HIGH-FAT DIET-INDUCED OBESITY MODULATES PREGNANCY GUT MICROBIOTA AND ALTERS MATERNAL INTESTINAL ADAPTATIONS TO PREGNANCY

HIGH-FAT DIET-INDUCED OBESITY MODULATES PREGNANCY GUT MICROBIOTA AND ALTERS MATERNAL INTESTINAL ADAPTATIONS TO PREGNANCY

By

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Abstract

Maternal obesity is a key predictor of childhood obesity. It has been shown to cause changes in maternal adaptation to pregnancy that result in an adverse *in utero* environment, inducing increased risk of obesity and metabolic disease in the offspring. Perturbation of gut microbiota over the course of pregnancy has been implicated in maternal metabolic adaptations. We investigated how high-fat (HF) diet-induced obesity before and during pregnancy affects gut microbiota and maternal intestinal barrier function, and whether altered maternal gut adaptations to pregnancy influence placental development. Microbiota of HF mice was modified by diet, and further modulated by pregnancy. Changes in mucin-degrading and short chain fatty acid (SCFA) producing bacteria were found in microbiota from HF pregnant mice. SCFA receptor, GPR41, expression was reduced in the duodenum and jejunum of HF dams. Intestinal barrier function was impaired in HF pregnancies as indicated by increased paracellular intestinal permeability measured by passage of FITC-Dextran and increased concentration of bacterial lipopolysaccharide in the maternal serum, observations that were consistent with reduced gene expression of tight junction proteins in the small intestine of HF dams. Diet-induced maternal obesity altered gene expression of inflammatory and immune cell marker genes in different gut sections. However, no change in TNFα and IL-6 in maternal serum suggested that HF diet-induced obesity was not associated with systemic maternal inflammation. Female placenta from HF pregnancies were smaller in size, as calculated by measuring the cross sectional area of the placenta, which may be regulated by increased apoptosis. Augmented placental inflammation was not observed in HF placentas. Changes in maternal intestinal adaptations appear to be modified by diet-induced obesity before and during pregnancy, in a manner that reduced maternal barrier function and increased intestinal immune cell markers but these changes appeared not be associated with placental pro-inflammatory status.

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Abbreviations

BM	Before mating
BMI	Body mass index
cDNA	Complimentary DNA
CI	Confidence interval
DIO	Diet-induced obesity
DMEM	Dulbecco's Modified Eagle's Medium
DOHaD	Developmental origins of health and disease
DSS	Dextran sodium sulfate
E	Embryonic
EtOH	Ethanol
FAT	Fatty acid transport proteins
FITC	Fluorescein isothiosyanate
GC-MS	Gas Chromatography Mass Spectrometry
GF	Germ-free
GDM	Gestational diabetes mellitus
GLP-1	Glucagon-like peptide-1
GLUT	Glucose transporting proteins
GPR	G-protein coupled receptors
GALT	Gut-associated lymphoid tissue
HBD	HEK Blue Detection
HEK293	Human embryonic kidney 293
HF	High-fat
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
hr	Hour(s)
IRS	Insulin receptor substrate
IFN-γ	Interferon gamma
IκBα	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor α
IKK	IκB kinase
IL	Interleukin
IRAK	IL-1 receptor-associated kinase
ICP	Intrahepatic cholestasis of pregnancy
KO	Knockout
LPS	Lipopolysaccharide
MDCK	Madin-Darby Canine Kidney Epithelial
mTOR	Mammalian target of rapamycin
min	Minute(s)
MLN	Mesenteric lymph nodes
MCP-1	Monocyte chemoattractant protein-1
MTBSTFA	N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide
MyD88	Myeloid differentiation factor 88

NEFA	Non-esterified fatty acids
ob/ob	Genetically obese
OTU	Operational taxonomic units
NF-Kb	Nuclear factor-kappaB
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate buffered solution
PCR	Polymerase Chain Reaction
PCoA	Principal Coordinates Analysis
PERMANOVA	Permutational multivariate analysis of variance
PYY	Peptide tyrosine-tyrosine
PPARγ	Peroxisome proliferator-activated receptor
PP	Peyer's patches
QIIME	Quantitative Insights Into Microbial Ecology
qPCR	Quantitative Polymerase Chain Reaction
rpm	Rotations per minute
SCFA	Short chain fatty acids
SDS	Sodium dodecyl sulfate
sec	Second(s)
Th1	T helper 1
Th2	T helper 2
TIR	Toll/IL-1R domain
TLR	Toll-like receptors
TNFα	Tumor necrosis factor α
TER	Transepithelial electrical resistance
UBC	Ubiquitin C
WT	Wildtype
ZO	Zonula occludens

Declaration of Academic Achievement

The present study was primarily designed, executed, analyzed and written by the author of this thesis, under the supervision and guidance of Dr. Deborah M. Sloboda with the following exceptions:

Sections of Chapter 1.0 Introduction were adapted from the following published review article: Of the bugs that shape us: maternal obesity, the gut microbiome, and long-term disease risk; written by Wajiha Gohir, Elyanne M. Ratcliffe, and Deborah M. Sloboda, published in Pediatric Research, 2015 Jan;77(1-2):196-204. doi: 10.1038/pr.2014.169. Wajiha Gohir was the primary author of the review article. Animal work was performed by Kaitlyn Chan, Caroline Moore, Jessica Wallace and Wajiha Gohir. Placentae were cut, stained and analyzed by Brendan Patterson. Kate Kennedy assisted with the assay used to measure lipopolysaccharide in maternal serum. Dr. Deborah M. Sloboda contributed significantly to the study's design, and analysis and interpretation of data.

1.0 INTRODUCTION

Obesity is a global epidemic that affects more than 500 million people worldwide[1]. The rapidly increasing global prevalence can no longer be solely attributed to genetic factors[2]. The difficulty in minimizing the rising rate of obesity and its co-morbidities with lifestyle changes has resulted in the exploration of other pathways that may contribute to disease risk. There is no doubt that obesity is a result of complex, multifaceted causal pathways and thus the quest for a single magic bullet is becoming a thing of the past.

