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Exploring the Prenatal Microbiome in *Mus*

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by

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ABSTRACT OF THE DISSERTATION

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Sarah Ann Gardner

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The developmental environment, including the microbiome, can strongly influence offspring phenotypes. Investigating how microbes interact with developing offspring is the first step in understanding how the prenatal microbiome impacts physiological, immunological, and behavioral phenotypes in adults. In eutherian mammals, the current general consensus is that the placenta does not have a stable microbial community, and that the first microbial inoculation of embryos occurs during birth as offspring move through the vaginal tract. The main critique of early studies of the prenatal microbiome was centered around the high likelihood of contamination in the low-biomass samples. My dissertation addresses if and how species identity and embryonic genotype affect the prenatal microbes in two different mouse systems: first, with a hybrid cross between the house mouse (*Mus musculus domesticus*) and its sympatric congener, the Algerian mouse (*Mus spretus*), and second within *M. m. domesticus*, using two wild-derived strains that have maintained population-specific microbiomes after multiple generations of lab-

rearing. Finally, in my last chapter, I utilized multiple methods to determine if live microbes are present in the gut of developing embryos. I found that there was no difference between embryonic guts or placental microbial communities based on embryonic genotype after contaminant removal. However, I observed an effect of embryonic genotype on beta diversity in embryonic guts and placenta in chapter two. Surprisingly, I also found an effect of embryonic genotype on the microbial community composition of maternal guts. Additionally, chapter two investigated which maternal source was the most likely contributor to the embryonic gut microbial community, including the oral cavity, blood, gut, and vaginal tract. Similar to previous reports, my data support the idea that microbes pass through the maternal gut to the circulatory system and, ultimately, from maternal blood flow via the placenta to the embryonic gut. In chapter three, I did not find any evidence of live microbes when attempting to quantify short chain fatty acids (SCFAs), the main products of anaerobic microbes in the adult gut. Despite a lack of evidence of SCFAs in the embryonic gut, prenatal exposure to microbial DNA may still affect embryonic development.

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Introduction

All eukaryotic organisms have evolved in the presence of microbes, with some forming close symbioses important for the normal development and survival of the host (McFall Ngai et al. 2013). The microbiota refers to the complex ecological community of commensal, symbiotic, and pathogenic microorganisms that regulate diverse host processes. The collective genome of this community, and often the community itself, is referred to as the microbiome. The community composition of the microbiome can be shaped by both genetic and environmental factors, including diet (reviewed in Fischbach and Sonnenburg 2011) and interactions with other individuals within the same population (Knowles et al. 2019); however, the extent to which one factor contributes to shaping the community over another varies across contexts and taxa. Although the community composition of the microbiome is subject to shift over the course of an organism's lifetime (Yatsuneko et al. 2012), the foundational microbiome has long-lasting physiological and behavioral effects on the host. In addition, the mechanism by which the microbiome is vertically transmitted (passed down from mother to offspring) and when this initial transfer occurs varies across taxa. Finding when the initial transfer occurs is critical to understanding the potential for offspring phenotypes to be indelibly shaped by the microbiome.

The presence of a prenatal microbiome in humans has been a subject of debate for over a century. The idea that humans develop in a sterile *in utero* environment was first introduced by Theodor Escherich in the late 1800s based on his discovery that the meconium (a newborn's first postpartum bowel movement derived from swallowed

amniotic fluid during gestation) did not contain bacteria (Escherich 1889). This finding was supported by French obstetrician, Henry Tissier, who proposed the sterile womb paradigm in 1900, which states that the developmental environment in humans is sterile and that newborns are first inoculated with commensal microbes during birth when passing through the vaginal tract (Tissier 1900).

More recent evidence in humans suggests that microbes are present during gestation in the placenta (Aagaard et al. 2014), amniotic fluid (Collado et al. 2016), and meconium (Jimenez et al. 2008; Stinson, Keelan, and Payne 2019). However, the presence of microbes in the human placenta remains particularly contentious and there is currently no support for a stable placental microbial community throughout pregnancy (Bushman 2019; de Goffau et al. 2019; Theis et al. 2019). Instead, the placenta may act as a transfer point for microbes from mothers to offspring. There is now robust support for prenatal inoculation in mice and humans (Younge et al. 2019), and evidence that microbes in the fetal gut derive from multiple maternal sources.

The initial colonization of the microbiome is important, as this occurs during a critical period of development for offspring that has long-term impacts on fitness. In humans, perturbations to the early-life microbiome (for example, antibiotic treatments during pregnancy or as a neonate) can impact metabolic (Ajslev et al. 2011) and immune function (Gonzalez-Perez et al. 2016; reviewed in Koleva et al. 2015). The microbiome also has a high potential to affect behavior, as the microbiome and the central nervous system are intertwined in early-life development (reviewed in Sylvia and Demas 2018). Together, these individual effects indicate that the developmental origins of the

microbiome and life-long host phenotypes are linked; however, these relationships are not fully understood, in part because of the lack of attention to the prenatal microbiome as a functional community.

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CHAPTER 1

Characterizing the prenatal microbiome in mouse (*Mus*) hybrids

Abstract

The gut microbiome was historically thought to establish during parturition in eutherian mammals, however evidence collected over the past decade suggests that non-pathogenic microbes first interact with embryos during gestation. It is an open question as to what determines which microbes that are able to establish in prenatal tissues (the placenta and embryonic gut), but embryonic genotype may play a role. Moreover, the effect of host genotype on microbial community composition scales to the species level, however the extent to which this is true for the prenatal microbiome is unknown. To test the effect of species identity on the prenatal, we crossed two closely related mouse species, *Mus musculus domesticus* (the house mouse) and *Mus spretus* (the Algerian mouse), in which hybrid placentas are undersized when *M. m. domesticus* is the maternal species. We present two hypotheses for the community composition the hybrid prenatal microbiome: 1) because the microbiome is vertically transferred from mothers to offspring, hybrid and *M. m. domesticus* placentae and embryonic guts will show similar community compositions that are distinct from *M. spretus* or 2) if there is an effect of species identity on the prenatal microbiome, then hybrids will be distinct from both parental species. *M. m. domesticus* (n = 10, 1/sex/5 litters), *M. spretus* (n = 10, 1/sex/5 litters), and hybrid (n = 9, 1/sex/5 litters) placentas and embryonic guts were collected at approximately embryonic day 17.5, a time point in late pregnancy. All tissues were stored in RNAlater in -20°C until DNA extractions. Microbial DNA was then extracted from each sample and sequenced (V4 region of the 16S rRNA gene). The effect of species identity on microbial diversity and abundance in the prenatal microbiome were analyzed using

ANOVA for alpha diversity and PERMANOVA for beta diversity. We found evidence of microbes in the placenta and embryonic guts. Whereas the adult gut microbiota of *M. m. domesticus* and *M. spretus* were distinct, species identity did not affect alpha or beta diversity in placental or embryonic gut samples. Given the life-long impact of the gut microbiome on health and disease, if and how microbes in the prenatal environment affect postnatal phenotypes is an important area for future work.

1. Introduction

All eukaryotic organisms evolve in the presence of microbes, with some forming close symbioses critical for development and survival of the host (McFall-Ngai et al. 2013). In sexually reproducing animals, the foundational gut microbiome (the first microbes to colonize the gut) is vertically transferred from mothers to offspring; between-species divergence in microbial community composition is established early in an offspring's life and maintained through various factors, including host genetics (Moeller et al. 2019; Suzuki et al. 2019) and diet (Daniel et al. 2014; Org et al. 2015). Although the community composition of the microbiome is subject to change over the course of an organism's lifetime (Yatsunenko et al. 2012; Schloss et al. 2012), the foundational microbiome has long-lasting physiological and behavioral effects on the host (Donald and Finlay 2023; Arrieta et al. 2014).

In eutherian mammals, the microbiome was historically thought to first establish during parturition and grow in complexity over the course of the postnatal period (Tissier 1900; Koenig et al. 2011). However, evidence collected over the past decade suggests that non-pathogenic microbes first interact with embryos during gestation in humans and other eutherian mammals (reviewed in Funkhouser and Bordenstein 2013; Perez-Muñoz et al. 2017). This trend is also seen in vertebrate egg-laying taxa (e.g. Trevelline et al. 2018, Dietz et al. 2019) where microbes are transmitted to offspring before the eggs are laid. The initial establishment of the microbiome occurs during a critical period of development for offspring that has long-term impacts on offspring fitness, which has been demonstrated through studies of germ-free animals or the application of antibiotics

during pregnancy. Germ-free mice (mice that lack internal microbiota and are maintained in a microbe-free environment) have reduced ability to fight infection throughout their life (Hapfelmeier et al. 2010) and show reduced anxiety-like behaviors modulated by abnormalities in the hypothalamic-pituitary-adrenal axis (HPA axis) and altered expression of serotonin receptors (Neufeld et al. 2011). In humans, perturbations to the early-life microbiome (for example, antibiotic treatments during pregnancy or as a neonate) can impact metabolic (Ajslev et al. 2011) and immune function (Gonzalez-Perez et al. 2016; reviewed in Koleva et al. 2015).

While evidence supporting microbial presence in prenatal systems continues to grow, multiple studies report no evidence of microbial communities in human placental tissue (Leiby et al. 2018, Theis et al. 2019). Taken together, these contrasting results suggest that the placenta does not harbor a unique group of metabolically active microbes, but instead serves as a route for maternal microbes to inoculate the embryonic gut (Collado et al. 2016). It is unclear what determines which microbes are able to pass through the placenta to establish in the embryonic gut. However, because the placenta is largely derived from the embryo, embryonic genotype may play a role.

The variance of microbiome composition explained by host genetics differs across studies in both mice and humans, however the contribution of host genotype is consistently significant (Goodrich et al. 2014; Org et al. 2015; Suzuki et al. 2019). Host genetics can directly impact the identity and relative abundance of microbes inhabiting the gut (Benson et al. 2010), and indirectly shape community composition by modulating the immune response (Suzuki et al. 2019). The effect of host genotype on microbial

community composition scales to the species level. For example, two species of hydra (*Hydra oligactis* and *Hydra vulgaris*) maintained as lab stocks with identical growth conditions and diets for nearly 30 years showed distinct differences in microbiota composition, as did wild-caught individuals of the same species (Fraune and Bosch 2007). Moeller et al. (2018) found that population differences in the gut microbiota composition of wild-caught house mice were retained after 10 generations in the same lab environment. In wild populations, the gut microbiota of co-occurring small mammals were more similar between conspecifics from different collection sites than between congeners from the same site (Knowles et al. 2019). Thus, species identity can supersede environmental factors in determining adult microbial community composition. However, the extent to which this is true for the mammalian prenatal microbiome is unknown.

To test the effect of species identity on the prenatal microbiome, two closely related mouse species were crossed: the house mouse (*Mus musculus domesticus*) and the Algerian mouse (*Mus spretus*). In this cross, hybrid placentas are undersized when *M. m. domesticus* is the maternal species (Zechner et al. 1996). We focus solely on hybrid offspring produced in this direction of the cross. In addition, hybrid placentas show abnormalities in cellular organization and gene expression compared to both parental species (Zechner et al. 1996; Arévalo and Campbell 2020; Arévalo et al. 2021); these abnormalities may cause deficits in microbial transfer across the placenta. We made two alternative predictions for the comparison between the community composition of the hybrid prenatal microbiome (collectively, the placenta and embryonic gut) and the within species prenatal microbiome. 1) The hybrid prenatal microbiome and the *M. m.*

domesticus prenatal microbiome will have similar community compositions that are distinct from the *M. spretus* prenatal microbiome, indicating that the composition of the prenatal microbiome is driven by vertical transmission from mothers to offspring or 2) the hybrid prenatal community composition will be distinct from both parental species, indicating an effect of offspring genotype on the composition of the prenatal microbiome. Given the abnormal structure of hybrid placentas in this cross, we predicted that the difference in hybrid placental microbes, if present, would manifest as reduced overall abundance in both the placenta and embryonic gut compared to the within species crosses.

2. Methods

2. 1 Animals

Wild-derived inbred mice were maintained on a 12-hour light/dark cycle with lights on at 9:00am. All mice were provided with *ad libitum* food (LabDiet 5001 Rodent Diet) and water. *M. m. domesticus* was represented by the WSB/EiJ strain (Jackson Laboratory) and *M. spretus* was represented by the SFM/Pas strain (Montpellier Wild Mice Genetic Repository). Three crosses were conducted in this experiment (maternal species listed first): *M. m. domesticus* x *M. m. domesticus*, *M. m. domesticus* x *M. spretus* (hybrid cross), and *M. spretus* x *M. spretus*. Mice were paired for approximately 48 hours (two full nights, split the morning of the second day). The second night was considered embryonic day 0. *M. m. domesticus* (n = 10, 1/sex/5 litters), *M. spretus* (n = 10, 1/sex/5 litters), and hybrid (n = 9, 1/sex/4 litters plus one litter of only females) embryos and placentas were collected at approximately embryonic day 17.5, a time point in late pregnancy when the placenta is fully developed (Theiler 1989). Total sample size for each tissue is included in Table 1. Crosses and tissue collection took place at Oklahoma State University (OSU), 2016-2018.

The gut microbiomes of both parental species were tested for the retention of species differences when maintained in a standard lab environment. Previous research has shown that population differences between wild-caught mice persist in captivity for more than 10 generations (Moeller et al. 2018) and we wanted to confirm this was the case with the two species used in the hybrid cross. Non-pregnant adult female *M. spretus* cecal samples were collected after the colony had been moved from OSU to the University of

California Riverside (UCR) in 2019. Adult female *M. m. domesticus* guts were collected from the Good Lab mouse colony at the University of Montana. All animal procedures were approved by the IACUCs at OSU and UCR under protocols AS-1-41 and 20180069, respectively.

2.2 Microbial DNA extraction

All tissues were stored in RNAlater at -20°C until extraction. Prior to extraction, embryonic gut and placental samples were treated with 20µL Proteinase K and 100µL 10% SDS solution for 60 minutes in order to lyse tissue and maximize the microbial DNA that could be extracted. Following tissue lysing, samples were transferred to bead beating tubes and vortexed for an additional 20 minutes. DNA extractions were conducted using ZymoBIOMICS DNA Miniprep Kit (Zymo Research) according to manufacturer's instructions. One negative control sample collected in tandem with DNA extraction was included for placental samples, and 5 negative controls were included for embryonic gut samples. An additional 2 negative controls were collected in tandem with adult gut DNA extractions.

2.3 16s rRNA Gene Sequencing

Amplicon production and sequencing was done at Novogene (Sacramento, CA). Briefly, the V4 region of the 16S rRNA gene was amplified for all samples using the 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') primers. Libraries were produced with the Illumina TruSeq DNA PCR-Free Library Preparation Kit (Illumina, USA) and 250bp paired-end reads were generated on the Illumina NovaSeq 6000 platform. Primer sequences were trimmed from reads and reads

were removed if they were less than 60bp, contained 10% or more uncertain nucleotides, or if low quality nucleotides made up more than 50% of the read.

2.4 Data Processing and Analysis

All sequences were processed in QIIME2 (version 2019.7; Bolyen et al. 2019). Raw sequences were joined and denoised using the deblur pipeline (Amir et al. 2017). Taxonomic classification was conducted using the SILVA 132 database (Quast et al. 2013, Yilmaz et al. 2014) and trained using the feature-classifier tool (Bokulich et al. 2018). Sequences were aligned using MAFFT (Katoh et al. 2002) and subsequently generated into a midpoint-rooted tree with FastTree (Price et al. 2010). Representative sequences from deblur, taxonomic identification from MAFFT, and phylogenetic tree from FastTree were exported from QIIME2 for further analysis in R (version 4.2.3, "Shortstop Beagle").

QIIME2 artifacts were imported into R using the qiime2R package (version 0.99.6; Bisanz 2018) and analyzed using the phyloseq (version 1.41.1; McMurdie and Holmes 2013), microViz (version 0.10.0; Barnett et al. 2021) and microbiome (version 1.20.0; Lahti et al. 2017) packages. Contaminants in negative controls were identified using the decontam package in R (version 1.18.0; Davis 2017) and were removed from samples. Additionally, any sequences identified as mitochondria, chloroplast, or unidentified were removed from samples. Singletons were filtered and removed if one read was present in only one sample.

Differences in alpha diversity (diversity within individuals) between genotypes were analyzed with Analysis of Variance (ANOVA) models with both litter genotype and

maternal genotype as fixed effects. The effect of genotype on community composition was visualized with non-metric multidimensional scaling (NMDS) using Bray-Curtis dissimilarities for adult gut samples and Weighted and Unweighted UniFrac distances for embryonic samples. Bray-Curtis dissimilarities measure differences in community composition weighted by the abundances of operational taxonomic units (OTUs). Unweighted UniFrac measures phylogenetic distances between communities based solely on OTU presence/absence, whereas weighted UniFrac weights branch length with OTU abundance. Differences in community composition between genotypes were evaluated with a permutational multivariate analysis of variance (PERMANOVA) with litter genotype and maternal genotype as fixed effects.

3. Results

3.1 Adult guts show species-level differences in microbial community

To test for the maintenance of species differences in gut microbial community composition, non-pregnant adult female *M. spretus* (n = 5) and *M. m. domesticus* (n = 5) cecal samples were sequenced and processed. The two species' gut microbiomes differed in all evaluated metrics: species richness (Figure 1a; ANOVA: $F = 6.91$, $p = 0.0302$), Shannon diversity (Figure 1b; ANOVA: $F = 23.23$, $p = 0.0132$), and beta diversity using Bray-Curtis dissimilarities (Figure 1c; PERMANOVA: $p = 0.0078$; NMDS stress = 0.047).

3.2 No effect of species or genotype on placental and embryonic gut microbiota

Placental samples (*M. m. domesticus* n = 10, *M. spretus* n = 10, hybrid n = 9) did not differ in species richness (Figure 2a; ANOVA: $F = 1.817$, $p = 0.183$) or Shannon diversity (Figure 2b; ANOVA: $F = 0.648$, $p = 0.532$) based on litter genotype. Similarly, embryonic guts (*M. m. domesticus* n = 10, *M. spretus* n = 10, hybrid n = 9) did not differ in species richness (Figure 3a; ANOVA: $F = 1.931$, $p = 0.166$) or Shannon diversity (Figure 3b; ANOVA: $F = 0.006$, $p = 0.994$) based on litter genotype. Using both weighted and unweighted UniFrac distances, there was no effect of genotype on placental microbiota (Figure 2c and 2d; UniFrac PERMANOVA: $F = 1.305$, $p = 0.1498$, NMDS stress = 0.0923; weighted UniFrac PERMANOVA: $F = 1.262$, $p = 0.195$, NMDS stress = 0.113) or on embryonic gut microbiota (Figure 3c and 3d; UniFrac PERMANOVA: $F = 0.976$, $p = 0.541$, NMDS stress = 0.0764; weighted UniFrac PERMANOVA: $F = 0.596$, $p = 0.752$, NMDS stress = 0.235).

