hierarchical levels and to calculate alpha and beta diversity in QIIME (Caporaso et al., 2010); these tables were also used to perform linear discriminant analysis of effect size (LEfSe) (Segata and Huttenhower, 2011). Alpha (within-community) diversity was calculated using a variety of metrics that account for different aspects of richness and abundance. Measures of diversity included number of operational taxonomic units (OTUs) for richness; the Shannon diversity index, which combines OTU richness and abundance into a single evenness value that is higher in communities where abundance is distributed equally among taxa; and the Simpson's diversity index, which further weighs abundance of the more dominant taxa (Magurran, 2004). Beta (between-community) diversity was calculated from Bray-Curtis dissimilarity matrices and visualized using Principal Coordinates Analysis (PCoA) plots. Beta diversity was also calculated using weighted and unweighted UniFrac, Gower and Canberra methods, which all gave the same conclusions (data not shown). Bray-Curtis distances and beta-dispersion (data not shown) were compared to assess variation within and between groups.

Statistical analysis

GraphPad Prism 6 software was used to plot abundance data and determine statistical significance (alpha = 0.05) using parametric and non-parametric tests, as appropriate. For multivariate data, R software (v3.4.1 with R package vegan v2.4-4) was used to produce principal coordinate analysis (PCoA) plots from the Bray-Curtis distance matrix and p values and variance (R^2) were determined using Adonis (permutational analysis of variance, PERMANOVA) and confirmed using Anosim (analysis of similarity) methods (Oksanen et al., 2017).

DATA AND SOFTWARE AVAILABILITY

The accession number for the sequence data reported in this paper is European Nucleotide Archive study number PRJEB28381.