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Contribution of the Microbiome on Reproductive Outcomes During Pregnancy

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CONTRIBUTION OF THE MICROBIOME ON REPRODUCTIVE OUTCOMES DURING PREGNANCY

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LIST OF ABBREVIATIONS

BPH/5	Blood Pressure High Subline-5 mouse strain
C57	C57BL/6 mouse strain
PE	Preeclampsia
WAT	White adipose tissue
FGR	Fetal growth restriction
OTU	Operational taxonomic units
ASV	Amplicon sequencing variants
RUPP	Reduced uterine perfusion pressure model
TLRs	Toll-like receptors
DOHaD	Development origins of health and disease theory
PERMANOVA	Permutational multivariate analysis of variance
FDR	False discovery rate
SCFAs	Short chain fatty acids
FFAR	Free fatty acid receptors
GLP-1	Glucagon like peptide 1
PYY	Peptide YY
PAMP	Pathogen associated molecular pattern
LPS	Lipopolysaccharide
IBD	Inflammatory bowel disease
IL-15	Interleukin 15
IL-6	Interleukin 6
TNFa	Tumor necrosis factor alpha

MAP	Mean arterial pressure
BAT	Brown adipose tissue
H&E	Hematoxylin and eosin
COX-2	Cyclooxygenase-2
PTGS-2	Prostaglandin synthase 2
LDA	Linear discriminant analysis

ABSTRACT

A series of translational comparative studies were performed to investigate the effects of the maternal microbiome on reproductive function and outcomes. With the launch of the human microbiome project in 2007, the next generation sequencing of microbiomes is booming. It is not only in the human medicine field, but also in veterinary species. This study specifically investigates the fecal microbiome in blood pressure high sublime 5 (BPH/5) mouse model that spontaneously develops preeclampsia (PE) and obesity. Additionally, the equine reproductive tract microbiome was explored and compared to microbiome of other host body sites. The BPH/5 mouse is a translational model that demonstrates the main features of human PE, a hypertensive disorder of pregnancy with high morbidity and mortality in both the mother and offspring. Gestating in a preeclamptic environment is associated with lifelong risk of cardiometabolic disease in the offspring. Adult BPH/5 female mice have an adverse cardiometabolic phenotype, hypertension, obesity with increased white adipose tissue (WAT), hyperphagia, and dyslipidemia that is exaggerated by pregnancy. Interestingly in humans, gut microbial dysbiosis is found in obese as well as hypertensive patients. Therefore, our first study was to characterize the maternal fecal microbiome associated with the development of PE in the BPH/5 mouse model to determine if gut dysbiosis is present. Our study is novel because our mouse model does not need dietary, medical, or surgical intervention to develop signs of PE. This allows for investigations and interventions to begin prior to pregnancy or early gestation when the disease originates. Our second study aimed to identify whether the male offspring born to preeclamptic dams share the same cardiometabolic disease as their female offspring counterparts. The next step in this research was to implement an intervention. A feeding paradigm was used to prevent maternal obesity and determine the effects on offspring outcomes. The final aim was a comparative equine study to characterize the placental

microbiome and determine associations between other body sites' microbial communities. Future studies would be to characterize BPH/5 and diseased equine placentas that may harbor microbial dysbiosis and leads to adverse pregnancy outcomes in dam and offspring.

INTRODUCTION

Pregnancy causes physiologic changes in all maternal organ systems. For example, cardiac output increases up to 50% within the first 6 weeks of gestation due to the increased demands of the uteroplacental circulation. Heart rate and stroke volume are additionally found to increase during pregnancy. In contrast, systemic vascular resistance decreases through lower blood viscosity and angiotensin. Any impairment in these normal physiologic changes in pregnancy can result in disease, which may harm the mother and fetus.

Preeclampsia (PE), a disease of late-gestation pregnancy occurs when there is a malfunction of cardiovascular regulation resulting in increased blood pressure during pregnancy. Other symptoms accompany these changes, such as proteinuria, renal insufficiency, thrombocytopenia, hepatic dysfunction, and pulmonary edema. PE not only affects the mother, but the disease also can have detrimental effects on the offspring, an outcome that will be investigated in subsequent chapters. Offspring are often premature or stillborn, exhibit fetal growth restriction (FGR), and are small for gestational age (1). Beyond the perinatal effects of being born to PE mothers, the associated lifelong consequences can be devastating for the offspring. Fetal programming, or *in utero* alterations occurring due to the impacts from the maternal environment, can influence fetal growth and development, leading to lifelong disease for the offspring (2). In humans, PE-associated fetal programming can result in increased cardiovascular complications, including hypertension, ischemic heart disease, stroke (3), and metabolic disease in the offspring as they age (4–6). These PE outcomes are in relation with the Developmental Origins of Health and Disease theory (DOHaD), by demonstrating that an unfavorable maternal environment will lead to pathogenic conditions in the offspring, which will increase the risk of chronic disease later in life.

Pre-conception maternal obesity increases the likelihood of developing PE by 30% (7). A novel study suggests that the maternal gut microbiome may contribute to PE by exaggerating the inflammatory response (8). The original theory was microbial dysbiosis characterized by low diversity, disruption in the Firmicutes to Bacteroidetes ratio, or overgrowth of pathogenic bacteria may lead to the development of maternal obesity. A common sequela of maternal obesity and gut dysbiosis during pregnancy is the "leaky gut syndrome" (9–11). Leaky gut syndrome is defined as the weakening or failure of the intestinal epithelial barrier and is caused by stress, chronic inflammation, and dysbiosis of the gut microflora (11, 12). Leaky gut syndrome could increase the risk of translocation of bacteria and may contribute to a heightened state of inflammation during pregnancy and abnormal placental development (9). Since the interplay between gut microbiome and obesity is much more complex than previously thought, more research needs to be done to examine the effects of diet, pre- and probiotics, antibiotics, surgery, and fecal transfaunation (13).

This study will utilize the blood pressure high sub-line 5 (BPH/5) mouse model to investigate how a dysfunction in the development of pregnancy leads to the pathogenesis of PE. Within this strain there is sexual dimorphism, the BPH/5 female presents excessive catch-up growth after birth, hyperphagia, obesity, cardiomegaly, increased blood pressure, and hyperleptinemia with leptin resistance. These clinical signs develop spontaneously without surgical or medical intervention, making this an ideal model for studying early gestation and pre-pregnancy factors, particularly obesity, that affect dams and offspring. In contrast, no studies specifically described the effects of in-utero PE exposure on the BPH/5 male offspring.

In this study, a weight loss intervention was implemented to combat the maternal obesity that is naturally occurring in these BPH/5 female mice. A pair-feeding paradigm was used to match the food intake of the pregnant BPH/5 mice to the normal intake of the lean C57 controls of similar

age and day of gestation. The BPH/5 offspring outcomes were subsequently investigated after attenuation of maternal obesity during pregnancy in the following chapters.

Studies involving BPH/5 mouse model are not limited to translational investigations of human obesity and PE. The BPH/5 mouse also can be used as a model for equine studies. Given the nature of the comparative study, there are similarities of the mouse model to equine reproductive diseases such as placentitis. The much shorter gestation length in a mouse is beneficial for sampling and data acquisition compared with the horse gestation length (21 days vs averaging 330 days, respectively). This shorter gestation specifically makes pregnancy and offspring outcome studies more feasible. Another component of this mouse model linking it to the equine model of placentitis is the inflammatory environment of their pregnancies. The BPH/5 have increased pro-inflammatory cytokines in their implantation sites, demonstrating a pregnancy harbored in an inflammatory environment. Equine placentitis also is known to have increased inflammation that results in pre-mature delivery of an unhealthy foal; a similar fetal outcome is characteristic of the BPH/5 mouse model.

The overall goal of this research is to investigate the effects of the maternal microbiome on pregnancy outcomes and offspring health. Aim one characterized the maternal microbiome associated with the development of PE in the BPH/5 mouse model. To expand on that study, the next aim determined if the male offspring born to PE dams demonstrated a cardiovascular phenotype similar to their female counterparts. Previous research has only investigated the BPH/5 female offspring, leaving the effects on the male undetermined. The following aim used a maternal weight loss intervention to improve offspring outcomes. Finally, the last aim comparatively characterized the equine placental microbiome and determined if other body sites' microbiome contribute to the development of the placental microbiome. Research looking into a PE specific

mouse model and the equine reproductive tract is novel. Initial exploration studies such as these are needed to lay the foundation for future work in this field.

CHAPTER 1. REVIEW OF THE LITERATURE: INFLUENCE OF THE MATERNAL MICROBIOME ON THE HYPERTENSIVE DISORDER OF PREGNANCY, PREECLAMPSIA

1.1 Introduction

Preeclampsia (PE) is characterized by late gestational hypertension (systolic ≥ 140 mmHg or diastolic ≥ 90 mmHg) and signs such as proteinuria, renal insufficiency, thrombocytopenia, hepatic dysfunction, and pulmonary edema (4). When PE is left untreated, it can result in morbidity and mortality to the mother and baby. The only known treatment is delivery of both the fetus and the placenta, often resulting in premature birth of growth restricted neonates, which leads to deleterious consequences. PE can result in cardiovascular complications for the mother, as well as cardiometabolic disease to the offspring later in life (4). Since the treatment for PE involves removing the placenta, it is thought that an abnormal placenta plays a causal role in the pathogenesis of PE (14–18).

PE is known to effect between 2-8% of pregnancies worldwide (1). The precise mechanisms that cause PE are still unknown, and there is no definitive way to predict when or if a mother will develop it. A number of maternal risk factors have been recognized to identify high-risk pregnant women, including preconception obesity, chronic hypertension, family history, and more. It is hypothesized that an increase in adipose tissue, which is a rich source of pro-inflammatory cytokines and complement proteins, causes an aggravated systemic inflammatory response, angiogenic imbalances in circulation and the placenta, and abnormal placental development resulting in PE (19).

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The placenta, a once thought ‘sterile’ site, is responsible for the maintenance of pregnancy in mammals. Recent technologies, such as next generation sequencing, now provide evidence that the placenta harbors an unique microbiome of its own (20). A microbiome is a collective genomic community of symbiotic, commensal, and sometimes pathogenic microorganisms that reside in an environment, such as a body cavity. These communities can consist of bacteria, archaea, fungi, protists, and viruses. The microbiome is therefore the collective genomes of the microbiota community members, and the study of this has been termed metagenomics. The study of an environment’s metagenome, where scientists utilize the principles of molecular biology and genetics, is referred to as metagenetics. The host and microbiome relationship is considered mutualistic symbiosis. Studies have shown that the human body provides the sustenance for the microbes and in return the microbes execute essential functions for the host; an example would be the gastrointestinal tract microbiome providing defense against enteric infections (21). Genomic DNA can be extracted from swabs, bodily fluids, feces, or tissue from a biopsy. Metagenetic samples should be collected as sterilely as possible before sequencing to limit the potential for contamination. Different hypervariable regions (V1-V9) of the 16s ribosomal RNA gene can be amplified for next generation sequencing. The results are grouped into operational taxonomic units (OTUs) based on their similarities to a known microbiome database such as SILVA (22) or greengenes (23). Using bioinformatics, scientists read sequence length based on a specific depth, filter out chimeras, and process the large data set to ensure more accurate results.

The microbiome has been found to be involved in processes that contribute to abnormal placentation, including periodontal disease, cardiometabolic complications, and excessive gestational weight gain. This review will investigate the role of the microbiome in the developing placenta and its potential contribution to PE.

1.2 The normal placental microbiome

The placenta is responsible for the maternal/fetal exchange of nutrient and waste products. Aagaard and colleagues were the first to characterize the human placental microbiome using Illumina sequencing (20). This study examined the placenta from over 300 patients with healthy and adverse pregnancies. The healthy human placental microbiome has been found to consist of the following phyla: Firmicutes, Tenericutes, Proteobacteria, Bacteroidetes, and Fusobacteria. More specifically *Fusobacterium spp, Neisseria lactamica, Neisseria polysaccharea, Rhodococcus erythropolis, Propionibacterium acne, Streptomyces overmitilis, Bacteroides spp., Prevotella tannerae*, and *Escherichia* spp. (Table 1). The human placental microbiome has been closely correlated to the maternal microbiome of the oral cavity, as opposed to the microbiome of the nearby vagina, using Bray-Curtis similarity matrix analysis in a study from Baylor College of Medicine (20). In an Australian study, 37 overweight and obese pregnant women were employed to examine their oral, fecal, and placental microbiome using 16S rRNA sequencing methods (24). This study was concurrent with the previous study, grouping the placental microbiome most similarly with the oral microbiome, instead of the gut or vaginal microbiome as previously thought. At the family level, Actinomycetaceae (Actinobacteria), Micrococcaceae (Actinobacteria), Oxalobacteraceae (Proteobacteria), Neisseriaceae (Proteobacteria), Pasteurellaceae (Proteobacteria), and Pseudomonadaceae (Proteobacteria) were shared between the obese mother's placental and their oral microbiome. The similarity decreased at the lower taxonomic levels, and phylogenetic analyses showed that the human placental microbiome does cluster independently from the oral microbiome resulting in a distinct placental microbial community at the lower taxonomic levels (24). Studies examining the full microbiome of the PE placenta are lacking.

Table 1.1 Healthy placental microbiome vs preeclampsia-related microbiome

	Healthy Placental Microbiome	PE-related Microbiome*
Genus species	Clostridium (Firmicutes) Corynebacterium sp. (Actinobacteria) Enterococcus spp. (Firmicutes) Lactobacillus crispatus (Firmicutes) Propionibacterium acnes (Actinobacteria) Staphylococcus spp (Firmicutes) Acinetobacter spp. (Proteobacteria)Bacillus (Firmicutes) Bacteroidales (Bacteroidetes) Bifidobacterium spp. (Actinobacteria) Enterobacter spp. (Proteobacteria) Escherichia coli (Proteobacteria) Fuscobacterium spp. (Fusobacteria) Gardnerella spp. (Actinobacteria) Haemophilus parainfluenzae (Proteobacteria) Lachnospiraceae (Firmicutes) Lactococcus (Firmicutes) Lysinibacillus (Firmicutes) Neisseria spp. (Proteobacteria) Nitrobacter (Proteobacteria) Prevotella melaninogenica (Bacteroidetes) Rhodococcus (Actinobacteria) Solibacillus (Firmicutes) Sporosarcina (Firmicutes) Streptococcaceae (Firmicutes) Streptococcus agalactiae (Firmicutes) unclassified Bacillales (Firmicutes) Ureaplasma parvum (Firmicutes) Ureaplasma urealyticum (Firmicutes) Veillonellaceae (Firmicutes)	Bacillus cereus (Firmicutes) Listeria sp (Firmicutes) Salmonella sp (Proteobacteria) Escherichia sp (Proteobacteria) Klesiella pneumonia (Proteobacteria) Anoxybacillus sp (Firmicutes)Variovorax sp (Proteobacteria) Prevotella sp (Bacteroidetes) Porphyromonas sp (Bacteroidetes) Dialister sp (Firmicutes) Actinobacillus actinomycestemconitans (Actinobacteria) Fusobacterium nucleatum (Fusobacteria) Tannerella forsynthesis (Bacteroidetes) Treponema dentocola (Spirochaetes)
Reference	(20, 25)	(8, 26)

*The bacteria listed are the current bacteria found in PE placentas to our current knowledge.

The function of the placental microbiome is predicted to regulate tryptophan, fatty acid metabolism, and benzoate degradation. Placental tryptophan metabolism is known to be important for neurodevelopment of the fetus (27). Catabolism of tryptophan is linked to the creation of neurodevelopmental feto-maternal immune tolerance (28). Furthermore, it has been postulated that the placental microbiome may have antimicrobial properties that serve as a natural selective mechanism to prevent colonization of foreign and pathogenic bacteria (29). Fatty acid metabolism may aid in the role of supplying energy yielding substrates to the fetus (24). The findings for the placenta microbiome were published in 2014 (20); since then approximately 100 new studies have been published investigating the placental microbiome and its metagenetic data. Studies have examined the uterine and placental microbiota in humans (20), cows (30), dogs (31), and horses (32), and all have been found to have a unique microbiome. A recent study found that the uterine microbiome may help modulate the local immune system in preparation for embryo implantation and placenta formation (33), which may have direct effects on the development of PE.

The placenta is considered the barrier to protect the fetus from pathogens whilst providing nutrients and waste removal during pregnancy. Symbiotic and pathogenic bacteria invade the blood stream of the mother and can potentially translocate to the placenta (34). So how do the bacteria get there? The current proposed hypothesis is that placental colonization happens in three possible ways. First, vertical translocation from vagina (35), secondly, hematogenous spread from the gut (36), and lastly, hematogenous spread from the oral cavity (37). Interestingly, *Veillonella* (Firmicutes), *Streptococcus* (Firmicutes), and *Prevotella* (Bacteroidetes) were found in all three cavities (oral, placental, and gut) (24). It was postulated that they were hematogenously translocated during pregnancy due to increased leakiness of the gut and the oral cavity as it has more tendency to bleed during pregnancy, thus promoting the idea that these bacteria are

transferred in the bloodstream. These changes are associated with hormonal fluctuations, such as rising progesterone and estrogen (9), and cardiovascular changes during pregnancy (24). *Fusobacterium nucleatum* (Fusobacteria) has been isolated from the amniotic fluid, placenta, and chorioamnionic membranes after preterm labor from two women who gave birth prematurely prior to rupture of the fetal membranes (38, 39). To test the hematogenous transfer hypothesis, *F. nucleatum* was administered to pregnant mice intravenously by the tail vein and resulted in preterm delivery and stillbirths. Under normal circumstances, *F. nucleatum* is found in the oral cavity. The bacteria after the intravenous injection of this study were isolated in the endothelium, surrounding tissues, and the amniotic fluid. This may suggest the hematogenous nature of this bacterial transfer (38).

1.3 Conflicting Data in the Field of Placental Microbiome

There have been recent studies that challenge the neonatal and placental microbiome dogma. Perez-Munoz et al. stated that their data does not support the existence of microbiomes within the healthy fetoplacental environment. They further indicated that the current methodology (next generation sequencing) is faulty, the low biomass creates a sample too small to accurately detect bacterial DNA, the potential for contamination is high, and is seen in numerous studies (36). Lauder et al. using both PCR and Illumina sequencing matched a set of contamination controls to compare the healthy placental samples and found that the placental samples contained low and indistinguishable 16S rRNA copy numbers when compared to extraction blanks using two different methods of DNA extraction. They also found no community separation using PERMANOVA of Bray-Curtis and UniFrac distances between the contaminated control samples and the placental samples (40). A follow-up study completed by Leiby et al. examining placentas from spontaneous preterm births found no distinction between background negative control and

placenta samples. Instead artifact and erroneous read classification and barcode misattribution were found within the samples (41). A possible explanation from the previous authors is that the placenta is sterile until the rupture of the membranes during delivery. The microbiome found within cesarean sections is thought to be due to contamination, commonly from the DNA extraction kit (40). It is important to keep challenging the scientific literature, but this information goes against the current trend of data supporting the placental microbiome. There is a need to further examine both sides to assess the accuracy of the core microbiome of the placenta in healthy full-term pregnancies.

1.4 The microbiome of surrounding reproductive tract that may affect placenta

The pregnant reproductive tract has natural defenses to invading bacteria, including the closed cervix and vulva. Immune response and the symbiotic environment also play a role in keeping the uterus healthy to support pregnancy. While organisms can still invade the uterus from the vagina and cervix, other mechanisms of placental contamination from extra-placental body sites also are possible. Dissemination from other body cavities through hematogenous transfer, as stated above, is possible as well as organisms in the peritoneum can translocate through the fallopian tube and iatrogenic causes through invasive procedures (42).

Preterm birth and neonatal death have been identified linked with intrauterine infections ascending from the lower genital tract (43). Preterm birth also has been associated with vaginal microbiome instability. A suppression of the vaginal microbial richness and diversity during the first two trimesters resulting in preterm births may suggest that proper vaginal microbial colonization is needed to maintain a successful pregnancy (44). In the previously mentioned study by Stout and colleagues, eleven patients were diagnosed with PE necessitating preterm birth. Recent studies have shown that dysbiosis of the vaginal microbiome influences preterm birth in

humans (45, 46). The healthy vaginal microbiome helps prevent vaginosis, sexually transmitted bacterial infections, and urinary tract infections (47, 48). It is normal for the vaginal microbiome in healthy women to fluctuate with the menstrual cycle (13).

Lactobacillus (Firmicutes) is thought to play a major role in preventing overgrowth of pathogenic bacteria. Bacterial vaginosis is now characterized by a reduction in *Lactobacillus*, which would be a dysbiosis of the natural vaginal microbiome and could be detrimental to the pregnancy (50). During healthy pregnancies an overall increase in orders, Clostridiales (Firmicutes) and Actinomycetales (Actinobacteria), and genera, *Lactobacillus* and *Bacteroides*, within the vagina has been shown (51). *Lactobacillus* has been found to dominate the vaginal microbiome with greater than 70% of the total abundance (52).

The uterus and extrauterine structures also have been found to have a microbiome of their own, even though not as extensively studied as the vaginal microbiome. The human uterine microbiome has an abundance of *Lactobacillus, Bacteroides, Gardnerella and Prevotella.* The uterine microbiome shares similar taxa from the phyla Firmicutes and Actinobacteria with the vaginal microbiome (53). The amniotic fluid also has been found to contain microbes such as *Enterobacter* (Proteobacteria), *Escherchia/Shigella* (Proteobacteria), and *Propionibacterium* (Actinobacteria) (54). It was found that the amniotic fluid shared a low abundance of microbes, but had richness and diversity similar to the placenta (54). The exciting potential of the microbiome is understanding the function of the microorganisms in relation to health and disease and whether it is possible to promote a healthy microbiome that protects the body from negative health outcomes.

1.5 Specific microbiome found with preeclamptic women

PE is a common cardiometabolic complication of pregnancy affecting ~300,000 pregnancies yearly in the United States (55). Pre-conception maternal obesity increases the likelihood of developing PE by 30% (7). PE is associated with adverse pregnancy outcomes and has the potential for future cardiometabolic disease for both mother and offspring later in life, including stroke, hypertension, and metabolic syndrome. PE is associated with several maternal co-morbidities, which increase the risk of cesarean delivery and preterm birth (56). A novel study suggests that the maternal gut microbiome may contribute to PE by exaggerating the inflammatory response (8). The original theory was an overgrowth of Bacteroidetes or Firmicutes may lead to the development of maternal obesity. A common sequela of maternal obesity is gut dysbiosis and "leaky gut syndrome" (9–11). Leaky gut syndrome is defined as the weakening or failure of the intestinal epithelial barrier and is caused by stress, chronic inflammation, and dysbiosis of the gut microflora (11, 12) during pregnancy Together, this may contribute to a heightened state of inflammation during pregnancy, which may contribute to abnormal placental development and PE (Figure 1.1) (9). Since the complex interplay link between gut microbiome and obesity is much more complex than previously thought, more research needs to be done to examine the effects of diet, pre- and probiotics, antibiotics, surgery, and fecal transfaunation (13). Maternal obesity has been found to be a key predictor of childhood obesity and metabolic complications of the offspring. While the mechanisms are poorly understood, researchers believe the microbiome alters metabolism and modulates the weight gain in both the mother and the offspring (57).

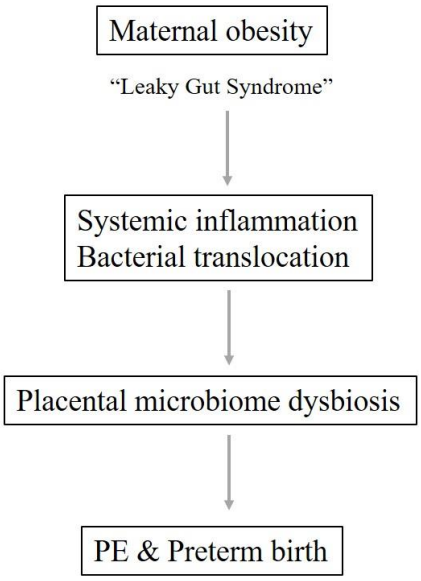


Figure 1.1 Hypothesis for the effects of maternal obesity on adverse pregnancy outcomes. Beginning with maternal obesity, and potentially concurrent caused by leaky gut syndrome, we hypothesize that this leads to systemic inflammation and bacterial translocation to the placenta. As a result, the placenta microbiome enters an inflammatory dysbiotic state, which may cause preeclampsia and preterm birth. PE=preeclampsia.

Bacillus cereus (Firmicutes), *Listeria* (Firmicutes), *Salmonella* (Proteobacteria), *Escherichia* (Proteobacteria), *Klebsiella pneumonia* (Proteobacteria), *Anoxybacillus* (Firmicutes), *Variovorax* (Proteobacteria),, *Prevotella* (Bacteroidetes), *Porphyromonas* (Bacteroidetes), and *Dialister* (Firmicutes) were found in seven placental samples from mothers with PE (Table 1.1). The mothers in this study had infectious agents in both the placenta and amniotic fluid, but not detectable in the venous blood or urine at time of delivery (8). Interestingly, some of the bacteria listed above are known or related to pathogens in the reproductive tract, gastrointestinal tract, and respiratory tract that may have resulted from hematogenous transfer. When the serum was examined from women diagnosed with PE, *Chlamydia pneumonia* (Chlamydiae), *Sneathia amnii* (Fusobacteria), *Porphyromonas gingivalis* (Bacteroidetes), *Tannerella forsythensis* (Bacteroidetes), and *Eikenella corrodens* (Proteobacteria) were detected. These pathogenic bacteria were believed to have translocated from previous infections in the respiratory tract, other parts of the reproductive tract, and the periodontal cavity (8). The theory rationale behind the

pathogenic infection theory is that a single infectious event could trigger an inflammatory response contributing to the development of PE since it is believed that the abnormal placenta in PE develops because of pro-inflammatory cytokines and complement proteins in response to inflammation (19, 58, 59). Other researchers believe that PE could be triggered by a heavy infectious burden that increases inflammatory cells and anti-angiogenic factors leading to defective placentation, which is a key pathologic feature of PE (Figure 1.2) (60–62). Another study by Kell and Kenny found that dormant microbial infection that overgrow in abundance are responsible for the chronic inflammation and the PE sequelae (62) .