The developing organism is plastic, where genotypic responses to changing environments has led to the hypothesis that disease risk is the result of complex gene-environment interactions[3]. It is well known that our current environment, which is shaped by diet, physical activity, drugs and alcohol use and other lifestyle factors, has direct consequences on our overall health and well-being. Of critical importance is the recognition that our "environment" also includes the period encompassing the developmental milieu within which gametes, germ/stem and somatic cells take shape and differentiate into established organ systems. It is proposed that the inherent developmental plasticity of an organism during critical windows of development, particularly the prenatal period, allows it to respond to cues that will ultimately determine the mature (adult) phenotype. This concept is now recognized as the developmental origins of health and disease (DOHaD). Under certain circumstances when there is a mismatch between prenatal and postnatal environment, these adaptations may lead to increased disease risk[4]. In this regard, epidemiological and experimental data has

inextricably shown a relationship between the *in utero* environment and the risk of developing chronic disease, such as obesity, later in life[4].

The *in utero* environment is highly sensitive to changes in maternal physiology. Over the course of a healthy pregnancy, the mother makes critical metabolic, cardiovascular, respiratory, endocrine and skeletomuscular adaptations to accommodate the growth of the placenta and the fetus[5]. Prenatal adversity can modify maternal adaptations to pregnancy, resulting in a suboptimal *in utero* environment in which the fetus develops. One of the earliest studies implicating the prenatal environment to the development of obesity came from data collected from the Dutch Hunger Winter of WWII[6]. Women who were exposed to famine during early gestation gave birth to offspring that had normal birth weight but higher risk of developing obesity and cardiovascular disease later in life. While women who were exposed to famine during late pregnancy gave birth to offspring that were small at birth and had lower rates of obesity later in life. This provided evidence that insults during key periods of development could have a lasting impact on offspring health. However, in the recent years, as a result of increasing prevalence of obesity during pregnancy, more attention is now being paid to understanding the role of maternal obesity in the programming of childhood obesity. The *in utero* environment has been shown to be particularly sensitive to maternal diet and metabolic status. Maternal obesity and over-nutrition during pregnancy are recognized as early life programming factors, and their impact on the *in utero* environment has been extensively studied in clinical settings and animal models.

1.1 Maternal Obesity

Maternal obesity is currently one of the leading factors predicting childhood obesity[7]. It is associated with abnormal feto-placental function[8], offspring obesity risk[7], and increased disease risk overall. Animal studies using high fat diet-induced maternal obesity have made significant advances in our understanding of the underlying mechanisms linking maternal obesity with offspring obesity and metabolic dysfunction. However, which one (or combination) of the identified signaling pathways is best for targeted interventions to reduce childhood and adolescent obesity and metabolic compromise remains unclear.

1.1.1 Impact of maternal obesity: Epidemiological evidence

Obese women are at risk for a multitude of pregnancy complications, including increased risk of miscarriage, stillbirth, gestational diabetes, hypertension, and pre-eclampsia as well as increased risk of C-section delivery[9]. It has been reported that the incidence of both pre-eclampsia and gestational hypertension increases linearly with increasing maternal body mass index (BMI) and that morbidly obese women have a three-fold higher risk of experiencing obstetrical complications (OR 4.21; 95% CI 3.45, 5.13 pre-clampsia and OR 3.66; 95% CI 3.23, 4.15 for gestational hypertension)[10]. Maternal obesity is also associated with significant changes in endocrine and metabolic function where circulating levels of short chain fatty acids (SCFA), as well as pro-inflammatory cytokines are elevated in mothers that are obese during pregnancy[11,12].

Maternal obesity not only impacts the mother's health during gestation, but has both short and long-term ramifications for her offspring[13]. Maternal obesity significantly

increases risk for neural tube defects and other congenital anomalies in neonates[14,15]. Both obesity prior to and during pregnancy are associated with fetal macrosomia. The incidence of high birth weight increases linearly with increasing maternal BMI and, relative to a reference group, both obese and morbidly obese women show a two-fold greater risk of delivering a baby weighing >4000 g [OR 2.01 (95% CI 1.83, 2.22) and OR 2.62 (95% CI 2.30, 2.98), respectively][10]. Fetal macrosomia is also associated with changes in body composition. In 2006, Sewell et *al*. showed that offspring of obese women have mean fat mass of 416 g, which was significantly higher when compared to 334 g of infants born to mothers with a normal BMI[16-18]. Catalano et *al*. found fetuses of obese mothers had greater percent body fat and had already developed insulin resistance *in utero*[19].

Maternal obesity and excess gestational weight gain is associated with an increased incidence of childhood obesity and metabolic syndrome[20]. Macrosomia itself is associated with an increased risk of developing obesity and metabolic syndrome[21] which appears to persist for multiple generations[22]. Mortality rates, overall, have been shown to be increased in offspring of obese mothers (BMI >30) compared with mothers with normal BMI (after adjustment for confounding variables; hazard ratio 1.35, 95% CI 1.17 to 1.55)[23].

1.1.2 The impact of maternal obesity: Experimental evidence

Animal models are routinely used to study the effects of maternal obesity and pinpoint specific mechanisms underlying adverse outcomes. In rodents, maternal high-fat (HF)

diet during pregnancy and lactation has been shown to induce obesity and hepatic steatosis in the offspring (irrespective of postnatal diet)[24], insulin and leptin resistance[25], hypertension[26], and hepatic inflammation in offspring[27]. Offspring of rats fed HF diet during pregnancy and lactation, and control diet after weaning tend to have lower basal metabolic rates and higher fat mass. When fed a HF diet post-weaning, the offspring tend to become obese in adulthood.

There has been evidence for increased sex-specific incidence for obesity and metabolic dysfunction and thus sex has also become a focus for some studies[28]. Diet-induced obesity (DIO) during pregnancy in rodents generates sex-specific effects on placental growth and fetal metabolic phenotype[29], gene expression and epigenetic regulation[30] – consistent with human data[31]. In mice, DIO during pregnancy was associated with inhibition of placental nutrient sensor, mammalian target of rapamycin (mTOR), signaling and reduced placental efficiency[32] and increased trans-placental transport of nutrients, such as glucose and neutral amino acids, resulting in increased fetal growth[33].