When comparing the community composition of the placental and embryonic gut microbiomes, the two tissues differed in both species richness (Figure 4a; ANOVA: $F = 34.41$, $p < 0.001$) and Shannon diversity (Figure 4b; ANOVA: $F = 97.41$, $p < 0.001$). Additionally, using weighted UniFrac distances, the placental and embryonic gut microbiomes were significantly different in beta diversity (Figure 4c; PERMANOVA: $F = 50.42$, $p < 0.001$, NMDS stress = 0.0464). The top ten taxa present in the embryonic gut and placenta are listed in Table 2. Of the 20 taxa in the placenta and embryonic gut, 13 were identified as likely contaminants as they are typically found in environments other than the gut or vaginal tract microbiome.

Offspring sex did not have an effect on either species richness (ANOVA: $F = 0.005$, $p = 0.944$), or Shannon diversity (ANOVA: $F = 0.421$, $p = 0.522$) in the placenta. Offspring sex also did not affect species richness (ANOVA: $F = 0.11$, $p = 0.743$), or Shannon diversity (ANOVA: $F = 2.224$, $p = 0.148$) in the embryonic gut.

3.3 Adult and embryonic guts differ in community composition

The embryonic and adult gut microbiomes differed significantly in species richness (Figure 5a; ANOVA: $F = 3.86$, $p = 0.0305$), but not Shannon diversity (Figure 5b; ANOVA: $F = 1.56$, $p = 0.224$). The two groups also differed in beta diversity when using both unweighted (Figure 5c; PERMANOVA: $F = 22.736$, $p < 0.001$, NMDS stress = 0.044) and weighted UniFrac distances (Figure 5d; PERMANOVA: $F = 44.4$, $p < 0.001$, NMDS stress = 0.069).

4. Discussion

In this chapter, we found evidence for the presence of microbes in the placenta and embryonic guts of embryos collected during late stage of pregnancy in two species of *Mus*, and in hybrids produced from one direction of the cross between the two species. Whereas the adult gut microbiota of *M. m. domesticus* and *M. spretus* were distinct, species identity did not affect alpha or beta diversity in placental or embryonic gut samples. We tested two alternative predictions for the comparison between the prenatal microbiome (collectively, the placenta and embryonic gut) of hybrids and that of the offspring of within-species crosses. Because the microbiome is vertically transferred from mothers to offspring, we predicted that the two genotypes with the same maternal species - hybrids and *M. m. domesticus* – would have similar prenatal microbiomes that were distinct from the *M. spretus* prenatal microbiome. Alternatively, if offspring genotype influences the prenatal microbiome, then hybrids would be distinct from both parental species. Neither of these predictions was supported by the data: the hybrid prenatal microbiome composition was statistically indistinguishable from that of both *M. m. domesticus* and *M. spretus*. Moreover, despite documented abnormalities in hybrid placenta size and structure, the abundance of prenatal microbiota was not reduced in hybrids relative to within-species offspring. We consider three non-mutually exclusive explanations for the apparent homogeneity of microbial taxa in placenta and embryonic gut sampled from two species and their hybrid.

First, offspring genotype may not impact which microbes are able to reach the developing embryo or may only impact diversity in the gut microbiome postpartum. This

idea seems initially unlikely in light of the work conducted on adult hybrid gut microbiota, where hybrids are either intermediate or distinct from their parental species. The gut microbiome is expected to shift as host populations diverge, either through coevolutionary dynamics or variable environmental influences, or a combination of both factors (Brooks et al. 2016). Further, hybrid individuals are hypothesized to carry deleterious combinations of parental microbes, analogous to genetic Dobzhansky-Muller incompatibilities (Brooks et al. 2016). However, strong evidence for incompatibilities in hybrid gut microbiota is currently limited to one study. In the cross between house mouse subspecies, *M. m. domesticus* and *M. m. musculus*, hybrid gut microbiota were distinct from both parental lineages, and showed associated differences in immune gene expression and increased gut pathology (Wang et al. 2015). While this study showed a clear effect of hybridization on the gut microbiome, consistent differences between parents and hybrid offspring are lacking in other studies in natural hybrid systems. For example, a recent study of naturally occurring hybrids between two warbler species found that, whereas species identity accounted for some variation in the microbiome, microbial richness did not vary between hybrids and parental species (Baiz et al. 2024). Likewise, in a hybrid zone between two woodrat species (*Neotoma lepida* and *N. bryanti*), hybrid gut microbiota were intermediate relative to the parental species when looking at beta diversity, but microbial richness was correlated with dietary richness rather than genotype (Nielsen et al. 2022). Given these mixed results in studies of adult hybrid gut microbiota, it may not be surprising that our hybrids did not differ from the two parental species. However, the homogeneity of microbes in the prenatal tissues

across all genotypes was an unexpected result, given that adult *M. m. domesticus* and *M. spretus* guts were distinct in both alpha and beta diversity.

To our knowledge, no prior studies have investigated the effect of offspring genotype on microbes present in prenatal tissues. However, extensive research on the postnatal development of microbiota in diverse species demonstrates that the gut microbiome is very sensitive to the environment in which offspring are reared. While we observed no species differences in the prenatal tissues, further research is needed to determine if and how the gut microbiome may develop species differences as offspring interact with their environment and mothers postpartum.

Another factor that may have contributed a lack of an effect of species identity on prenatal samples was my use of proxies for the parental species. While we observed differences between the parental species, it is possible that the proxies were more distinct from each other than the parents themselves were. The proxy parental guts were from two separate facilities, unlike the parents of the offspring used in this experiment. The parental species used in the experiment were maintained in the same lab space with the same standard diet, which could have homogenized the parental species' gut microbiome and, in turn, impacted the microbial richness in the embryonic guts.

Second, the functional role of microbes in the embryonic gut may be independent of taxonomic identity. As the presence of a microbial community in any prenatal tissue is still contested (Blaser et al. 2021), the functional purpose of a prenatal microbiome is largely unstudied. One suggested function of the placental microbiome is that it jump-starts the colonization of the embryonic gut before the embryo passes through the vaginal

tract during birth (Jiménez et al. 2008, Aagaard et al. 2014, Collado et al. 2016, Wassenaar and Panigrahi 2014). In my data, the placenta and embryonic gut shared a subset of microbes, and the placenta had more observed species than the embryonic gut. This pattern is in line with the suggestion of Collado et al. (2016), that the placenta acts as a filter for selective transfer of maternal microbes to embryos. To my knowledge, the selective ability of the placenta to transfer microbes has not been directly tested, but labelled bacteria introduced in maternal guts were subsequently found in the placenta (Jiménez et al. 2005). Moving forward, it will be useful to test if the placenta can selectively transfer beneficial microbes or exclude pathogenic microbes from the developing embryo.

The initial colonization of the embryonic gut is important as the early-arriving microbes can exert priority effects, wherein the establishment of new species in a microbial community depends on the timing of their arrival and presence of other species (Debray et al 2022). Priority effects can affect the community composition of the developing microbiome through a number of mechanisms that can either assist or inhibit subsequent microbes. Microbial taxa present in the embryonic gut may reflect one such mechanism by which early-arriving microbial species modify conditions that can prevent the establishment of later-arriving species. In the embryonic gut, four of the top ten taxa present belonged to the phylum *Firmicutes*, members of which can produce short-chain fatty acids (SCFAs). SCFAs stimulate the production of antimicrobial peptides in the gut that can affect the ability of pathogens to colonize the gut (Chang et al. 2020). Of the taxa present in our data set that could produce SCFAs, *Lactobacillus salivarius* is a probiotic

bacterium that is commonly found in the gastrointestinal tract and functions to suppress pathogenic bacteria (Messaoudi et al. 2013). While some of the microbes identified in the placenta and embryonic gut were of biological significance, there were also contaminants present in the data set.

Third, it is possible that contamination in low biomass samples (samples with low microbial relative to host DNA) masked any signal of genotypic differences. The debate as to whether or not embryos are exposed to microbes during gestation is ongoing and there is evidence for (Stinson, Keelan, and Payne 2019; Martinez et al. 2018; Younge et al. 2019; Borghi et al. 2019; Rackaitye et al. 2020; Mishra et al. 2021) and against (de Goffau et al. 2019; de Goffau et al. 2021; Theis et al. 2019; Theis et al. 2020; Panzer et al. 2023) this proposition. The main argument against the presence of a functional prenatal microbiome is the prevalence of contamination in low biomass samples, including the placenta and embryonic gut (Kennedy et al. 2023). In this study, sample processing included negative controls to ensure that any contamination that occurred during DNA extraction and sequencing was accounted for and could be removed during data processing. However, residual contamination in the samples is still a possibility, as the embryonic tissues were not collected under aseptic conditions. Even after contaminant sequence removal using the *decontam* package, additional probable contaminants were identified manually in both placental and embryonic gut samples. This could contribute to the lack of an effect of species identity on the microbiota, especially as we detected robust species differences in the adult guts, which contain abundant microbial DNA. It is possible that the soil or other environmentally-found microbes

identified in my data could have originated from maternal food and water sources that were subsequently transmitted to offspring tissues (Martinez et al. 2018). Nonetheless, future work on the effect of species identity on the prenatal microbiome should include strict dissection protocols to minimize any potential contamination.

Whereas the effect of host species identity on the adult microbiome community composition is evident, it remains unclear whether species identity affects the composition of the first microbial constituents of the prenatal gut. To my knowledge, this study represents the first time that the effect of host species identity has been tested for any measure of a prenatal microbial community. More generally, this work contributes to the growing number of studies that provide evidence for microbes in prenatal tissues (Stinson, Keelan, and Payne 2019; Martinez et al. 2018; Younge et al. 2019; Borghi et al. 2019; Rackaitye et al. 2020; Mishra et al. 2021). Given the life-long impact of the gut microbiome on health and disease, if and how microbes in the prenatal environment affect postnatal phenotypes is an important area for future work.

Table 1.1

Tissue	**Crosses (*n*)**
Controls	4
Embryonic gut	*M. m. domesticus* (10) Hybrid (9) *M. spretus* (10)
Placenta	*M. m. domesticus* (10) Hybrid (9) *M. spretus* (10)
Maternal gut	*M. m. domesticus* (5) *M. spretus* (5)

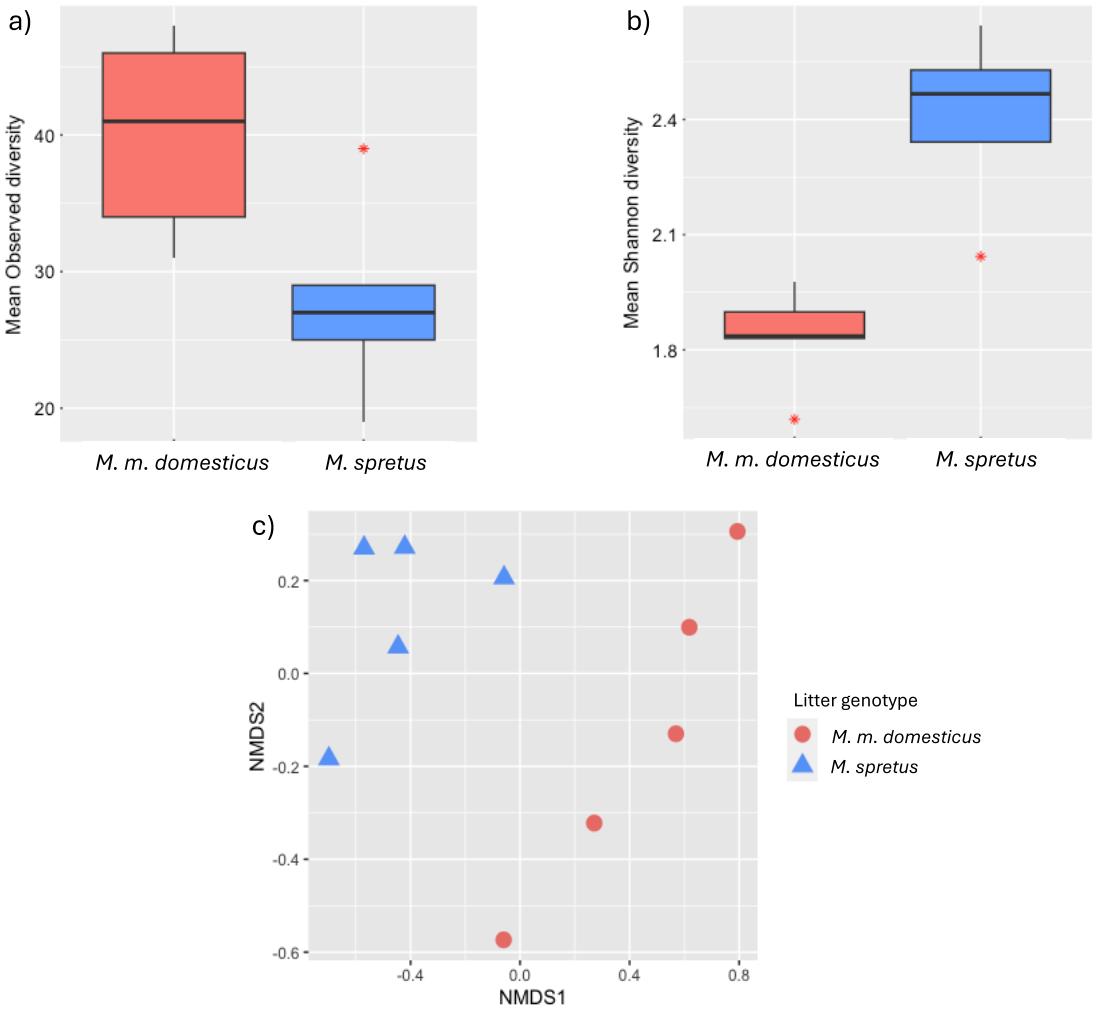
Sample sizes for all tissues and negative controls. Each sample size is split into species. Maternal guts are representative females of the same species of females used in experimental crosses but are not the parents of the embryos used in this experiment.

Table 1.2

Microbial taxon	Phylum	Tissue present	Biological relevance?
Bifidobacterium	Actinobacteria	Placenta	Ubiquitous in the gut; isolated from the vagina and mouth in mammals
Phyllobacterium	Proteobacteria	Placenta	Contaminant
Lactobacillus	Firmicutes	Placenta	Associated with gut and vaginal microbiota
Bacillus	Firmicutes	Placenta	Contaminant
Bacillus	Firmicutes	Placenta	Contaminant
Pseudomonas sp. Leaf434	Proteobacteria	Placenta	Contaminant
Stenotrophomonas	Proteobacteria	Placenta	Contaminant
Lactobacillus	Firmicutes	Placenta	Associated with gut and vaginal microbiota
Pseudomonas	Proteobacteria	Placenta	Contaminant
Bacillus	Firmicutes	Placenta	Contaminant
Rhodanobacter	Proteobacteria	Embryonic gut	Contaminant
Lactococcus	Firmicutes	Embryonic gut	Contaminant
Staphylococcus	Firmicutes	Embryonic gut	Contaminant
Dyella	Proteobacteria	Embryonic gut	Contaminant
Lactobacillus salivarius	Firmicutes	Embryonic gut	Gut microbe
Azospirillum	Proteobacteria	Embryonic gut	Contaminant
Enterobacteriaceae	Proteobacteria	Embryonic gut	Gut microbe
Lachnospiraceae	Firmicutes	Embryonic gut	Gut microbe
Brevundimonas	Proteobacteria	Embryonic gut	Contaminant
Haemophilus	Proteobacteria	Embryonic gut	Associated with mouth, vagina, and gut

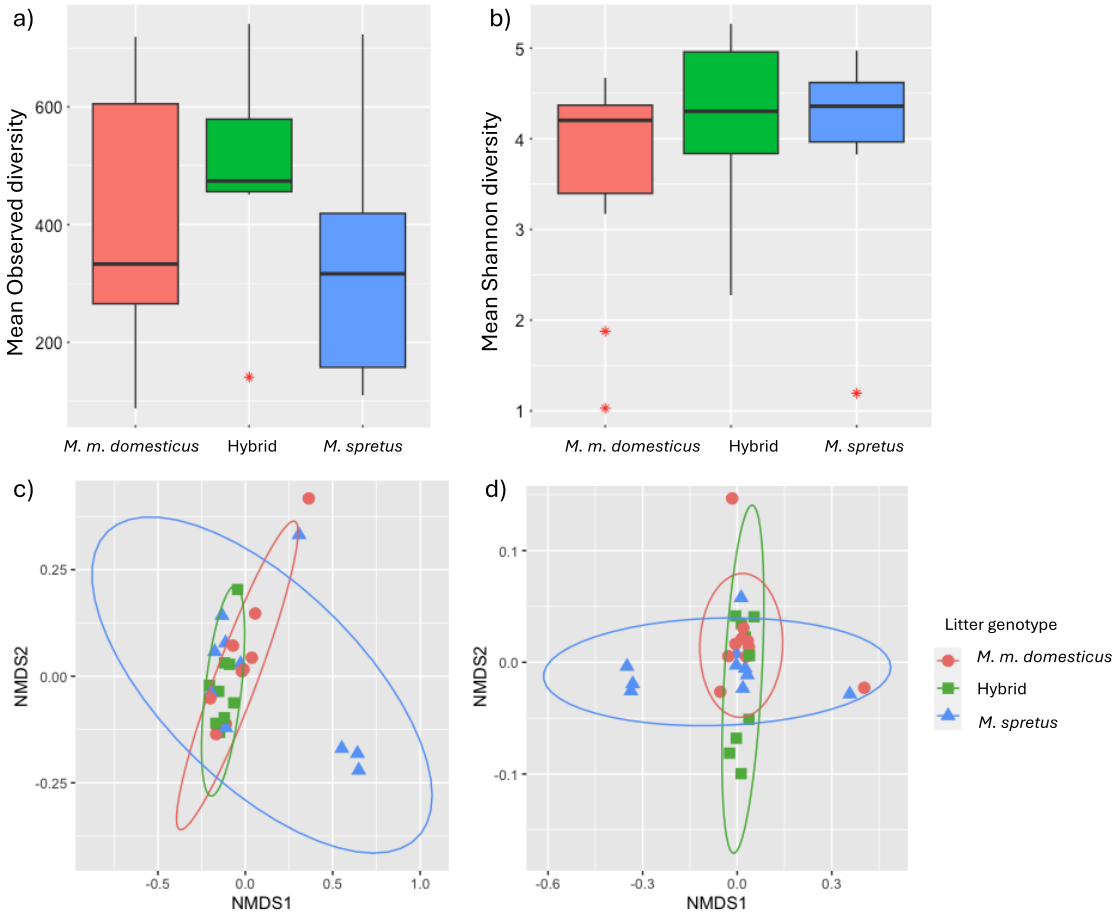
Table containing the top ten taxa found in the placenta and embryonic gut, as well as the phylum that the microbe is associated with. Biological relevance was included to differentiate between likely contamination that remained in samples after contaminant sequences were removed from the data set during data processing and microbes that were likely to be present in the prenatal microbiome.