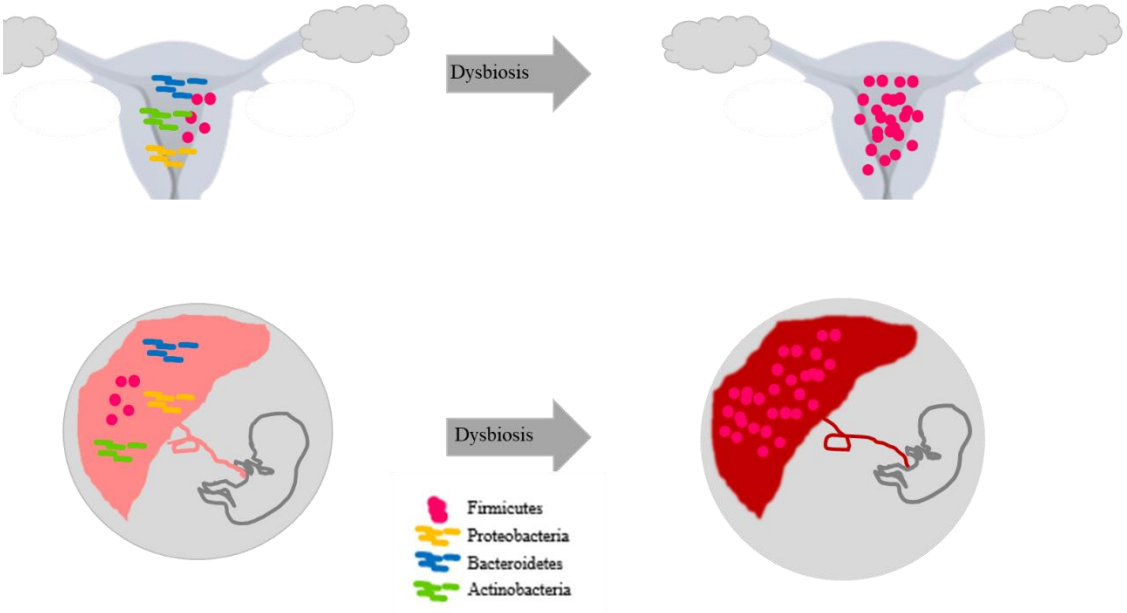


Figure 1.2 Female reproductive tract dysbiosis before and during pregnancy in health and disease. (A) Dysbiosis in the uterine and/or (B) placental microbiome that can lead to preeclampsia (PE) and fetal growth restriction. The uterus and placenta (left panels) represent a healthy microbial environment. The images on the right represent dysbiosis and an overgrowth of the phyla Firmicutes. This may lead to PE and fetal growth restriction represented by the inflamed placenta and small fetus.

In another study by Barak and colleagues using PCR, they identified *Actinobacillus actinomycestemcomitans* (Proteobacteria), *Fusobacterium nucleatum* (Fusobacteria),

Porphyromonas gingivalis (Bacteroidetes), *Prevotella intermedia* (Bacteroidetes), *Tannerella forsynthia* (Bacteroidetes), and *Treponema denticola* (Spirochaetes) in 16 PE placentas (26). Fifty percent of these samples were positive for more than one of these periopathogenic organisms, furthering the idea that periodontal disease contributes to the increased risk of developing PE (26). Contreras et al. concurred with these findings that periopathogenic bacteria are present in PE patients (63). Murthy et al. also found a correlation between periodontal disease and preterm birth with low birth weights (64).

1.6 The effects of lifestyle on adverse pregnancy outcomes and microbiome

Preterm birth is characterized by birth prior to 37 weeks gestation; which effects 1 out of every 9 babies born in the Unites States (65). Preterm birth can have detrimental effects on organ function and cause disability among infants (66). The dysbiotic maternal microbiome may have been found to influence neonatal health by promoting preterm birth, cardiometabolic complications during pregnancy such as PE, gestational diabetes, and excessive weight gain (67). Pre-existing maternal characteristics conditions such as age, African American race, higher body mass index (BMI), as well as pre-existing conditions, including pre-conception diabetes, chronic hypertension, renal disease, autoantibody disease, and family history have all been linked to the development of PE (Figure 1.3) (1). Preterm placenta have been shown to have an altered placental microbiome, and its metabolic profile has been shown to vary with gestational weight gain (68).

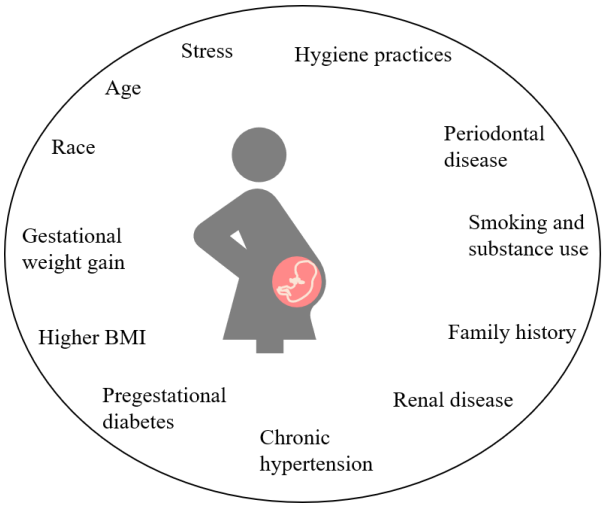


Figure 1.3 Factors that affect the placental microbiome that can lead to the development of preeclampsia (PE). Hygiene practices such as frequent sexual intercourse, multiple sex partners, oral sex, douching, or use of spermicides are all considered risk factors for microbial dysbiosis. African Americans are predisposed to development of PE. BMI= body mass index.

High BMI has both been linked with preterm birth and PE (69). Of the women in the United States who are childbearing age, two-thirds of them are considered overweight or obese and nearly half of these women have excessive gestational weight gain once pregnant (70). Obesity during pregnancy and excessive gestation weight gain increases the risk of PE, which can lead to preterm birth and small birth size for gestational age infants. The gut microbiome has been linked to obesity and gestation weight gain (70–72). This is becoming a more pressing issue with the increased obesity rate in the world. In a study examining the placental microbiome of 13 overweight and 24 obese mothers, Firmicutes and Proteobacteria dominated the samples (80% of total microbial abundance) (24). The most dominant organisms found in the phylum Firmicutes included *Streptococcus, Lactobacillus,* and *Veillonela. Pseuodomonas, Haempohilus* and *Actinetobacter* dominated the phylum Proteobacteria (24). A study completed by Lv et al. found an increased abundance of *Blautia* (Firmicutes), *Ruminococcus2* (Firmicutes), *Bilophila* (Proteobacteria), and *Fusobacterium* (Fusobacteria) in the gut microbiome of PE antepartum patients. These researchers

also found a decrease of *Gemmiger* (Firmicutes), *Akkermansia* (Verrucomicrobia), *Dialister* (Firmicutes), and *Methanobrevibacter* (Euryarchaeota) (73).

A traditional western diet has been linked with imbalance of the gut microbiome increasing bacterial counts of *Clostridium innocuum* (Firmicutes), *Eubacterium dolichum* (Firmicutes), *Catenibacterium mitsuokai* (Firmicutes), and *Enterococcus* spp (Firmicutes), and decreasing *Bifidobacterium* (Actinobacteria) and Bacteroidetes (74). Research shows changing the microbiome composition back towards a non-obese microbiome is successful by calorie-restriction and exercise. (75). To combat obesity, consumption of probiotic- enriched foods by the obese mother has been associated with lower preterm birth and PE rates. A Norwegian study examined the effects of probiotics containing Lactobacilli on maternal PE (76). They found that it reduced the overall risk of developing PE, but this study did not examine the effects of the probiotics on the placental or vaginal microbiome (76).

Additionally, smoking, substance abuse, and some hygiene practices are known to change the vaginal, oral, and gut microbiome, thus potentially contributing to preterm births (67). Smoking specifically creates a pro-inflammatory response and alters the oral microbiome that may contribute to PE (77). Behaviors such as frequent sexual intercourse, multiple sex partners, oral sex, douching, or use of spermicides alter the vaginal microbiome, creating a dysbiosis that can increase the risk of pathogenic bacteria colonization and inflammation (78).

The oral microbiome also has been linked to the development of PE and preterm births (79). Oral dysbiosis caused by dental caries, gingivitis, and periodontitis have been linked to cause inflammatory immune responses that lead to adverse pregnancy outcomes (79, 80). In a rodent study, mice were challenged with *Porphyromonas gingivalis* (Bacteroidetes), a common oral pathogen. This led to decreased fetal weights, increased fetal reabsorption, and fetal growth

restriction (81). Since the microbiome of the placenta is more similar to the oral cavity, it is hypothesized that oral pathogenic microbes that disseminate hematogenously cause intrauterine infection when periodontal infection is present (82).

Stress is also a large contributing factor in disease. Stress has been found to increase pro-inflammatory cytokines and cortisol (83). These inflammatory responses increase the risk of preterm birth (84). Chronic stress causes chronic inflammation, which can lead to preterm birth, PE, and gestational diabetes mellitus (85). There also has been a link between stress and bacterial vaginosis (86). The gut microbiome is affected by stress also by increasing the translocation of bacterial cell components from the gut into the blood stream. An animal model suggests that stress causes an increase in circulating inflammatory cytokines and activation of the hypothalamic-pituitary-adrenal axis, which has been associated with preterm births also (67).

Development of PE during pregnancy can have lifelong effects on the mother with an 8-fold increase in developing cardiovascular disease afterwards (87). The diseases found include hypertension, dyslipidemia, insulin resistance, endothelial dysfunction, and vascular impairments. Side effects of these diseases have been shown to last for months to years (88). The offspring of preeclamptic mothers that survive have an increased risk of stroke, coronary heart disease, and metabolic syndrome in adult life (89). The size of the fetus and the gestational weight gain of the mother have been found to influence the infant's developing intestinal microbiome. Preeclamptic and preterm infants have been found to lack *Bifidobacterium* (Actinobacteria) and *Lactobacillus* (Firmicutes) and are instead dominated by Proteobacteria (90). This could mean that babies born to PE mothers may not be able to develop a proper gastrointestinal (GI) microbiota and may be subjected to GI disorders later in life.

1.7 The rodent model for preeclampsia and its microbiome

Ethically and logistically, it is difficult to study placental developmental events of the first trimester in women. Therefore, adequate animal models are necessary to study key processes at the maternal-fetal interface that play a causal role in the pathogenesis of PE. Rodent models also have been used to study the translocation of bacteria to the placenta from the oral cavity (37), as well as intestinal labeled bacteria into the placenta (91). Challenging the pregnant mice with *Campylobacter rectus* (Proteobacteria) resulted in abnormal placental architecture and inflammation. This increased pup mortality (92).

Rodents have been widely used to study pregnancy disorders, including PE. Some examples of rodent models relevant for studying PE are reduced uterine perfusion pressure (RUPP), BPH/5, adaptive transfer model, and a chronic infusion of arginine model (18). A rat model of placental ischemia, which occurs in PE, also has been developed and termed the RUPP model (18). This model is created by placing silver clips around the aorta, right and left uterine arcade to reduce blood flow to the placenta by approximately by 40% (93). The RUPP model provokes systemic alterations in distinct immune cell populations, such as T-helper 17 cells. By injecting T-helper 17 cells harvested from the spleen of RUPP rats into the peritoneal cavity of normal pregnant rats, a maternal and fetal PE phenotype is observed (93). Conditions that alter the TH17/T regulatory cell balance, such as inflammatory bowel disease, obesity, and ulcerative colitis, are associated with an altered gut microbiota (94). Therefore, investigating the microbiome in the TH17 adaptive transfer model of PE would be useful (95, 96). Additionally, an adaptive transfer model has been created by the intravenous injection of activated Th1-switched splenocytes on day 10 and 12 of pregnancy (97). This induced an increase in maternal blood pressure and urinary protein excretion in this model. Also, the chronic infusion of arginine vasopressin at the

onset of pregnancy has led to the development of PE like symptoms in C57BL/6 strain mice (98). The BPH/5 mouse was discovered to spontaneously develop maternal and fetal features of PE, including late gestational hypertension and fetal growth restriction, respectively (99). BPH/5 female mice have mildly elevated baseline blood pressure prior to pregnancy, which is a known factor in the development of PE in women. Therefore, the BPH/5 model can be considered a model of superimposed PE along with the Dahl Salt-Sensitive Rat (100). These rodent models have played a vital role thus far in PE research; further studies need to be completed to determine their specific placental microbiome and its effects on PE. Our current hypothesis is that a dysbiosis can be caused by an overgrowth of certain bacteria, which may contribute to the death of the healthy bacteria. Proliferation of microorganisms and subsequent death, particularly gram negative bacteria, creates an overall activation of inflammatory signaling pathways, such as complement factors and toll-like receptors (TLRs), which have been implicated in the pathogenesis of PE (101–103). Rodent models have the potential to be a good candidate to study the microbiome's role in PE.

1.8 Conclusion

The new and exciting microbiome field supports the symbiotic nature of the placental microbiome and its interactions with maternal/fetal health and the maintenance of pregnancy. Diagnosing dysbiosis of the maternal microbiome before birth could aid in the treatment of adverse outcomes such as PE. The potential for a probiotic, perhaps using *Lactobacilli*, can aid in the inhibition of overgrowth of bacterial species and maintain a healthy uterine environment.

There are some pitfalls when examining microbiome sequencing data including the potential detection of dead organisms, low microbial biomass in the samples, and the high potential for DNA contamination through sampling techniques and processing. Although the dead microbes

are not replicating within the host, they still represent ligands that the host cells recognize. Therefore, they are subjected to host immune response because of physiologic interaction with the host (29).

The theories behind the potential effect of the microbiome on PE involved a dysbiosis of the healthy microbial flora. The disruption leads to the overgrowth of pathogenic infection that in turn triggers an inflammatory immune response, including complement and pro-inflammatory cytokines that leads to the development of PE. This immune response within the pregnant mother leads to a defective placenta, which is causative in the pathogenesis, the primary pathogen of PE. This hypothesis has been demonstrated by the periodontal infection hematogenous translocating to the placenta and the mother developing PE. Another theory of the effects of the microbiome, which includes resident bacteria and viruses, may involve the bacterial gene transfer into the host genome similar to the viral gene transfer of endogenous beta retroviruses (104) and their contribution to the development of multiple sclerosis, amyotrophic lateral sclerosis, and schizophrenia (105).

It is not clear when the placental microbiome is seeded during pregnancy. The current theory is that bacteria translocate vertically through the vagina or hematogenously from the mouth or gut; this would mean the microbiome in these sites may directly affect the placental microbiome and its health. PE can present as early or late onset and can be mild, moderate, or severe; these differences may be due to varying maternal characteristics and risk factors such as the dysbiosis that is a sequela from leaky gut syndrome or periodontal disease. Further studies are needed to assess the microbial populations and dynamic to better understand their fetoplacental role involving PE. Importantly, PE creates a significant risk for the development of cardiovascular

disease for the mother and offspring. The associations between the placental microbiome and adverse pregnancy outcomes, such as PE, still need to be investigated.

CHAPTER 2. LINKING THE MATERNAL MICROBIOME WITH THE ONSET OF PREECLAMPSIA, IN THE BPH/5 MOUSE MODEL

2.1 Introduction

PE is a devastating pregnancy-specific disorder that affects approximately 300,000 women in the United States every year (7). It is characterized by new onset hypertension during the second half of pregnancy (systolic ≥ 140 mmHg or diastolic ≥ 90 mmHg) and signs such as proteinuria, renal insufficiency, thrombocytopenia, hepatic dysfunction, and pulmonary edema (7). There is no known cure for PE, and the only effective treatment is delivery of the fetus and placenta, which is often preterm and has harmful consequences. Women with an increased body mass index (>35 kg/$m^{2)}$ prior to pregnancy have a 30% increased risk of developing PE compared to lean counterparts (7). Of the women who are childbearing age, two-thirds are considered overweight or obese (106). Pre-conception obesity has been linked to maternal microbiome dysbiosis and leads to a pro-inflammatory state, which is harmful to the pregnancy (9). A common sequela of maternal obesity is dysbiosis of the gastrointestinal (GI) tract and leaky gut syndrome. Leaky gut syndrome is defined as the weakening or failure of the epithelial barrier of the GI tract during pregnancy caused by stress, chronic inflammation, and dysbiosis of the gut microbiome (9). Together, this contributes to a heightened state of inflammation, which may contribute to abnormal placental development and PE (9).

The maternal microbiome has influence during pregnancy, negatively if the microbial community progresses to a dysbiotic state. Microbial dysbiosis can lead to unwanted inflammation. Pregnancies that begin in a state of heightened inflammation are linked to adverse outcomes, such as gestational diabetes, preterm birth, and PE (9). The theories behind the potential effect of the microbiome on the development of PE involved a dysbiosis of the healthy microbial flora. The disruption leads to the overgrowth of pathogenic bacteria, which in turn triggers an

inflammatory immune response, including complement and pro-inflammatory cytokines that contribute to the development of PE (107). This immune response within the pregnant mother may lead to a defective placenta, which is involved in the etiology of PE (107). This is only one hypothesis on how the microbiome influences the development of PE. It has been demonstrated by the periodontal infection with bacteria hematogenously translocating to the placenta and the mother developing PE (26). Diagnosing dysbiosis of the maternal microbiome prior to pregnancy or during early gestation could aid in the prevention of adverse outcomes such as PE.

Factors such as disease and diets can affect the gut microbiome and metabolism in a negative manner. Short chain fatty acids (SFCAs) are gut microbiome derived metabolites that fuel the host metabolism and also are used as a signaling mechanism. GPR 41 and 43 are free fatty acid receptors (FFAR) and are key receptors for SCFAs. Previous literature has demonstrated the importance of SCFAs and FFAR on host energy homeostasis (108), insulin signaling (108), fat deposition (108), and even embryo development (109). According to the developmental origins of health and disease theory (DOHaD), regulation of disease susceptibility begins in utero (110). However, the underlying mechanisms of the relation of the diseased mothers' microbial dysbiosis and adverse pregnancy outcomes are still unknown.

In this study, the BPH/5 mouse model was used. BPH/5 mice are a well-known strain that spontaneously develop a PE-like phenotype during pregnancy. BPH/5 female mice have mildly elevated baseline blood pressure prior to pregnancy, which is a known risk factor in the development of PE in women (99). BPH/5 also demonstrate a predisposition for obesity with increased white adipose tissue (WAT), pro-inflammatory reproductive WAT, and leptin resistance (111). Similar to PE in women who experience perinatal morbidity/mortality, BPH/5 offspring have low-birth weight compared to normotensive C57 control mice, indicative of FGR; litter size

is severely compromised due to fetal demise (99). Additionally, pro-inflammatory mediators are upregulated systemically and in the placenta of BPH/5 mice (112, 113). Although a well-known model of PE, the maternal fecal microbiome of BPH/5 has yet to be investigated. We hypothesize that obese mice with a PE-like phenotype will demonstrate gut dysbiosis and have a perturbed GPR pathways with increased local inflammation compared to lean normotensive C57 controls.

2.2 Methods

2.2.1 Animal experiments

Adult (8-12 weeks of age) female BPH/5 (n=13 pregnant, n=12 non-pregnant) and C57 (n=8 pregnant, n=6 non-pregnant) mice were used in this study. BPH/5 mice were a gift from Dr. Robin Davisson, Cornell University. Mice were housed in standard cages placed in a temperature- and humidity-controlled facility, maintained on a 12-hour light/dark cycle, and fed standard mouse chow with water available ad libitum. All mice used in this study were housed for multiple generations within the same room. C57 mice have been used as control mice in previous BPH/5 studies, as they were used in the original eight-way cross to derive the BPH/5 strain (99, 113, 114). Intrastrain timed matings were performed, and detection of a copulatory plug was designated as embryonic day 0.5 (e0.5). All animals studied were approved by the Louisiana State University School of Veterinary Medicine and Pennington Biomedical Research Center IACUC committee.

2.2.2 Samples collected

Feces, colon, and serum samples were collected from BPH/5 and C57 non-pregnant females and pregnant dams. To collect feces, mice were placed individually in sterile, empty cages and allowed to defecate voluntarily. Samples were placed in sterile tubes and stored at –80 °C until further analysis.

2.2.3 DNA sequencing

Microbial DNA was extracted from fecal samples using the Qiagen DNeasy PowerSoil extraction kits (Qiagen, USA) according to manufacturer's protocol. The V4 variable region of the 16S rRNA gene was amplified with PCR primers 515f/806r in a 30 cycle PCR using the DreamTaq Hot Start PCR Master Mix Kit (Thermoscieniftic, Waltham, MA). PCR was performed in 20 µl volumes and included: 2 µl (7.5 µM concentration) of forward and reverse primers, 12.5 µl of Hot Start Taq 2X Master Mix (New England BioLabs Inc., Ipswich, MA., USA), 3.5 µl of deionized water, and 2 µl of sample DNA. Thermal cycle conditions were 95˚C for 3 min for the initial denaturing step, followed by 30 cycles of 95˚C for 30 s, 50˚C for 1 min, and 72˚C for 1 min. PCR products were checked on a 2% agarose gel for correct product size formation (approx. 350 bp). Michigan State University Genomics Core performed library preparation prior to Illumina MiSeq sequencing following the manufacturer's guidelines (115). Reagent controls using certified DNA free water were run through library preparation and PCR and did not generate libraries. For quality control, samples submitted for sequencing included a random blank sample of technical replicates.

2.2.4 Bioinformatics

Initial quality screening, demultiplexing, amplicon sequence variant (ASV) inference and chimera removal were performed using the DADA2 package (116). ASVs were classified using the Silva Release 132 16S rRNA database (22, 117). Microbial Community analysis (Alpha and Beta Diversity) was performed using the vegan R package (118). Permutational multivariate analysis of variance (PERMANOVA) (119) was performed using vegan package Adonis function. To determine differentially abundant ASVs, the ASV table was first trimmed to only include ASVs with a median abundance greater than two across all samples. ANOVAs were performed on the trimmed ASV table using centered log ratio transformed abundances. ANOVA p-values were

corrected using the False Discovery Rate (FDR) method of Benjamin and Hochberg (120). Linear discriminate analysis effect size (LEfSe) (121) analysis was performed to identify the taxa characterizing the differences among groups.

2.2.5 SCFA determination

Fecal and serum SCFAs were measured using gas chromatography (Thermo Trace 1310), coupled to a flame ionization detector (Thermo) by Microbiome Insights Inc. Our SCFA column is 'Thermo TG-WAXMS A GC Column, 30 m, 0.32 mm, 0.25 µm, which is previously described methodology of Zhao et al. (122). The concentration of SCFA was determined using external standard calibration over an appropriate concentration range.

2.2.6 Colon RNA isolation and quantitative reverse transcription PCR

Total RNA was extracted from colon tissues using TRIzol according to manufacturer's instructions (Qiagen, Hilden, Germany). RNA quality and quantity were assessed by spectrophotometry (Nano Drop). 1000 ng cDNA was reverse transcribed using the qScript cDNA kit (Quanta BioSciences, Beverly, MA). Each qPCR was performed in triplicate with an ABI 7500 Fast Thermocycler (Applied Bioscience) using SYBR Green (Quanta BioSciences) using 25 ng cDNA. Real-time PCR cycling conditions were as follows: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, 58°C for 30 s, and 72°C for 1 min. The 18S rRNA gene was used as an internal control. Each sample was tested in triplicate for the average Ct value. Relative mRNA expression was calculated after normalization to the 18S rRNA reference gene using the 2-Ct method. Primers used are listed below *18s* (CCGGGCTTCTATTTTGTTGGT, TAGCGGCGCAATACGAATG (107)) (123), *GPR 41* (GTGACCATGGGGACAAGCTTC, CCCTGGCTGTAGGTTGCATT)(109), *GPR43* (GGCTTCTACAGCAGCATCTA, AAGCACACCAGGAAATTAAG)(109), *IL-15* (CTGCAAGTCTCTCCCAATTCTC,

CCTCCTGTAGGCTGGTTATCT)(114) and *IL-6* (TGGCTAAGGACCAAGACCATCCAA, AACGCACTAGGTTTGCCGAGTAGA) (111).

2.2.7 Statistical analysis

Statistical analysis was performed using GraphPad Prism version 9. Shapiro-Wilk test was used to check for normality. Student's t-test was used to compare the two groups. All figures were presented as means± SEM. p values <0.05 were considered significant.

2.3 Results

2.3.1 Microbial community profiles are different between obese and hypertensive non-pregnant BPH/5 and lean normotensive control non-pregnant C57.

Of the 39 samples processed and sequenced, all achieved a threshold above 10,000 reads per sample after quality filtering. The BPH/5 non-pregnant female showed significantly different microbial communities when using Bray-Curtis dissimilarity matrix (p=0.001) (Figure 2.1A). To assess changes in diversity of the gut microbiome Shannon's index was evaluated. Non-pregnant BPH/5 had a significantly more diverse gut microbiome compared to non-pregnant C57 (p=0.0068) (Figure 2.1B). The Firmicutes to Bacteroidetes ratio, which is an indicator of obesity in humans (Magne et al., 2020), was significantly different between groups, with non-pregnant BPH/5 showing a lower ratio than the lean control (p=0.005) (Figure 2.1C).

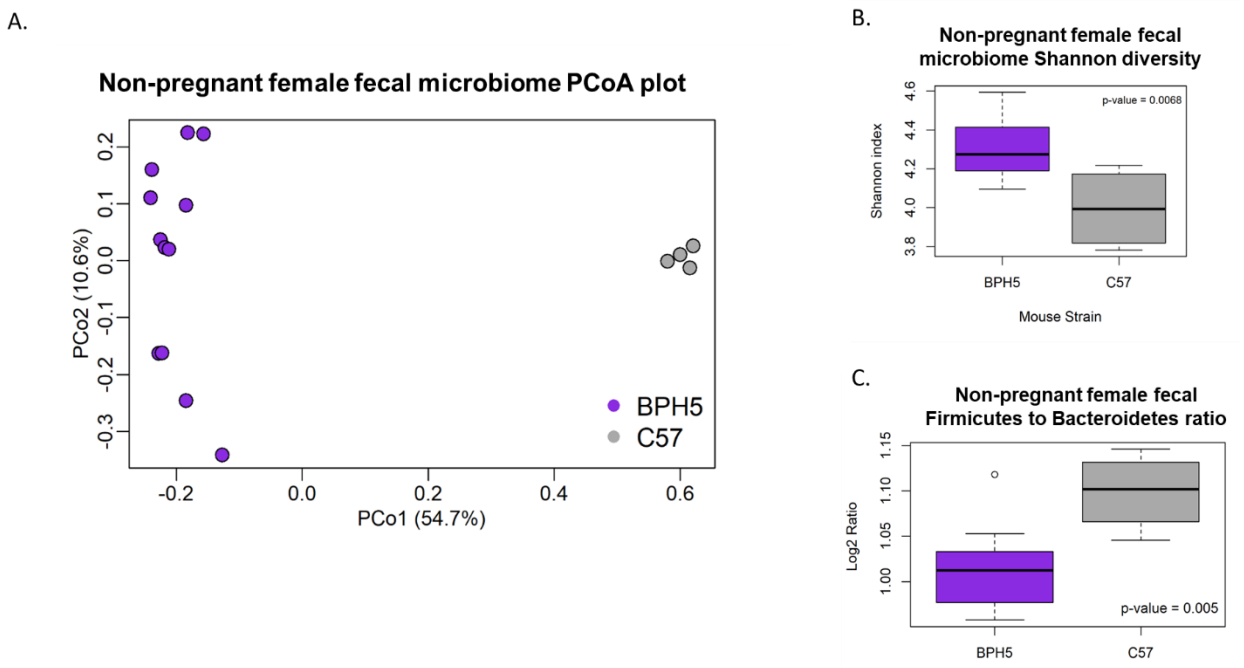


Figure 2.1 Markers of gut dysbiosis in BPH/5 mice model. A). Principal Coordinate analysis noting differences in beta-diversity of non-pregnant BPH/5 and C57 B). Alpha diversity is increased in BPH/5 non-pregnant mice compared to C57 controls. C). Non-pregnant BPH/5 have a decreased Firmicutes to Bacteroidetes ratio compared to C57 non-pregnant controls.