In sheep, fetuses from DIO ewes are macrosomic, hyperglycemic, and hyperinsulinemic[34] and show decreased insulin signaling in skeletal muscle associated with increased intramuscular triglycerides and higher expression of fatty acid transporters[35]. In sheep, maternal obesity results in enhanced placental content of fatty acid transport proteins and transcription factors that regulate lipid metabolism (such as PPARγ)[36], increased levels of toll-like receptors 2 and 4 (TLR2, TLR4), macrophage

markers and pro-inflammatory cytokines as well as upstream transcription factors in signaling pathways associated with inflammation[37].

Studies in non-human primates have further delineated the role of maternal obesity in increasing disease risk in the offspring. In 2010, Grayson et *al*. demonstrated in non-human primates that HF diet before and during pregnancy results in fetuses with lower body weight in early third trimester, followed by catch up growth and increased adiposity postnatally, regardless of postnatal diet[16]. In Japanese macaques, HF diet alone, without the maternal obesity phenotype, induced increased placental cytokine and TLR4 expression. HF diet also reduced uterine blood flow volume, resulting in placental dysfunction and stillbirth[38].

1.1.3 Mechanistic link between maternal and offspring obesity

Many studies have described phenotypic and physiological changes that occur in the mother, fetus, and offspring due to maternal obesity. The mechanisms by which these changes occur are difficult to ascertain due to the complex nature of both pregnancy and obesity.

Obesity is a medical condition caused by energy imbalance, where increased energy intake and decreased energy expenditure lead to excessive increase in adipose tissue. Obesity induces insulin resistance and is associated with chronic low-grade inflammation[39]. A fundamental characteristic of obesity is the presence of higher number of macrophages in adipose tissue[40]. Macrophages produce inflammatory mediators, such as pro-inflammatory cytokines, which contribute to the insulin resistance observed in

obesity[40]. Macrophages have been defined to have two different polarizations, M1 and M2[41]. Pro-inflammatory mediators, such as pathogen-associated molecular patterns (PAMPs), like lipopolysaccharide (LPS), or T helper 1 (Th1) cytokines, such as interferon gamma (IFN-γ) induce M1 macrophages, which are referred to as "classically activated". These macrophages increase production of pro-inflammatory cytokines and induce Th1 responses[39]. M2 macrophages, referred as "alternatively activated", are polarized by interleukin (IL) 4 and IL13 *in vitro*[42]. They increase production of anti-inflammatory cytokines, invoke T helper 2 (Th2) responses and are involved in tissue remodeling and resolution of inflammation[42]. There is a bias towards M1 macrophage activation (or differentiation) during obesity[43]. M1 macrophages express higher levels of toll-like receptor 4 (TLR4) and TLR2 that recognize PAMPs and activate nuclear factor-kappaB (NF-kB), a transcription factor which controls the expression of an array of inflammatory cytokine genes, including tumor necrosis factor α (TNFα), IL-6, IL-1β and IL-12[39].

Interestingly, pregnancy is associated with a prevalence of Th2 immune response for majority of gestation, followed by a shift towards Th1 in late gestation[44,45]. The balance between Th1 and Th2 cytokines is crucial for a healthy pregnancy, and when disturbed it results in adverse pregnancy outcomes, like spontaneous abortion, intrauterine growth restriction and preeclampsia[45]. Pregnancy is also associated with insulin resistance, a characteristic it shares with obesity. Thus, the role of low-grade chronic inflammation in obese pregnancy has been studied as a possible mechanistic link between maternal and offspring obesity that triggers an adverse *in utero* environment.

Despite the fact that normal pregnancy is characterized by heightened Th2 activity, several studies have reported that pregnancy complicated with obesity is associated with heightened inflammation and Th1 activity[12,46,47]. The observation that the placenta during obese pregnancy is infiltrated by pro-inflammatory M1 macrophages has led to the hypothesis that maternal obesity results in increased placental inflammation causing the fetus to develop in an inflammatory milieu[37].

1.1.3.1 Placental function during pregnancy

In order to understand how placental inflammation can impact fetal development and result in fetal programming of obesity, a review of placental function is necessary. The placenta is a highly specialized, multi-functional organ that supports the growth and development of the fetus[48]. Its main functions are to coordinate the exchange of nutrients and waste products between the mother and the fetus, and produce and metabolize hormones that are required for the maintenance of pregnancy and fetal growth[48]. Fetal growth is dependent on the nutrients it receives from the mother through the placenta. Placental capacity to transfer nutrients is dependent on a number of factors including as placental size and morphology, blood flow, nutrient transporters on the placenta, concentration of nutrients in the maternal circulation and the consumption and production of nutrients by the placenta itself[49].

Placental size affects nutrient transport by changes in its surface area, which impact nutrient diffusion and nutrient transporter expression[50]. Compromised placental growth has been shown to result in adverse fetal phenotypes, such as intrauterine growth

restriction[51]. Several studies have directly correlated placental size to fetal weight, affirming the impact of placental size on fetal development[52,53]. Overall placental morphology also plays a key role in facilitating optimal nutrients to the fetus. Placental chorionic villi are in direct contact with the maternal blood. Changes in the structure and/or size of the synctiotrophoblast, which forms a barrier that prevents maternal immune cells from infiltrating the fetus, can alter nutrient exchange leading to changes in fetal and placental weight[48].