Figure 1.1



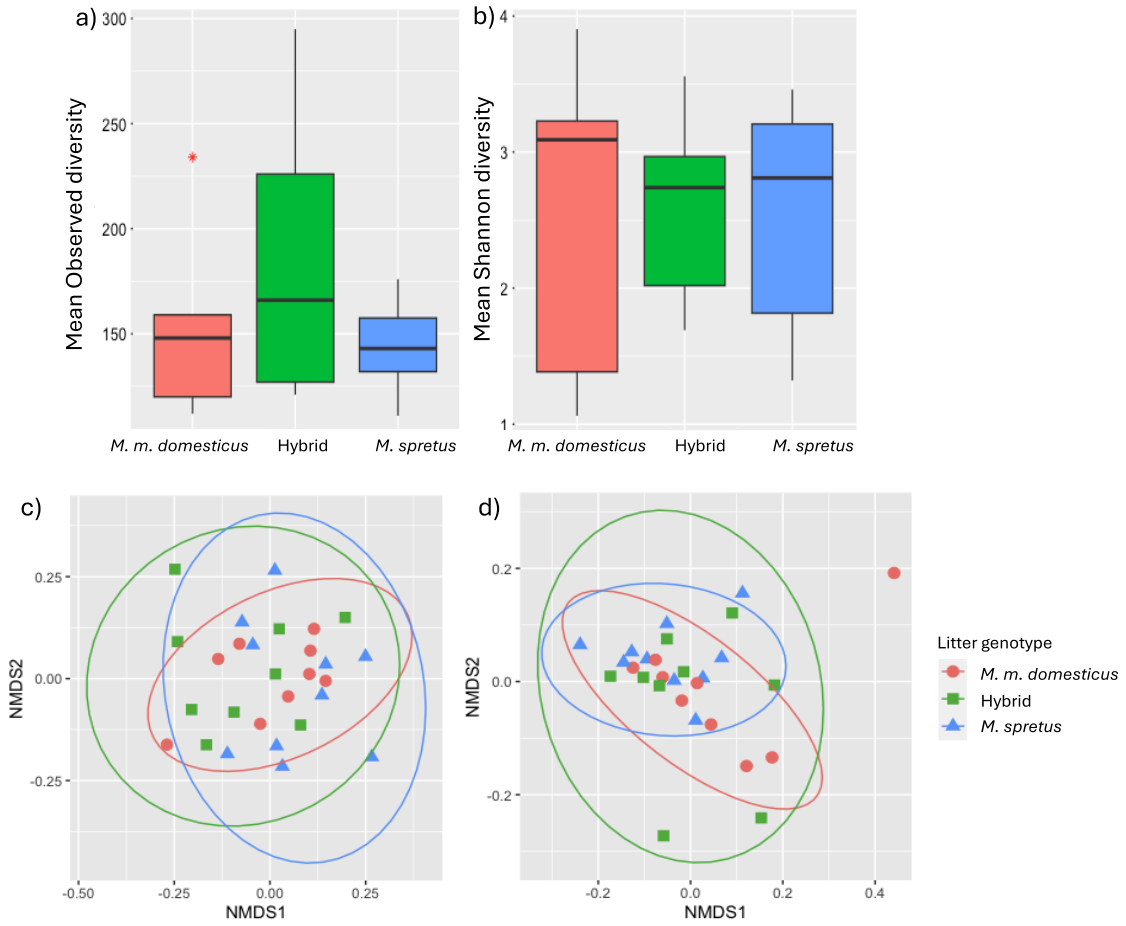
Diversity measures in adult *M. m. domesticus* and *M. spretus*. Box and whisker plots for two measures of alpha diversity: a) species richness and b) Shannon's diversity. c) NMDS plot using Bray-Curtis dissimilarities.

Figure 1.2



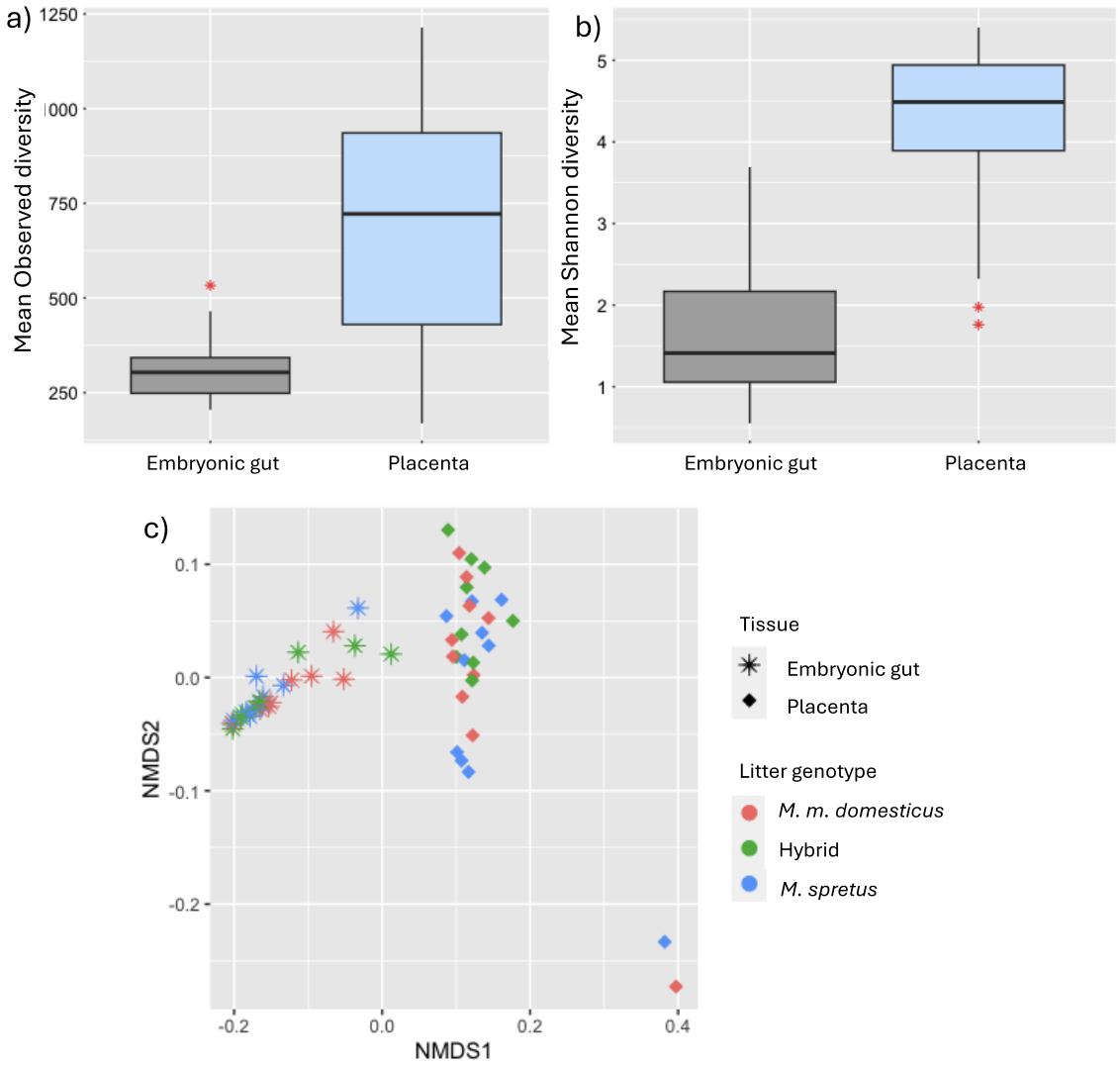
Diversity measures in *M. m. domesticus*, hybrid, and *M. spretus* placental tissue. Box and whisker plots for two measures of alpha diversity: a) species richness and b) Shannon's diversity. NMDS plots using c) UniFrac distances and d) weighted UniFrac distances.

Figure 1.3



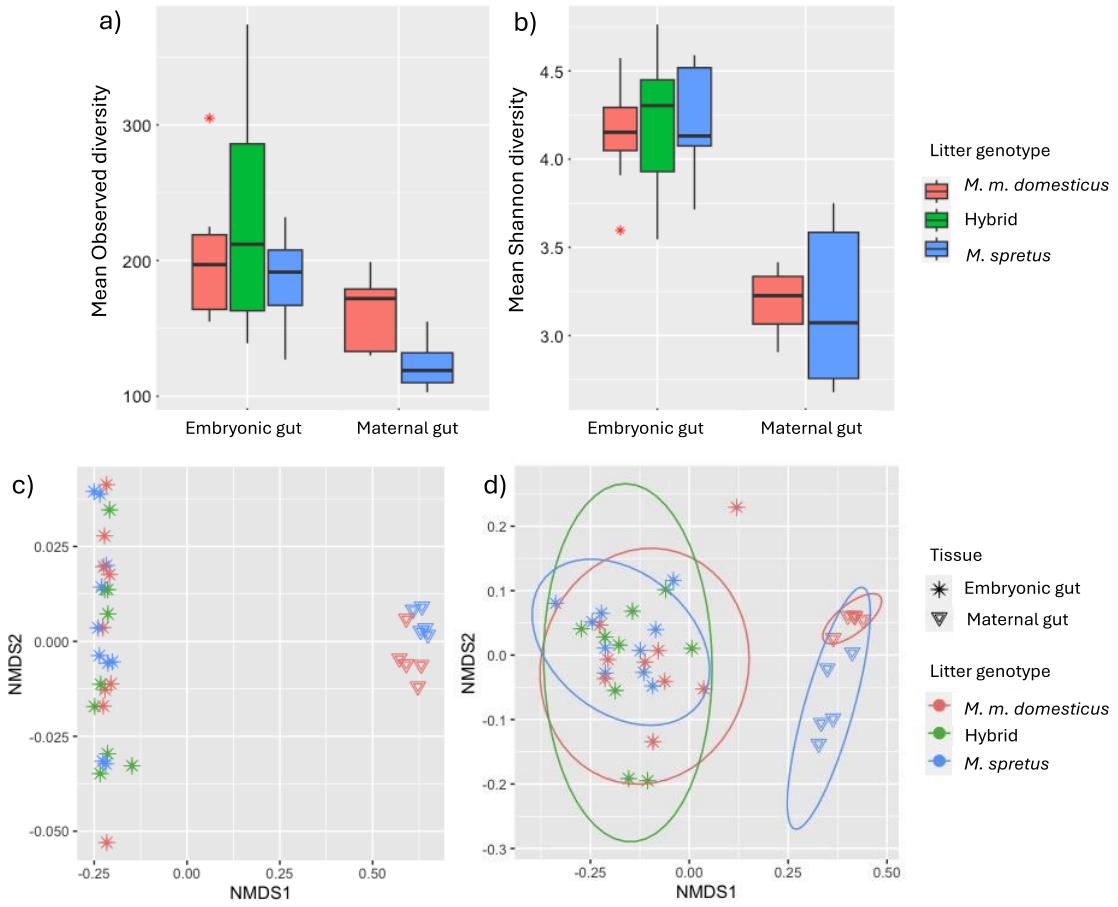
Diversity measures in the embryonic guts of *M. m. domesticus*, hybrid, and *M. spretus* embryos. Box and whisker plots for two measures of alpha diversity: a) species richness and b) Shannon's diversity. NMDS plots using c) UniFrac distances and d) weighted UniFrac distances.

Figure 1.4



Comparison between placental and embryonic gut microbiota. Box and whisker plots for two measures of alpha diversity: a) species richness and b) Shannon's diversity. NMDS plots using c) weighted UniFrac distances.

Figure 1.5



Comparison of embryonic and adult gut microbiota. Box and whisker plots for two measures of alpha diversity: a) species richness and b) Shannon's diversity. NMDS plots using c) UniFrac distances and d) weighted UniFrac distances.

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Chapter 2

Defining the origin of the prenatal gut microbiome and the effect of the embryonic genotype on its composition

Abstract

In mammals, the microbiome is historically thought to establish in the postnatal period after exposure to the maternal vaginal tract. However, recent evidence in mice suggests that offspring are exposed to maternal microbes during gestation in part via the placenta. Vertical transmission of the microbiome (from mothers to offspring) can be considered a maternal effect. Adult gut microbiota are sensitive to changes in the host environment, however genotype can also influence the diversity and abundance of microbes in a given tissue. I address two questions in this chapter. First, how do maternal and embryonic genotypes interact to influence the composition of microbes present in the fetal environment? Second, which maternal microbial community contributed the most to the prenatal microbiome? To address these questions in the house mouse (*Mus musculus domesticus*), four crosses were generated from two wild-derived inbred stains of *M. m. domesticus* (descendants of mice captured in Tucson, AZ (AZ) and Edmonton, Canada (ED)). I collected tissue samples from the placenta and embryonic gut, as well as a suite of maternal samples including the oral cavity, blood, gut, and vaginal tract. Microbial DNA was then extracted from each sample and sequenced (V4 region of the 16S rRNA gene). The effects of maternal and embryonic genotype, as well as tissue type, on microbial diversity and abundance in the prenatal microbiome were analyzed using ANOVA for measures of alpha diversity and PERMANOVA for measures of beta diversity. I found a strong effect of embryonic genotype on the community composition of the placenta and embryonic gut, as well as a surprising effect on maternal gut community composition. In contract, maternal genotype did not affect beta diversity in

the placenta or embryonic gut, and only affected one metric of alpha diversity in embryonic guts. The placenta and embryonic gut were similar across alpha diversity measures when comparing all tissues. Additionally, the placenta was not statistically distinguishable from maternal gut and blood samples. Together, these results suggest that microbes originating in the maternal gut reach the placenta via the maternal circulatory system and are then transferred to the embryonic gut. While the prenatal microbiome remains a contentious subject, this experiment contributes to the body of literature showing evidence for the presence of microbes in prenatal tissues.

1. Introduction

Historically, the initial seeding of the mammalian gut microbiome was thought to occur in the vaginal tract during birth, and during the early post-natal period. However, in the past decade, multiple studies have independently suggested that this is not the case in eutherian mammals (reviewed in Funkhouser and Bordenstein 2013; Perez-Muñoz et al. 2017) – particularly in mice (Martinez et al. 2018; Younge et al. 2019) and humans (Jiménez et al. 2005; Aagaard et al. 2014; Collado et al. 2016). The microbes that an individual acquires *in utero* likely serve a similar purpose to microbes acquired in early postnatal life: to prime the fetal immune system (Li et al. 2019, Mishra et al 2021). This includes the generation of immune cells within the embryonic gut (Stras et al. 2019) and modulation of the community composition of the developing postnatal gut microbiome (Martínez et al. 2018; Roswell et al. 2021). While the utility of a prenatal microbiome is clear, how microbial DNA reaches the developing embryo is an open question with multiple maternal sources potentially contributing. In species with uniparental maternal care, the only paternal contribution to offspring microbial community composition is alleles that may limit or promote particular microbial species' abundances within the microbiome. The extent to which parental genomes interact to affect the initial microbial colonization of the offspring gut is unclear, in part due to the debate surrounding the timing of colonization.

The most likely route by which maternal microbes could reach the uterine environment is by translocation from the gut into the maternal bloodstream. Despite immunological defenses in place to stop microbial escape from the gut into the

circulatory system, microbial translocation occurs more often in pregnant than in nonpregnant mice (Perez et al. 2007). Blood-borne microbes are most likely transferred to the embryo through the placenta as this is the primary site of maternal-fetal exchange. In mammals with hemochorionic placentas (e.g. humans, mice), maternal-fetal exchange is facilitated by maternal blood flow into the fetus-derived trophoblast tissue (Dilworth and Sibley 2013). Thus, if prenatal microbes are acquired from maternal circulation, the placenta is the probable site of transfer. However, the placenta is not necessarily the sole source of maternal microbial transfer to offspring in utero. Additional sources of maternal microbes – including those present in the uterus before pregnancy and others that ascend from the vaginal tract during gestation (Benner et al. 2018) – can influence the community composition of the prenatal microbiome. Martinez et al. (2018) identified the placenta, the maternal vaginal tract, and oral cavity as origins of fetal intestinal bacterial DNA and hypothesized that the placental contribution comprises microbes from the vaginal tract, oral cavity, and microbes translocated from the maternal gut to the circulatory system. Although this study identified the placenta as a major contributor to the microbial community of the fetal intestine, over 80% of the bacterial DNA found in the fetal intestine were of unknown origin suggesting that additional sources may have contributed, including potential contaminants (Martinez et al. 2018).

The community composition of the adult gut microbiome is a phenotype that is shaped by both host genotype and environmental factors; however, the community composition of the early life gut microbiome is largely maternally-driven (Vaishampayan et al. 2010). Adult gut microbiota are sensitive to changes in the environment (e.g.

antibiotic treatment; Dethlefsen et al. 2008). However, host genotype often influences the diversity and abundance of the microbes in a given tissue (reviewed in Spor, Koren, and Ley 2011). Importantly, no study to date has tested for an effect of genotype on the prenatal microbiome.

As the microbiome is vertically transmitted from mother to offspring, the initial community received by offspring can also be considered a maternal effect (sensu Wolf and Wade 2009). During healthy pregnancies, the microbiota of the vaginal tract (Aagaard et al. 2012) and the maternal gut (Koren et al. 2012; Mueller et al. 2015; Jašarević et al. 2017; DiGiulio et al. 2015) undergo shifts in their community structures. These shifts occur during critical windows of fetal development that are affected by the maternal microbiome, either through direct transmission of microbes or by products of the maternal microbiome (Xiao et al. 2022; reviewed in Miko et al. 2022). Based on similarities between the microbial community in the meconium and maternal sources of microbes, it has been hypothesized that microbes are selectively transmitted during the initial colonization of offspring (Collado et al. 2016). This could act as a maternal effect if the selective transmission is driven by mothers, however selectivity could be mediated by offspring as microbes pass through the placenta on the way to the embryonic gut and the placenta is largely derived from the embryo. How maternal and embryonic genotype interact to influence the composition of microbes present in the fetal environment is a question that has yet to be directly addressed.

With these gaps in knowledge in mind, this study uses two wild-derived strains of house mouse (*Mus musculus domesticus*) to address two main questions. 1) What are the

effects of embryonic genotype versus maternal genotype on the initial seeding of the prenatal microbiome? The effect of embryonic genotype on the community composition of embryonic gut microbiota could manifest in a number of ways. First, there may be no effect of genotype, whereby the community composition of the embryonic gut is strictly a maternal effect. In this instance, I would expect that the embryonic gut microbiome would differ by maternal strain. However, if the paternally inherited genome has an effect on the embryonic gut microbiome (for example, limiting the microbes that successfully inoculate the embryonic gut), then I would expect offspring from inter-strain crosses to have distinct community compositions. 2) Which maternal microbial community contributes the most to the prenatal microbiome? I characterized the bacterial community structure of potential maternal sources of the prenatal microbiome (oral cavity, gut, blood, and vaginal tract), together with the placenta and fetal gut, in crosses within and between two inbred strains of house mouse (*M. m. domesticus*) derived from different populations in North America. Based on prior studies, I predicted that the maternal gut would be the primary source of prenatal microbes (Martinez et al. 2018).