2.3.2 BPH/5 gut microbiome changes with the onset of pregnancy

BPH/5 mice spontaneously develop PE during late gestation pregnancy (111). The microbial communities were compared from the BPH/5/ non-pregnant mouse to the e18.5 late gestation BPH/5 dam to determine if alteration to the gut microbiome were present. Beta diversity was significantly different with the onset of pregnancy (p=0.008) (Figure2.2a). Whereas the alpha diversity did not change (p=0.115) (Figure 2.2b). Using an LDA plot, the top 25 taxa were charted. Acetatifactor, Ruminiclostridium, Lachnospiraceae_FCS_020_group, Verrucomicrobia, and Akkermansia had a notable increase with the onset of pregnancy. While Candidatus_Stoquefichus, Ruminococcaceae_UCG_013, members of Entercoccaceae, Muribaculum, Eisenbergiella, members of Prevotellaceae, Alphaproteobacteria, Rhodospirillales, Parabacteroides, Tannerellaceae, and members of Proteobacteria were decreased (Figure 2.3).

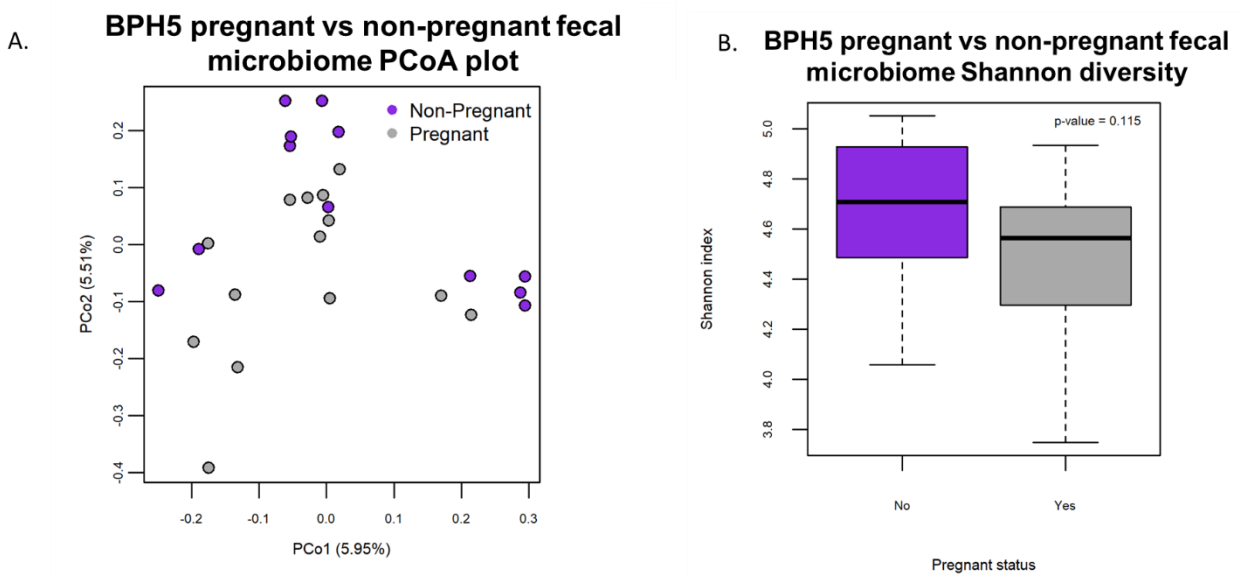


Figure 2.2 Beta-diversity changes with the onset of pregnancy in BPH/5 model, but alpha diversity does not. A). Principal coordinate analysis showed differences in beta-diversity of BPH/5 pregnant and BPH/5 non-pregnant mice. B). Alpha diversity was not different between pregnant and non-pregnant BPH/5.

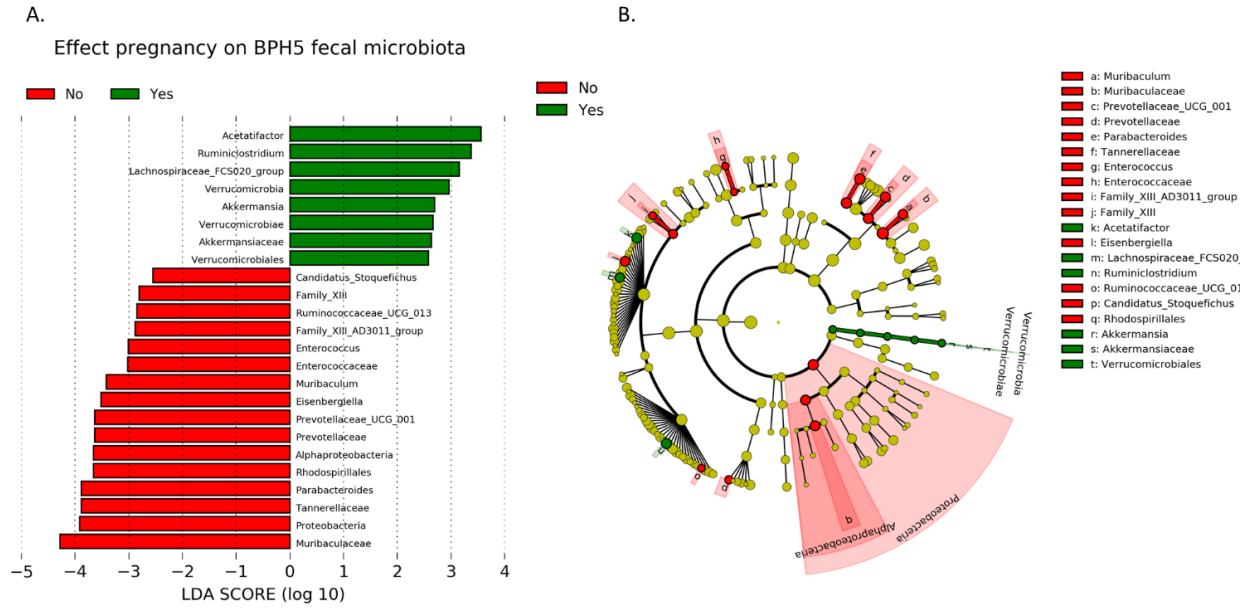
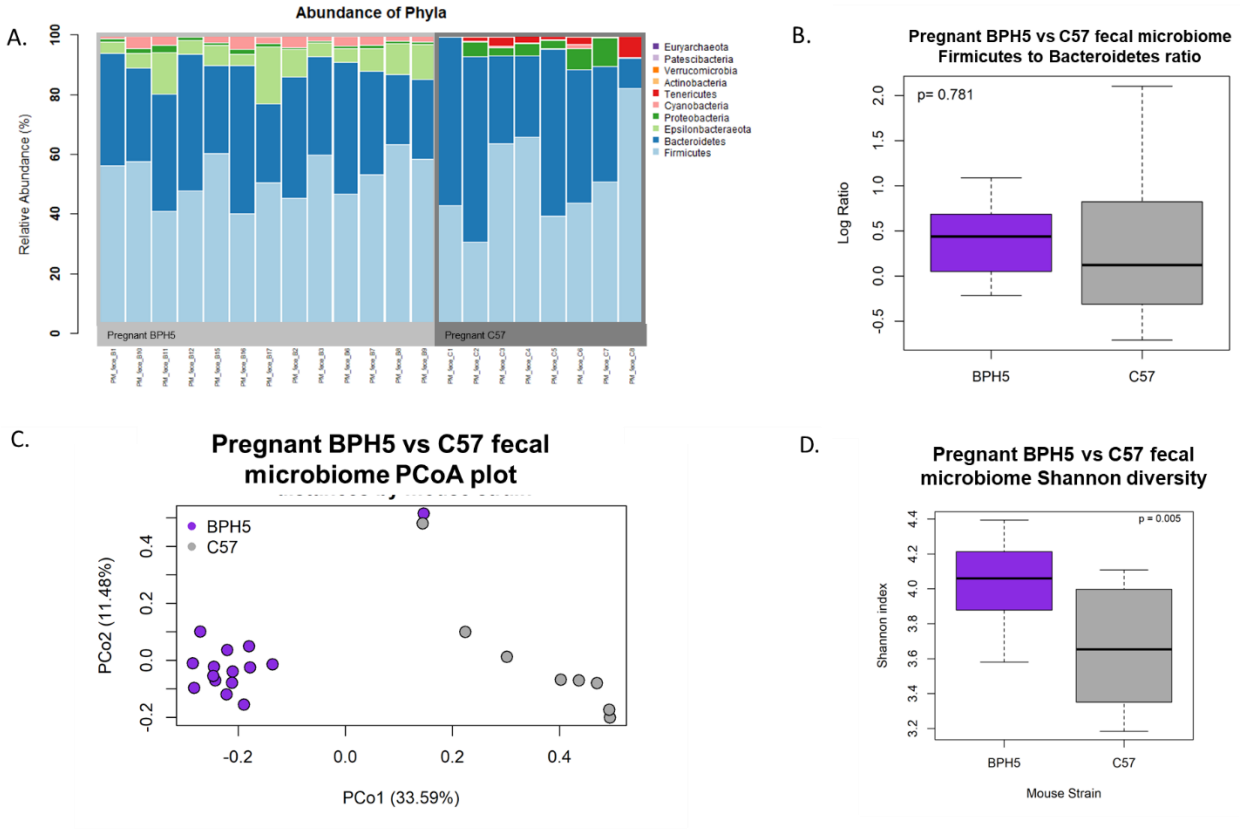


Figure 2.3 Linear discriminate analysis mapping the changes in gut microbiome with the onset of pregnancy in BPH/5 mice.

2.3.3 Differences in the gut microbial communities during pregnancy in the obese preeclamptic BPH/5 compared to lean normotensive C57

At the phyla level, all the samples were dominated by Firmicutes, Bacteroidetes, Epsilonbacteraeota, Cyanobacteria, and Proteobacteria (Figure 2.4a). The relative abundance of Firmicutes remained similar, while Bacteroidetes was 35.43% in the BPH/5 and 40.6% in the C57. Although, the Firmicutes to Bacteroidetes ratio was not significantly different between groups (Figure 2.4c). A notable difference in the phyla relative abundance was in the Epsilonbactaeota with BPH/5 having 8.11% and C57 0.01% respectively. Cyanobacteria also had a prominent change 2.70% and 0.04% respectively. Proteobacteria (1.15% BPH/5 and 3.90% C57), Tenericutes (0.10% BPH/5 and 2.24% C57), and Actinobacteria (0.09% BPH/5 and 0.48% C57) had minor changes (Figure 2.4e).

Microbial community composition of the pregnant BPH/5 compared to the C57 controls were significantly different at late gestation ($p=0.001$, PERMANOVA with Bray-Curtis dissimilarity of 16S Amplicon Sequence Variant's relative abundance) (Figure 2.4c). Alpha diversity also differed between pregnant BPH/5 dams being increased compared to lean C57 dams ($p=0.005$) (Figure 2.4b).



E.

Phyla	Pregnant BPH5	Pregnant C57	P-value
Firmicutes	52.31%	52.30%	0.99
Bacteroidetes	35.43%	40.6%	0.375
Epsilonbactaeota	8.11%	0.01%	0.00009*
Cyanobacteria	2.70%	0.04%	0.006*
Proteobacteria	1.15%	3.90%	0.00004*
Tenericutes	0.10%	2.24%	0.002*
Actinobacteria	0.09%	0.48%	0.000003*

Figure 2.4 Major phyla changes when comparing pregnant BPH/5 vs pregnant C57. A). Bar graph of the relative abundance of phyla in the pregnant BPH/5 and C57. B). No difference was found in the Firmicutes to Bacteroidetes ratio during pregnancy. C). PCo plot using beta diversity of pregnant BPH/5 compared to normotensive controls. D). Pregnant BPH/5 had a significant increase in alpha diversity compared to normotensive controls. E). Table of relative abundances at the phyla level of pregnant BPH/5 and C57.

2.3.4 Changes at the genus level during pregnancy of BPH/5 compared to C57

Looking specifically at the BPH/5 model, fecal samples were dominated by *Alistipes*, *Lachnospiraceae_NK4A136*, *Helicobacter*, *Bacteroides*, and *Lactobacillus* (Figure 2.5). With the

most notable during pregnancy differences in the *Alistipes* (23.31% BPH/5 and 10.07% C57), *Bacteroides spp* (7.03% BPH/5 and 15.38% C57), *Lactobacillus* (6.36% BPH/5 and 16.53% C57), *Helicobacter spp* (10.39% BPH/5 and 0.02% C57), *Parasulterrella spp* (1.79% BPH/5 and 4.85% C57), and *Parabacteroides spp* (1.79% BPH/5 and 3.51% C57) (Figure 2.5ab).

A.

Genus	Pregnant BPH5	Pregnant C57	P-value
Lachnospiraceae_NK4A136_group	20.75%	17.98%	0.507
Alistipes spp	23.31%	10.07%	0.0004*
Bacteroides spp	7.03%	15.38%	0.007*
Lactobacillus spp	6.36%	16.53%	0.008*
Helicobacter spp	10.39%	0.02%	.0007*
A2	2.65%	4.11%	0.472
Lachnoclostridium spp	1.28%	3.60%	0.07
Roseburia spp	1.54%	3.03%	0.242
Lachnospiraceae-UCG_001	2.98%	0.50%	0.114
Parasulterrella spp	1.79%	4.85%	0.005*
Parabacteroides spp	1.79%	3.51%	0.01*
Lachnospiraceae-UCG_001	2.98%	0.50%	0.114
Rikenellaceace_RC9_gut_group	1.78%	0.001%	0.05

B.

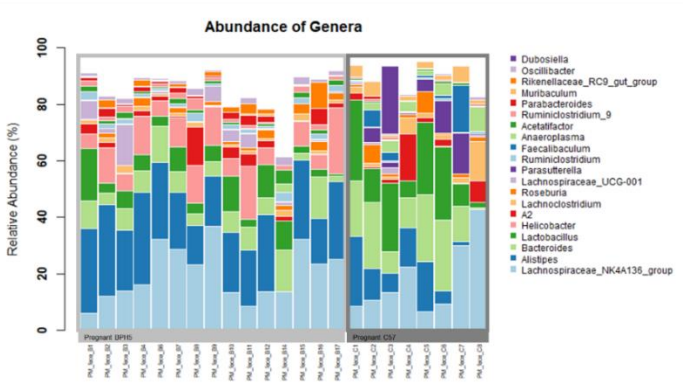


Figure 2.5 Major changes at genus level between pregnant BPH/5 and C57. A). Table at the genus level of the relative abundances of pregnant BPH/5 and C57. B). Bar graph depicting the genera of pregnant BPH/5 and C57.

2.3.5 SCFAs and colonic inflammation in the BPH/5 pregnant mouse

SCFA are the byproducts of microbial metabolism and bind to G-protein coupled receptors such as GPR 41/43 to carry out downstream analysis. GPR41 was significantly reduced in the BPH/5 dams during pregnancy (Figure 2.6a), while GPR43 was not different between strains. Colonic expression of IL-15 was upregulated in the BPH/5 during pregnancy, while IL-6 was not (Figure 2.6b). No significant differences were found between BPH/5 and C57 fecal SCFA level during pregnancy (Figure 2.6c-g). The ratio of acetic acid, propionic acid, and butyric acid was 6:1:1, respectively in both strains.

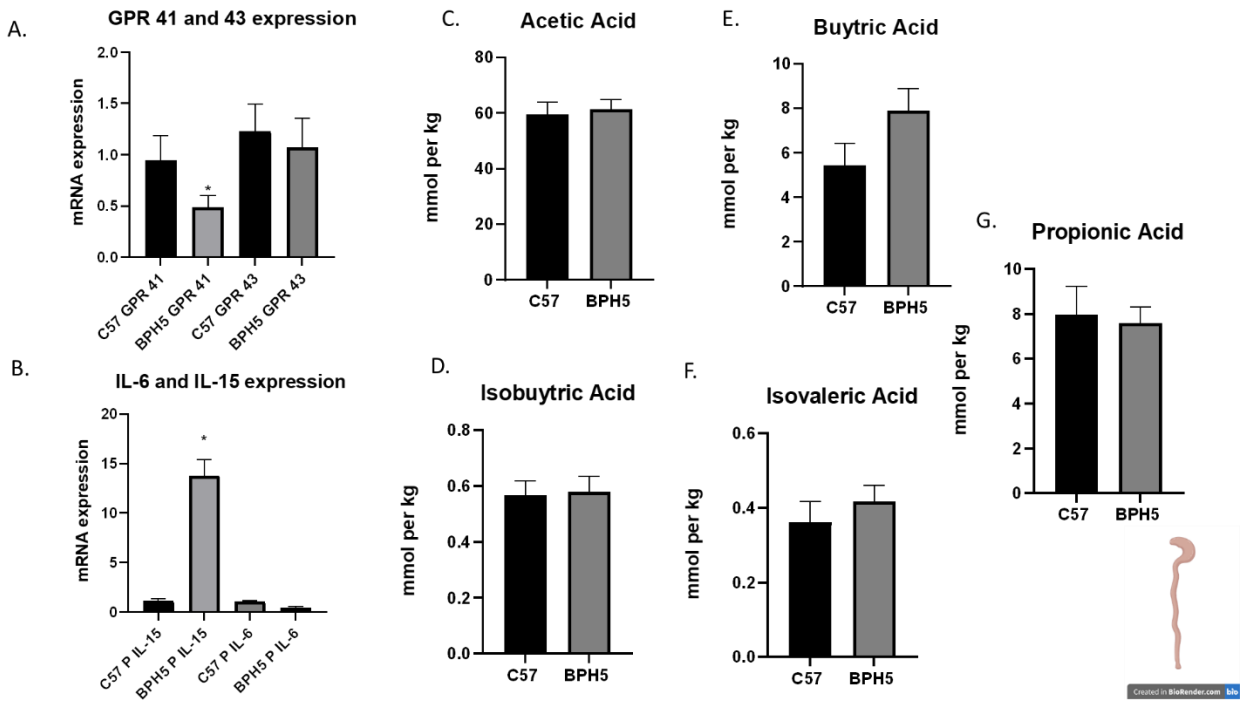


Figure 2.6 Short chain fatty acid profile, GPR41/43, and IL-15 expression in the colon during pregnancy A).BPH/5 pregnancy dams show a decrease in GPR 41 expression in the colon, while GPR 43 is similar to C57 pregnant controls B). BPH/5 pregnant dam shown an increase of IL-15 expression in the colon compared to pregnant C57 lean controls, whereas no difference is shown in IL-6. C). Acetic acid during pregnancy in the feces. D). Isobuytric acid during pregnancy in the feces. E). Butyric acid during pregnancy in the feces. F). Isovaleric acid during pregnancy in the feces. G). Propionic acid during pregnancy in the feces. (*$p<0.05$).

2.3.6 SCFAs in circulation

When looking at SCFA in circulation acetic acid and butyric acid were significantly decreased in the pregnant BPH/5 (Figure 2.7ac). While isobutyric acid and isovaleric acid were significantly increased (Figure 2.7bd). Propionic acid showed no difference between strains (Figure 2.7e).

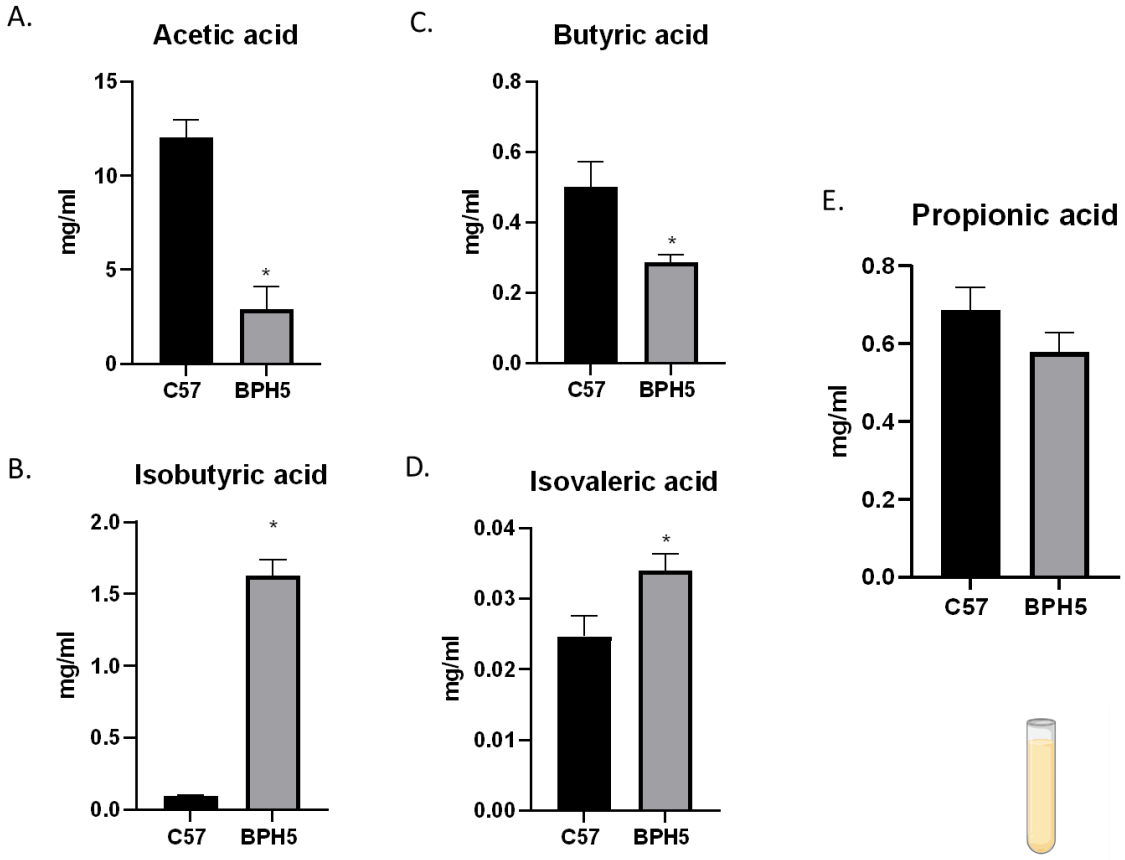


Figure 2.7 Short chain fatty acid in the serum during pregnancy. A). Acetic acid during pregnancy in the serum. B). Isobuytric acid during pregnancy in the serum. C). Butyric acid during pregnancy in the serum. D). Isovaleric acid during pregnancy in the serum. E). Propionic acid during pregnancy in the serum. (*$p<0.05$).

2.4 Discussion

The overall goal of this study was to characterize the BPH/5 gut microbiome and describe the microbial changes that occur with the onset of pregnancy to determine if the BPH/5 mouse model develops a gut dysbiosis. The main findings are 1). The BPH/5 mouse model does demonstrate a gut dysbiosis with differences in diversity compared to the lean C57 model. 2). The female BPH/5 non-pregnant mouse exhibits a gut dysbiosis prior to pregnancy and changes occur in the BPH/5 mouse model with the onset of pregnancy. 3). Differences were found in SCFAs in the serum but not within the feces; BPH/5 had significantly less GPR 41 mRNA found in the colon

compared to normotensive controls. 4). BPH/5 mice have increased markers of inflammation in the colon. We reported that the non-pregnant BPH/5 female mouse demonstrates gut microbial dysbiosis by increased alpha diversity, altered beta diversity, and decreased Firmicutes to Bacteroidetes ratio. Similar results were found with the BPH/5 pregnant mouse compared to normotensive pregnant mice, specifically altered alpha and beta diversity. Our results were congruent with previous findings of the Dahl S rat that linked hypertension and the gut microbiome (125, 126). Factors such as environment, location, and diet may lead to minor changes within the gut microbiome between studies.

Multiple studies describe the link of hypertension and the gut microbiome in other animal models and additionally in humans (126–132). Current research has explored the theory relating gut microbial dysbiosis to the development of PE stating that the gut dysbiosis increases the inflammatory response, and pregnancies that begin in an heightened state of inflammation are at great risk for the development of PE (133). The BPH/5 mouse model demonstrates gut dysbiosis and increased inflammation at the local colonic level, as well as in the adipose tissues and implantation sites as previously described (114). The placenta plays a critical role in PE pregnancies with some suggesting that placental microbial dysbiosis is associated to the development of PE, but this is yet to be determined in the BPH/5 mice model. Besides the fecal microbiome, the oral cavity and the vagina also have been hypothesized to contribute to the development of PE (133). Further research is needed to determine the contribution of inflammation derived from other body site's microbial community and its role in the development of PE.

Significant changes did occur in the BPH/5 model with the onset of pregnancy, such as changes in community structure using beta-diversity and increases in specific bacterial families. Healthy human pregnancies and normotensive mice demonstrate a shift in the gut microbiome that

relates to pregnancy specific metabolic changes that allow adequate fetal growth and balance energy homeostasis (125, 134). Normal pregnancy is suggested to be associated with a decrease in alpha diversity, increase in beta diversity, and increase in Proteobacteria phylum (134). Our findings demonstrate that the BPH/5 shows markers of dysbiosis by failing to change alpha diversity and decreasing Proteobacteria with the onset of pregnancy. This demonstrates a failure in the gut microbiome to adapt with pregnancy starting before pregnancy and may lead to increased inflammation and adverse outcomes. Future investigations are needed to functionally link gut dysbiosis with impaired placentation, increased hypertension, and fetal growth restriction in this model (99).

Ishimwe et al. suggested that transient pregnancy-specific dysbiosis may be accompanied by specific changes at the genera level including genus as such *Helicobacter* (125). The BPH/5 mouse model also demonstrated an increase in *Helicobacter* compared to normotensive controls. This is particularly intriguing because other studies in rodent models have demonstrated that specific bacterial strains can influence phenotypic changes characteristic of PE (92). For example, *Campylobacter rectus* causes abnormal placental development, fetal growth restriction, and increased pup mortality in mice (92) while *Porphyromonas gingivalis* was shown to increase inflammation and fetal growth restriction in mice (135). Although some of these bacteria are mutualistic inhabitants, an overgrowth may lead them to become pathogenic within the same host.

Short chain fatty acids (SFCAs) are gut microbiome-derived metabolites that fuel the host metabolism and have been found to be key mediators between dysbiosis and disease. Some SCFAs are considered anti-inflammatory (propionate, butyrate, and acetate), while others are pro-inflammatory (lactate). Anti-inflammatory SCFA have been shown to lower BP (136–138). Human studies also have shown a correlation between BP and SCFAs (139, 140). Supplementation

of propionate attenuated hypertension and systemic inflammation in mice (141). Even though SCFAs were not found to be significantly different between BPH/5 and normotensive C57 in the feces, anti-inflammatory SCFAs (butyrate and acetate) are decreased in the serum. Branched SCFAs, isovaleric and isobutyric, were increased in the serum of pregnant BPH/5 mice. This may contribute to BPH/5 gestating in a heighten state of inflammation due to the lack of negative inhibition, especially a decrease in butyrate since it is an essential microbial metabolite with a vital role as a modulator of proper immune function in the host. It has been shown that children lacking butyrate are more susceptible to allergic disease (142) and Type 1 Diabetes (143). Butyrate is reduced also in a low fiber diet, which can subsequently induce inflammation (77). In the BPH/5 model decreased butyrate may play a role in increased colonic inflammation and potentially inflammation at other distant body sites. Additionally, acetate is known to be produced by bacteria such as Bifidobacteria, Lactobacilli, and *Ruminococcus* (144). It binds to GPR 41 and 43 within the colon and is the main substrate in the synthesis of cholesterol (144). Because BPH/5 female mice have hypercholesterolemia, this paradoxical decrease in acetate may not contribute to dyslipidemia observed in this model. However, it may have a direct effect on other tissues such as … hypothalamus, placenta and fetus?