Apart from placental structural features, the nutrients transferred to the fetus via the placenta are dependent on what is available in the maternal circulation. Glucose is an important nutrient for both the placenta and the fetus, however the primary glucose source for the fetus is maternal since fetal glucose production is very low[54]. In order to facilitate maternal trans-placental transfer of glucose to the fetus maternal basal glucose concentrations remain high during pregnancy (particularly late in pregnancy). Development of insulin resistance during pregnancy has been accredited to the increase production of human placental lactogen from the placenta with advancing gestation[55]. However, more recently adipokines, such as TNFα, secreted by adipose tissue have been shown to reduce insulin sensitivity by increasing phosphorylation of insulin receptor substrate (IRS)-1[56-58]. Insulin resistance, as a result, is a necessary maternal metabolic adaptation to provide glucose to the growing fetus and placenta. Placental transport of glucose is facilitated by glucose transporting proteins (GLUTs), primarily GLUT1 and GLUT3[59,60]. Fatty acids are also an important nutrient required for fetal brain and adipose development. Non-esterified fatty acids (NEFAs) and esterified fatty acids in triglycerides

carried by lipoproteins are the two main types of fatty acids taken up by the placenta and transported to the fetus. Fatty acid transport is facilitated by fatty acid transport proteins (FATs) and by simple diffusion driven by the maternal to fetal concentration gradient [36,61]. Amino acids are also transported to the fetus through a number of transporters that mediate their uptake against the concentration gradient[62]. Maternal amino acid concentrations are lower than fetal, indicating continuous transport to the placenta and fetus[62,63]. Neutral amino acids, such as alanine, glycine, and serine, are transported by system A amino acid transporter, that allows transport against their gradient by transporting sodium at the same time[64]. System L transporter is sodium independent, and it allows transport of branched and aromatic amino acids[65].

1.1.3.2 Placenta in obese pregnancy

Maternal obesity affects both placental morphology and function. Obese women are more likely to have larger placentas and larger placenta-to-birth weight ratio. Placental efficiency, described as grams of fetus produced per grams of placenta, is reduced in placenta from overweight and obese women[10]. Overweight to morbidly obese women are also progressively less likely to have a small placenta and more likely to have a large placenta[10]. Placentas of obese women demonstrate decreased sodium-dependent neutral amino acid transporter (System A) expression[66]. *In vitro*, IL-1β has been shown to inhibit insulin-stimulated System A amino acid uptake in human placental trophoblast cells[67], suggesting pro-inflammatory cytokine, IL-1β, may reduce the effects of maternal insulin on placental function. Obese women show placental dysregulation of redox balance[68],

decreased fatty acid transport and increased placental lipoprotein lipase activity which could be responsible for placental triglyceride accumulation[69], although this contrasts to placenta-derived data from animal models studying diet-induced maternal obesity. Recent data suggests that maternal BMI is associated with activation of placental inflammatory pathways, and that the activation of pro-inflammatory markers MCP-1 and TNFα, is a possible link between elevated pro-inflammatory cytokines in maternal plasma and activation of placental inflammatory pathways potentially influencing placental function[12]. Consistent with this, others report that placentae from obese women develop exaggerated inflammatory responses with increased macrophage infiltration and pro-inflammatory cytokines[70] and this pro-inflammatory environment may have the capacity to modulate placental nutrient transfer. The mechanism by which maternal obesity initiates placental inflammation is still under investigation.

An aspect that has not been investigated is whether maternal microbiota plays a role in the placental inflammation associated with maternal obesity. Pattern recognition receptors, such as toll-like receptors, which recognize microbial pathogens and initiate production of pro-inflammatory cytokines, are present in the placenta[71]. The expression levels of TLR2 and TLR4 are increased during maternal obesity, thus may play a direct role in initiating inflammation in the placenta during obese pregnancy[37]. TLRs are also activated by saturated fatty acids. Over the course of gestation plasma free fatty acid levels increase 2-fold[72], while during obese pregnancy maternal fatty acid levels have been strongly related to excess fetal growth[73]. Changes in fatty acid levels in obese

women could also be contributing to placental inflammation through activation of TLRs by saturated fatty acids.

1.2 Gut microbiota

Recently, the gut microbiota has become one of most studied factors influencing disease risk. The gut microbiota is modulated by several factors, such as diet, age and health status[74]. It is now thought that the responses of commensal bacteria, residing symbiotically within our gut, to changing nutritional, endocrine, and infectious stimuli likely contribute to long-term metabolic dysfunction, obesity and chronic disease risk.

1.2.1 Role of gut microbiota in health

The human gut hosts trillions of microbes; collectively, these microbes constitute our microbiota, and the genes they encode are known as the microbiome[75]. The gut microbiota has coevolved with vertebrates over many millennia and developed a mutualistic relationship with their hosts[76]. It contributes significantly to metabolic processes that occur in the gut; the successful establishment of commensal gut microbiota is essential for metabolizing indigestible polysaccharides and producing essential nutrients for the host[77]. In addition to regulating host metabolism, commensal bacteria and their metabolites play a key role in the development and function of the mammalian immune system, and the maintenance of the intestinal epithelial barrier[78,79].

The intestinal mucosal immune system is composed of the gut-associated lymphoid tissue (GALT), which contains the mesenteric lymph nodes (MLN) and Peyer's

patches (PP), as well as more diffusely spread out immune cells in the lamina propria of the intestinal tract[80]. Studies using germ-free (GF) animals, that are devoid of microbiota, have established that bacterial colonization of the gut is necessary for the development of the mucosal immune system. GF mice have smaller hypoplastic PPs, fewer intraepithelial lymphocytes and they exhibit irregular levels of cytokines[81]. GF animals also have impaired CD4+ T cell maturation[82]. CD4+ T cells play a crucial role in regulating immune response and are subdivided into Th1 and Th2 subtypes[83]. The balance between Th1 and Th2 is critical for homeostasis. Impaired CD4+ T cell maturation can be remediated by colonization of GF mice with polysaccharide A of *Bacteroides fragilis,* which can activate CD4+ T cells and re-establish Th1/Th2 balance[84].

The commensal gut microbiota also plays a role in intestinal barrier function. The intestinal barrier is a complex structure that separates the gut lumen from the host and includes a complex system of molecular and structural components that shield the host from invasion by pathogenic bacteria[85]. This includes, a mucosal epithelial layer, as well as humoral defence modulated by defensins and IgA, immunological defence modulated by a complex immune system and cellular components, as well as the gut microbiota[85]. The gut microbiota prevents colonization by pathogenic bacteria by supressing their proliferation through a number of mechanisms. Both commensal bacteria and pathogenic bacteria require a niche environment to grow and thrive in, thus pathogenic bacteria must outcompete commensal bacteria in order to proliferate[86]. Gut microbiota produces antimicrobial peptides and proteins, as well as metabolites, such as SCFAs that inhibit the growth of pathogenic bacteria. Commensal bacteria can also stop growth of pathogenic

bacteria by utilizing resources that pathogenic bacteria require for survival. As well, they can also suppress growth by altering the environmental conditions within the gut, for example consuming residual oxygen to suppress growth of aerobic pathogens[87].