2. Methods

2. 1 Study system

Two wild-derived inbred strains of *M. m. domesticus* were used in this experiment: the descendants of mice captured in Tucson, AZ, (AZ) and Edmonton, Alberta, Canada (ED). The compositionally distinct, high-diversity gut microbiomes of the founders were retained through >10 generations of maintenance in the same colony under standard housing conditions (Moeller et al. 2018). Both strains were acquired from the Nachman Lab at the University of California, Berkeley and originally sent to Oklahoma State University in 2018. The colony was moved to the University of California, Riverside (UCR) in 2019, and moved between vivaria on the UCR campus in 2022. A second cohort of AZ mice was acquired from the Nachman Lab in 2020. Throughout the moves, the mice were consistently fed the same diet (LabDiet 5001 Rodent Diet).

Experimental pregnancies were generated using a standard timed mating protocol. Adult, nulliparous females were paired with sexually experienced males 1-2 hours before lights off. Females were subsequently checked for vaginal plugs each morning at lights on. The day a plug was observed (embryonic day 0), the pair was split and the female was moved to a clean cage. If a plug was not observed after 48 hours paired with the male, females were split from the male and monitored for pregnancy. Females were euthanized and samples were collected at embryonic day 17.5. Four crosses were generated (female strain listed first): AZxAZ, AZxED, EDxED, EDxAZ. Target sample sizes were 5 pregnancies/litter genotype (hereafter, AZ for AZxAZ cross, AE for AZxED

cross, ED for EDxED cross, and EA for EDxAZ cross); final sample sizes for each cross and tissue type are shown in Table 1.

2.2 Tissue collection

Pregnant females were euthanized by cervical dislocation. The dam's ventrum was disinfected with Betadine, followed by isopropyl alcohol before any samples were collected. Blood was collected by cardiac puncture. The uterus was dissected, and subsequent dissection of placentas, embryonic guts, and amniotic fluid collection were done in a laminar flow hood to minimize the probability of contamination with maternal or environmental microbes. Placentas and embryonic guts were dissected and stored individually. Placental and embryonic gut samples were flash frozen in liquid nitrogen and stored in -80°C until DNA extraction.

Maternal oral samples were collected by swabbing the oral cavity for 30 seconds. Vaginal samples were collected by flushing 3x with 100µl sterile saline solution. The maternal gut was dissected last to minimize the possibility of cross contamination from high to lower biomass tissue samples. All maternal experimental tissues were flash frozen in liquid nitrogen and stored at -80°C until DNA extraction. Embryonic tissue collected for sexing was stored in RNAlater at -20°C. Embryo sex was determined with a PCR assay (Arévalo and Campbell 2020).

2.3 Microbial DNA extraction and sequencing

Prior to extraction, embryonic gut and placental samples were treated with 20µL Proteinase K and 100µL 10% SDS solution for 60 minutes in order to lyse tissue and

maximize the microbial DNA that could be extracted. Following tissue lysing, samples were transferred to bead beating tubes and vortexed for an additional 20 minutes. DNA extractions were conducted using the ZymoBIOMICS DNA Miniprep Kit (Zymo Research) according to manufacturer's instructions. Negative control samples were collected in tandem with each DNA extraction, resulting in a total of 20 control samples (Table 1).

Amplicon production and sequencing was done at Novogene (Sacramento, CA, USA). Briefly, the V4 region of the 16S rRNA gene was amplified for all samples using the 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') primers. Libraries were prepared with the Illumina TruSeq DNA PCR-Free Library Preparation Kit and 250bp paired-end reads were generated on the Illumina NovaSeq 6000 platform. Primer sequences were trimmed from reads and reads were removed if they were less than 60bp, contained 10% or more uncertain nucleotides, or if low quality nucleotides made up more than 50% of the read.

2.4 Data Processing and Analysis

All sequences were processed in QIIME2 (version 2019.7; Bolyen et al. 2019). Raw sequences were joined and denoised using the deblur pipeline (Amir et al. 2017). Taxonomic classification was conducted using the SILVA 132 database (Quast et al. 2013, Yilmaz et al. 2014) and trained using the feature-classifier tool (Bokulich et al. 2018). Sequences were aligned using MAFFT (Katoh et al. 2002) and subsequently generated into a midpoint-rooted tree with FastTree (Price et al. 2010). Representative sequences from deblur, taxonomic identification from MAFFT, and the phylogenetic tree

from FastTree were exported from QIIME2 for further analysis in R (version 4.2.3, "Shortstop Beagle").

QIIME2 artifacts were imported into R using the qiime2R package (version 0.99.6; Bisanz 2018) and analyzed using the phyloseq (version 1.41.1; McMurdie and Holmes 2013), microViz (version 0.10.0; Barnett et al. 2021) and microbiome (version 1.20.0; Lahti et al. 2017) packages. Contaminants in negative controls were identified using the decontam package in R (version 1.18.0; Davis 2017) and were removed from samples. Any sequences identified as mitochondria, chloroplast, or unidentified were also removed. Singletons were filtered and removed if one read was present in only one sample.

Differences in alpha diversity (diversity within individuals) between genotypes were analyzed with analysis of variance (ANOVA) models with both litter genotype and maternal genotype as individual effects. Post-hoc tests were run with Tukey's HSD correction. The effect of genotype on community composition was visualized with non-metric multidimensional scaling (NMDS) using Bray-Curtis dissimilarities, as well as weighted and unweighted UniFrac distances. Differences in community composition between genotypes were evaluated with a permutational multivariate analysis of variance (PERMANOVA) and post-hoc pairwise comparisons were generated for significant effects with the pairwiseadonis package (Martinez Arbizu 2020). Litter genotype was used to test for the effect of embryonic genotype on community composition, and maternal genotype was used to test for a maternal effect. Taxonomic identifications are presented as the lowest level provided from the SILVA 132 classification.

3. Results

3.1 Maternal guts differ by strain and litter genotype

When comparing ED and AZ adult female gut microbiota, there were no differences in metrics of alpha diversity: species richness (Figure 1a, ANOVA: $F = 2.781$, $p = 0.108$), Shannon's diversity (Figure 1b, ANOVA: $F = 0.02$, $p = 0.89$), and Simpson's inverse index (Figure 1c, ANOVA: $F = 0.92$, $p = 0.347$). This pattern changed when separating females by the genotype of the litter they were carrying. When categorized by litter genotype, female guts differed in Shannon's diversity (Figure 1e, ANOVA: $F = 11.52$, $p < 0.001$) and Simpson's inverse index (Figure 1f, ANOVA: $F = 11.72$, $p < 0.001$), but not species richness (Figure 1d, ANOVA: $F = 2.456$, $p = 0.0887$). Post-hoc comparisons using Tukey's HSD showed the same trend in both Shannon's diversity and Simpson's inverse index. ED and AZ females were distinct from females of the same strain that produced a litter of a different genotype (ED-EA: Shannon's diversity $p = 0.00076$, Simpson's inverse index $p = 0.0011$; AZ-AE: Shannon's diversity $p = 0.0059$, Simpson's inverse index $p = 0.0059$), but ED and AZ females did not differ (Shannon's diversity $p = 0.81$, Simpson's inverse index $p = 0.99$) nor did EA from AE (Shannon's diversity $p = 0.99$, Simpson's inverse index $p = 0.91$).

Unlike alpha diversity, ED and AZ female gut microbiota differed in beta diversity using Bray-Curtis dissimilarities (Figure 2a, PERMANOVA: $p < 0.001$, NMDS stress: 0.0938), UniFrac distances (Figure 2b, PERMANOVA: $p = 0.0019$, NMDS stress: 0.105), and weighted UniFrac distances (Figure 2c, PERMANOVA: $p < 0.001$, NMDS stress: 0.100). Differences persisted for each distance measure when the females were

separated by their litters' genotype (Bray-Curtis dissimilarities: Figure 2d, PERMANOVA: $p < 0.001$; UniFrac distances: Figure 2e, PERMANOVA: $p < 0.001$; weighted UniFrac distances: Figure 2f, PERMANOVA: $p < 0.001$). However, across post-hoc comparisons of each distance metric, the only pair that was consistently different was AZ and ED females (Bray-Curtis $p_{adj} = 0.012$, UniFrac $p_{adj} = 0.012$, weighted UniFrac $p_{adj} = 0.006$).

3.2 Characteristics of the embryonic gut microbiota

When testing for an effect of maternal genotype (AZ or ED) on alpha diversity metrics, embryonic guts did not differ in species richness (Figure 3a, ANOVA: $F = 0.157$, $p = 0.694$) or Shannon's diversity (Figure 3b, ANOVA: $F = 0.8$, $p = 0.377$). Simpson's inverse index was marginally higher in the guts of embryos with an ED mother (Figure 3c, ANOVA: $F = 4.347$, $p = 0.0438$). As for maternal guts, the effect of embryo genotype (AZ, AE, ED, or EA) on alpha diversity metrics was consistently significant: species richness (Figure 3d, ANOVA: $F = 18.19$, $p < 0.001$), Shannon's diversity (Figure 3e, ANOVA: $F = 14.88$, $p < 0.001$), and Simpson's inverse index (Figure 3f, ANOVA: $F = 7.697$, $p < 0.001$). Post-hoc pairwise comparisons of species richness revealed that ED embryos had significantly higher species richness than EA (Tukey's HSD: $p < 0.001$) and AE ($p = 0.0015$), but not AZ ($p = 0.435$). AZ embryos also had significantly higher species richness than EA ($p = 0.0011$). Post-hoc pairwise comparisons of Shannon's diversity showed a similar pattern, in which ED embryonic guts differed from EA ($p < 0.001$) and AE ($p = 0.001$), whereas AZ embryonic guts only differed from EA ($p =$

0.0146). Finally, post-hoc comparisons of Simpson's inverse index showed that ED embryonic guts differed from AE ($p = 0.0042$) and EA ($p = 0.0013$).

There was no effect of maternal genotype on any beta diversity distance metric for embryonic gut (Bray-Curtis PERMANOVA: $p = 0.15$; UniFrac PERMANOVA: 0.126; weighted UniFrac PERMANOVA: $p = 0.304$). In contrast, there was a significant effect of embryo genotype on all distance measures for embryonic gut (Bray-Curtis Figure 4a, PERMANOVA: $p < 0.001$, NMDS stress = 0.106; UniFrac Figure 4b, PERMANOVA: $p < 0.001$, NMDS stress = 0.079; weighted UniFrac Figure 4c, PERMANOVA: $p < 0.001$, NMDS stress = 0.112). Across post-hoc pairwise comparisons, the only consistent difference across all beta diversity metrics was between ED and EA embryos (Bray-Curtis $p_{adj} = 0.006$, UniFrac $p_{adj} = 0.006$, weighted UniFrac $p_{adj} = 0.006$)

Of the top ten taxa present in all embryonic gut samples, six were commonly found in gut or vaginal tract microbiota including two identified as *Lactobacillus*, *Lactobacillus iners* AB-1, *Faecalibacterium*, *Subdoligranulum*, and *Actinomyces*. The remaining four were identified as contaminants (*Aeromonas*, *Bradyrizobium*, *Nitrososphaeracaea*, and *Actinobacter*). Total abundances of phyla present in embryonic gut samples are shown in Figure 10.

3.4 Maternal and embryonic guts differ in community composition

Maternal and embryonic guts differed in species richness (Figure 5a, ANOVA: $F = 13.97$, $p < 0.001$) and Shannon's diversity (Figure 5b, ANOVA: $F = 5.63$, $p = 0.021$), but not Simpson's inverse index (Figure 5c, ANOVA: $F = 0.352$, $p = 0.555$). Likewise, maternal

and embryonic guts differed in all beta diversity metrices: Bray-Curtis distances (Figure 5d, PERMANOVA: $p < 0.001$, NMDS stress = 0.13), UniFrac distances (Figure 5e, PERMANOVA: $p < 0.001$, NMDS stress = 0.10), and weighted UniFrac distances (Figure 5f, PERMANOVA: $p < 0.001$, NMDS stress = 0.074).

3.3 Characteristics of the placental microbiome

Maternal genotype did not have an effect on species richness (Figure 6a, ANOVA: $F = 0.905$, $p = 0.347$), Shannon's diversity (Figure 6b, ANOVA: $F = 1.653$, $p = 0.206$), or Simpson's inverse index (Figure 6c, ANOVA: $F = 1.913$, $p = 0.175$). Similar to my findings in Chapter 1, the placenta showed no differences in alpha diversity based on embryonic genotype. There were no significant effects of embryonic genotype for species richness (Figure 6d, ANOVA: $F = 2.165$, $p = 0.109$), Shannon's diversity (Figure 6e, ANOVA: $F = 0.781$, $p = 0.512$), and Simpson's inverse index (Figure 6f, ANOVA: $F = 1.758$, $p = 0.172$).

Maternal genotype did not have a significant effect on beta diversity with any distance metric (Bray-Curtis: PERMANOVA $p = 0.069$; UniFrac: PERMANOVA $p = 0.074$; weighted UniFrac: PERMANOVA $p = 0.24$). There was a significant effect of embryonic genotype on placental beta diversity when using Bray-Curtis distances (Figure 7a, PERMANOVA: $p < 0.001$; NMDS stress = 0.196) and UniFrac distances (Figure 7b, PERMANOVA: $p < 0.001$; NMDS stress = 0.121), but not weighted UniFrac distances (PERMANOVA: $p = 0.0537$; NMDS stress = 0.16). For both distance measures, ED was consistently distinct from EA (Bray-Curtis $p_{adj} = 0.006$, UniFrac $p_{adj} = 0.012$) and AE (Bray-Curtis $p_{adj} = 0.048$, UniFrac $p_{adj} = 0.042$).

Of the top ten taxa present in placental tissue, four are commonly found in feces or intestines. These taxa included *Blautia* (which appeared twice), *Acidaminococcus*, and *Megamonas*. The remaining six taxa were environmental microbes. Total abundances of phyla present in placental samples are shown in Figure 11.

3.5 Comparison of maternal sources of microbes and prenatal community compositions

In order to evaluate the most probable maternal source of microbes in prenatal tissues, I looked at between-tissue comparisons of alpha diversity with Tukey's HSD test. The full table of corrected p-values for species richness, Shannon's diversity, and Simpson's inverse index are provided in Table 2.

Tissue type significantly affected species richness (Figure 8a, ANOVA: $F = 6.6$, $p < 0.001$), Shannon's diversity (Figure 8b, ANOVA: $F = 28.56$, $p < 0.001$), and Simpson's inverse index (Figure 8c, ANOVA: $F = 4.395$, $p = 0.00086$). Pairwise comparisons of observed number of OTUs showed that embryonic gut was distinct from all maternal sources in at least one measure of alpha diversity, but did not significantly differ from the placenta for any measure of alpha diversity (species richness: $p = 0.056$, Shannon's diversity: $p = 0.93$, Simpson's inverse index: $p = 1.00$). The placenta did not differ from the maternal gut (species richness: $p = 0.52$, Shannon's diversity: $p = 0.99$, Simpson's inverse index: $p = 0.95$) or blood (species richness: $p = 0.38$, Shannon's diversity: $p = 1.00$, Simpson's inverse index: $p = 0.99$) for any measure of the alpha diversity, which is in line with the prediction that microbes escape the gut and reach the placenta through the circulatory system. The maternal gut and blood samples also did not differ from each

other for any measure of alpha diversity (species richness: $p = 0.99$, Shannon's diversity: $p = 0.99$, Simpson's inverse index: $p = 0.99$).

The effect of tissue was significant across all samples when calculating beta diversity with Bray-Curtis dissimilarities (Figure 9a, PERMANOVA: $p < 0.001$; NMDS stress = 0.21), UniFrac distances (Figure 9b, PERMANOVA: $p < 0.001$; NMDS stress = 0.19), and weighted UniFrac distances (Figure 9c, PERMANOVA: $p < 0.001$; NMDS stress = 0.17). Similar to alpha diversity, the embryonic gut was distinct from every potential maternal source of microbes but was not significantly different from the placenta using Bray-Curtis dissimilarities ($p = 0.41$) and weighted UniFrac distances ($p = 0.63$). The placenta was distinct from all maternal sources except for blood using Bray-Curtis dissimilarities ($p = 0.48$) and weighted UniFrac distances ($p = 0.84$). The full list of post-hoc pairwise comparisons is available in Table 3.

4. Discussion

I addressed two main questions in this chapter. First, I investigated the potential contributions of embryonic genotype and maternal genotype (independent of embryonic genotype) to the initial seeding of the prenatal microbiome. There was robust evidence for an effect of embryonic genotype on the community composition of the placenta and the embryonic gut. In contrast, maternal genotype (AZ or ED) did not affect beta diversity in placenta or embryonic gut, and only affected one metric of alpha diversity in embryonic guts. These results suggest that interactions between maternal and paternal genomes from different populations shape the community composition of the prenatal microbiome. Surprisingly, alpha diversity for maternal guts differed by the genotype of the litter they produced, not by maternal genotype. This effect of litter genotype on mothers may be due to microbes acquired from sires during co-housing and mating, or to interactions between mothers and offspring during gestation. These non-mutually exclusive possibilities are discussed below.

Second, I evaluated which maternal microbial community contributed the most to the prenatal microbiome. Across alpha diversity measures, the embryonic gut was similar to the placenta. The placenta was, in turn, statistically indistinguishable from maternal gut and blood but distinct from maternal oral cavity and vaginal tract. This suggests that the prenatal microbiome derives primarily from maternal gut microbes that reach the placenta via the maternal circulatory system and is consistent with previous studies (Martinez et al. 2018; Younge et al. 2019).

Herein, I discuss the potential influences of mate and litter genotypes on the maternal gut microbiome, how embryonic genotype may affect the composition of microbes in the placenta and embryonic gut differently, and compare these results with prior studies of the murine prenatal microbiome.

Paternal and embryonic influences on maternal gut microbiota

The gut microbiota of ED and AZ mothers were significantly differentiated for all measures of beta diversity but were indistinguishable for alpha diversity. The latter result was surprising as a study conducted during the development of the ED and AZ strains found that their gut microbiota remained distinct after ten generations under standard laboratory conditions (Moeller et al. 2018). However, the same study found that dissimilarity between the strains decreased across generations, presumably due to horizontal transmission of microbes between strains maintained in the same colony (Moeller et al. 2018). Given that the ED and AZ strains have now been in lab environments for more than a decade (Dumont et al. 2024), lack of difference in the number and phylogenetic diversity of microbial species may be explained by continued mixing and homogenization of gut microbes in a shared environment.