Free fatty acid receptors (FFAR), GPR 41 and 43, are key receptors for SCFAs. They aid in the regulation of host energy homeostasis in the sympathetic nervous system, adipose tissue, pancreas, and intestines. Our findings indicate differences in GPR41 mRNA isolated from the distal colon, whereas GPR43 mRNA was not different from normotensive controls. In a healthy gut, the GI tract is associated with higher mucus thickness, production of antimicrobial signals, and an appropriate amount of SCFA production. The SCFAs bind to GPR41/43, which are expressed on the enteroendocrine L cells that stimulate the production of gut peptides such as

glucagon-like peptide-1 (GLP-1) and peptide YY (PYY) (145). This results in reduced food intake and improved glucose metabolism within a healthy gut (145). In the BPH/5 model, fecal SCFAs production does not appear to be altered but GPR 41 signaling is reduced, which could lead to decreased uptake of SCFA in the serum and decreased secretion of GLP-1 and/or PYY. This reduction may lead to a leakage of pathogen associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) and trigger low-grade inflammation within the GI tract. In the BPH/5 model, GPR41 may be the link between disparate fecal and serum SCFA levels, acetate, and butyrate.

BPH/5 are known to have increased inflammation in their adipose tissue and implantation sites (114); we also found an increase in inflammatory markers in the colon represented by an increase in interleukin 15 (IL-15) mRNA expression. IL-15 is a pleiotropic cytokine expressed by dendritic cells, macrophages, fibroblasts, and epithelial cells, following inflammatory stimuli is upregulated in macrophages and enterocytes (146). In the context of PE, IL-15 was increased in the serum of preeclamptic mothers compared with healthy controls (147). These findings provide evidence that decreased GPR41, and increased IL-15 may be involved in poor pregnancy outcomes that may be influenced by gut microbial dysbiosis.

A similar theory linking gut dysbiosis and increased inflammation has been proposed in patients with inflammatory bowel disease (IBD) (148). It is believed that this is a result from gut-microbiome alterations with reduced butyrate production. IL-15 also is upregulated in the colonic tissue in IBD infected individuals and is thought to play a role in the pathogenesis of IBD (148). More specifically in a study by Meisel et al., IL-15 overexpression restructures the composition of the microbiota with a decrease in butyrate producing bacteria that is associated with a reduction in butyrate levels across all intestinal compartments. Altogether, this study reveals that IL-15

impacted butyrate-producing bacteria and lowers butyrate levels, which represent events that promote intestinal inflammatory disease (148).

The importance of studies such as these to define specific pathobionts behind gut microbial dysbiosis in the etiology of PE remain vital. A major advantage of the BPH/5 model is that it develops PE spontaneously without surgical or pharmacological intervention. Thus, we are able to study the microbial changes longitudinally without intervention. This will be advantageous in future studies that test the effect of probiotics or replacement of SCFAs and the effects on maternal and fetal PE outcomes. There is still a great deal to be discovered about the role of the maternal microbiome and the development of PE, including the influence of the maternal microbiome from other body sites. This study further contributes to the hypothesis that gut dysbiosis leads to an increase in inflammatory responses and results in adverse pregnancy outcomes. The gut dysbiosis may be a key mechanism that affects GPR41 signaling, inflammation, and contributes to the BPH/5 phenotype (Figure 2.8). Future directions would be to develop a rapid screening technique using feces as a biomarker for maternal gut dysbiosis to monitor bacterial community shifts during pregnancy. The long term goal would be to identify microbial dysbiosis in high-risk women prior to pregnancy or in early gestation to help prevent adverse pregnancy outcomes.

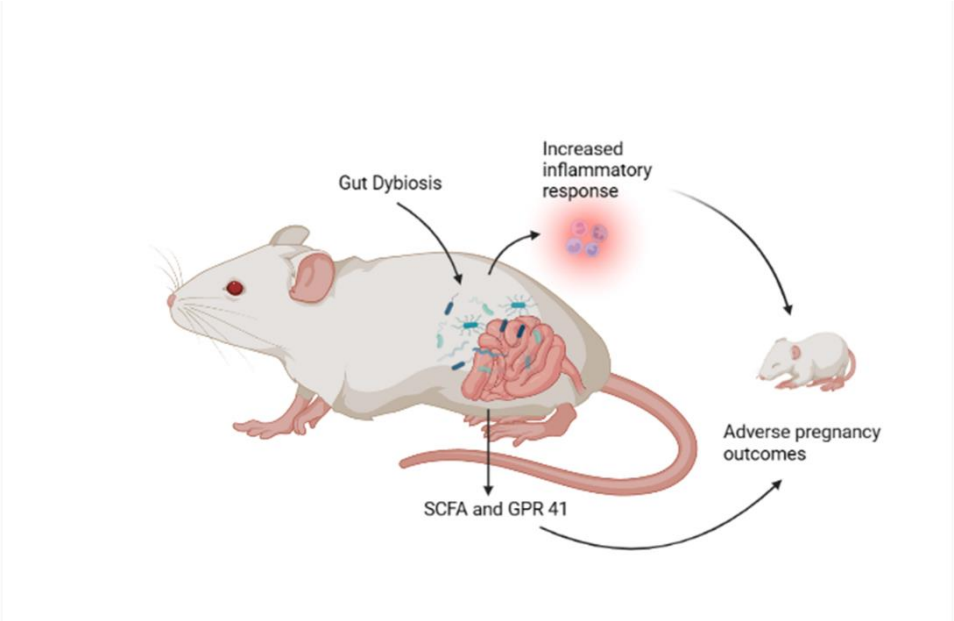


Figure 2.8 Depiction of the effects of gut dysbiosis on inflammation and pregnancy outcomes in the BPH/5 mouse. Image made with Bio Render.

CHAPTER 3. CARDIOMETABOLIC PHENOTYPIC DIFFERENCES IN MALE OFFSPRING BORN TO OBESE PREECLAMPTIC-LIKE BPH/5 MICE

3.1 Introduction

Preeclampsia (PE) is characterized by maternal hypertension occurring after 20 weeks of gestation (systolic ≥ 140 mmHg or diastolic ≥ 90 mmHg) along with another accompanying sign/symptom, including proteinuria, renal insufficiency, thrombocytopenia, hepatic dysfunction, and/or pulmonary edema (4). PE affects up to 300,000 women worldwide making it a leading cause of maternal and fetal morbidity and mortality (1). The treatment is often delivery of the fetus and the placenta, which can have deleterious consequences on both the mother and baby (149). Offspring are often premature or stillborn, exhibit fetal growth restriction (FGR), and are small for gestational age (1). Beyond the perinatal effects of being born to PE mothers, the lifelong consequences can be devastating for the offspring. Fetal programming, in utero alterations occurring due to the maternal environment, can affect fetal growth and development leading to lifelong effects on the offspring (2). PE-associated fetal programming can result in increased cardiovascular complications, including hypertension, ischemic heart disease, stroke (3), and metabolic disease in the offspring as they age (4–6). PE outcomes are in line with the Developmental Origins of Health and Disease theory, by demonstrating that an unfavorable uterine environment will lead to pathogenic conditions in the offspring that will increase the risk of chronic disease later in life (150).

Kalie F. Beckers et al. "Cardiometabolic phenotypic differences in male offspring born to obese preeclamptic-like BPH/5 mice." Frontiers in Pediatrics: Neonatology. (2021)

Furthermore, poor prenatal nutrition is associated with low birth weights, which are linked to changes in adult body composition including altered fat distribution, reduced muscle mass, and low bone mineral content (2). One theory is that in response to an adverse intrauterine environment the fetus adapts for survival.

Fetal responses include altered metabolic homeostasis, downregulation of growth, and endocrine changes (151, 152). These adaptive changes may be beneficial to the fetus short-term, while long-term they are detrimental to the offspring health. Changes include compensatory growth, diet-induced obesity, hyperphagia, and other factors (152, 153).

Obesity has reached epidemic proportions in the majority of the developed world, with over 50% of women childbearing age being overweight or obese (154). In 2018, over 40% of the men in the United States were considered obese (155). Previous research links maternal and early life nutrients to the development of long-term metabolic disorders including alteration to organogenesis, tissue development, and metabolism. These changes predispose offspring to obesity, metabolic, and cardiovascular disease later in life (156–158). There are both human and animal models that have evidence linking maternal obesity to 'programming' the offspring to develop cardiometabolic disease in adulthood (159–163). It also has been determined that the adverse fetal programming is more pronounced with obesity versus malnutrition (164). The BPH/5 mouse model, originally described by Davisson et al. (99), spontaneously develops PE. Therefore, it is possible to study the generational effects of PE using BPH/5 mice. Specifically, BPH/5 females have mildly elevated blood pressure prior to pregnancy, which is a known risk factor for the development of PE in women (99). In late gestation, the dams display elevated mean arterial pressure (MAP) and proteinuria, endothelial dysfunction, and renal glomerulosclerosis (99). Similar to PE in women, BPH/5 offspring have lower birth weights compared to normotensive

C57 control mice, indicative of FGR, and litter size is severely compromised due to fetal demise. BPH/5 show placental abnormalities, such as upregulation of pro-inflammatory mediators (112, 113).

Most of the cardiovascular outcome studies focus on women/female offspring born to PE mothers because of the transgenerational effect they play in the life cycle of PE. This study aims to examine the importance of PE fetal programming on male offspring in the BPH/5 model. Previous research by Sutton et al., studied the effects of in utero PE on the female offspring of obese BPH/5 (111). Female BPH/5 offspring are born smaller than C57 controls and exhibit excessive catchup growth and hyperphagia. Sutton et al. also demonstrated that BPH/5 female mice have a predisposition for obesity with increased white adipose tissue (WAT) accumulation, pro-inflammatory reproductive WAT, and leptin dysfunction (111). We hypothesized that male BPH/5 offspring born to PE-like dams will demonstrate cardiovascular and metabolic phenotypes similar to BPH/5 females.

3.2 Materials and Methods

3.2.1 Animal experiments

The Louisiana State University Institutional Animal Care and Use Committee approved all animal experiments. Body weights were assessed in prepubertal (2-3 weeks) BPH/5 (n=9) and C57 (n=15) and adult (8 weeks – 6 months) BPH/5 (n=32) and C57 (n=17). All males within a given litter from at least 3 litters were used for animal experiments. Normal chow (Purina [Neenah, WI] rodent chow: 23% crude protein, 4.5% crude fat, 6% crude fiber, and 8% ash) food intake was measured for 12 consecutive days from adult C57 and BPH/5 male mice after 2 days of accumulation. Using a gram scale, body weights, visceral WAT (peri-gonadal and peri-renal depots), inguinal subcutaneous WAT, subscapular brown adipose tissue (BAT), hearts with left

ventricle and LV dissected, kidneys, and livers were weighed from BPH/5 and C57 age-matched adult males. All WAT and BAT depots were dissected free from surrounding tissues according to published methods in mice (165). Tissues were flash frozen in liquid nitrogen for downstream analyses.

3.2.2 Radio telemetric measurement of blood pressure and heart rate

Adult male BPH/5 (n=4) and C57BL/6 (n=16) mice underwent carotid implantation of telemetry (Data Sciences International) according to published methods (99). Briefly, male mice were anesthetized for placement of a telemeter in the left carotid artery and transmitter body in the subcutaneous space. Mice were allowed to recover for 10 days, followed by 4 days of heart rate (beats per minute) and mean arterial pressure (MAP) recording.

3.2.3 Histology

The heart, kidney, liver, and peri-renal and subcutaneous WAT were fixed in 10% formalin, paraffin embedded sectioned and stained using hematoxylin and eosin (H&E) by the Louisiana Animal Disease Diagnostic Laboratory standards and analyzed by a board-certified veterinary pathologist. A blinded single operator measured adipocyte area in 6 randomly selected frames per mouse (n=3/strain) using ImageJ (NIH).

3.2.4 Quantitative PCR (qPCR)

Total RNA was extracted from peri-renal and inguinal subcutaneous WAT using TRIzol according to manufacturer's instructions (Qiagen, Hilden, Germany). RNA quality and quantity were assessed by spectrophotometry (Nano Drop). 1000 ng cDNA was reverse transcribed using the qScript cDNA kit (Quanta BioSciences, Beverly, MA). Each qPCR was performed in triplicate with an ABI 7500 Fast Thermocycler (Applied Bioscience) using SYBR Green (Quanta

BioSciences) using 25 ng cDNA. The following forward and reverse primers were used, respectively: *TNFa* (GAACTGGCAGAAGAGGCACT, AGGGTCTGGGCCATAGAACT (111)), *IL-6* (TGGCTAAGGACCAAGACCATCCAA, AACGCACTAGGTTTGCCGAGTAGA (111)), *Ptgs-2* (ACTGGGCCATGGAGTGGACTTAAA, AACTGCAGGTTCTCAGGGATGTGA (113)) expression level. Data was analyzed using the ΔΔ Ct method, and results were normalized to *18s* (CCGGGCTTCTATTTTGTTGGT, TAGCGGCGCAATACGAATG (107)) (123).

3.2.5 Statistical analysis

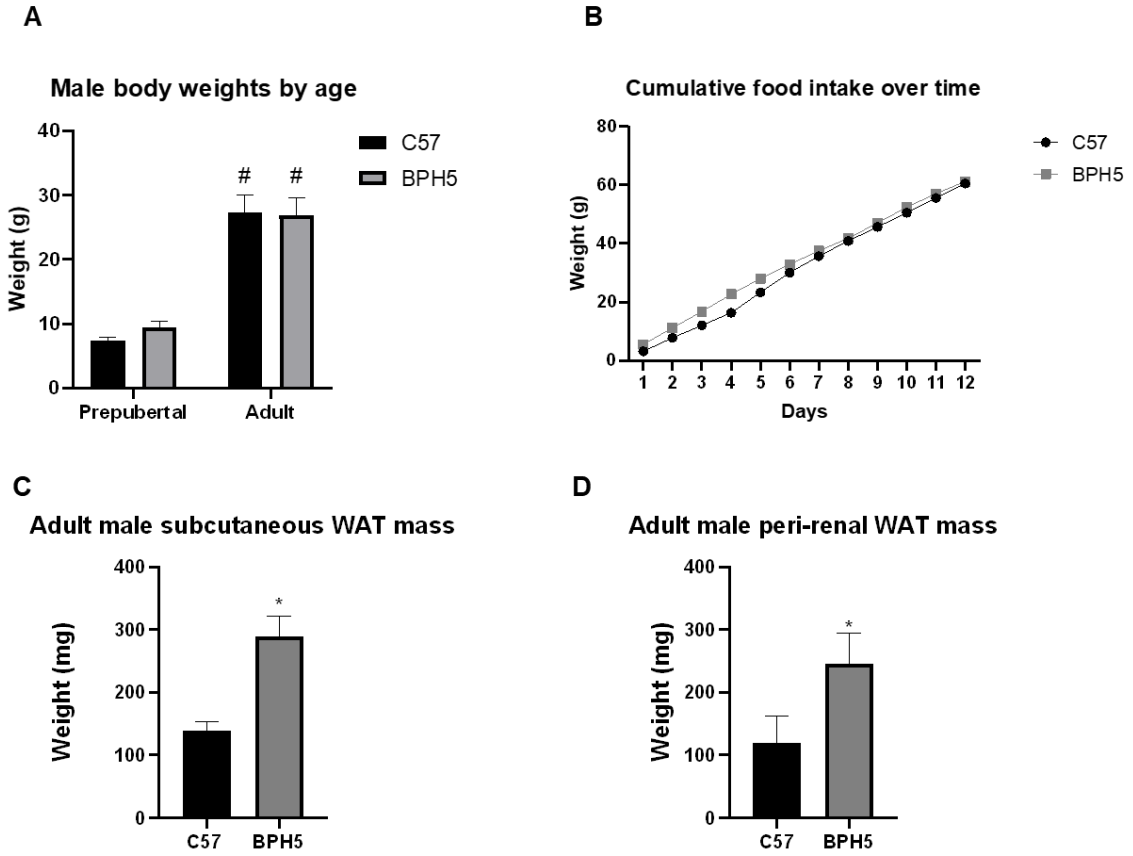
Statistical analysis was performed using GraphPad Prism. Shapiro-Wilk test was used to check for normality. Two-way ANOVA with Tukey's post hoc test and/or a student's t-test were used. The p values < 0.05 are considered significant. Error bars within figures recorded in standard error mean (SEM).

3.3 Results

3.3.1 Adult BPH/5 male body weight and food intake are similar to control male mice, while visceral and subcutaneous adipose tissue depots are increased

In humans, infants with low birth weights have been shown to exhibit accelerated catch up growth and central pattern of fat distribution, reduced lean mass, and increased adiposity (166, 167). Offspring of preeclamptic-like BPH/5 dams previously have been described to have intrauterine FGR and have smaller birthweights when compared to C57 aged-matched counter-parts (99, 113, 168). It was previously described that female BPH/5 offspring exhibit accelerated catch up growth beginning with small for gestational age birth weights, then overweight by adulthood when compared to C57 female controls (12). In this study, male BPH/5 offspring have similar prepubertal and adult body weights when compared to C57 age-matched controls (Figure 3.1A; $p>0.05$). While male mice in both strains significantly increased body weight from the

prepubertal stage into adulthood (Figure 3.1A; $p<0.05$). BPH/5 adult female mice are hyperphagic when compared to adult C57 female mice (111). However, BPH/5 adult male mice show daily food intake and cumulative food intake over 12 days (Figure 3.1B) that is comparable to adult C57 male mice ($p>0.05$). Similar to findings in Sutton et al., BPH/5 males exhibited increased WAT mass in the subcutaneous (Figure 3.1C), and peri-renal depots (Figure 3.1D). This was not



associated with an increase in BPH/5 adipocyte area when measured histologically after H&E staining compared to age matched C57 adult males (data not shown). Peri-gonadal WAT was not significantly different in BPH/5 males compared to C57 aged-matched controls ($p>0.05$; Table 3.1) as was found in BPH/5 adult females. Furthermore, intrascapular BAT weights were not different when compared to controls (Table 3.1).

Figure 3.1 Adult BPH/5 male phenotypic differences in body weight, food intake, and white adipose tissue (WAT). (A) Body weights were measured in prepubertal and adult BPH/5 males, and a similar weight was demonstrated at both prepubertal and adulthood when compared to C57 males (n = 10–32/group). (B) Daily and cumulative food intake was measured for 12 days in both BPH/5 and C57 male and was found to be not significantly different (n = 5/group). (C) BPH/5 males have significantly increased subcutaneous WAT (n = 6–18/group). (D) BPH/5 males have more peri-renal WAT vs. C57 (n = 8/group) (*$p < 0.05$ vs. C57, #$p < 0.05$ vs. prepubertal weights of their respective strain).

Table 3.1 Kidney, liver, peri-gonadal white adipose tissue, and brown adipose tissue mass of BPH/5 and C57 adult male mice

Kidney	414	551	33.09 %	0.0014 *
Liver	1230	1450	17.8 %	0.0068 *
Peri-gonadal white adipose tissue (WAT)	434.4	470.1	8.2 %	0.34
Brown adipose tissue (BAT)	152.3	163.1	7.09 %	0.57

3.3.2 Adult BPH/5 male mice show evidence of cardiovascular disease

Low birth weights in humans have been associated with development of cardiovascular disease (4–6, 31). The adult male BPH/5 offspring have increased heart weights compared to age matched C57 mice, indicative of cardiomegaly (Figure 3.2A; $p<0.05$). On histological examination, BPH/5 exhibit mild abnormal fiber size variation with variously sized occasional larger nuclei (karyomegaly). There were also rare areas of cardiomyocyte necrosis with hypereosinophilic cytoplasm and pyknotic nuclei (Figure 3.2B). This evidence for cardiomyopathy with mild acute ischemic necrosis in BPH/5 adult males was not identified in age matched C57 adult males (Figure 3.2B). Cardiomegaly in adult BPH/5 males is accompanied by increased left ventricle mass as compared to age-matched control C57 mice (Figure 3.2C; $p<0.05$). Mean arterial pressure in adult BPH/5 males is increased compared to age-matched control C57 mice (Figure 3.2D; $p<0.05$), while average daily heart rates are decreased (Figure 3.2E; $p<0.05$). The BPH/5 male offspring also displayed increased liver weight compared to controls (17.8%) suggestive of hepatomegaly (Supp. Table 1; $p<0.05$). Finally, adult male BPH/5 offspring have increased kidney weight compared to adult male C57 controls (33%), suggestive of nephromegaly (Table 3.1; $p<0.05$). However, on histological examination no significant lesions were identified either in the liver or the kidney.

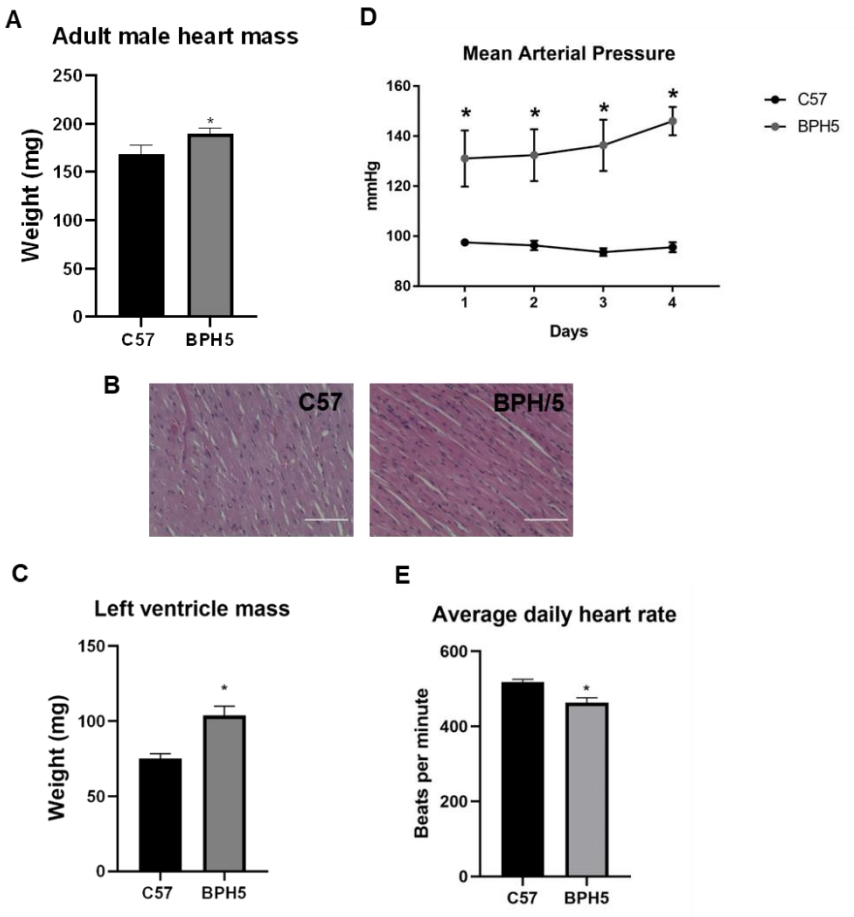


Figure 3.2 Adult BPH/5 male mice exhibit signs of cardiovascular disease. (A) BPH/5 male mice demonstrate increased heart mass compared to C57 controls. (B) Hematoxylin and eosin (H&E) stain of C57 (left) and BPH/5 (right) cardiac tissue. Scale bar = 100 μm. (C) BPH/5 male mice demonstrate increased mass of the left ventricle compared to C57 controls. (D) BPH/5 male mice demonstrate increased mean arterial pressure compared to C57 controls as measured over 4 consecutive days. (E) Daily average heart rates measured as beats per min were increased in BPH/5 adult males compared to C57 (n = 4–18/group; *p < 0.05 vs. C57).

3.3.3 Inflammatory mediators are increased in BPH/5 male white adipose tissue depots

Increased adiposity is linked to increased adipose tissue inflammation with upregulation of inflammatory cytokines (169). Reproductive WAT of adult BPH/5 female offspring displayed a seven- and four-fold increase in tumor necrosis factor alpha (*TNFa)* and interleukin-6 (*IL-6*) mRNA, respectively (26). Adult BPH/5 males showed an increase in *TNFa* relative mRNA in the peri-renal but not the subcutaneous WAT compared to age-matched C57 adult males (Figure 3.3A;

p<0.05). In subcutaneous WAT, there was a significant difference in prostaglandin synthase 2 (*Ptgs-2*) (Figure 3.3B) and *IL-6* mRNA expression (Figure 3.3C) compared to adult male C57 controls (p<0.05).

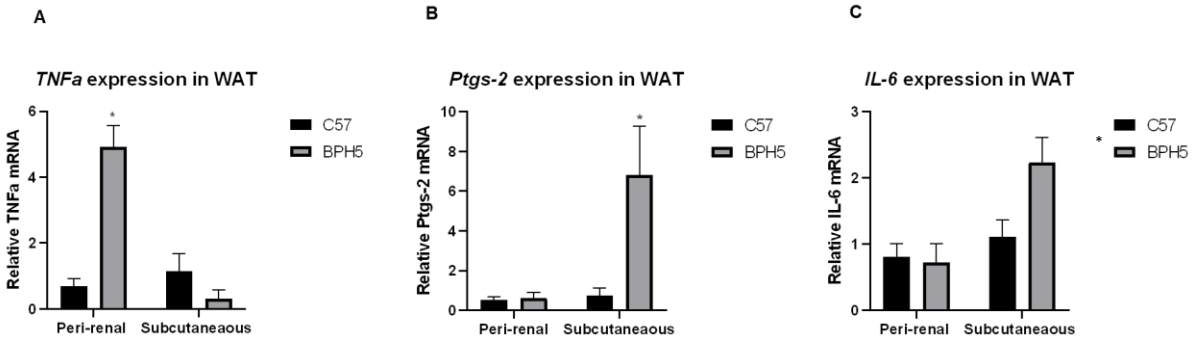


Figure 3.3 Adult BPH/5 male mice exhibit increased inflammatory mediators within peri-renal and subcutaneous white adipose tissue (WAT). (A) Using real-time PCR, adult BPH/5 males have significantly increased tumor necrosis factor alpha (TNFa) relative mRNA expression in the peri-renal WAT; (B) prostaglandin synthase 2 (Ptgs-2) relative mRNA expression was significantly increased in subcutaneous WAT; and (C) interleukin-6 (IL-6) mRNA expression was significantly increased in the subcutaneous WAT (n = 3–8/group; *p < 0.05 vs. C57).