Gut microbiota also controls the intestinal barrier by maintaining cell-to-cell junctions and expression of tight junction proteins that regulate intestinal permeability[88]. Intestinal permeability is a functional feature of the intestinal barrier which is defined by the flux rate of small molecules across the intestinal wall[85]. Tight junction complexes are composed of multiple transmembrane proteins that interact with actin cytoskeleton within the cell and mediate adhesion between cells[89]. Evidence suggesting gut microbiota plays a role in mediating intestinal permeability comes from studies that have shown that probiotic and commensal bacteria can attenuate intestinal permeability, by changing gene expression of tight junction proteins, such as zonula occludens (ZO), claudins and occludins[90]. Altered gene expression of tight junction proteins has been observed in multiple inflammatory diseases that are associated with perturbed gut microbiota, such as celiac disease[91], Crohn's disease[92], ulcerative colitis[93] and obesity[94]. Thus, the interaction between specific bacteria in the gut with tight junction proteins plays a critical role in regulating intestinal barrier function.

The gut microbiota has a vast and versatile metabolomics profile, far larger than that of human cells[95]. Microbiota metabolic processes play a key role in host nutrient acquisition[96]. When compared to convention mice, GF mice require higher caloric intake to maintain the same body weight, suggesting microbiota aids the host in extracting more energy out of the ingested nutrients[97,98]. Commensal microbes are able to digest nutrients

that are otherwise indigestible by the host, such as complex carbohydrates and dietary fibers. The short chain fatty acids (SCFA)s they produce from indigestible carbohydrates in the cecum and colon are a huge energy source for the mammalian host, as well as the resident microbes. SCFAs also serve as signal transduction molecules through activation of G-protein coupled receptors (GPRs) to regulate lipid and glucose metabolism[99,100]. GPR43 (also known as Ffar2) is mainly activated by acetate and propionate, and its activation promotes glucagon like peptide-1 (GLP-1) and peptide YY (PYY) secretion[101]. GLP-1 acts on multiple tissues to maintain energy homeostasis. It augments secretion of insulin by pancreatic-B cells, inhibits glucagon secretion and alters gastric motility[102]. GPR43 activation suppresses insulin signaling in adipose tissue, reducing fat accumulation, but not in muscles or liver[103]. Thus, SCFA activation of GPR43 plays a key role in regulating fat accumulation and maintaining glucose homeostasis. GPR41 (also known as Ffar3) is another SCFA receptor that is activated by propionate and butyrate and it also activates secretion of GLP-1 and PYY. Furthermore, GPR41 is highly expressed in sympathetic ganglia, and regulate the sympathetic nervous system[104].

1.2.2 Gut microbiota and obesity

Gut bacterial populations also play a critical role in regulating fat storage. Studies have shown that colonizing GF mice with microbiota extracted from conventional mice induces a dramatic increase in body fat, despite a decrease in food consumption, through *de novo* hepatic lipogenesis[98]. Even more convincing is the demonstration that colonization of germ-free mice with microbiota from both genetically obese and DIO

mice results in a greater increase in body fat than when colonized with microbiota from lean mice or mice fed a low-fat diet[105]. How this shift in adipose tissue accumulation occurs is the focus of much research.

Different bacterial populations use and produce different nutritional substrates and metabolites, and have varying capacities for energy harvest. Of these populations, the two most abundant phyla in the mammalian gut, *Firmicutes* and *Bacteroidetes* have received the most attention[105-107]. Genetically obese *ob/ob* mice demonstrate shifts in the proportions of *Firmicutes* and *Bacteroidetes* where *Bacteroidetes* were significantly reduced, while *Firmicutes* were increased in abundance in obese mice relative to lean mice[107]. Consistent with this, shotgun sequencing of the gut microbiome of obese mice demonstrates enrichment in genes encoding for enzymes involved in energy extraction, supporting the concept that these bacterial populations allow obese mice to harvest more energy from their diet[105].

Such observations involving fluctuations in the proportions of bacteria populations are not limited to animal models. Analysis of the core gut microbiome in obese and lean twins by sequencing of the 16S rRNA gene sequence, has shown that obesity is associated with a decrease in abundance of *Bacteroidetes* and altered expression of genes involved in lipid, carbohydrate and amino acid metabolic pathways that favour increased energy uptake and adipogenesis[108]. Nutrient manipulation in obese individuals, through fat-restricted or carbohydrate-restricted diets, showed an increased shift in *Bacteroidetes* and decreases in Firmicutes populations over time and these shifts were correlated with a loss of body weight[106] suggesting that these bacterial populations

may be calorically responsive[106]. Similarly in humans, the metabolic activity of the gut microbiota is increased in obese children compared to lean children, thus gut dysbiosis is not limited to obesity in adulthood: it has also been implicated in the etiology of childhood obesity[109]. As in the adult literature, there is evidence to suggest that not only are there shifts in fecal microbial populations in overweight children, but variations in bacteria exist at the species levels[110].