More surprising was the effect of embryonic genotype on maternal gut microbiome alpha diversity for females of the same strain. Specifically, ED females carrying a litter sired by an ED male were significantly differentiated from ED females carrying a litter sired by an AZ male and the same was true for AZ females. There was also an effect of embryonic genotype on microbial beta diversity in maternal guts, but this effect was weaker than that of maternal strain.

We consider two non-mutually exclusive sources of the effect of embryonic genotype on maternal gut alpha diversity: 1) the identity of males that females were crossed to, and 2) differences in maternal-embryonic interactions mediated by litter genotype.

While it is unknown if male gut microbiota community composition followed the same pattern as the females in this experiment, cohousing with a male of a different strain may alter female gut microbiota composition. Cohousing laboratory mice leads to the homogenization of gut microbiota in a very short time period, with changes first appearing after three days due to coprophagy (Caruso et al. 2019). However, a recent study investigating the mode by which pre-mating dysbiosis in fathers can affect offspring showed that mothers' postpartum microbiome was not impacted by fathers (Argaw-Denboba et al. 2024).

Another way in which males may impact the female microbiome is through seminal fluid. The microbiome of the seminal fluid interacts with the female reproductive tract during and after copulation (reviewed in Kilama et al. 2024). However, the majority of work on this topic focuses on how the seminal fluid microbiome influences the microbiota of the female reproductive tract, not the gut. Specifically, the proposed interaction between the seminal fluid microbiome and the microbiome of the vagina and uterus affects embryonic development by modulating the immune response within the female reproductive tract (Luecke et al. 2022; Schoenmakers et al. 2019). Future work exploring how the seminal fluid microbiome interacts with females should address if and how peripheral maternal microbial communities might be affected by the seminal fluid microbiome.

While mates may impact the female gut microbiome during reproduction and in early stages of embryonic development, the embryo can affect maternal systems for longer periods of time through endocrine signals produced by the placenta. The placenta mediates mother-offspring interactions during gestation, and is the main site of microbial transfer to embryos. The placenta benefits developing offspring through nutrient transfer and waste removal, but it is also the source of endocrine signals that induce physiological and behavioral changes that prime pregnant females for parturition and the onset of maternal care. In healthy pregnancies, the maternal gut microbiome undergoes shifts as a result of the endocrine signals produced by the placenta (Elderman et al. 2018). For example, increased progesterone during pregnancy affects the composition of the maternal gut microbiome, especially the abundance of *Bifidobacterium* (Koren et al. 2012; Nuriel-Ohayon et al. 2019). Placental endocrine function varies based on embryonic genotype (reviewed in John 2022), which may differentially affect maternal gut microbiota. Further research needs to be conducted to determine whether the presence of a between-cross placenta (EA, AE genotypes) in these crosses resulted in altered concentrations of pregnancy hormones. Disruption of the maternal gut microbiota during pregnancy has negative outcomes for embryonic gut microbiota and immune function postpartum (Nyangahu et al. 2018). Thus, it is critical to understand if and how embryonic genotype affects the maternal gut microbiome as maternal gut dysbiosis has long-term negative consequences for offspring fitness.

Effect of embryonic genotype on prenatal microbiome

Whereas several prior studies characterized the prenatal microbiome in mice (Martinez et al. 2018; Younge et al. 2019; Li et al. 2021), this is, to my knowledge, the first experimental investigation of the effects of embryonic genotype on the prenatal gut microbiome. Across prior studies, the relative contribution of the placenta to the community composition of the embryonic gut is the highest compared to peripheral maternal sources (Martinez et al. 2018; Younge et al. 2019). Collado et al. (2016) suggested that the placenta acts as a filter for selective transfer of maternal microbes to embryos. However, I found that the placenta is not distinct from the embryonic gut when comparing all prenatal and maternal tissues (Table 2, Table 3), and that alpha diversity of the placenta does not differ between embryonic genotypes. This pattern suggests that the effect of embryonic genotype may instead play out directly in the embryonic gut. Variation in the maternal gut microbiome may contribute to differences between embryonic guts. As translocation from the maternal gut via maternal blood flow into the placenta is the highest relative contributor to the embryonic gut microbiome, diversity of the embryonic gut microbiota will be limited by which microbes are already present in maternal guts.

Genotype also affects the initial acquisition of the infant gut microbiome (reviewed in Spor, Koren, and Ley 2011; Enav et al. 2022). Species diversity and richness are very low in the gut microbiome of neonates shortly after birth, but increase over time. Low diversity of the neonatal gut microbiome suggests that microbes are selectively able to establish in the gut over time (Enav et al. 2022). Given that genotype can directly impact the identity and relative abundance of microbes in both neonatal

(Enav et al. 2022) and adult gut (Benson et al. 2010), it seems likely that embryonic genotype similarly affects microbial composition in the embryonic gut. Future work should investigate the mechanism by which embryonic genotype affects the community composition of microbes in the embryonic gut, specifically whether it is directly related to the microbes present in the maternal gut or if particular microbes are more easily able to establish in the embryonic gut due to genotypic differences.

Comparisons to previous studies of the prenatal microbiome

Only two other studies have investigated the prenatal microbiome in mice and compared prenatal samples to maternal sources. Martinez et al. (2018) compared fetal intestine (analogous to the embryonic gut) to newborn intestine, and to maternal samples from mouth, vagina, colon, and feces. Maternal samples had higher species richness than fetal samples and placenta. Additionally, the most abundant taxon in the fetal intestine was *Lachnospiracae*. (Martinez et al. 2018). Younge et al. (2019) investigated microbial composition of fetal samples sampled during mid- and late gestation, and tested potential maternal sources at both time points, including amniotic fluid, blood, feces, uterus, and vagina. Similar to Martinez et al. (2018), maternal feces had the highest value of Shannon's diversity across all fetal and maternal samples, however, there were no differences between maternal samples following correction for multiple comparisons. The placenta was the most likely contributor to fetal intestine microbiota in late gestation, followed by amniotic fluid. Fetal samples overlapped with amplicon sequence variants (ASVs) from placental and amniotic fluid including *Kurthia gibsonii* and *Escherichia/Shigella* ASVs (Younge et al. 2019).

In contrast to both studies, I found that embryonic gut and placental samples had higher species richness than the maternal gut. This disparity may be a result of a larger sample size for embryonic and maternal samples in this experiment, however, maternal samples also had higher species richness values than those reported in Martinez et al. (2018). In my experiment, maternal sites were mostly distinct from each other, aside from maternal gut and blood samples. The latter is consistent with the suggestion that microbes escape the maternal gut to the circulatory system (Perez et al. 2007). Both Martinez et al. (2018) and Younge et al. (2019) used maternal fecal samples as proxies for the maternal gut, which might account for the differences between our findings of alpha diversity. However, this seems unlikely as a large percentage of OTUs are shared between the lower GI tract and fecal samples in house mice (Suzuki and Nachman 2016).

Of the specific taxa present in the prenatal environment in previous studies, there were no overlaps between the major taxa found in the fetal intestine and embryonic guts in this experiment when classifying OTUs at taxonomic levels other than phylum. At the level of phylum, embryonic guts and placental samples were predominantly characterized by Actinobacteria, Firmicutes, and Proteobacteria. This finding is consistent with Martinez et al. (2018), wherein Proteobacteria and Firmicutes were predominant in the fetal intestine. Below the level of phyla, *Lachnospiraceae* was the most abundant family in fetal intestines in Martinez et al. (2018), and in my placental samples. *Lachnospiraceae* is commonly found in mammalian guts and represented two of the top ten taxa present in placental tissues (classified to the genus level as *Blautia*). *Blautia* occur widely in feces and intestines of mammals and have probiotic characteristics that

can impact microbiota composition (Liu et al. 2021). Additionally, multiple taxa in the embryonic gut were identified as *Lactobacillus*, which Younge et al. (2019) found in cultures from fetal and placental tissue. Taxa in the *Lactobacillus* genus have also been identified in the gut microbiome of human infants (Chu et al. 2017). Thus, although only two taxa overlapped between this experiment and prior studies of the prenatal microbiome in mice, these taxa are important members of the microbial community of the gut.

Disparity between taxa identified in studies of the prenatal microbiome is a point of contention. Some of the disparity could relate to differences in sequencing protocols (Theis et al. 2020) or data processing including the reference databases used for phylogenetic identification of microbial species (Ceccarani and Severgnini 2023). Future work on the prenatal microbiome should address the discordance in identified microbial taxa between studies. The discordance could be addressed by standardizing data production and analyses, or by utilizing multiple methodologies to test how consistently particular taxa are identified across methods.

Conclusion

To my knowledge, this is the first study to directly test the effect of embryonic genotype on diversity measures of the prenatal microbiome. In this chapter, I found robust evidence for an effect of embryonic genotype on the placenta and embryonic gut. Embryonic genotype also affected the maternal gut microbiota, which could be mediated by placental endocrine signals. I also report results that support previous sources indicating that the placenta is the most likely contributor of microbes in the embryonic

gut. It is unclear if microbial signals are indicative of live microbes in prenatal tissues, but chapter 3 will address this question. While the prenatal microbiome remains a contentious subject, the potential reciprocal microbial interactions between mothers and offspring during gestation, as well as fitness consequences of the reciprocal interactions between mothers and offspring, merit further study.

Table 2.1 Sample sizes for all tissues, crosses, and negative controls.

Tissue	**Crosses (*n*)**
Controls	20
Embryonic gut	ED x ED (16) ED x AZ (10) AZ x ED (8) AZ x AZ (6)
Placenta	ED x ED (16) ED x AZ (8) AZ x ED (8) AZ x AZ (8)
Maternal gut	ED x ED (13) ED x AZ (6) AZ x ED (4) AZ x AZ (4)
Blood	ED x ED (11) ED x AZ (6) AZ x ED (4) AZ x AZ (4)
Oral cavity	ED x ED (13) ED x AZ (4) AZ x ED (4) AZ x AZ (4)
Vaginal tract	ED x ED (11) ED x AZ (6) AZ x ED (4) AZ x AZ (4)

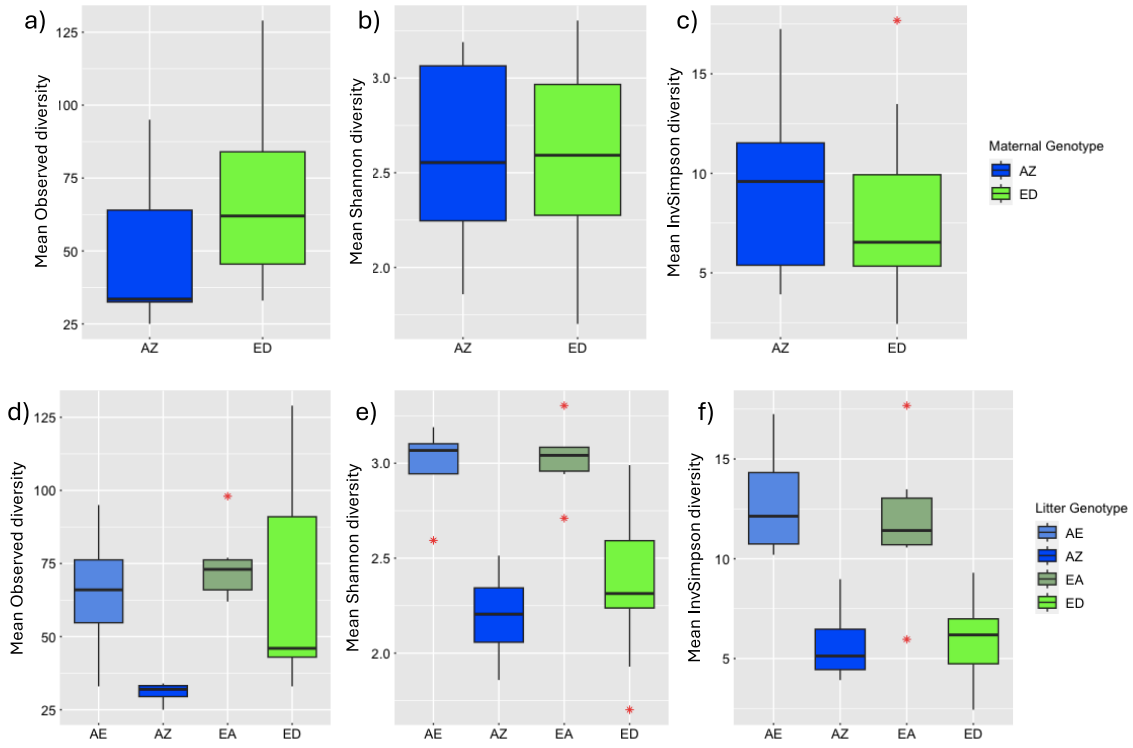
Table 2.2 Post-hoc pairwise comparisons with Tukey's correction for multiple comparisons of species richness, Shannon's diversity, and Simpson's inverse index across all tissue types. Bolded values indicate statistical significance ($\alpha = 0.05$).

Tissue comparison	**Observed species richness (p)**	**Shannon's diversity (p)**	**Simpson's inverse index (p)**
Embryonic gut - Blood	**0.00026**	0.950	1.00
Embryonic gut – Maternal gut	**0.00047**	0.890	0.966
Embryonic gut – Oral cavity	**0.0002**	**0.00**	**0.0017**
Embryonic gut – Placenta	0.056	0.931	1.00
Embryonic gut – Vaginal tract	**0.014**	**0.016**	0.392
Placenta – oral cavity	0.360	**0.00**	**0.0015**
Placenta – maternal gut	0.525	1.00	0.960
Placenta - blood	0.378	1.00	1.00
Placenta - vaginal tract	0.961	**0.00083**	0.373
Maternal gut - blood	1.00	1.00	0.989
Oral cavity - blood	1.00	**0.00**	**0.010**
Vaginal tract - blood	0.913	**0.0036**	0.584
Oral cavity – maternal gut	1.00	**0.00**	**0.0497**
Vaginal tract – maternal gut	0.972	**0.0014**	0.903
Vaginal tract – oral cavity	0.908	**0.0000033**	0.464

Table 2.3 Post-hoc pairwise comparisons between tissues. Comparisons were generated with the pairwise.adonis function for the significant effect of tissue type in a PERMANOVA using beta diversity calculated with Bray-Curtis dissimilarities, UniFrac distances, and weighted UniFrac distances. Bolded values indicate statistical significance ($\alpha = 0.05$).

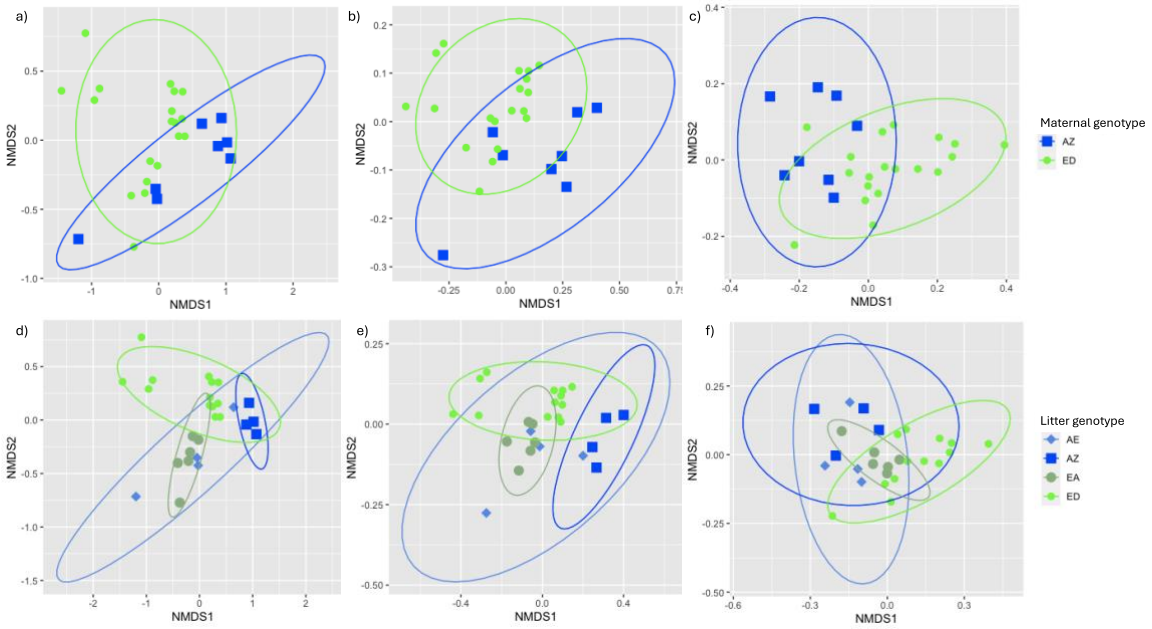
Tissue comparison	**Bray-Curtis (p_{adj})**	**UniFrac (p_{adj})**	**Weighted UniFrac (p_{adj})**
Embryonic gut - Blood	**0.015**	**0.015**	**0.015**
Embryonic gut – Maternal gut	**0.015**	**0.015**	**0.015**
Embryonic gut – Oral cavity	**0.015**	**0.015**	**0.015**
Embryonic gut – Placenta	0.405	**0.030**	0.630
Embryonic gut – Vaginal tract	**0.015**	**0.015**	**0.015**
Placenta – oral cavity	**0.015**	**0.015**	**0.015**
Placenta – maternal gut	**0.015**	**0.015**	**0.015**
Placenta - blood	0.480	**0.015**	0.840
Placenta - vaginal tract	**0.015**	**0.015**	**0.030**
Maternal gut - blood	**0.015**	**0.015**	**0.015**
Oral cavity - blood	**0.015**	**0.015**	**0.030**
Vaginal tract - blood	**0.015**	**0.045**	**0.015**
Oral cavity – maternal gut	**0.015**	**0.015**	**0.015**
Vaginal tract – maternal gut	**0.015**	**0.015**	**0.015**
Vaginal tract – oral cavity	**0.015**	**0.015**	**0.015**

Figure 2.1



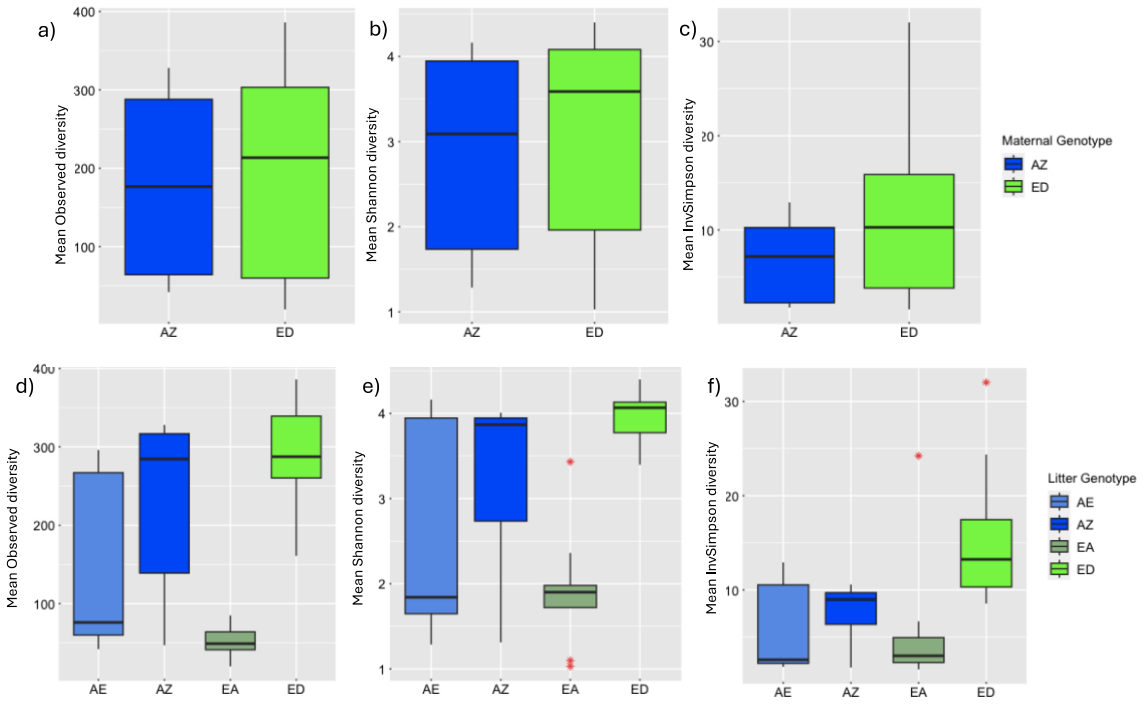
Alpha diversity of gut microbiome of two adult *M. m. domesticus* wild-derived inbred strains: the descendants of mice captured in Tucson, AZ (AZ) and Edmonton, Alberta, Canada (ED). On the top row, box and whisker plots showing mean (a) species richness, (b) Shannon's diversity, and (c) Simpson's inverse index comparing ED and AZ females. On the bottom row, box and whisker plots showing mean (d) species richness, (e) Shannon's diversity, and (f) Simpson's inverse index comparing females by the genotype of the litter they produced (with female strain listed first, AZ for AZxAZ cross, AE for AZxED cross, ED for EDxED cross, and EA for EDxAZ cross). There were no significant effects of maternal genotype on any metric of alpha diversity (a-c), whereas embryonic genotype significantly affected Shannon's diversity (e) and Simpson's inverse index (f).