3.4 Discussion

PE during pregnancy can be life threatening for both the mother and fetus, and cardiometabolic co-morbidities may persist long-term. The BPH/5 mouse model, which spontaneously develops PE, has been used to gain insight into the pathophysiology and outcomes of PE. This study focuses on the male offspring phenotype when exposed in utero to a maternal obesogenic environment. Genetic influence is important to take into consideration when analyzing the differences between offspring outcomes.

Differences in the pathophysiology of PE may occur depending on the sex of the fetus. For example, maternal endothelial dysfunction, characterized by peripheral microvascular vasoconstriction, was greater in preeclamptic pregnancies carrying a male fetus (170). The normal postnatal growth of male neonates from preeclamptic pregnancies suggests that fetal-placental

blood flow is maintained despite maternal hypertension and placental insufficiency (170). There is still a need for further studies examining the effects of placental insufficiency on postnatal body composition, specifically adipose tissue distribution, type, and glucose homeostasis. For male infants, those born to preeclamptic women had greater microvasculature blood flow at 6 hours postnatal while male infants of normotensive women exhibited increasing blood flow with time (170). It also was found that the PE driven maternal endothelial dysfunction was greater in the presence of a male fetus (171).

Solely studying this disease in humans presents various challenges, such as inability for in vivo manipulative studies. The Spontaneously Hypertensive Rat (SHR) is a model of essential hypertension in humans and one in which males have higher blood pressure (BP) than females as young adults (172), but both sexes are hypertensive compared with normotensive Sprague-Dawley rats. A rat model of placental ischemia, which occurs in PE, is the reduced uterine perfusion pressure (RUPP) model, and offspring of RUPP females exhibit intrauterine FGR (173, 174), as is also commonly found in offspring of women who develop PE. The male offspring of a RUPP pregnancy show increased BP with adulthood, whereas the female offspring do not (175). According to Reckelhoff et al., males demonstrate higher BP than females in hypertensive rat models (176). Similar differences may occur in these BPH/5 offspring contributing to the female showing obesity while the males apparently do not. Thus, investigating PE-driven cardiometabolic outcomes in male offspring is equally important as female offspring.

An unfavorable maternal environment, including obesity as well as malnutrition, has been shown to impact offspring into adulthood. The Dutch Winter famine, which occurred during World War II when food was limited, resulted in offspring having ~~an~~ increased abdominal fat distribution in adult male (177) demonstrating fetal programming likely occurred because of maternal

undernutrition. The fetuses from the famine were programmed in utero to have an altered fat distribution and metabolism. The offspring from the Dutch Famine cohort had a 3-fold increase of coronary heart disease (178). As previously mentioned, the effects of maternal obesity are more pronounced than malnutrition (164). This may be comparable to the BPH/5 offspring where the obese mother is an unfavorable environment for offspring development. This may lead to adverse offspring outcomes, including increased adiposity and cardiovascular disease.

BPH/5 preeclamptic mouse model are known to have offspring with low birth weights (99). The BPH/5 female offspring showed post pubertal accelerated catch up growth with adulthood obesity (111). Interestingly, male BPH/5 offspring may demonstrate an earlier accelerated catch up growth as BPH/5 males catch up to C57 male body weight by 2-3 weeks of age. Accelerated catch up growth has been associated with increased adiposity in both adult male and female rats (179). Furthermore, accelerated catch up growth also has been associated with hyperphagia leading to hyperleptinemia, hypertension, and obesity in adulthood, which is similar to BPH/5 females (111, 180). Therefore, we hypothesized and found that BPH/5 males do not demonstrate hyperphagia as females do and unlike BPH/5 females, maintain similar body weights to male C57 mice from the prepubertal stage and into adulthood. Studies investigating the lactational feeding period in BPH/5 would be of interest as postnatal overfeeding has been associated with hyperphagia and obesity later in life in both sexes (181, 182). Bol et al. demonstrated in overfed lactating mice, the male offspring showed an increased fat mass compared to controls (183).

Another contributing factor to the increased adiposity could be associated with developmental changes in the population of cells in the adipose tissue. Obesity can result from expansion of fat mass due to adipocyte hypertrophy by lipid accumulation or an exaggerated number of adipocytes (184). Offspring from diet-induced obese mothers showed increased fat

mass with larger adipocytes in adulthood (182). The BPH/5 male offspring demonstrated increased peri-renal and subcutaneous WAT mass, but not adipocyte area, and similar amounts of BAT when compared to age matched controls. It has been shown that an early reduction in BAT may perpetuate through the life cycle and suppress energy expenditure therefore promoting increased WAT and obesity (185). Therefore, BPH/5 males that do not demonstrate a reduced BAT may not exhibit the full obesogenic profile as do their female counterparts. In the newborn, the majority of the adipose tissue is BAT, which is used for thermoregulation in the extra-uterine environment (185). Adipose tissue first appears during mid-gestation and increases through late gestation, then becomes a mixture of both BAT and WAT. After birth, a majority of the BAT depot becomes WAT. The amount, location, and type of adipose tissue is effected by multiple factors that all play a role in the glucose homeostasis of the offspring (185). Further investigations into BAT function in BPH/5 males are ongoing.

Similarly to the unfavorable intrauterine environment of PE, infants born to diabetic mothers have been shown to demonstrate more adipose tissue when compared to controls (186). Fetal programming from obese mothers has been shown to result in hyperphagia, changes in cellular composition, lower energy expenditure or modification of the neurohormonal axis (186, 187). Thus, it is possible that fetal programming from an adverse maternal environment in this model could promote a pro-inflammatory milieu in the WAT, which may favor the development of the cardiovascular disease in this mouse model. Human and animal studies have shown that obesity is associated with low grade, chronic inflammation in the adipose tissue (188). Inflammation with the adipose tissue has been found to be a key contributor to the pathogenesis of metabolic syndrome and other cardiovascular disease (189, 190). It also has been linked to insulin resistance and type 2 diabetes (191, 192). TNFa and IL-6 are produced and secreted by

adipocytes and may be related to beta cell function (193). They also are produced by immune cells in the WAT, including adipose tissue macrophages (194). Ptgs-2 in WAT also has been associated with beta cell dysfunction and increased oxidative stress (193). A high fat diet fed to C57 mice to mimic obesity induced an upregulation in *IL-6, TNFa,* and *Ptgs-2* gene expression from adipose tissue (188). Increased inflammation of visceral adipose tissue might lead to an increase in the delivery of free fatty acids to the liver contributing more to the development of obesity (193). In the BPH/5 male mice, *TNFa* and *Ptgs-2* were increased similar to the studies above suggesting that these male mice may exhibit inflamed adipose tissue with oxidative stress that may contribute to cardiometabolic disease. Although there were not histological signs of liver disease, pro-inflammatory visceral and subcutaneous WAT depots in adult male BPH/5 mice may promote progressive liver disease as they age. These long-term studies in BPH/5 male and female mice would be valuable to better understand the chronic effects of maternal obesity-associated fetal programming as offspring age (Figure 3.4).

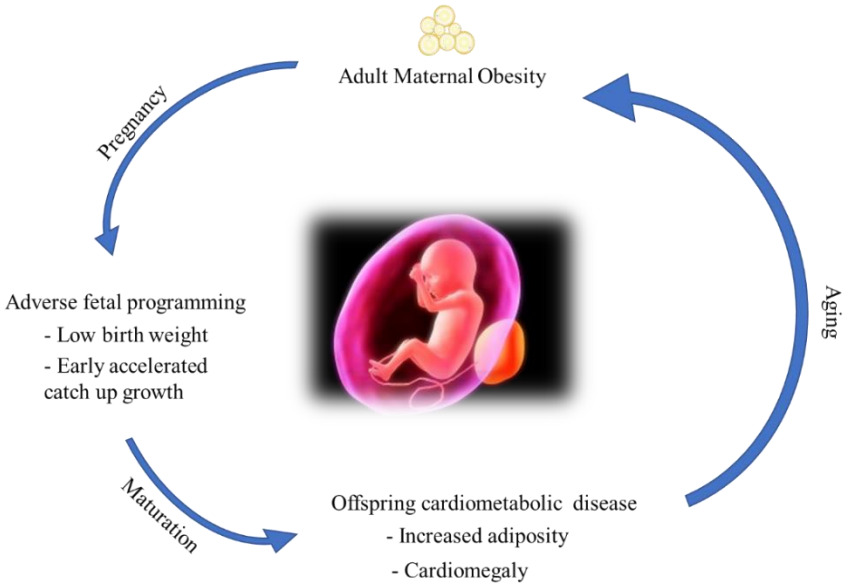


Figure 3.4: Working hypothesis for effects of maternal obesity on fetal programming. A depiction of the in-utero effects of maternal obesity on offspring demonstrated by BPH/5 mouse model. Adverse fetal programming attributing to low birth weight and early accelerated catch up growth.

Offspring cardiometabolic disease accrediting to increased adiposity and cardiomegaly found in this study. Image made with Creative Commons.

BPH/5 offspring demonstrate a cardiometabolic phenotype with markers such as central obesity and cardiovascular disease in the females. The males exhibit a unique phenotype showing increased visceral and subcutaneous adiposity and cardiovascular disease. In summary, BPH/5 males contrast the females as the female offspring strongly demonstrate the obese metabolic phenotype. In conclusion, maternal obesity and altered fetal programming may play a role in these sex dependent offspring outcomes into adulthood.

CHAPTER 4. SEX SPECIFIC EFFECTS OF MATERNAL WEIGHT LOSS ON OFFSPRING CARDIOMETABOLIC OUTCOMES IN THE OBESE PREECLAMPTIC-LIKE MOUSE MODEL, BPH/5

4.1 Introduction

Preeclampsia (PE) is characterized by late gestational onset of hypertension (systolic ≥ 140 mmHg or diastolic ≥ 90 mmHg), proteinuria, renal insufficiency, thrombocytopenia, hepatic dysfunction, and pulmonary edema (4). PE affects up to 2-8% of pregnancies worldwide, making it a leading cause of maternal and fetal morbidity and mortality (1). The only known treatment is delivery of the fetus and the placenta, which can have deleterious consequences on the mother and offspring. PE may lead to significant offspring co-morbidities, including small-for-gestational-age (SGA) neonates due to fetal growth restriction (FGR) and pre-term delivery-associated sepsis, and could ultimately lead to cardiovascular and metabolic disease later in life (1). Obese women are 6 times more likely to develop hypertension than their lean counterparts (195) and twice as likely to develop PE (196). Maternal obesity also increases the risk of pregnancy complications including gestational diabetes mellitus, preterm delivery, Caesarian section, SGA, and stillbirths (195, 197–202). In addition, maternal obesity predisposes offspring to cardiovascular, metabolic, and neurological disorders in adulthood (195, 203–205). This emphasizes the need for therapeutic interventions for obese mothers and their offspring. Studying the effects on the offspring in a sex dependent manner is equally as important. In the United States, 37.9% of men and 41.1% of women are considered obese.

Kalie F. Beckers et al. Sex specific effects of maternal weight loss on offspring cardiometabolic outcomes in the obese preeclamptic-like mouse model, BPH/5. Physiological Reports (2022).

According to WHO, while one in five women is diagnosed with hypertension, men have a one in four chance of developing the condition, making hypertension more prevalent and potentially a greater health risk in men (206). Therefore, an effect of sex is hypothesized in the development of these conditions.

The BPH/5 female mouse spontaneously develops obesity and hypertension pre-conception (111), and superimposed PE when challenged with pregnancy (99). The maternal obesogenic environment may play a role in offspring post-natal development and metabolism. However, the future developmental and transgenerational impacts of male and female offspring gestating in obese mothers with superimposed PE is not completely understood. Previous research demonstrates that BPH/5 offspring have sex dependent phenotypic differences. The BPH/5 female offspring present excessive catch up growth after birth, hyperphagia, obesity, cardiomegaly, increased blood pressure, and hyperleptinemia with leptin resistance (111). However, the adult male BPH/5 offspring are not hyperphagic and are not obese, but do exhibit increased white adipose tissue mass and cardiomegaly with left ventricular hypertrophy (207).

Herein, we propose the BPH/5 as a model for studying offspring born to PE dams in a sex dependent manner. A pair-feeding paradigm was used to match the food intake of the pregnant BPH/5 mice to the normal intake of the lean C57 controls of similar age and day of gestation. The BPH/5 offspring outcomes were then investigated after attenuation of maternal obesity during pregnancy. The metabolic imbalances and cardiac risk were investigated in the offspring of obese, ad libitum-fed, BPH/5 dams and compared to the offspring of lean, pair-fed, BPH/5 dams. Previous research has shown the benefits of pair-feeding dams, by decreasing adult body weight and reducing adiposity in BPH/5 female offspring (111). We hypothesized that cardiometabolic risk

differences exist between female and male BPH/5 offspring and that pair feeding BPH/5 dams during pregnancy would reprogram offspring to improve these outcomes.

4.2 Methods

4.2.1 Animal experiments

Adult (2-6 months of age) female and male BPH/5 mice were used in this study. BPH/5 mice were a gift from Dr. Robin Davisson, Cornell University and maintained as an in-house colony at Louisiana State University. Mice were housed in standard cages placed in a temperature and humidity-controlled facility, maintained on a 12-hour light/dark cycle, and fed standard mouse chow (Purina 5001) with water available ad libitum. Two groups of dams were used: 1) Ad libitum BPH/5 (AL) (n>6) and 2) Pair-fed BPH/5 (PF) (n=6). Group 2 was randomly selected BPH/5 dams that were pair-fed to mirror C57 food intake beginning at detection of copulatory plug, as previously described (114). C57 mice have been used as control mice in previous BPH/5 studies, as they were used in the original eight-way cross to derive the BPH/5 strain (99, 113, 114). Intrastrain timed matings were performed, and detection of a copulatory plug was designated as embryonic day 0.5 (e0.5). Two males and two females were randomly selected per litter across at least two litters of AL and PF dams. After parturition, these offspring were allowed to mature to adulthood, eating ad libitum. Four offspring groups were studied: a) BPH/5 female offspring born to ad libitum fed dams: AL/AL^{F}, b) BPH/5 male offspring born to ad libitum dams: AL/AL^{M}, c) BPH/5 female offspring born to pair-fed dams: PF/AL^{F}, and d) BPH/5 male offspring born to pair-fed dams: PF/AL^{M}. All animal studies were approved by the Louisiana State University School of Veterinary Medicine and Pennington Biomedical Research Center IACUC committees.

4.2.2 Pair-feeding dam's protocol

A cohort of pregnant BPH/5 female (n=6) mice were restricted to 3g of Purina rodent normal chow per day. Normal chow food intake of ad libitum-fed 6-wk-old nonpregnant C57 and BPH/5 mice was measured concurrently for 14 days. The pair-fed cohort of BPH/5 mice received

food intake matched to C57BL/6 counterparts (~25% less calories than their ad libitum-fed BPH/5 littermates during that time) (114).

4.2.3 Samples collected

Litter size, fetal and postnatal three-week-old body weights, adult body weights, and weights of liver, hearts, inguinal subcutaneous white adipose tissue (WAT), and visceral WAT from the peri-renal depot and the reproductive depot of both AL and PF groups of adult male and female BPH/5 age-matched (8-10 weeks) offspring. Litter size was used as inclusion criteria. Namely, body weights were measured from pups born to BPH/5 with litter sizes greater than or equal to 4 pups as PF BPH/5 dams did not have litters with less than 4 pups. Fetal livers were weighed and expressed as a ratio to body weight to rule out asymmetric growth (208). Offspring were fed an ad libitum normal chow diet until phenotypic analysis at adulthood. Additionally, fresh fecal samples were collected in all offspring groups between 8-12 weeks of age.

4.2.4 PCR of SRY from genomic

Tail snips (1.2cm) were collected post-mortem from BPH/5 (n=26) gestational day 18.5 (e18.5) pups. The tails were cut into smaller pieces and DNA was extracted using Qiagen DNeasy Blood & Tissue Kit according to manufacturer's (Qiagen, Hilden, Germany) protocols. DNA quantity and quality was assessed by spectrophotometry (Thermofisher Scientific, Nanodrop 200, Wilmington, DE, USA). To determine the sex of each pup, a final total reaction of 25 ul PCR was performed using 0.2 ul of each SRY (F-AACAACTGGGCTTTGCACATT, R-GTTTATCAGGTTTCTCTCTAGC) primer, 12.5 ul of Taq, 8.5 ul of RNAse-free H2O, and 2 ul of DNA. The PCR thermal conditions were adapted from a standard PCR genotyping protocol from the Mouse Metabolic Phenotyping Centers Protocols (https://mmpc.org/shared/document.aspx?id=260&doctype=Protocol). The cycle was as follows:

94°C for 5 min, 10 cycles of 94°C for 15s, 65°C for 30s, 72°C for 40s, 30 cycles of 94°C for 15s, 55°C for 30s, 72°C for 40s, 72°C for 15 min, and hold at 15°C. A 2% agarose gel was used to identify SRY positive bands with two male and female adult controls.

4.2.5 Leptin ELISA

Blood was collected via cardiac puncture, allowed to clot at room temperature for 90 min, centrifuged at 3,500 rpm for 20 min, and then stored at -80°C until it was assayed. A commercially available leptin ELISA was performed according to manufacturer's instructions (Cayman Chemicals, Ann Arbor, MI) with serum as previously described (111). The sensitivity of the assay was 50 pg/ml.

4.2.6 Quantitative reverse-transcription PCR of WAT

Total RNA was extracted from female and male visceral WAT and subcutaneous WAT using TRIzol according to manufacturer's (Qiagen, Hilden, Germany) guidelines to examine *TNFa* (F-GAACTGGCAGAAGAGGCACT, R-AGGGTCTGGGCCATAGAACT (111)), *IL-6* (F-TGGCTAAGGACCAAGACCATCCAA, R-AACGCACTAGGTTTGCCGAGTAGA (111)), and *Ptgs-2* (F-ACTGGGCCATGGAGTGGACTTAAA, R-AACTGCAGGTTCTCAGGGATGTGA (113)) expression levels. RNA quality and quantity were assessed by spectrophotometry (NanoDrop). One thousand nanograms was used for reverse transcription using the qScript cDNA kit (Quanta BioSciences, Beverly, MA). Each qPCR was performed in triplicate with an ABI 7500 Fast Thermocycler (Applied Bioscience) using SYBR Green (QuantaBioSciences) using 25 ng cDNA. Data were analyzed using the ΔΔCT method, and results were normalized to 18s gene (107, 113).

4.2.7 Radiotelemetric measurement of blood pressure and heart rate

Pair-fed dams, (n =4) and adult offspring born to pair-fed dams (n = 4) and ad libitum dams (n = 13) underwent carotid implantation of telemetry (Data Sciences International, Saint Paul, MN) according to published methods (99). Briefly, mice were anesthetized with isoflurane through inhalation for placement of a telemeter in the left carotid artery and transmitter body in the subcutaneous space. Mice were given carprofen (5 mg/kg subcutaneous) every 24 hours for two days post-surgery. Mice were allowed to recover for 7 days, followed by 4 days of heart rate (beats per min) and mean arterial pressure (MAP) recording in adult offspring mice and during the length of gestation in BPH/5 females mice as described (99). Heart rate and MAP were interpreted by an observer blinded to the study design.

4.2.8 DNA sequencing

Microbial DNA was extracted from fecal samples using the Qiagen DNeasy PowerSoil extraction kits (Qiagen, USA) according to manufacturer's protocol. The V4 variable region of the 16S rRNA gene was amplified with PCR primers 515/806 in a 30 cycle PCR using the DreamTaq Hot Start PCR Master Mix Kit (Thermoscieniftic, Waltham, MA). PCR was performed in 20 µl vol and included: 2 µl (7.5 µM concn) of forward and reverse primers, 12.5 µl of Hot Start Taq 2X Master Mix (New England BioLabs Inc., Ipswich, MA., USA), 3.5 µl of deionized water, and 2 µl of sample DNA. Thermal cycle conditions were 95˚C for 3 min for initial denaturing step, followed by 30 cycles of 95˚C for 30 s, 50˚C for 1 min, and 72˚C for 1 min. PCR products were checked on a 2% agarose gel for correct product size formation (approx. 350 bp). Michigan State University Genomics Core performed library preparation prior to Illumina MiSeq sequencing following the manufacturer's guidelines. Reagent controls using certified DNA free water were run through

library preparation and PCR and did not generate libraries. For quality control, samples submitted for sequencing included a random blank sample of technical replicates.

4.2.9 Bioinformatics

Initial quality screening, demultiplexing, amplicon sequence variant (ASV) inference, and chimera removal were performed using the DADA2 package. ASVs were classified using Greengenes v13.8 database (209). For contamination of libraries, background sequences were removed using Decontam package. DESeq2 was used to correct for different library read depths and to detect ASVs of differing abundances between treatment groups. Microbial Community analysis (alpha and beta diversity) was performed using vegan R package.

4.2.10 Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 9. Two-way ANOVA with Tukey's post hoc test and/or a student's t-test were used. Normality of residuals from the models were assessed and confirmed via Shapiro-Wilk tests and quantile-quantile (Q-Q) plots. All figures were presented as means± SEM. P values <0.05 were considered significant.

4.3 Results

4.3.1 Sex differences in offspring born to obese hypertensive BPH/5 dams is not apparent until adulthood

To study BPH/5 offspring outcomes in a sex dependent manner mechanistically, sex distribution and morphometrics were characterized. We have previously published that BPH/5 have symmetrical FGR (99, 113, 168), but for the first time we assessed this by sex. The offspring sex ratio (50.7% males and 48.8% females within a litter) observed no significant difference between BPH/5 male and female offspring born to ad libitum dams (Figure 4.1A). BPH/5 e18.5 fetuses were genotyped for expression of the SRY gene, indicative of male sex (210). No body weight differences were observed between BPH/5 males and females at e18.5 (Figure 4.1B) or at

weaning in prepubertal (~3 weeks of age) mice (Figure 4.1C). Thus, lactation had no effect on body weight between male and females, whereas a difference was not observed in adulthood (8 weeks of age), with comparable body weight between the BPH/5 males and females (Table 4.1), which have increased body weight compared to C57 age matched female mice (111).

Microbial community composition of fecal samples was investigated using PERMANOVA with Bray-Curtis dissimilarity of 16S Sequences variants detected (ASVs) relative abundance. Shannon diversity, measuring alpha diversity, showed there was no difference in male and female fecal communities. The community composition of BPH/5 ad libitum male and female offspring was not significantly different using a PERMANOVA to analyze Bray-Curtis dissimilarity and alpha diversity using Shannon diversity matrices (Figure 4.1 D&E). Likewise, no difference was found between sexes in the BPH/5 ad libitum offspring mean arterial pressure (Figure 4.1F).

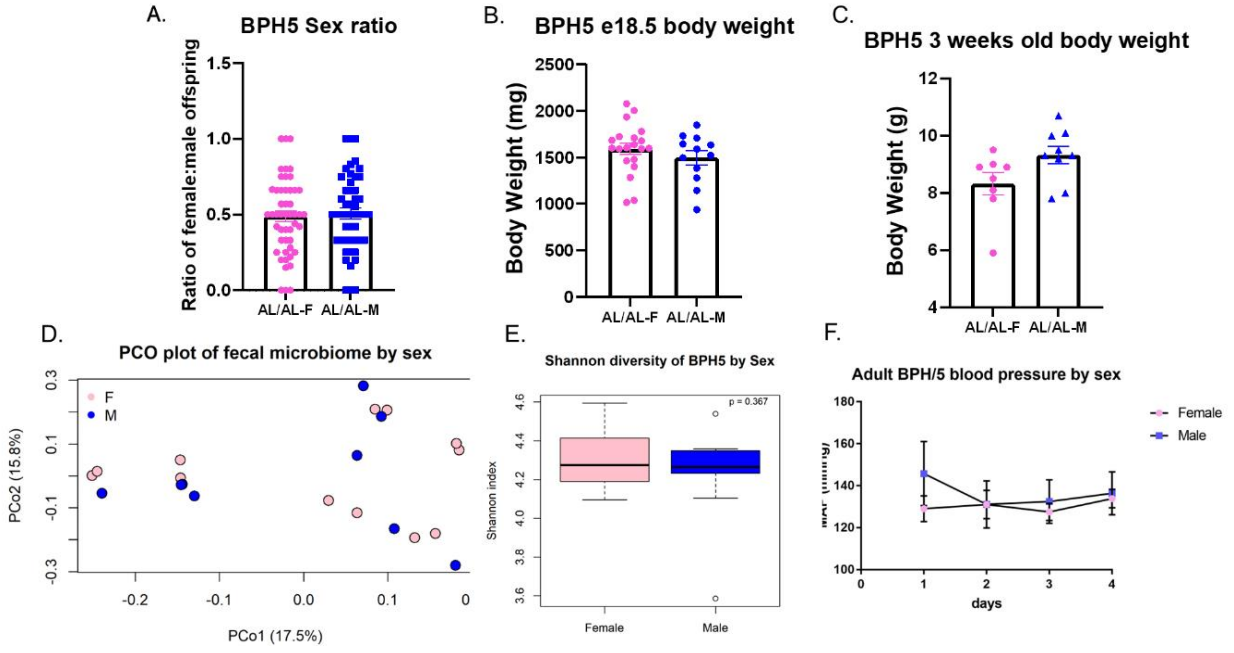


Figure 4.1 No differences were found between sexes in BPH/5 offspring in sex ratio, fetal body weight, body weight after lactational growth, fecal microbiome, or blood pressure demonstrating that BPH/5 is a good model to study offspring born to PE dams in a sex dependent manner A. Sex ratio was measured in embryonic day (e18.5) in BPH/5 AL/AL offspring, no difference was found

(n=49). B. Body weights were measured in e18.5day fetuses using SRY to identify sex, no difference was found (n=12-20). C. Body weights were measured in prepubertal BPH/5 AL/AL offspring similar weight was demonstrated in both males and females (n = 8–32). D. Microbial community composition of fecal samples was investigated using PERMANOVA with Bray-Curtis dissimilarity of 16S Sequences variants detected (ASVs) relative abundance, communities were found to be not significantly different (n=9-12). E. Shannon diversity, measuring alpha diversity, showed there was no difference in male and female fecal communities. (n=9-12). F. Blood pressure was measured using radiotelemetry devices over 4 days. Although BPH/5 AL/AL male and female both had increased mean arterial pressure, it was not significantly difference between sexes. $P < 0.05$.

4.3.2 Improved BPH/5 maternal and fetal pregnancy outcomes with pair-feeding

After introduction of the pair-feeding intervention to BPH/5 dams (PF/AL), e18.5 pregnancy outcomes were assessed and compared to ad libitum fed BPH/5 (AL/AL) (Figure 4.2A). The pair-feeding paradigm was used to match the calorie intake of the obese BPH/5 to the lean C57 control during pregnancy. Previous literature shows that BPH/5 are hyperphagic during early pregnancy, thus the pair-feeding intervention only imposes caloric restriction for this first 9 days of pregnancy (114). Gestational day 9.5 represents similarity to the first trimester in women and during placenta formation (18). We have previously published that BPH/5 have evidence of in utero fetal demise with decreased litter sizes at mid-gestation due to resorptions (18, 99). BPH/5 litter size was significantly higher in BPH/5 PF/AL (5.8±0.97) versus BPH/5 AL/AL (mean=3.2±0.65) (Figure 4.2B). Liver to body weight ratio was increased in offspring born to PF dams on e18.5 (Figure 4.2C). BPH/5 have previously shown an increase in late gestational blood pressure from non-pregnant baseline (18, 99); when pair-feeding was implemented we failed to see a rise from baseline mean arterial pressure at any timepoint during pregnancy (Figure 4.7).