1.2.3 "Obese" microbiota and inflammation

After the discovery that obesity is associated with altered gut microbiota[107,111], much attention has been paid to how these changes relate to the chronic low-grade inflammation and insulin resistance observed in obesity. Gram-negative bacterial wall component lipopolysaccaride (LPS) has been identified as one of the possible factors that initiate inflammation in the context of obesity or a HF diet. In control mice, plasma LPS concentrations follow a diurnal cycle, there is a gradual increase during the feeding period, followed by a sharp fall post-feeding[112]. HF diet feeding disrupts this diurnal cycle by causing continuous absorption of LPS, and resulting in elevated LPS concentration throughout the day, this is defined as metabolic endotoxemia[112]. HF diet intake increases fasting glycemia, whole body adipose and liver weight, circulating triglyceride concentrations and increases inflammation in metabolic tissues. Similar changes occur in control mice that received continuous subcutaneous infusion of LPS, suggesting increased LPS is sufficient to trigger metabolic inflammation[112]. Furthermore, CD14-deficient mice are protected against LPS and/or HF diet induced obesity and

metabolic inflammation[112]. CD14 and its co-receptor TLR4 are activated by binding of LPS. After LPS recognition, the Toll/IL-1R (TIR) domain of the TLR4 recruits intracellular signaling adapter protein Myeloid differentiation factor 88 (MyD88), and a subsequent kinase cascade triggers the activation of NFκB, which results in secretion of pro-inflammatory cytokines[113] (Fig. 1.3.3.1). Thus, increased LPS concentrations due to HF diet intake likely triggers inflammation through CD14/TLR4 signaling pathway, resulting in initiation of metabolic disease, such as obesity.

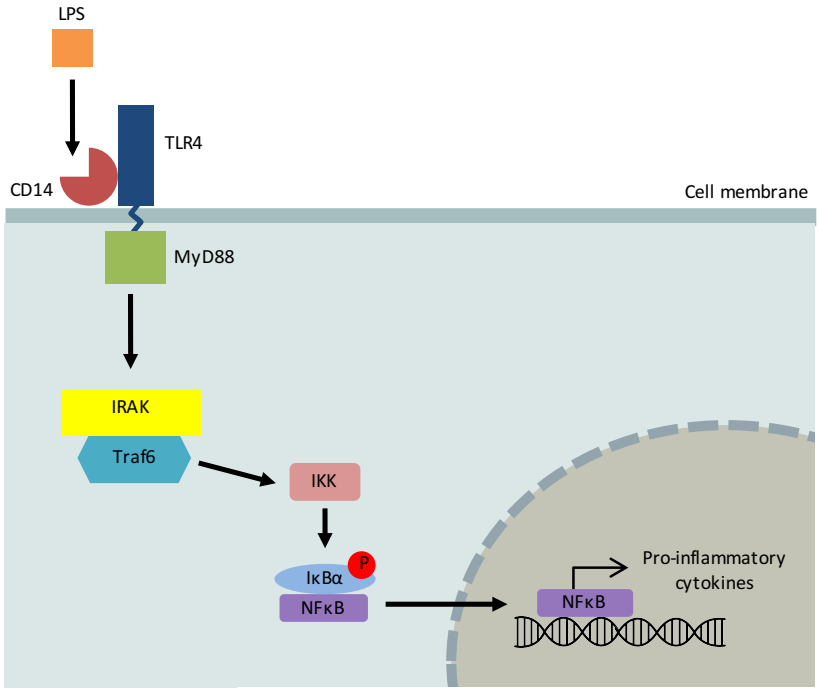

Figure 1.2.3.1: *Activation of CD4/TLR4 by bacterial component, lipopolysaccaride (LPS) initiates secretion of pro-inflammatory cytokines.*
Binding of LPS to TLR4 and its co-receptor CD14 activates a signalling cascade that results in the secretion of pro-inflammatory cytokines. After activation by LPS, the TIR domain of TLR4 recruits MyD88. MyD88 signals through Traf6, which is associated with IL-1 receptor-associated kinase (IRAK) activates IκB kinase (IKK), which phosphorylates nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor α (IκBα) resulting in the release of NF-κB. NF-κB moves into the nucleus and initiates transcription of pro-inflammatory cytokines.

Increased LPS diffusion during obesity has been proposed to be facilitated by impaired intestinal barrier function. HF DIO is associated with increased intestinal permeability and reduced gene expression of tight junction proteins, ZO-1 and occludin[114]. Antibiotic treatment of genetically obese (*ob*/*ob*) and DIO mice, to reduce the population of commensal bacteria in the gut has shown a reduction in cecal levels of LPS. Furthermore, antibiotic treatment of HF mice reduces intestinal permeability and inflammation[114]. A study using GF mice has shown HF diet does not induce ileal and colonic inflammation in GF mice in comparison to conventional mice[115]. This suggests it is the interaction between microbiota and HF diet that may culminate as inflammation, and that HF diet on its own is not sufficient to induce the inflammatory phenotype observed.

It is now clear that the gut microbiota is a contributor to the weight gain and altered metabolism that accompanies obesity[107,116]. Gut microbiota affect host metabolism by modulating energy extraction[117], immunity[118], and lipid metabolism[116], where both dietary factors[119] and genetics[107] influence gut microbial composition[120] and interactions between the gut microbiome and immunity likely contribute to diseases with aberrant inflammation, including obesity.

1.3 Gut Microbiota during pregnancy

Little attention has been paid to female and indeed pregnant female gut microbiota. Since pregnancy is a state of increased adiposity with insulin and leptin resistance and altered immunity, recent interest into the role of intestinal commensals in pregnancy adaptation

has emerged. The concept that maternal gut bacterial populations may change over the course of pregnancy to influence maternal and fetal metabolic development is novel and will no doubt expand the way we view maternal adaptation to pregnancy.

1.3.1 Maternal gut microbiota during healthy pregnancy

Maternal gut bacterial load increases over the course of pregnancy in humans[121]. There is a significant change in gut microbiota composition from the first to the third trimester of pregnancy, accompanied by increased bacterial diversity between mothers[122]. Although the composition and diversity of the gut microbiota during the first trimester is similar to the that of nonpregnant healthy women, with advancing gestation there is an increase in the abundance of *Proteobacteria* and *Actinobacteria*[122]. Since an increase in *Proteobacteria* has been observed in inflammatory bowel disease, it has been suggested that a similar dysbiosis of the gut occurs during the third trimester of pregnancy[123]. Proinflammatory cytokines (such as IL6 and TNFα) are notably increased in stool samples collected from women during the third trimester compared to the first trimester[122]. GF mice inoculated with pregnant third trimester microbiota show signs of increased inflammation and greater adipocity; consistent with observations in GF mice that were inoculated with microbiota from an obese individual[122]. Together, these observations suggest that bacterial population shifts in the final trimester of pregnancy may be associated with the known changes in insulin sensitivity, adiposity and immune response that accompany normal pregnancy progression[122].