Figure 2.2



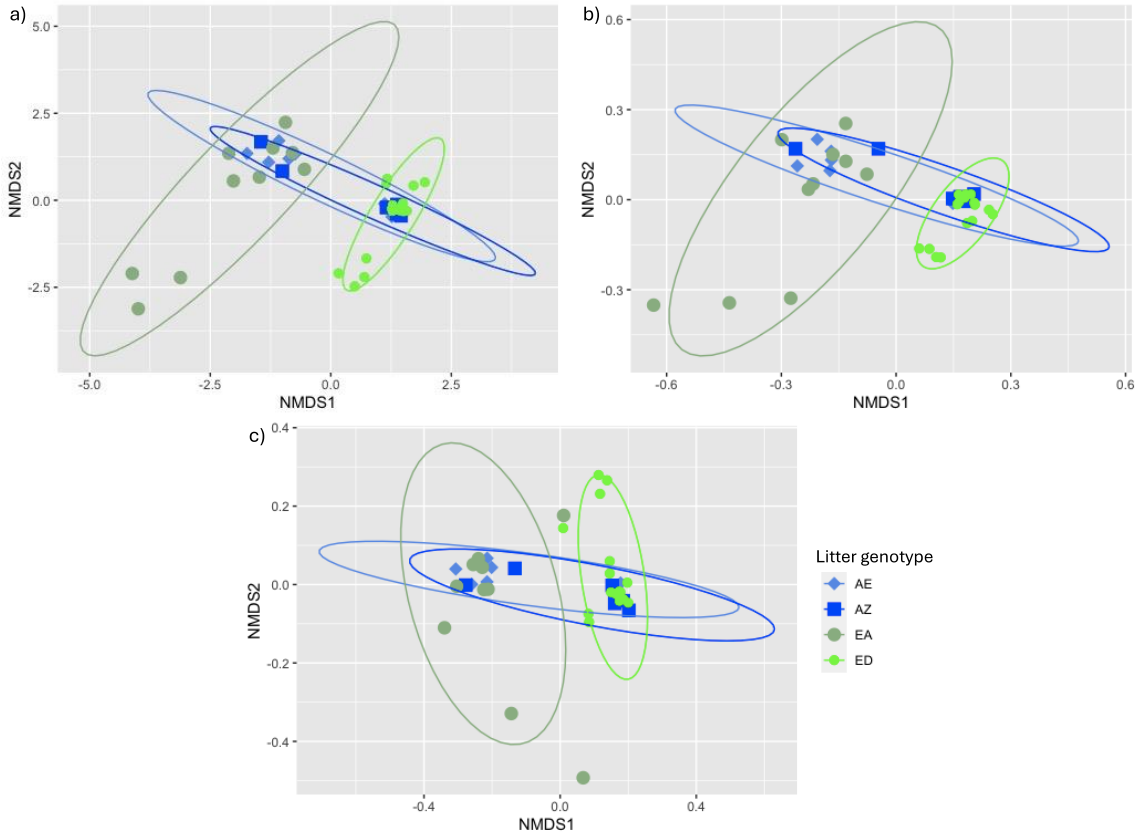
Beta diversity of gut microbiome of adult AZ and ED *M. m. domesticus* wild-derived inbred strains. On the top row, NMDS plots of the significant effect of maternal genotype on beta diversity calculated with (a) Bray-Curtis dissimilarities, (b) unweighted UniFrac distances, and (c) weighted UniFrac distances. On the bottom row, NMDS plots of the significant effect of embryonic genotype on beta diversity calculated with (d) Bray-Curtis dissimilarities, (e) unweighted UniFrac distances, and (f) weighted UniFrac distances.

Figure 2.3



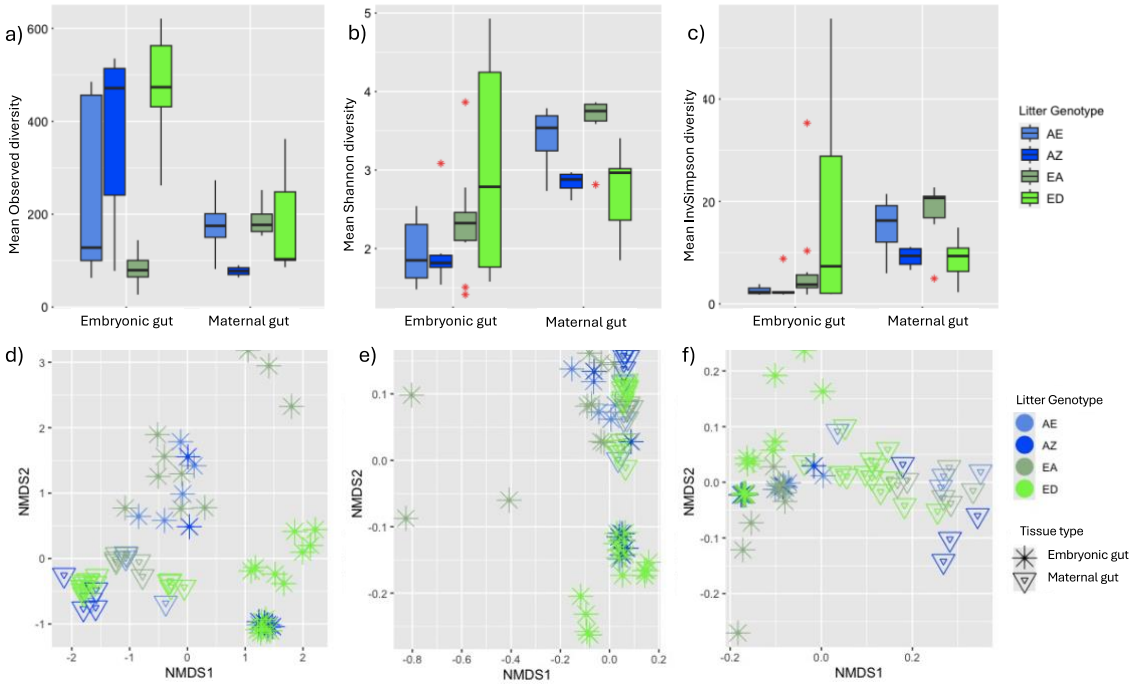
Alpha diversity of the embryonic gut: with female strain listed first, AZ for AZxAZ cross, AE for AZxED cross, ED for EDxED cross, and EA for EDxAZ cross. On the top row, box and whisker plots showing mean (a) species richness, (b) Shannon's diversity, and (c) Simpson's inverse index comparing the embryonic guts by maternal genotype. On the bottom row, box and whisker plots showing mean (d) species richness, (e) Shannon's diversity, and (f) Simpson's inverse index comparing embryos by embryonic genotype.

Figure 2.4



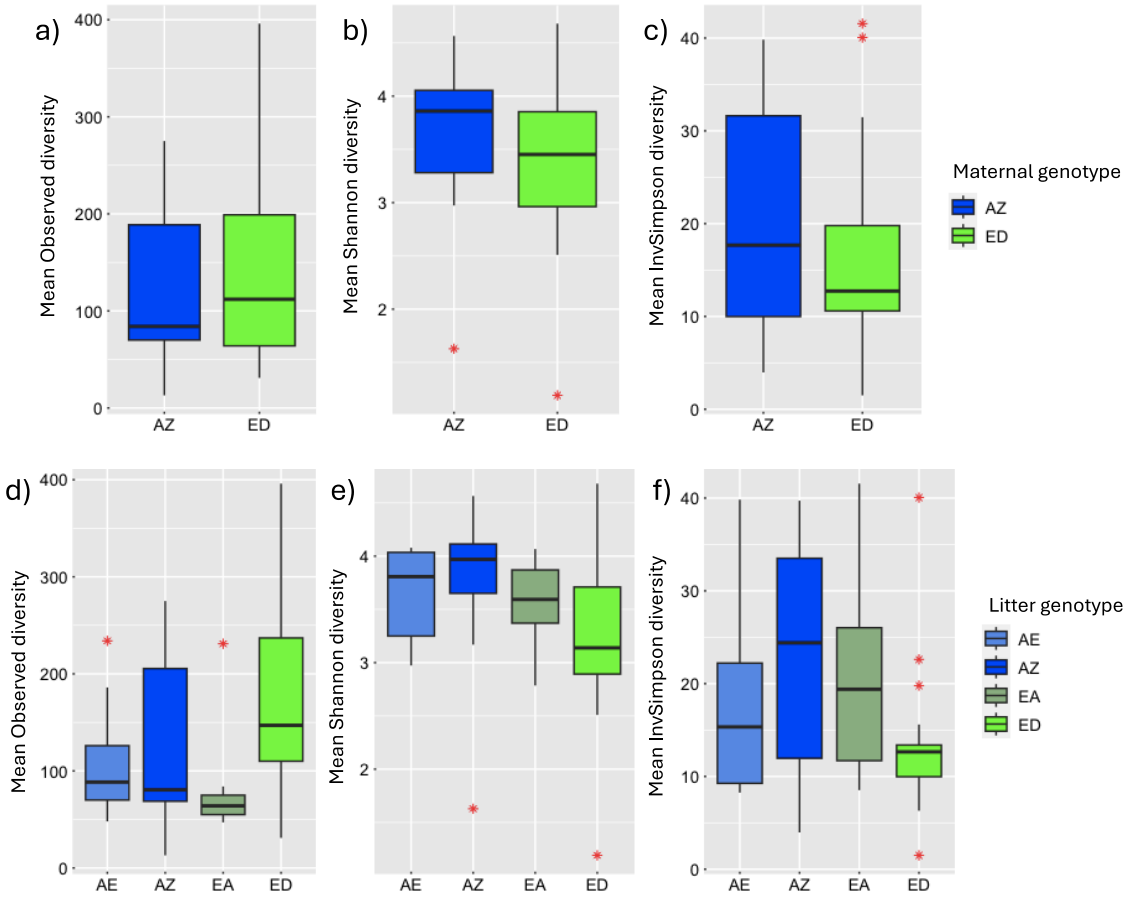
Significant effect of embryonic genotype on beta diversity in AZ, AE, EA, and ED embryonic guts. NMDS plots of beta diversity calculated with (a) Bray-Curtis dissimilarities, (b) UniFrac distances, and (c) weighted UniFrac distances where embryonic genotype showed a significant effect on community composition.

Figure 2.5



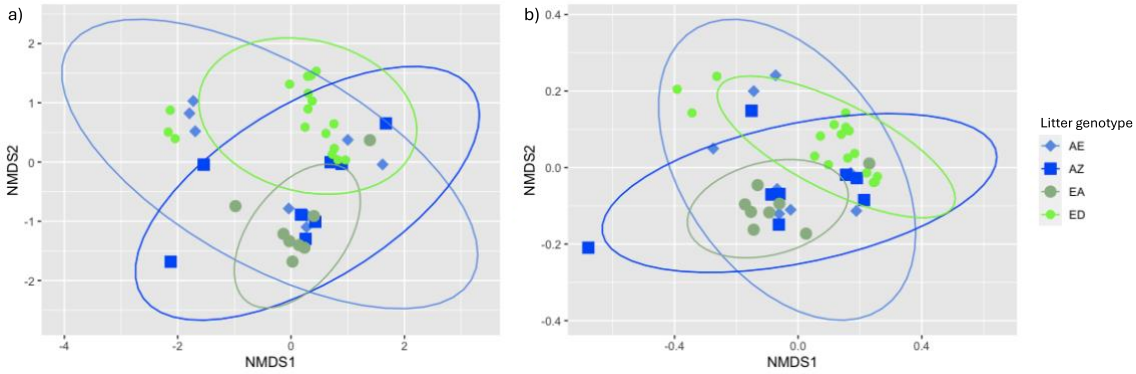
Comparison of diversity metrics between maternal and embryonic guts. Box and whisker plots showing mean (a) species richness, (b) Shannon's diversity, and (c) Simpson's inverse index. NMDS plots of beta diversity calculated with (d) Bray-Curtis dissimilarities, (e) UniFrac distances, and (f) weighted UniFrac distances.

Figure 2.6



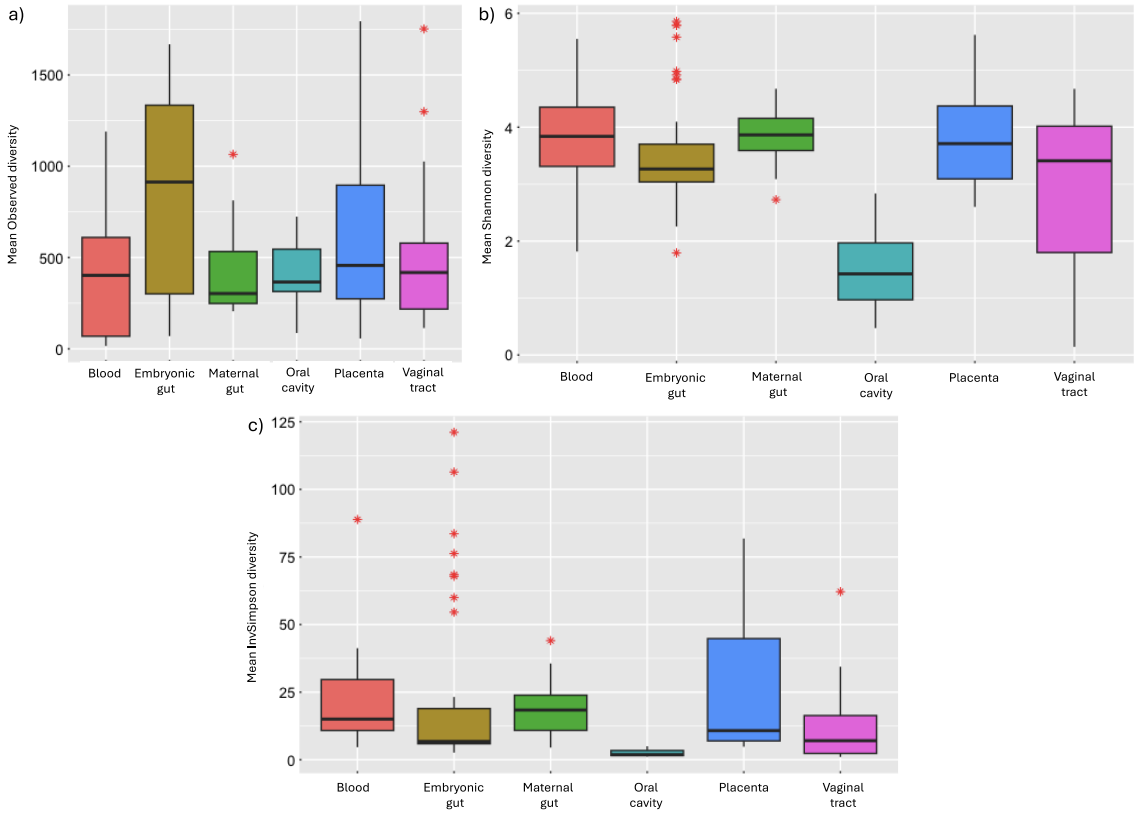
Alpha diversity in AZ, AE, EA, and ED placental tissue. Box and whisker plots showing mean (a) species richness, (b) Shannon's diversity, and (c) Simpson's inverse index comparing placentas by maternal genotype. Box and whisker plots showing mean (d) species richness, (e) Shannon's diversity, and (f) Simpson's inverse index comparing placentas by embryonic genotype.