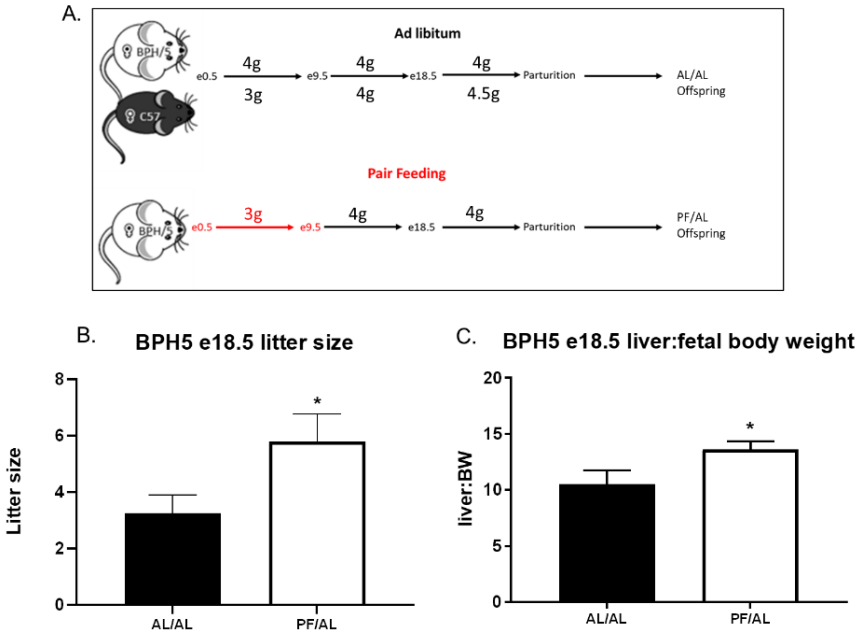


Figure 4.2 Pair feeding BPH/5 mice normalizes litter size and fetus weight by e18.5. A. Depiction of pair-feeding paradigm used in this study. With the BPH/5 pair-fed group matching the food intake of control C57 strain. B. Litter size of was measured at embryonic day e18.5, showing an increase in number of pups born to pair-fed dams. C. fetal symmetrical body weight was measured by using liver weight to body weight ratio at day e18.5 (n=3-9). $p < 0.05$.

4.3.3 Cardiometabolic phenotypic differences by sex found in offspring born to BPH/5 dams after maternal weight loss

When assessing pair fed BPH/5 outcomes by sex (female: PF/AL^{F}; male: PF/AL^{M}), adult body weight in the BPH/5 PF/AL^{F} and PF/AL^{M} demonstrated 15% and 17% reduction compared to BPH/5 AL/AL^{F} and AL/AL^{M}, respectively (Table 4.1). BPH/5 adult ad libitum fed offspring (AL/AL$^{F)}$ have evidence of cardiometabolic disease with increased pro-inflammatory visceral and subcutaneous WAT, hyperleptinemia, and heart enlargement with hypertension versus C57^{F} controls as previously published (111). BPH/5 AL/AL^{M} have increased visceral and subcutaneous WAT mass versus C57 controls males (207). Prevention of maternal obesity in the PF/AL^{F} and PF/AL^{M} offspring reduces WAT mass in the subcutaneous and visceral WAT depots, and the reproductive WAT is decreased only in BPH/5 PF/AL^{F} (Figure 4.3A-C). BPH/5 PF/AL^{F} offspring had reduced serum leptin levels when compared to BPH/5 AL/AL^{F} offspring (Figure 4.3D).

Cardiomegaly also was attenuated in PF/AL^{F} and PF/AL^{M} offspring (Figure 4.3E). BPH/5 AL/AL^{F} offspring were found to have significant left ventricular hypertrophy that was attenuated in PF/AL^{F} (Figure 4.3F).

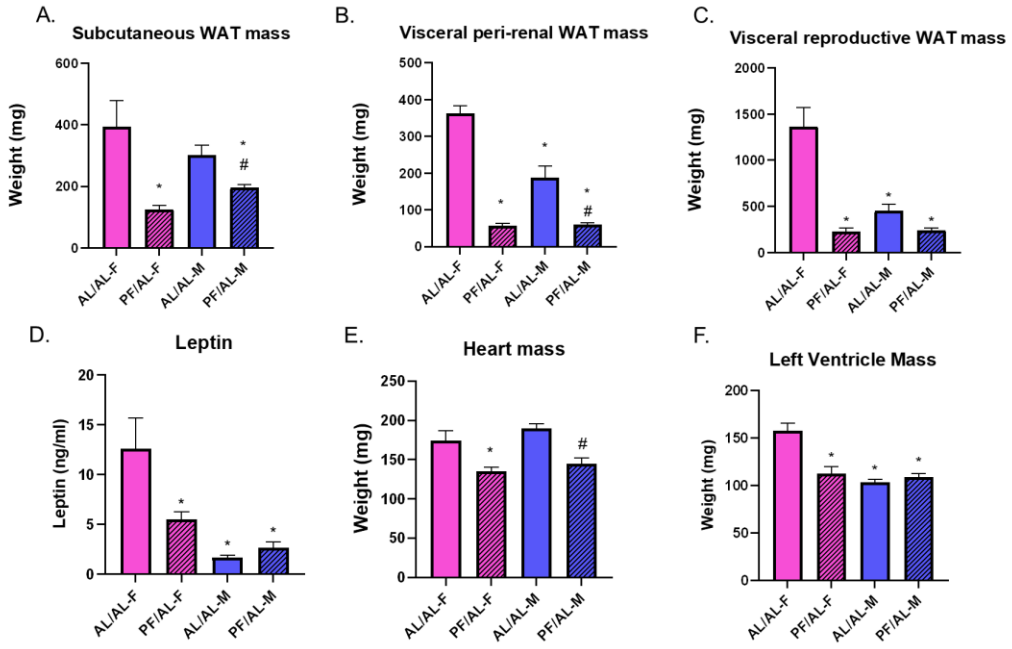


Figure 4.3 Pair-feeding the dams reduces WAT mass and heart mass in both female and male offspring, and leptin and left ventricle mass in females to improve offspring cardiometabolic phenotype. A. BPH/5 offspring born to pair-fed dams have significantly decreased subcutaneous WAT compared to AL/AL offspring. (n = 3–18/group). B. BPH/5 offspring born to pair-fed dams have significantly decreased visceral peri-renal WAT compared to AL/AL offspring, with AL/AL-M significantly less than AL/AL-F. (n = 3–18/group). C. BPH/5 offspring born to pair-fed dams have significantly decreased visceral reproductive WAT compared to AL/AL offspring, with AL/AL-M significantly less than AL/AL-F. (n = 3–18/group). D. Circulating leptin was measured and significantly reduced in PF/AL-F, while it remained low in the BPH/5 male offspring. (n=5-8). E. BPH/5 offspring born to pair-fed dams demonstrate a reduction in heart mass compared to AL/AL offspring of the same sex. F. Left ventricular mass was measured and significantly reduced in PF/AL-F, while it remained low in the BPH/5 male offspring. (n=3-8). ($^{*}p < 0.05$ vs. AL/AL-F, $^{\#}p < 0.05$ vs. AL/AL-M).

As previously shown, BPH/5 AL/AL^{F} offspring have an upregulation in visceral reproductive WAT *IL-6, PTGS-2,* and *TNFa* expression (111). BPH/5 PF/AL^{F} offspring demonstrated decreased *IL-6, PTGS-2,* and *TNFa* expression in visceral reproductive WAT (Figure 4.4A-C). While BPH/5 AL/AL^{M} showed an increase in visceral peri-renal *TNFa* and

subcutaneous WAT *Ptgs-2* and *IL-6* expression (207), no change was found in PF/AL^{M} offspring (data not shown).

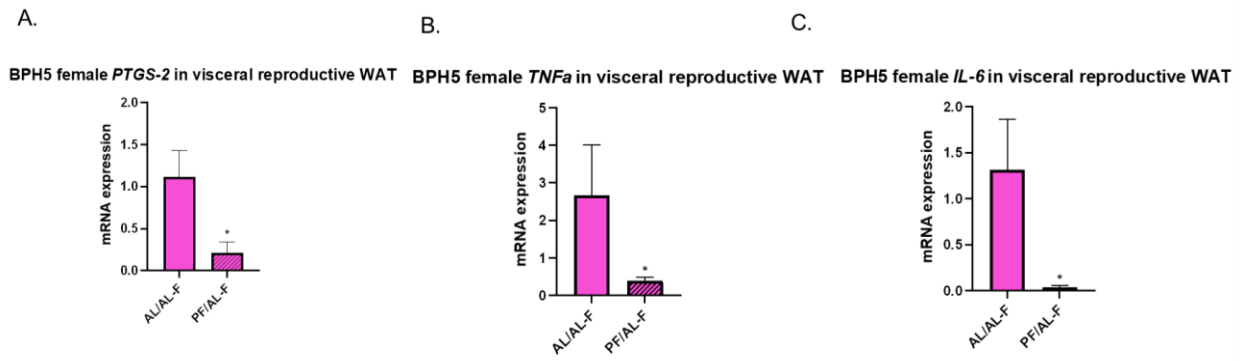


Figure 4.4 Pair-feeding the dams decreases inflammatory cytokine expressing in offspring. BPH/5 AL/AL-F offspring exhibit increased inflammatory mediators within visceral reproductive white adipose tissue (WAT). (A) Using real-time PCR, BPH/5 PF/AL have significantly decreased prostaglandin synthase 2 (*Ptgs-2*) relative mRNA expression compared to AL/AL-F; (B) Tumor necrosis factor *(TNFa)* relative mRNA expression was significantly decreased compared to AL/AL-F; and (C) interleukin-6 (*IL-6*) mRNA expression was significantly decreased compared to AL/AL-F. n=3-8, *p<0.05 when compared to AL/AL-F.

Both BPH/5 AL/AL^{F} and AL/AL^{M} offspring have MAP greater than 100mmHg (Figure 4.1F), which is reduced in the BPH/5 PF/AL^{F} and PF/AL^{M} offspring (Figure 4.5A, $p<0.05$). While blood pressure is decreased, the heart rate was not different (Figure 4.5B, $p>0.05$). Beta diversity analysis of offspring fecal microbial community samples, using a PERMANOVA with Bray-Curtis dissimilarity comparing BPH/5 AL/AL and PF/AL offspring, was significantly different (Figure 4.5C, $p=0.001$). No difference was found between groups using Shannon diversity examining alpha diversity or between the log ratio of Firmicutes to Bacteroidetes (Figure 4.5D-E, $p>0.05$).

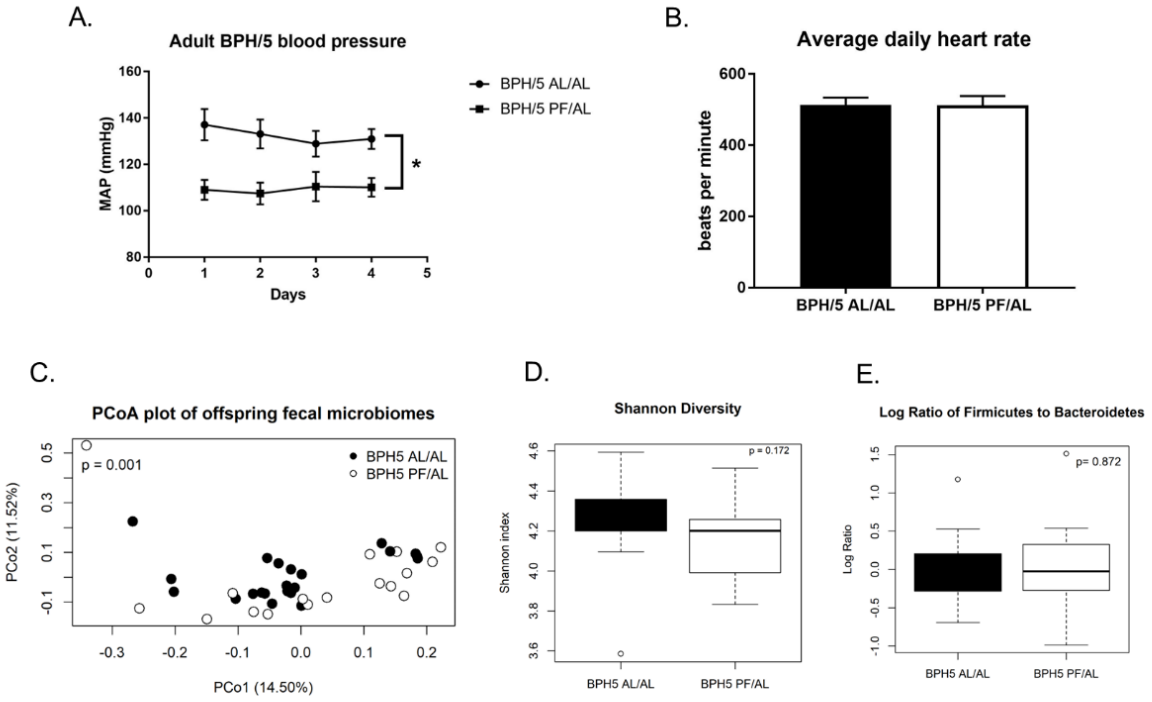


Figure 4.5 Pair-feeding the dams improves cardiac disease in offspring. A. BPH/5 PF/AL offspring demonstrate decreased mean arterial pressure compared to AL/AL offspring as measured over 4 consecutive days. B. Daily average heart rates measured as beats per min were not different. C. BPH/5 PF/AL offspring microbial community composition of fecal samples was investigated using PERMANOVA with Bray-Curtis dissimilarity of 16S Sequences variants detected (ASVs) relative abundance and the communities were found to be significantly different when compared to AL/AL offspring (n=9-12). D. Shannon diversity, measuring alpha diversity, showed there was no difference in PF/AL offspring and AL/AL in fecal samples. E. The log ratio of Firmicutes to Bacteroidetes, a known indictor of obesity, was not significantly different between PF/AL and AL/AL. n=9-12, $p<0.05$.

4.4 Discussion

Sex as a biological variable is an important consideration when testing physiological hypotheses. Herein we describe the sex differences in female and male BPH/5 mice. The BPH/5 mouse has been utilized for over 20 years as a model of superimposed PE; however, offspring differences in a sex dependent manner have not been directly compared. Utilizing spontaneous animal models of cardiometabolic disease allows for exploration of these genetic contributors to

hypertension in a sex-dependent manner. BPH/5 females have pre-existing cardiometabolic risk before pregnancy (hyperphagia, obesity and leptin resistance, and hypoestrogenemia) (111), and their phenotype is unique from BPH/5 males. Offspring of PE-like BPH/5 dams have intrauterine FGR and smaller birthweights when compared to C57 aged-matched counter-parts (99, 113, 168). BPH/5 female ad libitum fed (AL/AL^F) offspring exhibit accelerated catch up growth, i.e. small for gestational age at birth, then overweight by adulthood (111). BPH/5 male ad libitum fed (AL/AL^M) offspring have similar prepubertal and adult body weights when compared to C57 age-matched controls (207).

In this study, we first sought to determine the sex ratios of live BPH/5 offspring. There is an even number of BPH/5 female and male offspring born within a litter with similar bodyweights between both sexes at e18.5 and at weaning (3 weeks of age). Rodents, like most mammals, have a similar number of male to female offspring, although in the diseased state this ratio is altered, which promotes healthier males and helps preserve the species (211, 212). Interestingly in the case of BPH/5, which has been studied extensively as a model of hypertension, the sex ratio at birth is equivalent and thus allows for equal analysis of female and male offspring outcomes amongst littermates. Then, we determined for the first time, using SRY genotyping, that male and female pups at e18.5 are equally impacted by symmetrical FGR in utero, and this is maintained throughout the lactational period. Furthermore, by adulthood, BPH/5 female and male offspring have similar body weights, fecal microbiomes, and mean arterial pressure, which is in the hypertensive range. This direct comparison between BPH/5 female and male littermates confirms that BPH/5 females have evidence of adult onset obesity as previously reported (111), as opposed to males. Birth weight is inversely related to blood pressure in men (213) and women (214). Vos et al. showed that cardiovascular risk is two-fold greater in low birth weight (LBW) men relative to LBW

females in young adulthood (215), indicating a sex difference in susceptibility to cardiovascular risk may originate in fetal life. As with BPH/5 females, adult-onset obesity needs to be considered when predicting cardiometabolic disease risk in humans.

Infants with LBW exhibit accelerated catch up growth and a more central pattern of fat distribution, reduced lean mass, and increased adiposity (166, 167). BPH/5 females demonstrate accelerated catch up growth, while both BPH/5 females and males have increased adiposity (114, 207). Using our published feeding paradigm to promote maternal weight loss in BPH/5 pregnancy (114), we show that pair-feeding BPH/5 dams results in attenuation of LBW and increased litter size. Improved pregnancy outcomes in offspring with pair-feeding are continued into BPH/5 adulthood, PF/AL^{F} and PF/AL^{M} offspring have altered body weight at 8 weeks of age, where they show a significant reduction in body weight compared to the ad libitum offspring. The reduction in body weight may be attributed to significant loss of subcutaneous and visceral WAT in both male and female BPH/5 offspring. Further studies assessing BPH/5 offspring body composition are warranted. The difference in body weight may be attributed to differences in soft tissue density, but further research and advanced serial whole-body imaging is needed to assess lean versus fat mass as BPH/5 age.

Our BPH/5 dam pair-feeding paradigm suggests in utero fetal reprogramming of morphometrics to subsequently improve offspring outcomes and prevent obesity into adulthood. Despite similar gestational and postnatal environments, BPH/5 males are not obese nor hyperphagic and do not exhibit hyperleptinemia as females do (207). However, both BPH/5 females and males have heart enlargement and increased blood pressure (111, 207). Improving the BPH/5 maternal obesogenic environment by pair-feeding the dams, results in reduction of WAT depot mass and heart enlargement in both male and female adult offspring, whilst lowering

circulating leptin and left ventricular hypertrophy in females. Taken together, reversal of these deleterious offspring outcomes may reduce the cardiometabolic disease risk in BPH/5 offspring. Therefore, the developmental, pathological, and transgenerational effects of PE and obesity should be investigated separately by sex.

Obesity is a risk factor of PE possibly due to low-grade systemic inflammation (216). Tumor necrosis factor alpha (*TNFa*) is a proinflammatory factor produced by immune cells in adipose tissue (217, 218). Macrophages in WAT of obese individuals produce higher levels of *TNFa* and interleukin 6 (*IL-6*) than macrophages in lean individuals (217). *IL-6* is also a proinflammatory factor produced by macrophages and adipocytes in adipose tissue (217, 218). It is an inflammatory marker that is known to increase in obese individuals compared to lean subjects (217). It has been estimated that WAT contributes to approximately 30% of circulating *IL-6*, with visceral WAT producing higher levels of *IL-6* compared with subcutaneous WAT (219). Prostaglandin-endoperoxide synthase 2 (*Ptgs-2*) encodes cyclooxygenase-2 (*COX-2*) and is a proinflammatory factor produced by macrophages in adipose tissue. Like *IL-6* and *TNFa*, *Ptgs-2* also is upregulated in WAT of obese subjects (220). In this study, BPH/5 AL/AL^{F} and AL/AL^{M} offspring have comparable subcutaneous WAT mass that is attenuated in both sexes when born to pair-fed dams. BPH/5 AL/AL^{F} offspring have excessive visceral reproductive WAT with elevated levels of *Ptgs-2*, *TNFα,* and *IL-6* that are attenuated when BPH/5 dams are pair-fed during the first half of pregnancy. This suggests that further transcriptomic differences exist between sexes that may contribute to female obesity and early cardiovascular risk not observed in BPH/5 males.

Amarasekara et al. suggested that the maternal gut microbiome may contribute to the development of PE by exaggerating the inflammatory response (8). Intriguingly, BPH/5 adult PF/AL^{F} and PF/AL^{M} offspring had lower mean arterial pressure and altered fecal microbial

communities compared to ad libitum born offspring. The change in the fecal microbiome may be indicative of reprogrammed metabolism in BPH/5 offspring born to pair-fed dams. These together may serve to improve signs of systemic cardiovascular disease, including heart enlargement and hypertension, and possibly even PE in subsequent generations of BPH/5. While the mechanisms are poorly understood, researchers believe the microbiome alters metabolism and modulates the weight gain in both the mother and the offspring potentially due to the in utero environment (57).

Reprogramming BPH/5 male and female offspring through maternal weight loss may be done through several different mechanisms, including altered sex steroid hormone signaling, epi/genetics, and sex-specific modifications in placental nutrient transporters. Male and females sex and age specific differences in sex steroid hormones exist as they influence cardiovascular disease risk (221). For example, postmenopausal women with reduced estrogen and increased androgens are highly susceptible to hypertension (222), whereas a drop in androgens is associated with age-related hypertension in men (223). Elevated androgens may promote oxidative stress and hypertension in both males and females (224). We know BPH/5 adult females have lower circulating 17β estradiol when in proestrus (111), but androgens in both BPH/5 males and females have not been published. Furthermore, little is known regarding the genetics and epigenetics in BPH/5 mice. A recent genome wide study in BPH/5 females (225) revealed several genetic mutations shared between BPH/2 and BPH/5 that could contribute to the hypertensive phenotype, but functional studies are lacking. Finally, epigenetic modifications may exist on the X chromosome that may contribute to sex differences in BPH/5. Female offspring inherit two X chromosomes and inactivation of one is necessary to prevent overdose of X-linked genes. Several cardiometabolic diseases have been associated with X overdose and sex-biased gene expression

(226–228). This may contribute to the BPH/5 offspring differences found in this study and are currently under investigation in the laboratory.

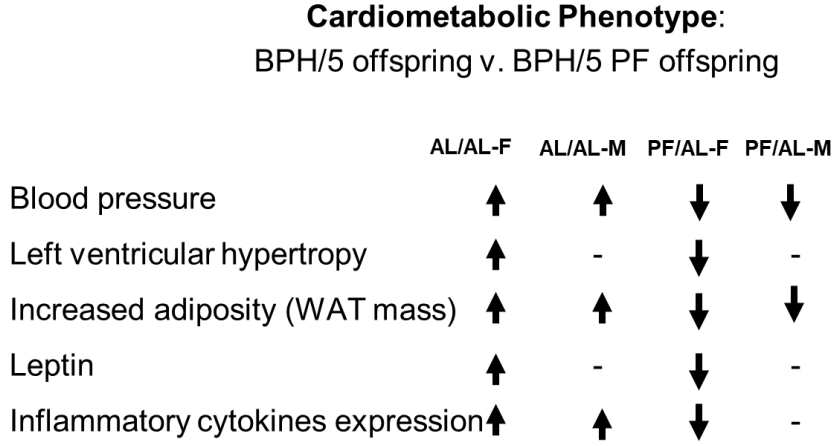


Figure 4.6 Summary of cardiometabolic phenotype comparing AL/AL-F, AL/AL-M, PF/AL-F, and PF/AL-M

In conclusion, the BPH/5 mouse model is an excellent mouse model to study the life cycle and offspring outcomes of PE by sex. BPH/5 male and female offspring both demonstrate cardiometabolic risk, hypertension, and heart enlargement, but have sex dependent differences in features of obesity (Figure 6). Overall, offspring born to pair-fed dams have reduced cardiometabolic phenotype. Future studies are warranted to investigate the influence of the X chromosome on female inheritance and sex steroids hormones as they impact these sex-dependent differences. Obesity coupled with PE contributes to the life cycle of obesity and cardiovascular risk observed in PE pregnancies. The different outcomes in BPH/5 may be due to in utero programming in an adverse environment. Additionally, further exploration on how the maternal gut microbiome effects fetal programming by fecal transfaunation from healthy lean control and embryo transfer into a healthy lean dam and vice versa.

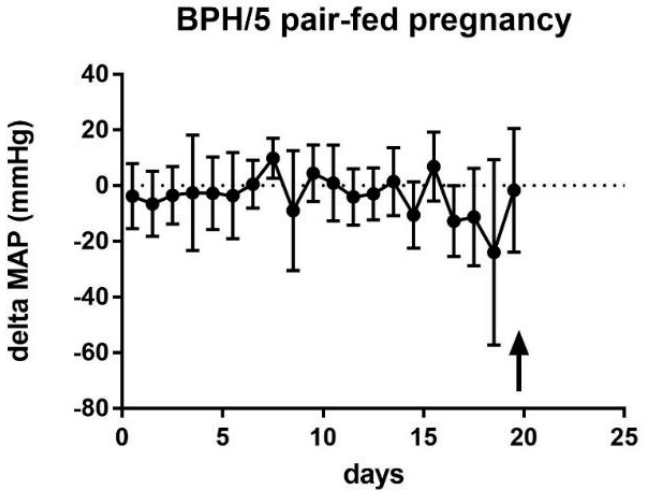


Figure 4.7 Change in BPH/5 pair-fed dams mean arterial pressure. The mean arterial pressure was measure throughout pregnancy in the pair-fed dams demonstrating no significant increase throughout the time of pregnancy. (n=4), $p<0.05$

TABLE 4.1

	AL/AL^F	AL/AL^M	PF/AL^F	PF/AL^M
Three-week-old Body Weight (g)	8.32±0.39	9.32±0.31	9.65±0.75	9.9±0.62
Adult Body Weight (g)	24.16 ± 0.750	26.31±0.662	20.59±0.519*	21.73±0.377 #
Visceral peri-renal WAT (mg)	363.7±19.41	187.4±32.91*	56.42±7.30 *	60.44 ±4.80*#
Subcutaneous WAT (mg)	393.0±85.12	301.7±31.66	125.5±13.12 *	196.1±10.03*#
Visceral reproductive WAT (mg)	1366±206.5	452.9±70.96*	229.7±39.29*	243.9±19.57*#
Heart Mass (mg)	166.8±12.77	190.2±5.25	135.1±5.09*	145.1±6.95#
Leptin (ng/ml)	12.6±3.10	1.693±0.18*	5.51±0.75*	3.628±0.60*
	*$p<0.05$ different from AL/AL^F			
	#$p<0.05$ different from AL/AL^M			

CHAPTER 5. COMPARISON OF THE METAGENETIC ANALYSIS OF THE MICROBIOME IN PREGNANT HORSES

5.1 Introduction

The placenta, a once thought sterile organ, has many functions to maintain a healthy pregnancy. Proper growth and function of the placenta are essential for development of the fetus (229). The functions of the placenta are to ensure the exchange of nutrients and waste products between the maternal and fetal circulatory systems (230, 231). The discovery was using next generation metagenomic sequencing techniques. The microbiome is referred to the combined genetic material of the microorganisms in a specific environment or body site, while metagenetics is the study of genes expressed by microorganisms recovered from a specific site. The host and microbiome relationship are considered mutualistic symbiosis (232). Studies have shown that the human body provides the sustenance for the microbes and in return the microbes execute essential functions for the host (145, 233). When the composition of the microbial environment is perturbed, disease can occur (129, 234, 235). This is referred to as microbial dysbiosis and can be defined as a reduction in microbial diversity and a combination of the loss of beneficial bacteria (236). Recently, a controversial theory of the placenta microbiome in humans has been reported (40, 41).