Although there is significant remodelling of the maternal gut microbiota from the first to third trimester[122], maternal microbial populations appear stable during the postnatal period[122,124]. Koren et *al*. reported that increased diversity persists in maternal microbiota for up to one month postpartum[122]. This observation was supported by Jost et *al*., who investigated gut microbiota during the perinatal period. They found that the maternal gut microbiome was dominated by the *Firmicutes* phylum, *Bacteroides* and *Bifidobacterium* and their density did not vary during the perinatal period[124]. Thus, it appears that the maternal gut does not immediately revert to its nonpregnant state post delievery during the lactation period. These persistent pregnancy-like microbial populations may serve to faciliate (or be manipulated by) maternal adapation to the new lactational environment, although there are no data describing how maternal gut microbiota during lactation influence maternal physiological function.

We have recently investigated changes in the mice gut microbiota over the course of pregnancy. The gut microbiota of pregnant mice were significantly altered compared to non=pregnant. There is an increase in the abundance of *Akkermansia*, *Clostridium*, *Bacteroides* and *Bifidobacterium* during pregnancy. Notably, the increase in *Akkermansia*, a mucin-degrading bacteria, occurs at the onset of pregnancy. In contrast, Fuller et *al*. recently reported that they observed no significant changes in the whole community over the course of gestation[125]. Although they did observe an increase in abundance of *Actinobacteria* on Day 3 post-partum and in *Bacteriodetes* on Day 20 post partum compared to pre-pregnancy levels[125]. The contradicting reports could be a result of different experimental conditions. Although both studies used C57BL/6 mice, the mice

were acquired from different sources and studied at different ages. Large variations in gut microbiota of genetically identical mice have been observed due to differences in age and housing conditions[126,127].

1.3.2 Maternal gut microbiota is affected by pre-pregnancy weight and diet

Preliminary studies have reported gut microbiota during pregnancy is affected by pre-pregnancy weight and weight gain over the course of pregnancy. In a 2010 study, gut microbiota composition during the first and third trimester was examined in 36 normal weight women (BMI < 25) and 18 overweight women (BMI > 30). Overweight pregnant women were found to have significantly higher levels of *Bacteroides* and *Staphylococcus*, where pregnancy weight gain was correlated with *Bacteroides* number[121]. In contrast, Santacruz and colleagues detected an increase in *Staphylococcus*, *Enterobacteriaceae* and *Escherichia coli* in overweight pregnant women and a decrease in *Bifidobacterium* and *Bacteroides*[128], not dissimilar to observations made in non-pregnant obese individuals. In mice, we have observed changes in pregnancy gut microbiota with a peri-conceptional HF diet. Distinctly different changes in microbial abundance were observed in HF mice compared to control in a gestational age-dependent manner[129]. We used PICRUSt in combination with our 16S rRNA gene sequencing data to predict changes in microbial metabolic pathways, where pregnant HF microbiota was enriched with genes that favor lipid metabolism, glycolysis and gluconeogenic metabolic pathways over the course of pregnancy[129]. Suggesting pregnant HF microbiota may be able to extract more energy from the diet, and thus maternal metabolic adaptations are altered during HF pregnancies.

SCFAs are one of the major metabolites produced by the gut microbiota during food fermentation, and levels of SCFAs depend on the composition of the gut microbiota[130]. Priyadharsini et *al*. have shown an association between maternal SCFA levels and metabolic parameters where serum acetate levels were associated positively with maternal weight gain during pregnancy[11]. In fact, evidence that SCFAs (particularly propionate and butyrate) contribute to inflammatory and metabolic disorders including obesity[130], suggests that obesity-induced changes in the pregnant gut microbiota may impart changes in metabolic pathways via SCFA production[11]. Studies looking at the effects of probiotic intake during pregnancy also provide evidence for a maternal microbiome-metabolism relationship. Laitinen and colleagues found that diet consultation along with administration of probiotics, such as *Lactobacillus rhamnosus GG* and *Bifidobacterium lactis Bb12,* during pregnancy improved maternal glucose tolerance and insulin sensitivity[131]. Probiotics and dietary counseling have also been associated with reduced risk of gestational diabetes mellitus (GDM)[132]. Although maternal overweight and excess weight gain during pregnancy is associated with aberrant gut microbiota, distinctly different from that of normal weight pregnant women, little is known about the impact of this dysbiosis on maternal or fetal metabolism during pregnancy. Obese microbiota, prior to pregnancy, could possibly alter maternal metabolic adaptation to pregnancy, causing augmented placental inflammation that could result in an adverse intrauterine environment, and impart increased risk of obesity in the offspring.

1.3.3 Do maternal gut adaptations influence placental and fetal development?

It is apparent that distinct adaptations take place in the maternal gut microbiota over pregnancy[133] and that maternal weight gain and BMI during this period influence maternal gut microbial populations[134]. It is likely that maternal gut microbiota modulates maternal obesity-induced changes in offspring metabolism since bacterial composition is critically dependent on nutrition[135]. Since the bacterial composition of the gut has been proposed to modulate weight gain, impair metabolism resulting in obesity[136] through its regulation of energy extraction[105], immunity[137] and lipid metabolism[136], it seems likely that the composition of the maternal gut microbiota would have downstream effects on pregnancy outcomes through effects on maternal adaptation to pregnancy, thus impacting placental function and finally, altering the fetal intrauterine environment.

Currently, it is unknown whether changes in maternal gut microbiota pregnancy influence maternal gut epithelial barrier function or play a role in the immune adaptations associated with pregnancy. As of now, there is only one study to suggest intestinal barrier is altered by pregnancy. Reyes et *al*. measured intestinal permeability in non-pregnant, healthy pregnant, and pregnant women with intrahepatic cholestasis of pregnancy (ICP) to link increased intestinal permeability with hepatic inflammation associated with ICP[138]. They showed that pregnant women had increased intestinal permeability compared to non pregnant women as assessed by the timed blood dual sugar lactulose/rhamnose (L/R) ratio intestinal permeability test. How pregnancy induces increased intestinal permeability is unknown. However, if pregnancy is associated with changes in the gut microbiota and intestinal permeability, it is likely that maternal obesity

would further modulate this change. Changes in pregnancy microbiota due to maternal obesity may also manifest as metabolic endotoxemia. Inflammation in metabolic tissues, such as in the liver and adipose, due to increased endotoxin translocation across the gut has been well documented. What remains unknown is whether this can also result in activation of inflammatory pathways in the placenta.