Figure 2.7



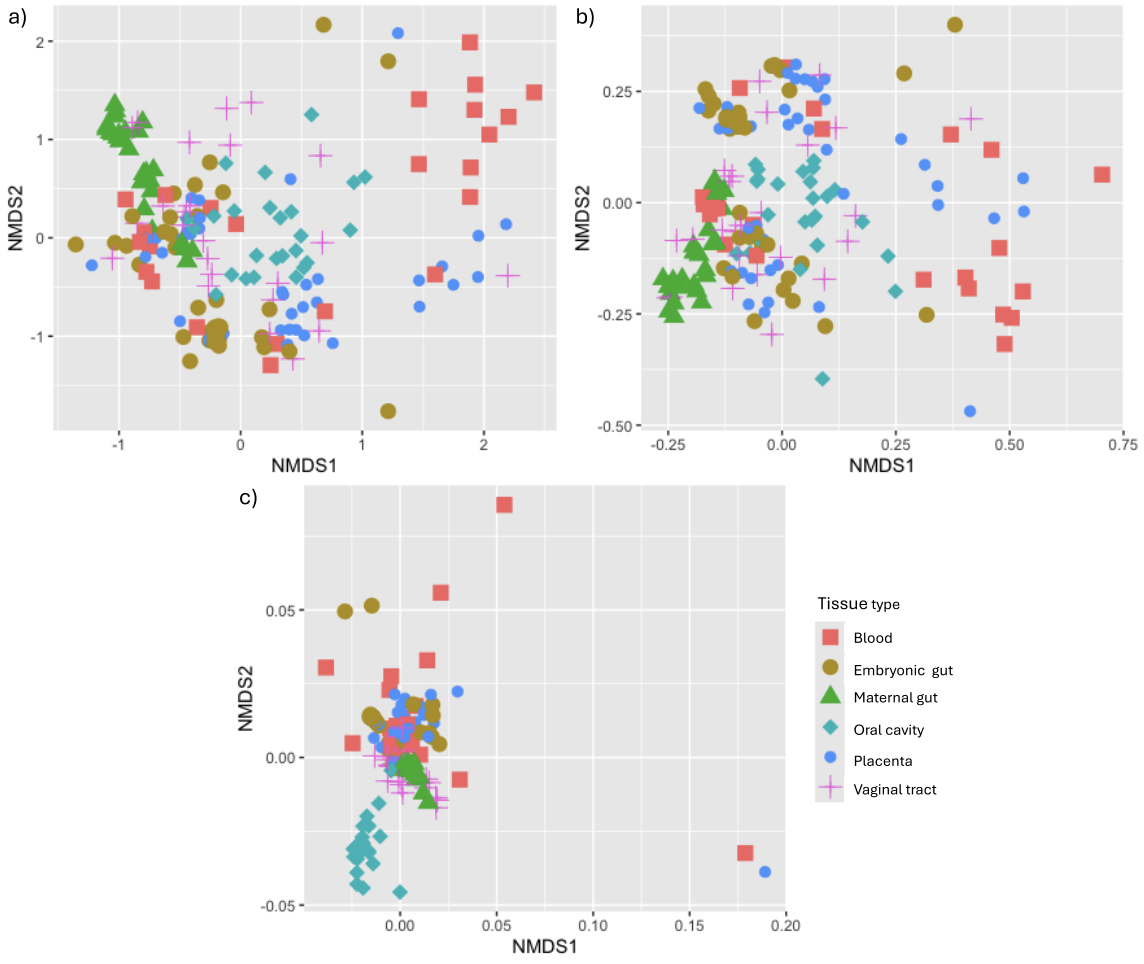
Beta diversity in AZ, AE, EA, and ED placental tissue. NMDS plots were generated using (a) Bray-Curtis dissimilarities and (b) UniFrac distances.

Figure 2.8



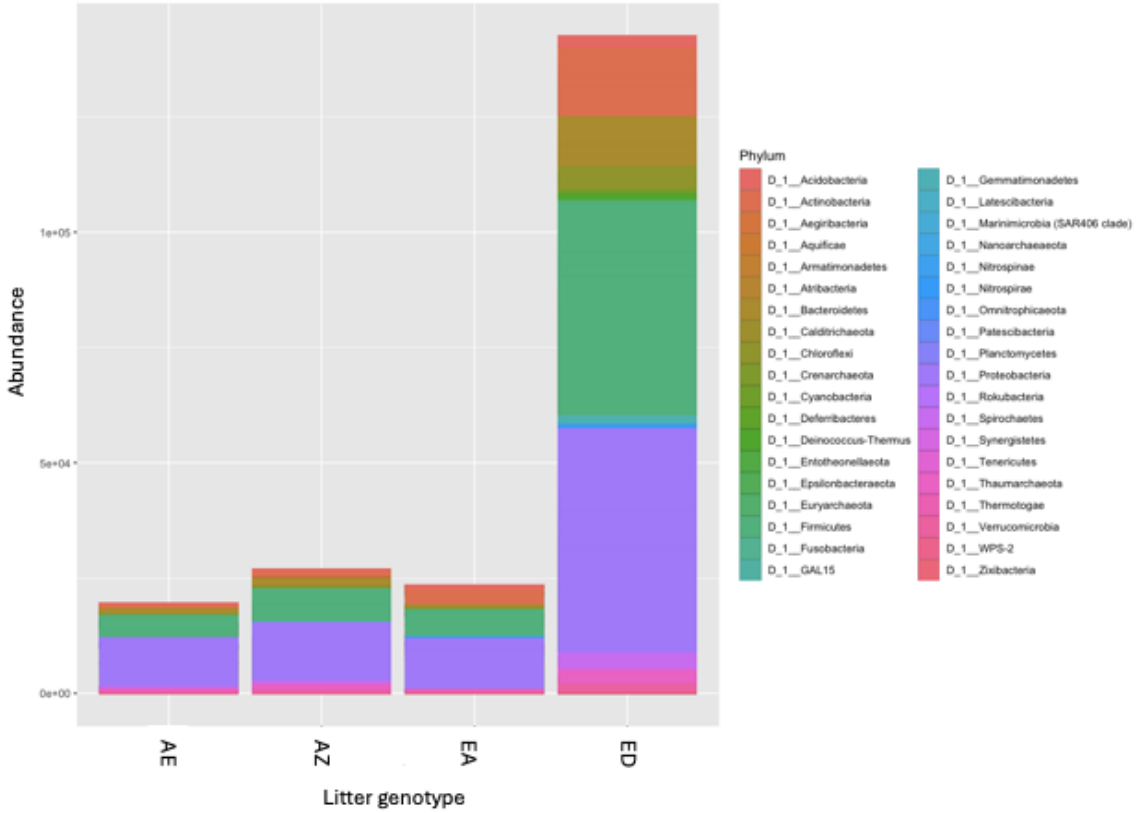
Alpha diversity across all tissues. Box and whisker plots showing mean (a) species richness, (b) Shannon's diversity, and (c) Simpson's inverse index for each prenatal tissue (embryonic gut and placenta) and maternal samples (blood, maternal gut, oral cavity, and vaginal tract). Maternal and embryonic genotype were not considered in this analysis.

Figure 2.9



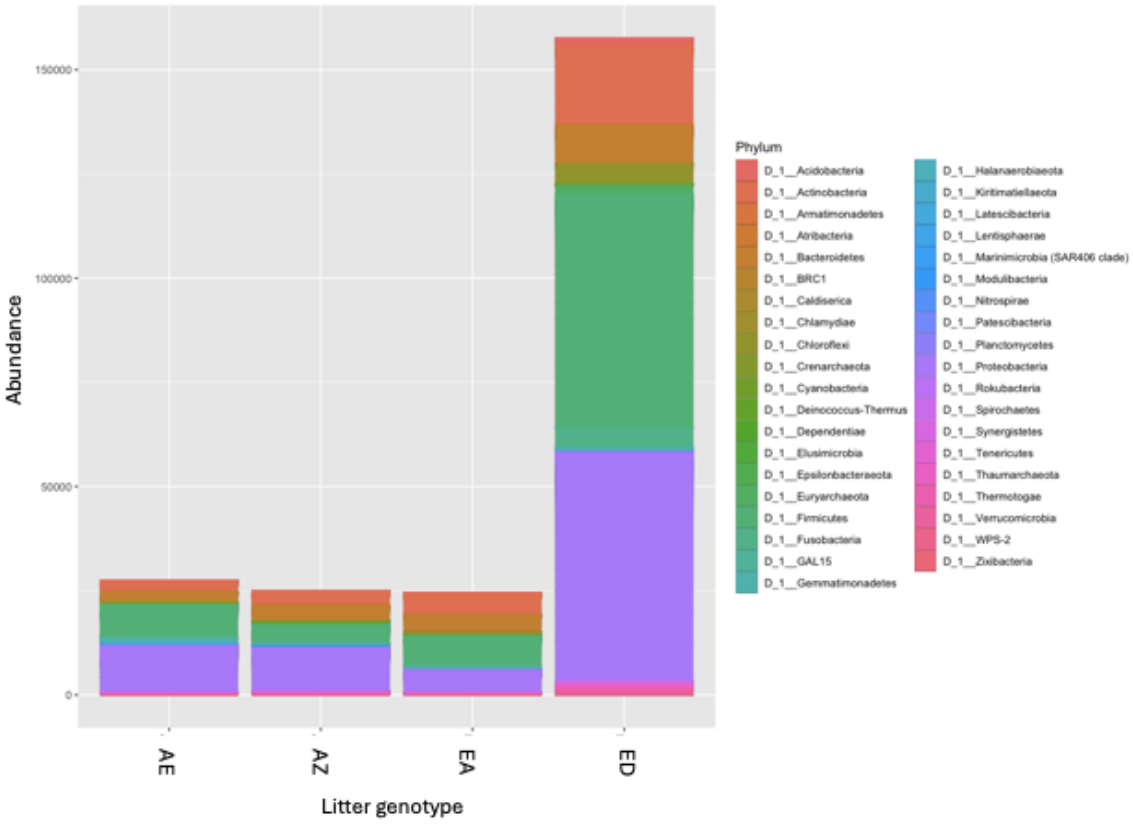
Beta diversity across all tissues. NMDS plots of beta diversity calculated with (a) Bray-Curtis dissimilarities, (b) UniFrac distances, and (c) weighted UniFrac distances for prenatal tissues (embryonic gut and placenta) and maternal samples (blood, maternal gut, oral cavity, and vaginal tract). Maternal and embryonic genotype were not considered in this analysis.

Figure 2.10



Abundances of bacterial phyla present in embryonic gut samples. The abundances for each sample are merged (OTUs summed across samples) by embryonic genotype. The most prevalent phyla are Firmicutes (teal), Proteobacteria (purple), and Actinobacteria (red).

Figure 2.11



Abundances of bacterial phyla present in placental samples. The abundances for each sample are merged (OTUs summed across samples) by embryonic genotype. Similar to embryonic guts, the most prevalent phyla are Firmicutes (teal), Proteobacteria (purple), and Actinobacteria (red).

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Chapter 3

Investigation into signatures of live microbes in embryonic guts using multiple methodologies

Abstract

Nearly two decades since the idea that eutherian mammals may not develop in sterile conditions was first proposed, the prenatal microbiome remains a contentious topic. The field has debated the validity of microbial signatures found in low biomass prenatal tissues, which are easily contaminated from environmental sources. In addition to concerns of contamination, it is unclear if microbes found in prenatal tissues are live or are fragments of microbial DNA transmitted via the maternal circulatory system. Short chain fatty acids (SCFAs) are the main products of anaerobic microbes in the adult gut and are therefore suitable markers for live microbes in the embryonic gut. We quantified microbial load in embryonic guts, adult guts, and negative controls. We first attempted to qualitatively assess SCFA presence in the embryonic gut using gas chromatography/mass spectroscopy (GC-MS) but only succeeded in determining the relative quantities of SCFAs. Due to the volatile nature of SCFAs, we wanted to confirm that the SCFAs identified in the GC-MS were true signals of SCFAs rather than derivatives of the SCFAs (e.g. acetyl groups rather than acetic acid). We therefore used nuclear magnetic resonance spectroscopy (NMR) to confirm the chemical conformation of putative SCFAs. H^1 NMR confirmed the presence of SCFAs in an adult gut sample but SCFAs were not detected in embryonic gut samples. Despite a lack of evidence of SCFAs in the embryonic gut, prenatal exposure to microbial DNA may still represent an important aspect of embryonic development.

1. Introduction

According to the sterile womb paradigm, vertebrate gestation occurs in sterile conditions and fetuses acquire microbes through both vertical (from a maternal source) and horizontal (from the environment and other individuals) transmission during and after birth. However, in the past decade, multiple studies have independently suggested that this is not the case in eutherian mammals (reviewed in Funkhouser and Bordenstein 2013; Perez-Muñoz et al. 2017) – particularly in mice (Martinez et al. 2018; Younge et al. 2019) and humans (Jiménez et al. 2005; Aagaard et al. 2014; Collado et al. 2016). The call for a more thorough examination of the sterile womb paradigm came with the advent of culture-independent, high throughput sequencing capabilities. However, many sequencing techniques are sensitive to contamination from the environment in which microbial samples are collected. In addition to concerns of contamination, critics of the "in utero colonization hypothesis" point out that detection of DNA using sequencing techniques is not an indication of live microbes, and thus does not demonstrate the existence of a prenatal "microbiome" (Kliman 2014). Now, in spite of increased sequencing capabilities, the major roadblocks to confirming the presence of a prenatal microbiome include the high risk of contamination in low biomass samples such as the placenta and the embryonic gut, as well as testing for evidence of metabolically active microbes.

Contamination in low microbial biomass tissues – due either to a lack of sterilization in the environment in which tissues are collected or to other outside contaminants, including those found in DNA extraction kits (Salter et al. 2014) – leads to

false positives in studies that do not include computational methods to remove contaminant signals. Because the first studies to provide sequence-based evidence of a placental microbiome (e.g. Aagaard et al. 2014) did not incorporate clear methodologies for contamination avoidance, the validity of their results has been questioned (Kliman 2014; Bushman 2019).

Outside of the issue of contamination, the debate over the presence of a prenatal microbiome continues today due to the lack of consistent findings between studies. While one recent study found strong support for a prenatal microbiome (including placental microbes) in mid-gestation in both humans and mice (Younge et al. 2019), another found no evidence of a placental microbiome in humans (de Goffau et al. 2019). Thus, despite the growing number of studies focused on the question of whether or not a prenatal microbiome is present in eutherian mammals, there is still no consensus.

More recent studies addressing the prenatal microbiome have included methods to test for live microbes in prenatal tissues, including cultures, microscopy (e.g. fluorescence *in situ* hybridization) and qPCR. Successfully cultured bacteria from the fetal gut of mid-gestation (embryonic day 14-16) mice include *Lactobacillus*, *Escherichia*, *Enterococcus*, *Bacteroides*, and *Bacillus* (Younge et al. 2019). The five taxa isolated from fetal tissues were also isolated from maternal fecal and vaginal samples (Younge et al. 2019), and are commonly found in the human neonatal gut microbiome (Chu et al. 2017). *Lactobacillus* was not isolated from human meconium samples, but a *Micrococcus* taxon was isolated after removing negative controls (Rackaityte et al. 2020). *Micrococcus* modulates mucosal immune function, which can then affect the ability of

other microbes to establish in the fetal gut (Duerkop, Vaishnava, and Hooper 2009). This was an important finding as it supports the hypothesis that the major function of prenatal microbes is to activate the mucosal immune system in developing embryos (Martinez et al. 2018; Stinson, Keelan, and Payne 2019). Fluorescence *in situ* hybridization probes identified low abundance microbes throughout the fetal intestine in late-gestation mice and human meconium samples (Younge et al. 2019; Rackaityte et al. 2020; Seferovic et al. 2019). Finally, qPCR has been used to quantify microbial load both in studies that confirm (Martinez et al. 2018) and reject (Theis et al. 2020a) the presence of prenatal microbes in mice. Theis et al. (2020a) found similar microbial loads in placental and fetal tissue samples, and in negative control samples. In addition, all maternal samples significantly exceeded microbial loads from negative controls (Theis et al. 2020a). Earlier investigations of microbial load in human placental tissues found the same negative result (Lauder et al. 2016; Leiby et al. 2018). In contrast, Martinez et al. (2018) found that microbial loads of mouse fetal intestines exceeded the microbial load of placental samples.

In the continued debate surrounding the prenatal microbiome, an important question remains as to the functionality of the microbes or microbial DNA present in the embryonic environment. In a recent review of expert opinions regarding the controversy of the prenatal microbiome, one source highlighted the importance of showing that this putative microbial community is metabolically active (Blaser et al. 2021). Stimulation of mucosal immune development is the proposed primary function of the prenatal gut microbiome (Stinson, Keelan, and Payne 2019). One line of evidence that supports this

proposed function is that first-pass meconium from infants delivered by non-emergency cesarean section contains short-chain fatty acids (SCFAs; Stinson, Keelan, and Payne 2019), immunostimulatory metabolites produced by commensal gut microbes (Corrêa et al. 2016; Luu and Visekruna 2019). Maternal SCFAs can pass from mothers to offspring and are important modulators of embryonic neurodevelopment (Vuong et al. 2020). Treatment with supplemental SCFAs promoted placental growth and vascular development in pregnant mice (Pronovost et al. 2023).

This study uses the same adult and embryonic gut samples collected in the previous chapter from wild-derived strains of house mouse (*Mus musculus domesticus*) to determine whether microbial DNA identified in embryonic guts is indicative of live microbes. As SCFAs are produced by metabolically active microbes and are important immunostimulants, we evaluated whether detectable SCFAs are produced in the embryonic gut.

2. Methods

2.1 Animals and Tissue Collection

Two wild-derived inbred strains of *M. m. domesticus* were used in this experiment: the descendants of mice captured in Tucson, AZ, (AZ) and Edmonton, Alberta, Canada (ED). Both strains were acquired from the Nachman Lab at the University of California, Berkeley and originally sent to Oklahoma State University in 2018. The colony was moved to the University of California, Riverside (UCR) in 2019. The colony was moved between vivaria on the UCR campus in 2022. An additional cohort of AZ mice were added to the colony in 2020. Throughout the moves, the mice were consistently fed the same diet (LabDiet 5001 Rodent Diet). Experimental pregnancies were generated using a standard timed mating protocol. Adult, nulliparous females were paired with sexually experienced males 1-2 hours before lights off. Females were subsequently checked for vaginal plugs each morning at lights on. The day a plug was observed (embryonic day 0), the pair was split and the female was moved to a clean cage. If a plug was not observed in females after 48 hours paired with the male, females were split from the male and monitored for pregnancy.

Females were euthanized and samples were collected at embryonic day 17.5. The uterus was dissected, and subsequent dissection of embryonic guts was done in a laminar flow hood to minimize the probability of contamination with maternal or environmental microbes. Embryonic guts were dissected and flash frozen in liquid nitrogen and stored in -80°C until DNA extraction or transport to the UCR Metabolomics Core Facility. The maternal gut was dissected last to minimize the possibility of cross contamination from

high to lower biomass tissue samples. All maternal experimental tissues were flash frozen in liquid nitrogen and stored at -80°C until DNA extraction or transport to the UCR Metabolomics Core Facility.

2.2 Microbial Load

Microbial load in embryonic gut samples was validated with qPCR (Cheng et al. 2011). Copy number (CN) of the 16S rRNA gene in microbial DNA extracted from embryonic whole gut (n = 3; ~30mg) and adult whole cecum (n = 3; ~300mg) was estimated with a standard curve generated from serial dilution of pure bacterial DNA. A negative extraction control sample was also included to account for microbes present in the DNA extraction kit (Salter et al. 2014).