The equine placenta is made up of two distinct membranes, the amnion, the opaque membrane that immediately surrounds the foal and the chorioallantois that joins to the uterus (237). The equine placenta is classified as diffuse. It involves the entire surface of the chorioallantois except for a small area adjacent to the cervix called the "cervical star," where attachment cannot occur (238). The placenta also is involved in immune tolerance of the semi-allogeneic fetus that is composed of maternal and paternal antigens (239). The placental of the horse has not been well described. Rationale to investigate the equine placental microbiome comes from non-pregnant reproductive tract studies described in the horse (240–242). These may influence that of the early

equine placenta. A distinct uterine microbiome has been reported between diestrual mares (Day 7 after ovulation) carrying an embryo compared to those that are open using metagenetics (240). Proteobacteria and Bacteroidetes were associated with culture positive samples at ovulation. Sphingobium (Proteobacteria) and Sphingobacteriales (Bacteroidetes) are associated with mares carrying embryos at Day 7 post ovulation, and Rhodocyclaceae and Enterobacteriaceae (Proteobacteria) are associated with mares not carrying embryos (240). Also, amniotic fluid taken at delivery from healthy equine pregnancies has yielded bacterial growth (243). Additionally, foal meconium on postnatal day 1 is dominated by the Firmicutes phyla consisting primarily of the genera *Enterococcus, Bacillus* and *Lactococcus* (244, 245).

Placentitis is a disease that can result from an uterine/placental microbial dysbiosis. It is the leading cause of infectious abortion in the horse and contributes to roughly 19% of all abortions in the United States (246). It continues to cause episodic abortions in addition to weak and/or growth restricted offspring (247). It has previously been shown that the equine placenta harbors a unique microbiome as collected at term delivery from healthy mares (248). This study aims to identify the core microbial communities in different body sites of the pregnant mare in early gestation to describe a core microbiome that may be perturbed in pathologic pregnancies such as in placentitis. We hypothesize that the equine placenta harbors a distinct resident microbiome in early pregnancy when characterized by metagenetics and that there will be a disparity in bacterial communities from the oral, vaginal, and fecal microbiome. Furthermore, we hypothesize metagenetics will reveal distinct communities and indicator taxa that characterize the healthy equine placenta.

5.2 Methods

5.2.1 Animal experiments

Five pregnant pony mares (age 4-8 years old) were used between 96-120 days of gestation upon necropsy. The pony mares underwent a health evaluation and were monitored by ultrasound by a board-certified theriogenologist throughout pregnancy. All pony mares were co-housed and naturally serviced by the same pony stallion. After euthanasia, swabs were sterilely collected from oral cavity, vagina, anus, and the allantoic portion of the allantochorion. The allantoic swab will be referred to as placental in the following sections. The uterus was removed in situ to aid in maintenance of sterility of the samples. Samples were placed in a sterile tube and stored at –80°C until further analysis. The blank samples were swabs taken from the environment by briefly exposing them to the environment in which the samples were collected. The blank swabs were immediately frozen and processed the same way as the body site samples.

5.2.2 DNA sequencing

Microbial DNA was extracted from different body site samples using the Qiagen DNeasy PowerSoil extraction kits (Qiagen, USA) according to manufacturer's protocol. The V4 variable region of the 16S rRNA gene was amplified with PCR primers 515f/806r (115) in a 30 cycle PCR using the DreamTaq Hot Start PCR Master Mix Kit (Thermoscieniftic, Waltham, MA). PCR was performed in 20 µl volume and included: 2 µl (7.5 µM concentration) of forward and reverse primers, 12.5 µl of Hot Start Taq 2X Master Mix (New England BioLabs Inc., Ipswich, MA, USA), 3.5 µl of deionized water, and 2 µl of sample DNA. Thermal cycle conditions were 95˚C for 3 min for initial denaturing step, followed by 30 cycles of 95˚C for 30 s, 50˚C for 1 min, and 72˚C for 1 min. PCR products were checked on a 2% agarose gel for correct product size formation (approx. 350 bp). Michigan State University Genomics Core performed library preparation prior to Illumina

MiSeq sequencing following the manufacturer's guidelines (115). Reagent controls using certified DNA free water were run through library preparation and PCR and did not generate libraries. For quality control, samples submitted for sequencing included a random blank sample of technical replicates.

5.2.3 Bioinformatics and statistics

Initial quality screening, demultiplexing, amplicon sequence variant (ASV) inference and chimera removal were performed using the DADA2 package (116). The decontam package was used to discern between the true bacterial sequences and potential contaminant DNA (249). Given the nature of this study with low microbial biomass, characterization requires contaminant removal to ensure that DNA from biological samples can be effectively distinguished from environmental blank contaminates and exogenous DNA. ASVs were classified using the Silva Release 132 16S rRNA database(22, 117). Microbial Community analysis (Alpha and Beta Diversity) was performed using the vegan R package (118). Permutational multivariate analysis of variance (PERMANOVA) (119) was performed using vegan package Adonis function. To determine differentially abundant ASVs, the ASV table was first trimmed to only include ASVs with a median abundance greater than two across all samples. We applied a probabilistic framework, Source Tracker (250), to assess whether the microbial communities from the placenta appear to source from other body site microbial community compositions. All statistical analyses were performed with JMP Pro 16.2.0 (SAS Institute Inc., Cary, NC). Graphs were generated using Prism 9 for Windows, Version 9.5.0 (GraphPad software, LLC, San Diego, CA). Relative abundance and alpha diversity were analyzed via mixed ANOVA and post-hoc Tukey tests with site as the fixed effect and each horse as the random effect. Logarithmic transformation was performed for data that did not meet the normality criteria. Normality of residuals from the models were accessed and

confirmed via Shapiro-Wilk tests and quantile-quantile (Q-Q) plots. Data are presented as mean ± SD. Significance was set at $p < 0.05$. All raw sequence reads and corresponding metadata can be found on the SRA website accession number SUB12947298.

5.3 Results

Of the 20 samples processed and sequenced, some samples demonstrated low sequence reads, but the overall average was 220,030 reads after quality filtering. After the use of the decontam package in R, only 47 contaminated reads were found using the frequency method, while no contaminated reads were found using the prevalence method. When assessing the microbial communities using alpha diversity, Shannon diversity matrix was significant, with the body sites being a compounding variable ($p=0.0008$); this meaning there was a difference in richness and evenness in the different microbial communities by site. When using Tukey's multiple comparisons, it was found that the placenta diversity index (2.91 ± 0.89) was not significantly different with the oral cavity (2.79 ± 0.95) when assessing alpha diversity alone ($p=0.8297$). Feces was found to be the most diverse (5.45 ± 0.40), followed by vagina (4.25 ± 0.84), while oral and placental were the least (oral-feces $p=0.0051$, placenta-feces $p=0.0206$. (Figure 5.1)

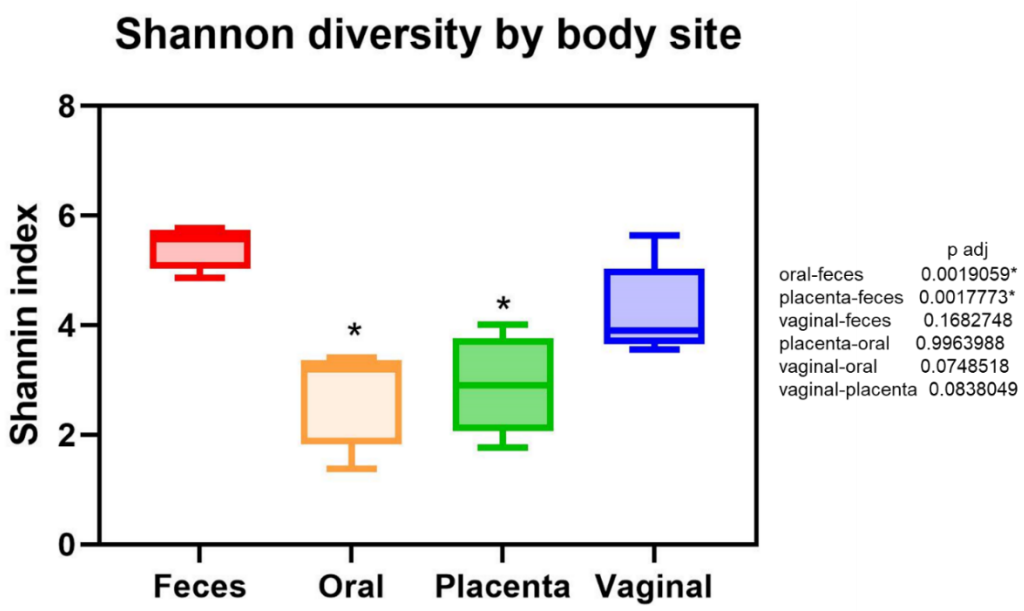
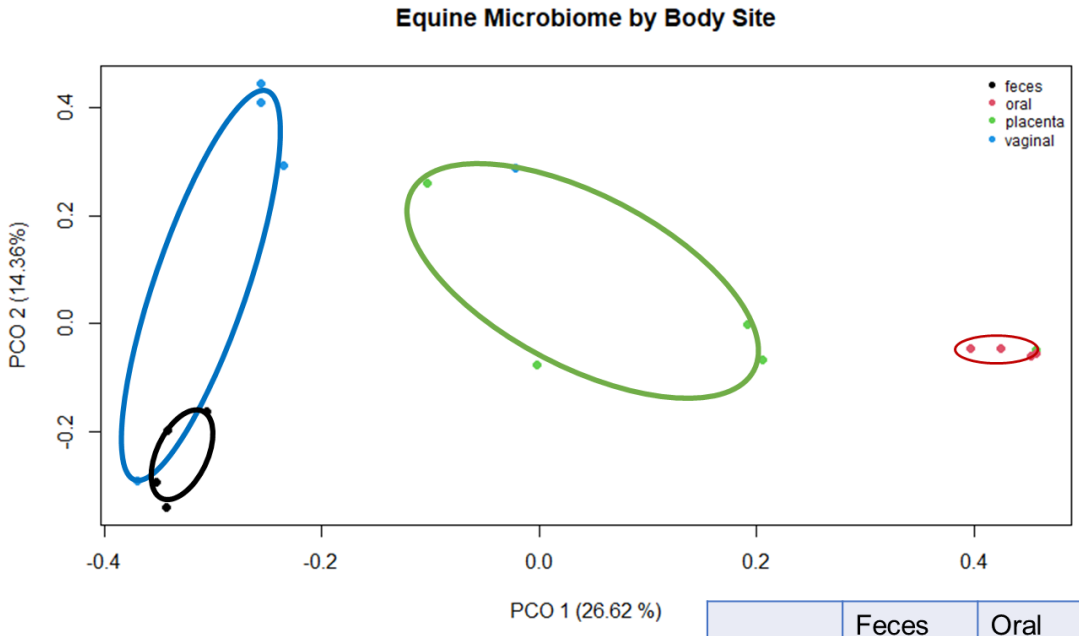


Figure 5.1 Alpha diversity measuring Shannon diversity was significant with the different body sites being a compounding variable (p=0.0008). * Significantly different from feces.

Microbial community composition of the pregnant ponies by body site was significantly different when assessing beta diversity (p=0.001, PERMANOVA with Bray-Curtis dissimilarity of 16S Amplicon Sequence Variant's relative abundance). The placenta was significantly different from feces (p=0.027), oral cavity (p=0.046), and the vagina (p=0.038) using Bray-Curtis dissimilarity (Figure 5.2).



	Feces	Oral	Placenta
Oral	0.039*	-	-
Placenta	0.027*	0.046*	-
Vaginal	0.054	0.027*	0.038*

Figure 5.2 Beta-diversity of equine microbiome by body site using PERMANOVA with Bray-Curtis dissimilarity of 16S Amplicon Sequence Variant's relative abundance. p=0.001.

When evaluating the different body sites at the phyla level, all were dominated by Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria (Figure 5.3). When analyzing at the genera level, the placenta was dominated by *Gemella*, *Rikenellaceae_RC9*, *Porphyromonas*, and *Streptococcus.* Similarly, the oral cavity was dominated by *Gemella*, *Porphyromonas*, *Streptococcus*, and *Alysiella*. The vagina consisted of *Rikenellceace_RC9*, *Porphyromonas*, *Campylobacter*, and *Streptococcus.* Finally, the fecal samples included *Rikenellaceae_RC9*, *Erysipelotrichaceae_UCG-004*, *Treponema_2*, and *Mycoplasma* (Figure 4). The individual horse variation was not different, but *Gemella* ($p < 0.0001$), *Campylobacteria* (p=0.0003), *Rikenellaceae* (p=0.0022), and *Erysipelotrichaceae_UCG_004* (p=0.0103) were different by body site (Figure

5.5), while *Porphyromoas*, *Streptococcus*, *Alysiella*, *Fusobacterium*, and *Bacteroides* were not ($p > 0.05$).

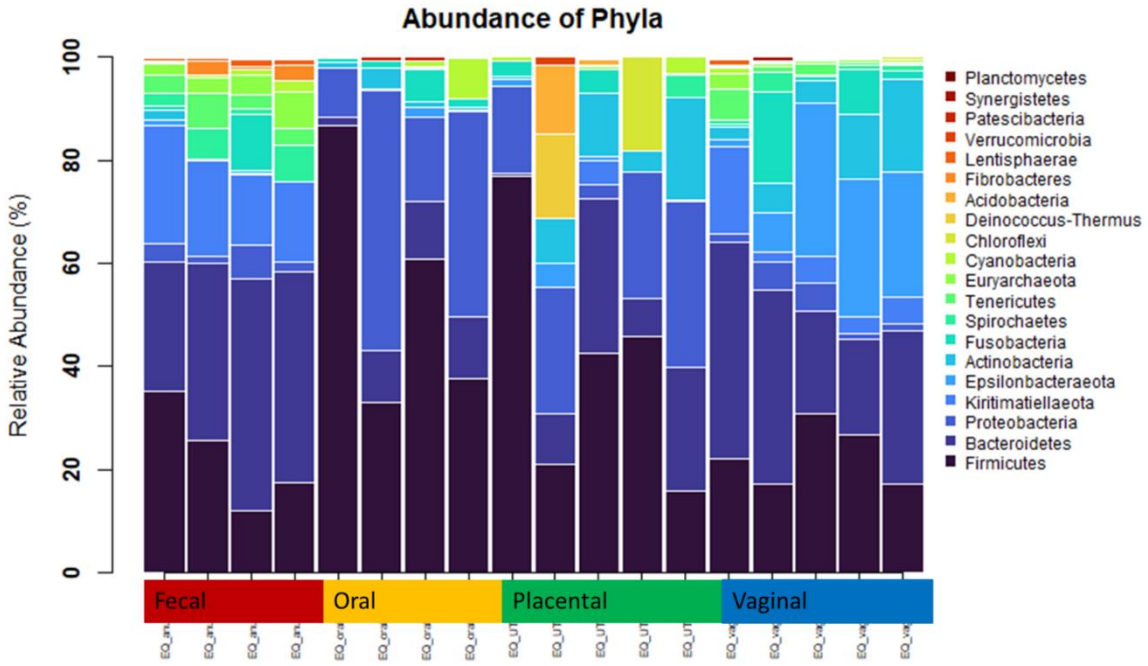


Figure 5.3 Relative abundance at the phyla level of equine microbiome at different body sites.

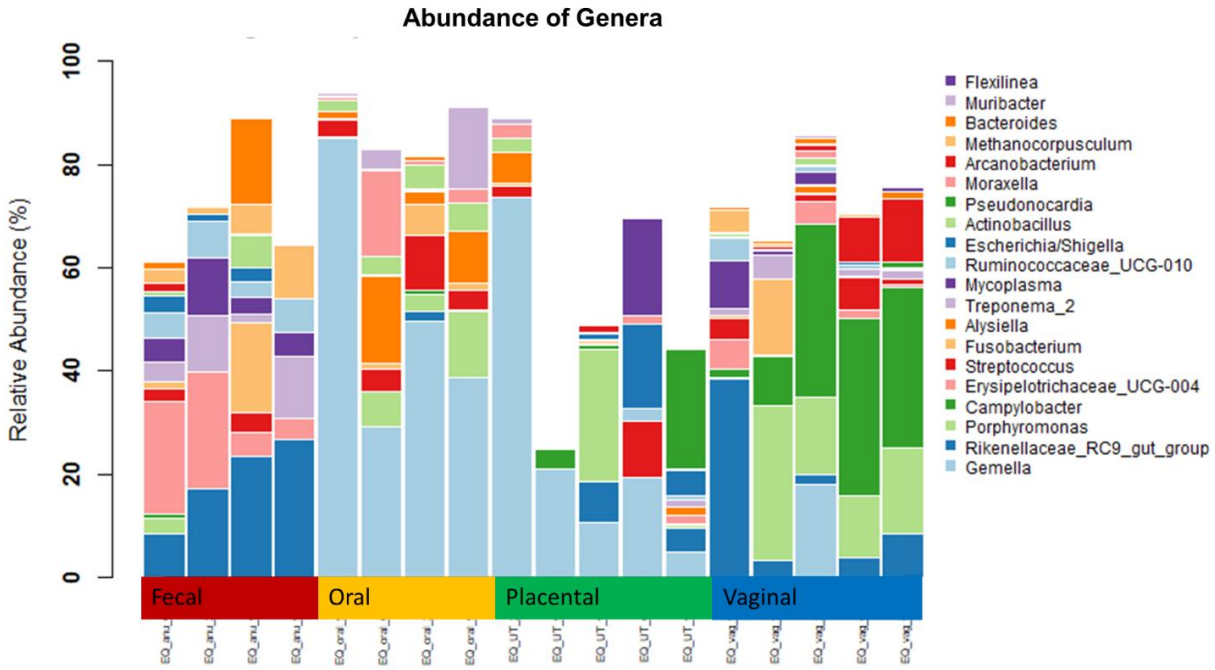


Figure 5.4 Relative abundance at the genus level of equine microbiome at different body sites.

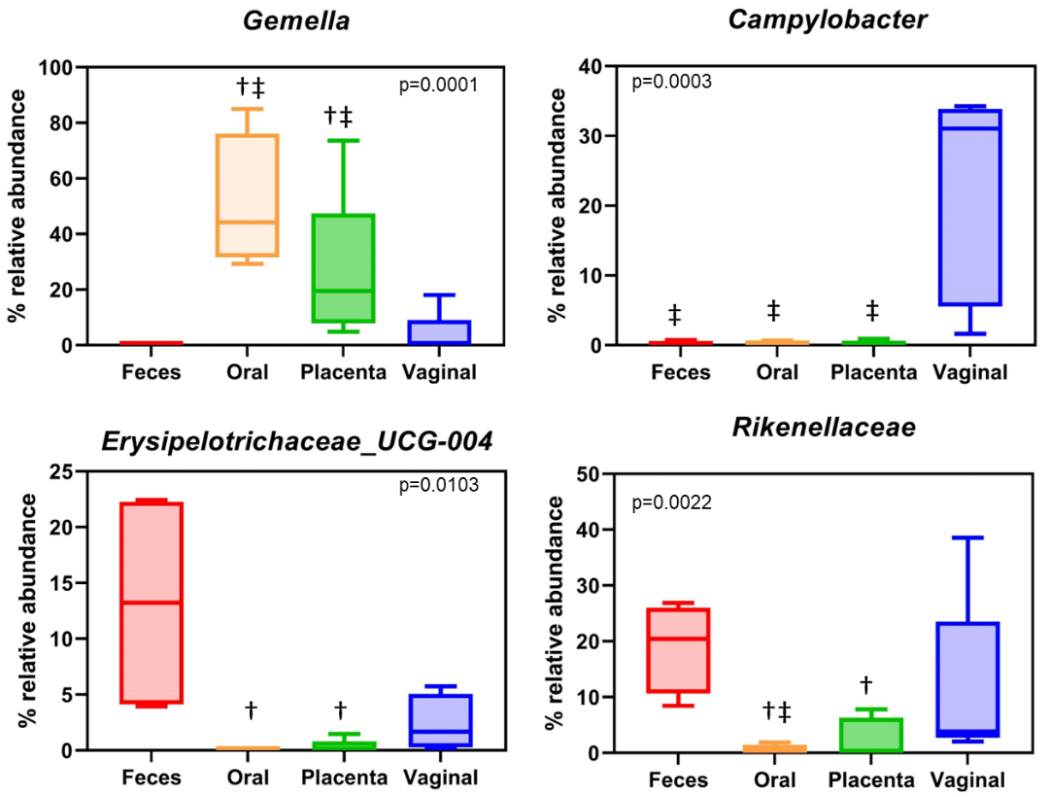
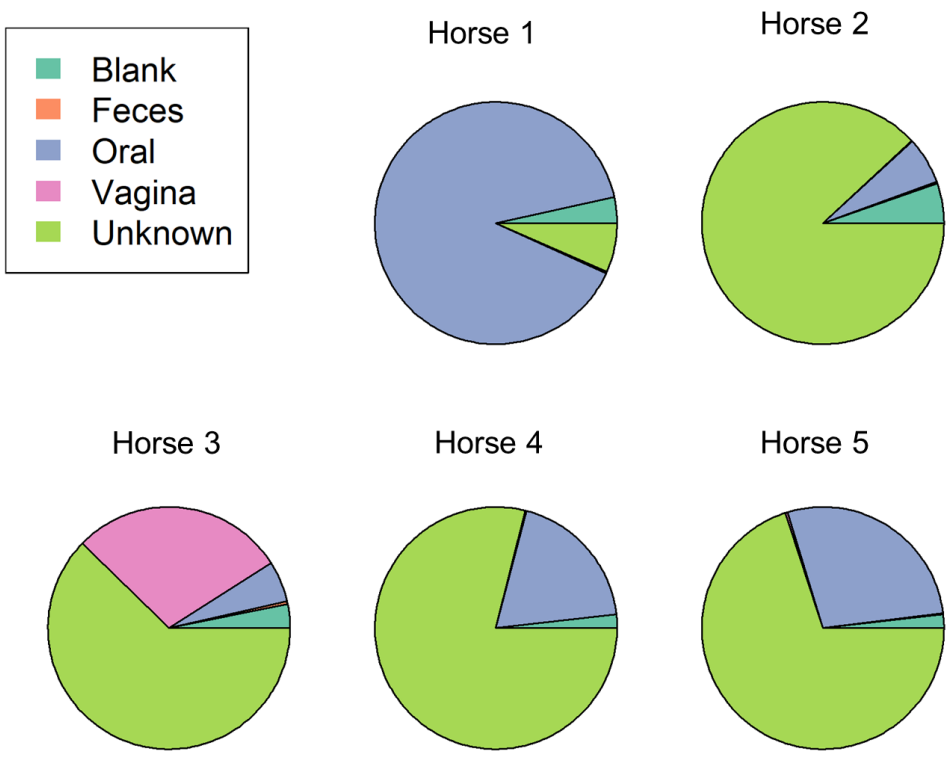


Figure 5.5 Top genera that are different by body site. A). Relative abundance differences by body site of Gemella. B). Relative abundance differences by body site of Campylobacter. C). Relative abundance differences by body site of Erysipelotrichaceae_UCG. D). Relative abundance differences by body site of Rikenellaceae. Using Tukey's multiple comparison * denote significantly different from the oral cavity, † denote significantly different from the feces, ‡ denote significantly different from the vagina.

Sourcetracker package in R was used to identify the potential source of the microbes found in the placental samples. This did vary by individual horse. Horse 1 placental samples were majority sourced from the oral cavity. Horse 2, 3, 4, and 5 were for the most part sourced from unknown bacterial origin, followed by the oral cavity for horse 2, 4, and 5. Conversely, horse 3 secondary source was found to be from the vagina (Figure 5.6).



Proportions	Blank	Feces	Oral	Vagina	Unknown
Horse 1	0.0342	0	0.8977	0.0022	0.0659
Horse 2	0.0535	0.0018	0.0628	0.0012	0.8807
Horse 3	0.0318	0.0041	0.0547	0.2861	0.6233
Horse 4	0.0182	0.0001	0.1913	0.0011	0.7893
Horse 5	0.018087	0.001739	0.277044	0.003304	0.6998261

Figure 5.6 Pie chart of the estimated placental samples bacterial sources identified by sourcetracker package in R.

5.4 Discussion

The overarching goal of this study was to characterize and describe the maternal microbiome of the pregnant horse by analyzing the feces, placenta, oral, and vaginal cavities and to determine their differences. We hypothesize that the equine placenta harbors a distinct resident microbiome in early pregnancy when characterized by metagenetics and that there will be a disparity in bacterial communities from the oral, vaginal, and fecal microbiome. Furthermore, we hypothesize metagenetics will reveal distinct communities and indicator taxa that characterize healthy placenta and extra-placental body sites in the mare. Main findings revealed that the

placenta was dominated by *Gemella, Rikenellaceae_RC9, Porphyromonas,* and *Streptococcus.* Differing, feces had the most diversity, while the oral and placenta similarly had the least. All body sites grouped differently when analyzed with beta diversity using Bray-Curtis dissimilarity. At the genus level some similarities were shown between body sites, even though the placenta did harbor its own unique microbiome.

In a previous report of the equine placenta during late gestation, uterine samples were similarly dominated by Firmicutes, Proteobacteria, Bacteroidetes, and Actinobacteria (248). In humans, the taxonomic profile of the placental microbiome was discovered to be most similar to the oral microbiome followed closely by the vaginal microbiome and further from the fecal microbiome (20). This finding was found to be consistent with our study with pregnant mares earlier in gestation. *Gemella* was found to be increased in the oral cavity, and some were found in the placenta, while not in the feces and vagina, highlighting a potential similarity between these two body sites. *Gemella* has been previously found in the healthy equine mouth (251). *Rikenellaceae* was found in all body sites with the highest abundance within the feces, which is consistent with other equine microbiome studies (252, 253). *Campylobacter* was found in high levels within the vagina but not in the other body sites. *Campylobacter* has been previously found also in the healthy equine vaginal microbiome (241, 244). *Erysipelotrichaceae_UCG* was found in the feces and in small amounts in the placenta and vaginal samples. *Erysipelotrichaceae_UCG* has been previously isolated in bovine and zebra intestinal tracts (254–256). An issue in the analysis of microbiome communities is they are typically comprised of several source "environments," including different contaminants as well as other microbial communities with which they interact. The estimated placental samples bacterial sources identified by sourcetracker package in R, found the oral cavity to be the major source contributor in horse 1 and secondarily

in horse 2, 4, and 5 (Figure 5.6). This is similar to a human study, which characterized the placenta being most closely related to the oral cavity (20). The estimated primary source from horse 2, 3, 4, and 5 were of unknown bacterial origin (Figure 5.6) meaning the potential of contamination in these samples is low, and there is still research needed to further our knowledge of how these bacterial communities interact with each other.

Diagnosing placental infection during equine pregnancy is extremely difficult since outward clinical signs are often not present. There are four types of equine placentitis: ascending, focal mucoid (nocardioform), diffuse (hematogenous), and multifocal (257). Ascending placentitis is the most common and is caused by bacteria ascending from the lower reproductive tract, vaginal and fecal microbe contamination (257). Bacteria rapidly colonize the chorioallantois, which promotes inflammation at the maternal-fetal interface. This, if left untreated, compromises oxygen and nutrient delivery to the developing foal. This cascade of events promotes abortion, premature delivery of a stillborn or weak foal. These severe consequences of placentitis make a rapid diagnosis essential. A quick and accurate screening method that is sensitive and specific is needed. Another potential issue is hematogenous bacterial translocation through the blood from extra-placental sites such as the oral cavity. Furthermore, focal bacterial placentitis cases, such as Nocardioform, do not follow the same pathogenesis of ascending placentitis cases, and their etiology is largely unknown. Understanding the relationship between the equine placenta and resident bacterial populations during healthy and diseased pregnancies could provide the opportunity to use extra-placental sources (oral, fecal and/or vaginal) as biomarkers for predicting placentitis and associated adverse outcomes. In nocardioform placentitis, the top bacteria found are *Amycolatopsis, Crossiella,* and *Streptomyces,* which are all members of the phyla Actinobacteria (257). In this study, *Streptomyces* spp was isolated in one placental and one vaginal

sample (Figure 5.7). *Pseudonocardia* spp, a close relative to *Amycolatopsis* and *Crossiella*, was isolated in two placental samples (Figure 5.7). The Pseudonocardiaceae family has been isolated in reproductive tracts of humans and cows (258, 259). This cohort of mare represents a healthy group, but developing a rapid screening method by using biomarkers may aid in diagnosing pregnant mares that will develop nocardioform placentitis earlier in gestation. Further investigations are required to evaluate clinical cases of equine placentitis, as well as the mare's uterine microbiome before pregnancy. Identification of bacterial targets in extra-placental body sites as causative in adverse pregnancy outcomes would revolutionize the way we manage pregnancy in the mare. A study by Heil et al. found that endometrial samples taken by three different sampling strategies (swab, low volume lavage, and biopsy) all yielded similar results (260). These results show the potential for using swabs from uterine and extra-placental site may provide early intervention and therapeutic properties.

There have been recent studies that challenge the placental microbiome dogma, specifically in humans. Using both PCR and Illumina sequencing, Lauder et al. matched a set of contamination controls to compare the healthy placental samples and found that the placental samples contained low and indistinguishable 16S rRNA copy numbers when compared to extraction blanks. Also, using PERMANOVA of Bray-Curtis and UniFrac distances no community separation was found (40). Perez-Munoz et al. stated that their data does not support the existence of the microbiomes within the healthy fetoplacental environment. They further indicated that the current methodology (Next Generation Sequencing) is faulty, the low biomass creates a sample too small to accurately detect bacterial DNA, potential for contamination is high, and is noted in numerous studies (261). A follow up study completed by Leiby et al. examining placentas from spontaneous preterm births found no distinction between background negative control and placenta samples (41). A possible

explanation from the previous authors is that the placenta is sterile until the rupture of the membranes during delivery. The microbiome found within Cesarean sections is thought to be due to contamination, commonly from the DNA extraction kit (40). There is a need to further examine both sides to assess the accuracy of the core microbiome of the placenta. Since the mares in this study were humanely euthanized, there was no contamination from rupture of membranes or birth. The uterus was aseptically removed in situ from the mare and opened. The swabs were sterilely collected and immediately frozen. Strict sterility and stringent bioinformatic filtering of the sequences was performed to acquire as little contamination as possible.

In conclusion, metagenetics did reveal distinct community differences in the oral, fecal, vaginal, and placenta cavities of the horse. We believe there is rationale to challenge the dogma of the placenta being sterile in horses. The equine placenta did show similarities in its microbial communities to the oral cavity when assessing alpha diversity. Further research needs to be completed to investigate how bacteria is translocated to the placenta from these other body sites and how it contributes to the development of placentitis. Continuation of this study would be to develop a screening method using next generation sequencing to rapidly identify microbial community dysbiosis to monitor mares that could potentially develop placentitis.

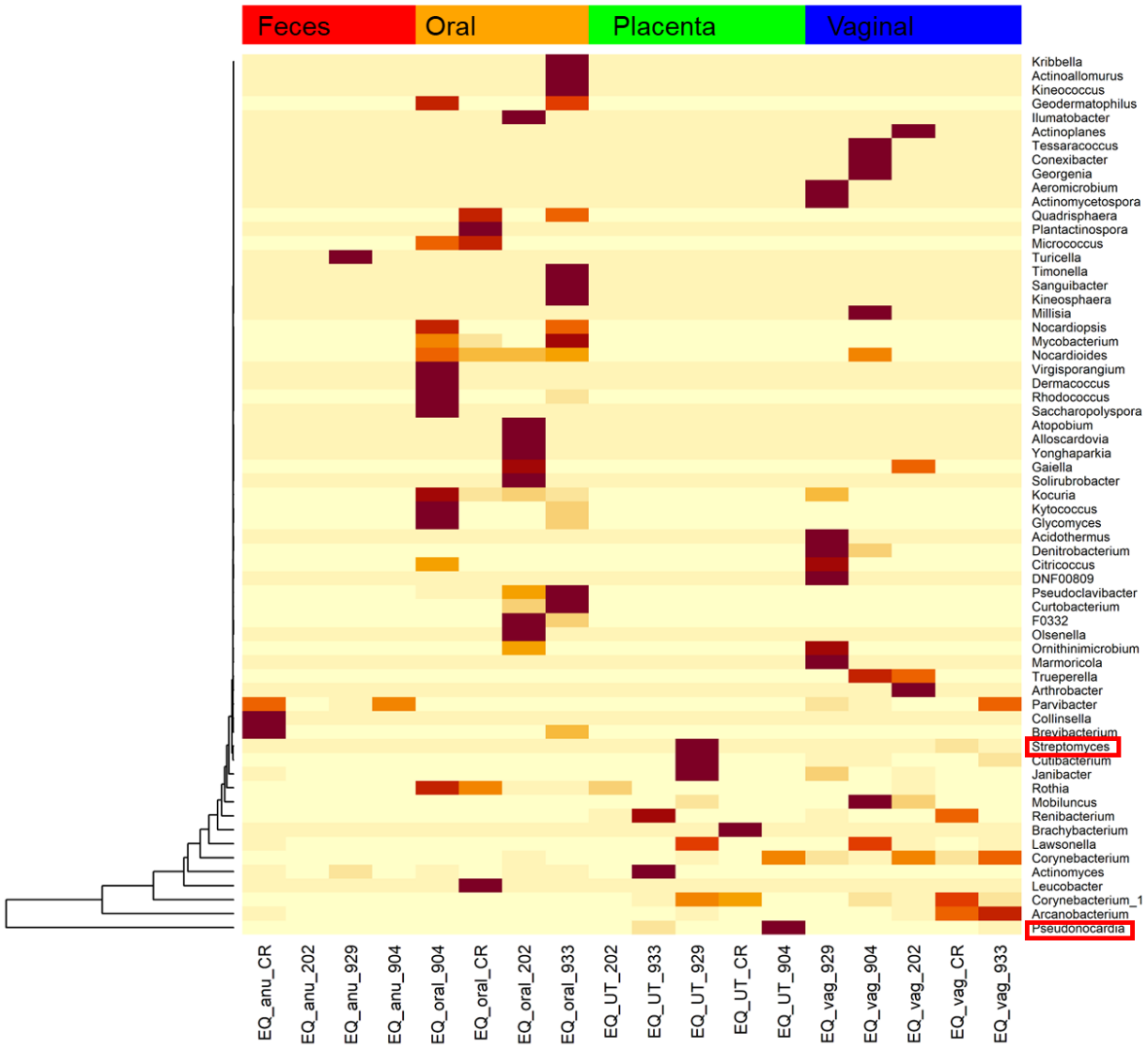


Figure 5.7 Heatmap of Actinobacteria phyla by body site. The red rectangles recognize the genera that are related to know nocardioform pathogens in the horse.

CHAPTER 6. SUMMARY AND CONCLUSIONS

In conclusion, this research investigated the effects of the maternal microbiome on pregnancy outcomes and offspring health. The overall conclusions are as followed 1). The BPH/5 mouse model demonstrated microbial gut dysbiosis prior to pregnancy, and their gut microbiome did not adapt with the onset of pregnancy. 2). BPH/5 male offspring exhibited a specific cardiometabolic phenotype different from female offspring. 3). Prevention of maternal obesity attenuated the cardiometabolic phenotype of offspring in a sex dependent manner. 4). Metagenetics revealed distinct microbial community differences in the oral, fecal, vaginal, and placenta cavities of the horse.

Overall objective of the initial chapter was to characterize the BPH/5 gut microbiome and describe the microbial changes that occur with the onset of pregnancy to determine if the BPH/5 mouse model develops a gut dysbiosis. BPH/5 mouse model demonstrated a gut dysbiosis with differences in diversity, Firmicutes to Bacteroidetes ratio, and microbial community composition compared to the lean C57 model. The female BPH/5 mouse exhibited gut dysbiosis prior to pregnancy and microbial communities did change with the onset of pregnancy. These features did not mimic normal physiologic changes to adapt with pregnancy. The BPH/5 mouse had a perturbed uptake of SCFAs, due to the difference in serum but not within the feces. BPH/5 had significantly less GPR41 mRNA found in the colon compared to normotensive controls, potentially contributing to differences in SCFA uptake. BPH/5 mice also had an increase in the pro-inflammatory cytokine IL-15 found in the colon. Historically, the BPH/5 model has been shown to have increased IL-15 in the implantation site. These findings further demonstrate that the BPH/5 model is gestating fetuses in an inflammatory environment. In summary, the BPH/5 female mouse demonstrated a gut microbial dysbiosis by increased alpha diversity, altered beta diversity, and altered Firmicutes

to Bacteroidetes ratio compared to normotensive pregnant mice. This maternal microbiome dysbiosis may contribute to increased inflammatory responses and exacerbate the development of PE, which contributes to poor offspring outcomes.

A fetus gestating in a perturbed uterine environment can cause disease later in life. BPH/5 offspring demonstrated a cardiometabolic phenotype with markers such as central obesity and cardiovascular disease in the females. The males exhibited a unique phenotype showing increased visceral and subcutaneous adiposity and cardiovascular disease: hypertension, and cardiomegaly. In summary, BPH/5 males contrast the females, given that female offspring demonstrated the obese metabolic phenotype in adulthood, while the males demonstrated cardiovascular disease without obesity or hyperleptinemia. Maternal obesity and altered fetal programming may play a role in these sex dependent offspring outcomes into adulthood.

Due to the spontaneous nature of PE in the BPH/5 mouse, it is a good model to study the life cycle and offspring outcomes. It is especially useful for the study of early pregnancy interventions. Dams in this study were pair-fed for the first 9 days of gestation to match the caloric intake of lean controls to prevent maternal obesity. Gestating in a healthier intrauterine environment was hypothesized to improve offspring outcomes. BPH/5 male and female offspring both demonstrated cardiometabolic risk, hypertension, and heart enlargement, but had sex dependent differences in features such as obesity when gestating with PE. Adiposity coupled with PE in mothers contributes to the life cycle of obesity and cardiovascular risk observed in their offspring. After intervention, both BPH/5 male and female offspring born to pair-fed dams have a reduction in adiposity and an altered gut microbiome, while only female offspring born to pair-fed dams have decreased circulating leptin and white adipose tissue inflammatory cytokines. These sexually dimorphic results suggest that reduction in the maternal obesogenic environment in early

pregnancy may play a greater role in female BPH/5 sex-dependent cardiometabolic outcomes than males. Reprograming females may mitigate the transgenerational progression of cardiometabolic disease. Thus, demonstrating early intervention can improve in-utero effects of offspring outcomes.

The final study comparatively investigated the equine placenta and determined that it harbors a distinct resident microbiome in early pregnancy when characterized by metagenetics. There was disparity in placental bacterial communities from the oral, vaginal, and fecal microbiome. The fecal microbiome had the most diversity, while the oral and placenta similarly had the least. All body sites grouped differently when analyzed with beta diversity using Bray-Curtis dissimilarity. At the genus level some similarities were shown between body sites including *Gemella* and *Porphyromonas,* even though the placenta did harbor its own unique microbiome in relation to diversity, relative abundance, and bacterial richness. The placenta was dominated by *Gemella, Rikenellaceae_RC9, Porphyromonas,* and *Streptococcus.* The predicted placental bacterial sources identified the oral cavity as a major contributor in one horse on this study, while the remaining samples' sources were of unknown origin, meaning the potential of contamination in these samples was low. This highlights the importance that there is still research needed in this field to further our knowledge of these bacterial communities and how they are contributing to the placenta. Developing a rapid screening method by using extra-body sites as biomarkers may aid in diagnosing pregnant mares that will develop placentitis, specifically nocardioform, earlier in gestation.

No translational model perfectly recapitulates a human. When comparing mouse verses horse models there are similarities and differences. The horse also could be a model for human pregnancy due to a similar gestation length and both being typically singleton pregnancies. There

also are similarities of a mouse model to equine reproductive diseases such as placentitis. The much shorter gestation length in a mouse is beneficial for sampling and data acquisition compared with the horse gestation length. This shorter gestation specifically makes pregnancy and offspring outcome studies more feasible. Another component of this mouse model linking it to the equine model of placentitis is the inflammatory environment of their pregnancies. The BPH/5 have increased pro-inflammatory cytokines in their maternal/fetal interface, demonstrating a pregnancy harbored in an inflammatory environment. Equine placentitis also is known to have increased inflammation that results in pre-mature delivery of an unhealthy foal; a similar fetal outcome is characteristic of the BPH/5 mouse model. Looking specifically at the BPH/5 mouse and equine placental microbiome similarities include similar richness and evenness within the samples (Figure 6.1). There was difference between species when looking at beta-diversity (Figure 6.2), which is expected due to the difference in the environment, genetics, and lifestyle between species. Research using this mouse model to study equine pregnancy may still be beneficial for testing interventions and pregnancy outcomes.

Limitations of these studies include the use of 16S rRNA sequencing. Although it is a good biomarker for next generation sequencing due to the conserved regions and plethora of research, it does have limitations. 16S is designed for taxonomic breadth meaning to differentiate as many organisms as possible. This means it is not as specific, i.e., strain/species identification are very limited. Also, the error rate will be higher than compared to whole genomic sequencing. Additionally, it is difficult to interrupt functional data from 16S, majority of the information provided is quantitative mainly taxonomic and relative abundances. Another limitation of this study is the use of real-time PCR; again this is using an inference that what is present represents what is functional. However, SCFA were measured by mass spectrometry that supported the

significance of GPR downstream signaling from the colon to circulation. Finally, the small sample size in the final chapter is a limitation. Using a G-power analysis with a large effect size, the appropriate power is achieved. In the equine study, euthanized pregnant pony mares at a specific time in gestation had to be utilized to avoid contamination of the placenta from other body sites and limited the number of viable candidates.

The overall conclusion is a healthy intrauterine environment is key to successful pregnancy and offspring outcomes. The maternal microbiome does influence that environment and ultimately the reproductive outcomes. Timing of intervention is important to improving offspring outcomes. Early intervention is crucial in the reduction of the inflammatory process during pregnancy in both the horse and mouse.

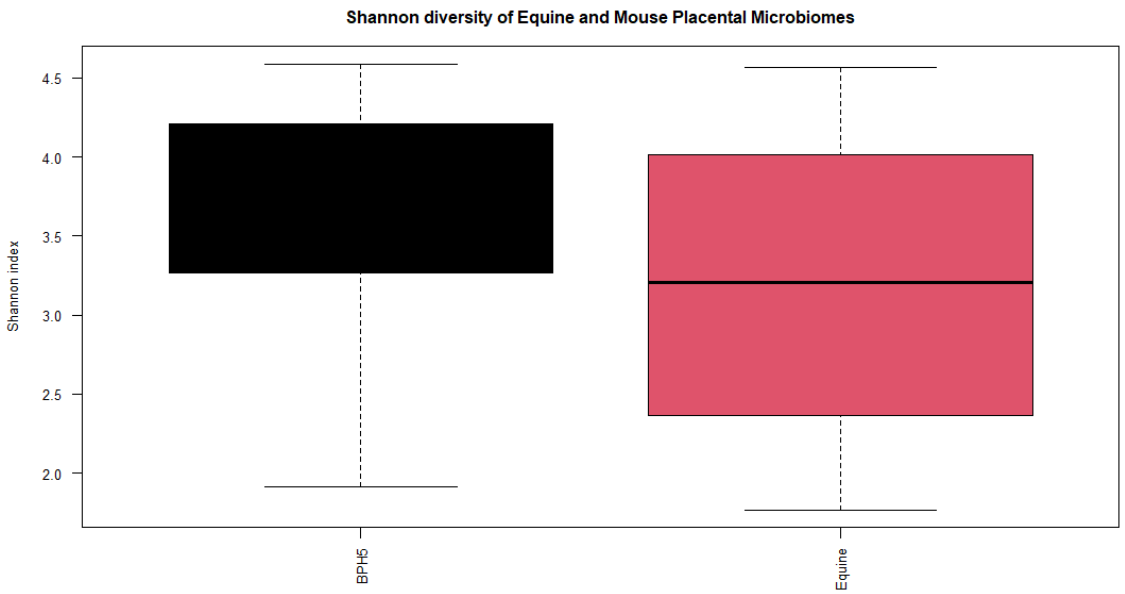


Figure 6.1 Shannon alpha diversity comparing the placental microbiome of the BPH/5 model to the equine

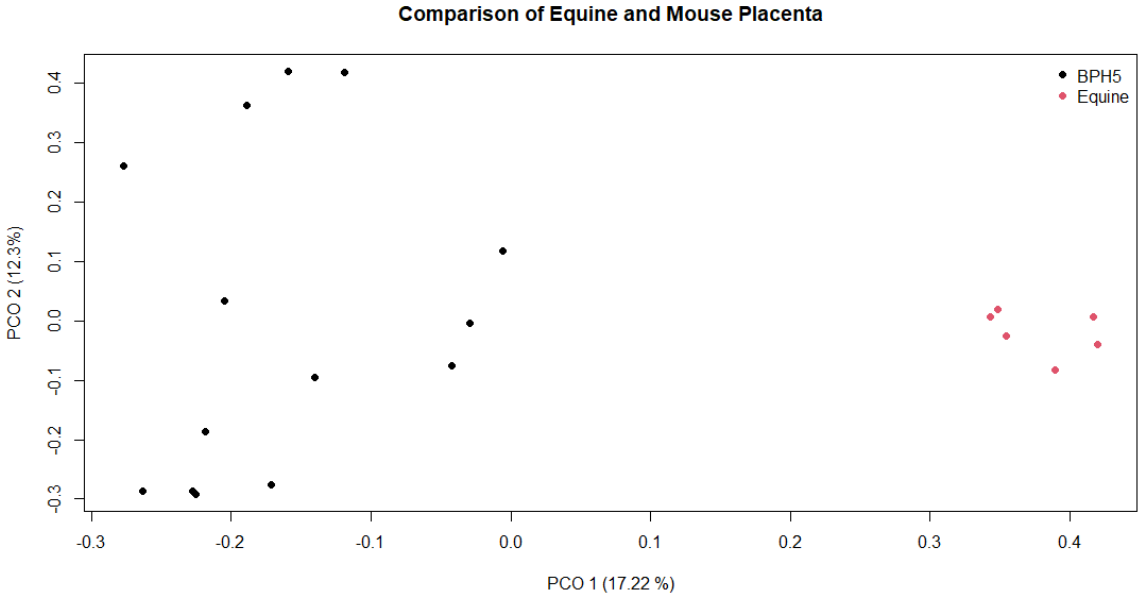


Figure 6.2 PCo plot of beta diversity comparing the placental microbiome of the BPH/5 model to the equine

APPENDIX. COPYRIGHT INFORMATION

Chapter 1. Copyright information

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REVIEW | *Microbiota and Cardiovascular Disease*

Maternal microbiome and the hypertensive disorder of pregnancy, preeclampsia

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Beckers KF, Sones JL. Maternal microbiome and the hypertensive disorder of pregnancy, preeclampsia. *Am J Physiol Heart Circ Physiol* 318: H1–H10, 2020. First published October 18, 2019; doi:10.1152/ajpheart.00469.2019.—Preeclampsia (PE) is a pregnancy-specific disorder that can be life threatening for both mother and baby. It is characterized by a new onset hypertension during the second half of pregnancy and affects ~300,000 women in the United States every year. There is no cure for PE, and the only effective treatment is delivery of the placenta and the fetus, which is often preterm. PE is believed to be a severe manifestation of placental dysfunction due to early angiogenic imbalances and inflammatory disturbances; however, the cause of this is unknown. The once thought "sterile" placenta now has been proposed to have a unique microbiome of its own. Under ideal conditions, the microbiome represents a balanced bacterial community that is important to the maintenance of a healthy environment. Dysbiosis of these communities may lead to inflammation that potentially contributes to adverse pregnancy outcomes, such as preterm birth and PE. Thus far, the female reproductive tract microbiome has been found to be influenced by periodontal disease, cardiometabolic complications, and maternal obesity, all of which have been identified as contributors to PE. This review will look at the maternal reproductive tract microbiome, evidence for and against, and its role in pregnancy and PE-related events as well as data from relevant mouse models that could be useful for further investigating the influence of the reproductive tract microbiome on the pathogenesis of PE.

dysbiosis; microbiome; placenta; preeclampsia; uterus

INTRODUCTION

Preeclampsia (PE) is characterized by late gestational hypertension (systolic ≥140 mmHg or diastolic ≥90 mmHg) and signs such as proteinuria, renal insufficiency, thrombocytopenia, hepatic dysfunction, and pulmonary edema (58). When PE is left untreated, it can result in morbidity and mortality to the mother and baby. The only known treatment is delivery of both the fetus and the placenta, often resulting in premature birth of growth-restricted neonates, which leads to deleterious consequences. PE can result in cardiovascular complications for the mother as well as cardiometabolic disease to the offspring later in life (58). Since the treatment for PE involves removing the placenta, it is thought that an abnormal placenta plays a causal role in the pathogenesis of PE (23, 76, 78, 79, 87).

PE is known to effect between 2 and 8% of pregnancies worldwide (50). The precise mechanisms that cause PE are still unknown, and there is no definitive way to predict when or if a mother will develop it. A number of maternal risk factors have been recognized to identify high-risk pregnant women, including preconception obesity, chronic hypertension, family history, and more. It is hypothesized that an increase in adipose tissue, which is a rich source of proinflammatory cytokines and complement proteins, causes an aggravated systemic inflammatory response, angiogenic imbalances in circulation and the placenta, and abnormal placental development, resulting in PE (69).

The placenta, a once thought "sterile" site, is responsible for the maintenance of pregnancy in mammals. Recent technologies, such as next generation sequencing, now provide evidence that the placenta harbors a unique microbiome of its own (1). A microbiome is a collective genomic community of symbiotic, commensal, and sometimes pathogenic microorganisms that reside in an environment, such as a body cavity. These communities can consist of bacteria, archaea, fungi, protists, and viruses. The microbiome is therefore the collective genomes of the microbiota community members, and the study of this has been termed metagenomics. The study of an environment's metagenome, where scientists utilize the principles of molecular biology and genetics, is referred to as metagenetics. The host and microbiome relationship is considered mutualistic symbiosis. Studies have shown that the human

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Chapter 3. Copyright information

frontiers in Pediatrics

BRIEF RESEARCH REPORT
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Cardiometabolic Phenotypic Differences in Male Offspring Born to Obese Preeclamptic-Like BPH/5 Mice

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Preeclampsia (PE) is a hypertensive disorder of pregnancy occurring in approximately 10% of women worldwide. While it is life threatening to both the mother and baby, the only effective treatment is delivery of the placenta and fetus, which is often preterm. Maternal obesity is a risk factor for PE, and the effects of both on offspring are long standing with increased incidence of cardiometabolic disease in adulthood. Obese BPH/5 mice spontaneously exhibit excessive gestational weight gain and late-gestational hypertension, similar to women with PE, along with fetal growth restriction and accelerated compensatory growth in female offspring. We hypothesized that BPH/5 male offspring will demonstrate cardiovascular and metabolic phenotypes similar to BPH/5 females. As previously described, BPH/5 females born to *ad libitum*-fed dams are overweight with hyperphagia and increased subcutaneous, peri-renal, and peri-gonadal white adipose tissue (WAT) and cardiomegaly compared to age-matched adult female controls. In this study, BPH/5 adult male mice have similar body weights and food intake compared to age-matched control mice but have increased inflammatory subcutaneous and peri-renal WAT and signs of cardiovascular disease: left ventricular hypertrophy and hypertension. Therefore, adult male BPH/5 do not completely phenocopy the cardiometabolic profile of female BPH/5 mice. Future investigations are necessary to understand the differences observed in BPH/5 male and female mice as they age. In conclusion, the impact of fetal programming due to PE has a transgenerational effect on both male and female offspring in the BPH/5 mouse model. The maternal obesogenic environment may play a role in PE pregnancy outcomes, including offspring health as they age.

Keywords: preeclampsia, fetal programming, obesity, sex differences, adiposity

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ORIGINAL ARTICLE

Physiological Reports

Sex-specific effects of maternal weight loss on offspring cardiometabolic outcomes in the obese preeclamptic-like mouse model, BPH/5

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Abstract

AbstractPreeclampsia (PE) is a hypertensive disorder that impacts 2–8% of pregnant women worldwide. It is characterized by new onset hypertension during the second half of gestation and is a leading cause of maternal and fetal morbidity/mortality. Maternal obesity increases the risk of PE and is a key predictor of childhood obesity and potentially offspring cardiometabolic complications in a sex-dependent manner. The influence of the maternal obesogenic environment, with superimposed PE, on offspring development into adulthood is unknown. Obese BPH/5 mice spontaneously exhibit late-gestational hypertension, fetal demise and growth restriction, and excessive gestational weight gain. BPH/5 females have improved pregnancy outcomes when maternal weight loss via pair-feeding is imposed beginning at conception. We hypothesized that phenotypic differences between female and male BPH/5 offspring can be influenced by pair feeding BPH/5 dams during pregnancy. BPH/5 pair-fed dams have improved litter sizes and increased fetal body weights. BPH/5 offspring born to ad libitum dams have similar sex ratios, body weights, and fecal microbiome as well as increased blood pressure that is reduced in the dam pair-fed offspring. Both BPH/5 male and female offspring born to pair-fed dams have a reduction in adiposity and an altered gut microbiome, while only female offspring born to pair-fed dams have decreased circulating leptin and white adipose tissue inflammatory cytokines. These sexually dimorphic results suggest that reduction in the maternal obesogenic environment in early pregnancy may play a greater role in female BPH/5 sex-dependent cardiometabolic outcomes than males. Reprograming females may mitigate the transgenerational progression of cardiometabolic disease.

KEYWORDS

hypertensionh, obesity, preeclampsia, sex as a biological variable

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VITA

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