2.3 Ultra-performance Liquid Chromatography-Mass Spectrometry (UPLC-MS) and Gas Chromatography/Mass Spectrometry (GC-MS) analyses

All assays were run in collaboration with the UCR Metabolomics Core Facility. We chose four SCFAs to target within the embryonic gut: acetic, propionic, butyric, and isobutyric acids. Acetic, propionic, and butyric acids are the most abundant acids produced in the human intestines (Ziętek et al. 2021). We also included isobutyric acid, which is produced as a result of branched amino acid fermentation and is produced in lower quantities in the gut than the other SCFAs of interest (Ziętek et al. 2021). SCFAs were first quantified in one adult sample to estimate the limits of detection for embryonic guts. Sample preparation and standard calibration were performed according to Han et al. (2014). Quantification of SCFAs was performed at the UCR Metabolomics Core on a Waters Xevo TQ-XS Triple Quadrupole Mass Spectrometer coupled to an I-class UPLC

system. Embryonic gut samples were similarly prepared according to Han et al. (2014), but did not yield any detectable SCFAs using the same preparation method as the adult gut sample.

Of the methods employed to quantify SCFAs, gas chromatography is used most often due to its ability to clearly separate compounds within samples and the high volatility of SCFAs (Cai et al. 2017). Additionally, gas chromatography is more sensitive than liquid chromatography to volatile compounds such as SCFAs. SCFAs were quantified using an additional cohort of embryonic gut samples (n = 19) using a Thermo 1300 coupled with Thermo Fisher ISQ7000 mass spectrometers. Samples were prepared using multiple derivatization methods in order to minimize any potential sample loss during sample preparation and, ultimately, we used a modification of sample preparation according to Bhatia et al. (2015).

2.4 Nuclear magnetic resonance spectroscopy (NMR) analysis

Assays were run in collaboration with the UCR Metabolomics Core and UCR Analytical Chemistry Instrumentation Facilities. To confirm the chemical structure of putative SCFAs identified from the GC-MS, embryonic and adult gut samples were analyzed using NMR spectroscopy. Biphasic extractions were performed according to Hollin et al. (2022) on adult gut, a single embryonic gut, and a pooled sample of five embryonic guts from the same litter. The polar fraction of the biphasic extraction was used for NMR to minimize background lipid signals from the resulting spectra. The ^{1}H NMR spectra were recorded on a Bruker Biospin 700 MHz NMR spectrometer (Bruker GmBH, Germany) equipped with a triple resonance cryoprobe.

3. Results

3.1 Microbial Load

Estimated mean CN/μL was $1.34x10^{10}$ for embryonic guts and $3.20x10^{12}$ for adult cecum (Figure 1). Given the order of magnitude difference between embryo and adult tissue input, this suggests that the microbial load in wild-derived mouse embryonic gut is less than two orders of magnitude lower than that in adult cecum. Importantly, estimated microbial load in embryonic guts was more than three orders of magnitude higher than background contamination in the extraction blank control (Figure 1).

3.2 UPLC-MS values and GC-MS relative proportions

Using UPLC-MS, all four SCFAs of interest were quantified from the adult gut sample. Acetic and butyric acids had the highest concentrations at 2.73 μmol/g tissue and 2.23 μmol/g tissue, respectively. Propionic acid was also present at 1.09 μmol/g tissue, and isobutyric acid was present in low concentrations of 0.024 μmol/g tissue. We were unable to detect signals of any SCFAs in embryonic guts using UPLC-MS.

Using GC-MS, we were able to measure the relative proportions of putative SCFAs of interest in embryonic gut samples. The relative proportions of each SCFA are shown in Figure 2. Acetic acid showed the highest relative proportion (93.1%) of all SCFAs in embryonic guts. The relative proportions of propionic (3.69%) and butyric (2.39%) acids were similar to each other, with isobutyric acid in the lowest relative proportion (<1%).

3.3 ^{1}H NMR Spectra

NMR spectroscopy was used to test the chemical structure of signals detected from GC-MS. Adult and embryonic samples were compared against standard spectra for each SCFA of interest. Peaks for acetic, propionic, and butyric acids were all present in adult samples (Figure 3). We did not see any peaks for any SCFA in the single embryonic gut sample (Figure 3) or peaks for acetic or butyric acids in the pooled embryonic gut sample (Figure 4).

4. Discussion

In this chapter, we quantified microbial signals in embryonic and adult gut samples, and investigated whether those signals were indicative of live microbes in the embryonic gut using SCFAs as biomarkers for microbial metabolic activity. We found that microbial load in embryonic guts was substantially higher than background using a negative extraction control. We were also able to compare relative concentrations of putative SCFAs through GC-MS. However, GC-MS results indicated a higher than expected relative concentration of acetic acid. This suggested that the detected signals might be derivatives of SCFAs (e.g. acetyl-CoA) rather than intact products of microbial metabolism. We therefore used NMR spectroscopy to determine the chemical structure of the molecules detected with GC-MS. Using NMR spectroscopy, we found no evidence of SCFAs in embryonic guts.

Microbial load

Microbial load in mouse embryonic gut, as quantified by 16S rRNA gene copy abundance, was higher in our samples than that of the technical control that only included kit solutions. Studies of the prenatal microbiome have increasingly included microbial load results in tandem with sequencing data. Studies that did not find evidence for microbial communities in prenatal tissues typically found microbial loads in prenatal tissues that were statistically indistinguishable from DNA extraction kit negative controls (Theis et al. 2019; Theis et al. 2020a; Theis et al. 2020b; Winters et al. 2022). In addition to low microbial load, Theis et al. (2020a) also reported that less than 3% of the placental and other fetal samples yielded a 16S rRNA amplicon library. Martinez et al. (2018)

found higher microbial loads in fetal intestines than placental tissue with both tissues expressing higher microbial loads than negative controls, and is the only other study that directly compared the placenta and fetal intestines in mice. It is unclear how this disparity has arisen between studies investigating microbial load in mice, as all report methods that minimize contamination.

UPLC-MS/GC-MS

We quantified SCFAs in adult gut samples using UPLC-MS. However, we were unable to do so in embryonic guts. Similarly, with GC-MS, we were only able to generate the relative proportions of our SCFAs of interest in embryonic guts. Moreover, the relative proportions of SCFAs were outside of the expected molar ratios based on previous reports for human colon, in which acetic, propionic, and butyric acids are typically seen in molar ratios of 60:20:18 (Wong et al. 2006). There have only been a few investigations of SCFA content in human meconium samples, and ratios of SCFAs differ between studies (Dobrowolska-Iwanek et al. 2020; Rasmussen et al. 1988). Dobrowolska-Iwanek et al. (2020) suggest that concentrations of the most common SCFAs vary widely in human neonates, leading to varied molar ratios. Rasmussen et al. (1988) reported a ratio of 89:5:5 for acetic, propionic, and butyric acids, which is closer to what we report in mouse embryonic guts (93:4:2). However, the much higher than expected proportion of acetic acid in embryonic guts found in the present study suggested that the observed signal might be derived from acetyl molecules other than acetic acid. Acetic acid can be converted into acetyl coenzyme A (acetyl-CoA), which is ubiquitous in the body (González Hernández et al. 2019). The assay was not sensitive enough to determine if the

detected SCFA molecules were from bacteria or products of cellular metabolism. Given the lack of sensitivity in molecule discrimination and the low concentrations of SCFA potentially present in embryonic tissues, quantification of SCFAs in fetal mouse tissues will require additional optimization.

NMR

Using NMR spectroscopy, we confirmed that the chemical signals found using GC-MS were SCFAs in adult gut samples, but did not find evidence for SCFAs in embryonic gut samples. NMR spectroscopy is sensitive to low concentrations of starting sample, in this instance the low biomass embryonic guts. After seeing no signal in a single embryonic gut sample (Figure 3), we pooled gut samples from five littermates to bring the sample mass closer to that of adult guts. Despite pooling embryonic samples, we still did not detect SCFA signal in embryonic guts (Figure 4). NMR spectroscopy has been used for qualitative and quantitative measures of SCFAs in adult mouse cecal and fecal samples (Jacobs et al. 2008; Cai et al. 2016), as well as in germ-free mouse samples (Cai et al. 2017). Germ-free mice have significantly lower levels of SCFAs as compared to conventionally raised lab mice, but still have detectable SCFA signal (Cai et al. 2017). With this evidence in mind, it is likely that the lack of SCFA signal in embryonic mouse guts is a true negative result, and not an artifact of the methodology used in this experiment.

Conclusions

The lack of a signal of SCFAs in embryonic guts suggests that the microbial load reported here, and results from Chapter 2, are indicative of microbial DNA, but not live

microbes, in the embryonic gut. Although this means that there is not a functioning microbial community in the embryonic guts assayed in this experiment, microbial DNA may still play an important functional role in developing embryos.

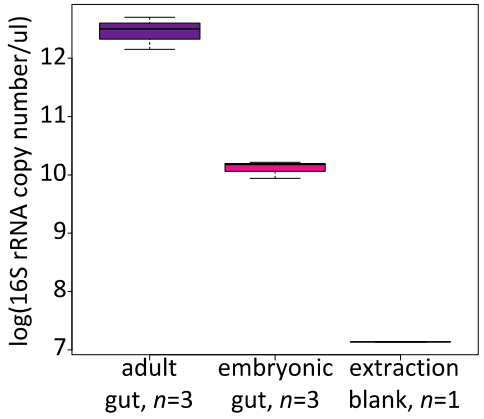
One possible explanation as to why we did not see signals of live microbes is that live microbes are only present relatively early in gestation. Younge et al. (2019) cultured fetal mouse tissues during mid (embryonic day 14-16) and late (embryonic day 17-18) gestation and only detected viable bacteria in the mid-gestation samples. Late gestation samples did not yield any viable cultures despite yielding bacterial DNA sequences. These results suggest that live microbes are transferred from mothers to offspring relatively early in gestation, while only microbial DNA remains close to parturition (Younge et al. 2019).

There is strong evidence that metabolites produced in the maternal gut, specifically SCFAs, reach developing embryos through the placenta. Maternal supplementation of SCFAs increased the size and vascularization of the placenta in mouse models (Pronovost et al. 2023). Additionally, supplementation of SCFAs increased the concentrations of butyric and propionic acids in fetal circulation (Pronovost et al. 2023). Maternal SCFAs and other by-products of the maternal gut microbiome are implicated in the formation of neural circuits in developing offspring (reviewed in Jašarević and Bale 2019). Comparative studies of germ-free mice and conventional mice also show that the lack of maternal gut microbiota leads to immune development deficiencies in offspring (Gomez de Agüero et al. 2016). While it is unclear if the

microbial DNA present in embryonic guts are acting to influence offspring phenotypes, it is clear that maternal gut microbiota are critical for offspring development.

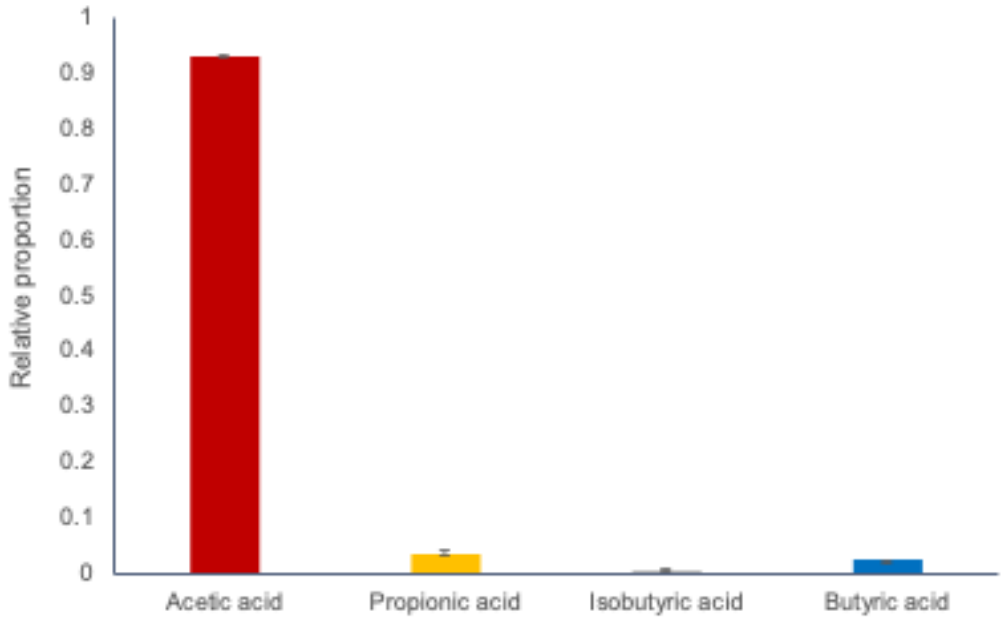
Moving forward, future work to evaluate the presence and functional role of a prenatal microbiome should follow previously described best practices to ensure contamination is minimized during sample collection, and removed during data processing. Current molecular methods may not be sufficient to definitively answer the question of whether a functional prenatal microbiome is present in all eutherian mammals (Rackaitye et al. 2020). However, the growing body of work that employs best practices for working with low biomass samples moves the field closer to an understanding of how microbes interact with embryos during gestation. The results of this chapter demonstrate that quantification of microbial metabolites will require very sensitive methods of detection, as values will be very small compared to adults. A recent commentary on the controversy of the prenatal microbiome suggests that continued rigorous sterilization methodologies and additional investigations into the localization of bacteria in fetal tissues and niche-level analyses of the maternal-fetal interface will aid in understanding the functional importance of fetal interactions with microbes (Silverstein and Mysorekar 2021). The results of this chapter add to the growing body of literature investigating the functional role of microbial DNA in the previously-supposed sterile fetal environment.

Figure 3.1



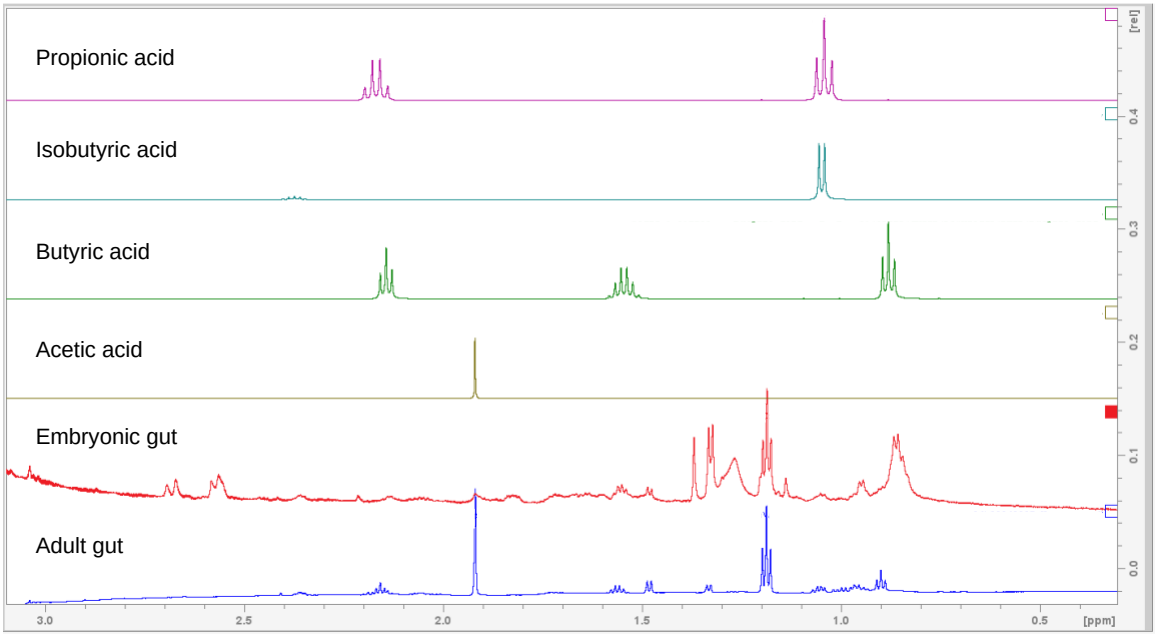
Microbial load in adult and embryonic samples. Box and whisker plots showing the log transformed copy number/uL of the 16S rRNA gene for adult and embryonic guts (n = 3/tissue) and for an extraction negative control.

Figure 3.2



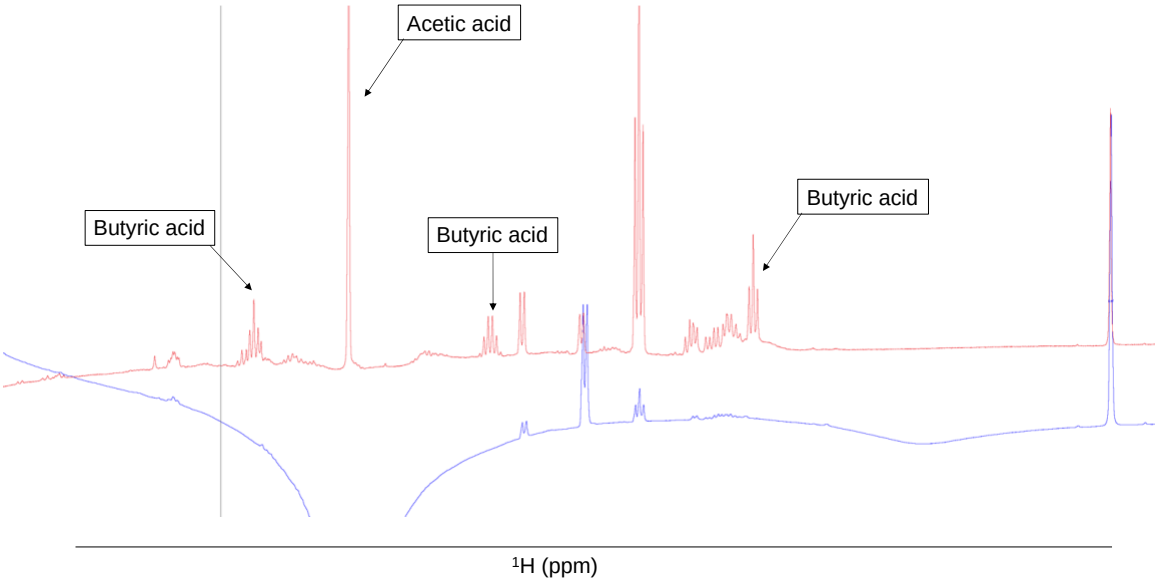
Relative concentrations of SCFAs in embryonic gut samples. Bar plots with standard deviation of relative proportions of SCFAs of interest (acetic, propionic, isobutyric, and butyric acids) in embryonic gut samples (n = 19).

Figure 3.3



^{1}H NMR spectra of adult and embryonic guts with standards for SCFAs of interest (acetic, propionic, butyric, and isobutyric acids). Embryonic gut spectra do not show any overlaps with the spectra for SCFAs. Adult gut spectra overlap with each SCFA.

Figure 3.4



Qualitative comparison of ^{1}H NMR spectra of adult (designated by the red line) and pooled embryonic gut sample (designated by the blue line). Peaks indicative of acetic and butyric acids are highlighted in the adult gut sample and do not overlap with any peaks in the pooled embryonic gut, which suggests that the SCFAs are not present in embryonic guts. Peaks present in the embryonic gut are representative of background noise in the sample.

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