Historically, bacteria and its components in the placental and fetal tissues have been associated with infection. Emerging evidence has confirmed the presence of bacteria in placenta and *in utero* environment during normal pregnancy. Moreover, it has been shown that the placenta harbours a microbiome more similar to that of the non-pregnant human oral microbiome[139]. These findings support other studies documenting the presence of Gram-negative bacteria in the placental samples in the absence of infection[140], and suggest the notion that the fetus may come in contact with maternal microbiota prior to birth. However, it is important to note that the presence of bacterial DNA is not the same as live bacteria, and thus far, live commensal bacterial populations have not been found to be present within the placenta. Furthermore, the source of these microbes that may exist in the placenta is unclear. Although, Aagaard et *al*. suggest that the maternal oral cavity may contribute to systemic bacterial counts that localize within the placenta—possibly during implantation[139]. Nevertheless, the concept that placental and fetal tissues are in contact with maternal microbes is gaining momentum. Whether changes in maternal gut microbiota due to obesity could alter the profile of bacteria that harbour placental and fetal tissues has not yet been explored.

1.4 Rational

For decades, genetics, unhealthy lifestyle and diet have been considered the main factors contributing to the development of obesity. The emergence of developmental origins of health and disease (DOHaD) hypothesis has provided an alternative explanation for the rapid rise in the prevalence of obesity to epidemic proportions. The DOHaD concept proposes that risk of developing a disease in adulthood is influenced by processes that occur during early fetal life[4]. The fetus makes physiological adaptations in response to cues it receives from the intrauterine environment during pregnancy, in order to maximize its rate of survival postnatally[4,141]. Thus, the trajectory of developing obesity may begin before birth due to the physiological changes the fetus makes in response to intrauterine insults. Multiple epidemiological studies and animal studies have shown a relationship between and onset of metabolic disease in the offspring[142].

Maternal obesity is a key predictor of childhood obesity and metabolic complications in adulthood. In Canada, the percentage of overweight or obese women rose from 34% in 1978 to 53% in 2004[143]. This increase has been accompanied by a sharp rise in adolescent obesity, from 2% to 10% among boys and 2% to 9% among girls[144]. The mechanistic link between maternal and offspring obesity has been extensively investigated in both clinical and experimental settings[4]. It is well known that obese women are at risk for several pregnancy-associated complications, such as increased risk of miscarriage, stillbirth, gestational diabetes, and preeclampsia[9]. Maternal obesity has been shown to result in fetal macrosomia and modify placental development[10]. Animal studies have shown maternal obesity induces offspring obesity, hepatic steatosis,

hypertension, and insulin and leptin resistance[24-27]. Furthermore, studies in rodents and sheep have observed increased placental inflammation during obese pregnancy[37,70]. It has been proposed that obesity during pregnancy results in an adverse intrauterine environment; however, the underlying mechanisms that cause maternal obesity to trigger development of obesity in the offspring remain unclear.

Recently, research into the gut microbiota has become one of most studied factors influencing metabolic disease risk. The trillions of microbial cells inhabiting the gut have a symbiotic and mutualistic relationship with their host[145]. The successful establishment of commensal gut microbiota is essential for metabolizing indigestible polysaccharides, producing essential nutrients and protecting against colonization by pathogenic bacteria[77]. In 2012, Koren et *al*. showed that gut microbiota is remodeled over the course of pregnancy in humans[122]. They observed that when germ-free mice were inoculated with third trimester microbiota, mice show signs of increased inflammation and greater adipocity consistent with observations in germ-free mice inoculated with microbiota from an obese individuals[122]. Although the mechanisms modifying gut microbial populations during pregnancy remain unclear, it is possible that changes in a mother's microbial makeup plays a role in the metabolic adaptations that must occur in order to accommodate the developing fetus. In a non-pregnant state, obesity is accompanied by dysbiosis of the gut microbiota, impaired intestinal barrier function and increasing intestinal permeability, resulting in metabolic endotoxaemia[112,114,146]. Whether similar processes occur during maternal obesity and how these intestinal adaptations would affect placental and fetal development is unknown.

Thus, using a mouse model of HF diet induced obesity, the following thesis aims to determine how an obesogenic maternal diet affects pregnancy microbiota and maternal intestinal barrier function and whether altered maternal gut adaptations to pregnancy influence placental development.

1.5 Hypothesis

We hypothesize that maternal diet-induced obesity modifies the composition of the maternal gut microbiota, and impairs maternal intestinal barrier function resulting in increased translocation of inflammation-inducing ligands into the maternal circulation and placental tissue. This may cause placental inflammation, culminating in changes to fetal metabolism that may underlie increased risk of chronic disease during adulthood.

1.6 Aim

The specific aims of this study were:

1. Determine changes in the maternal gut microbiota due to diet-induced obesity.
2. Evaluate intestinal barrier function during pregnancy and whether maternal intestinal adaptations during pregnancy are modified by HF diet.
3. Investigate whether maternal intestinal adaptations in the context of maternal obesity are associated with alterations in placental gene expression of pro-inflammatory cytokines.

2.0 MATERIALS AND METHODS

2.1 Animal model

All experiments were approved by the Animal Ethics 490 Committee at McMaster University (AUP 12-38-10). 3 week-old C57BL/6J mice were obtained from Jackson Laboratories. Mice were randomly assigned to two different nutritional groups: 1) control (Con, n=9; 17% fat, 29% protein, 54% carbohydrates, 3 kcal/g; Harlan 8640 Teklad 22/ 5 Rodent Diet) or 2) high-fat (HF) diet (HF; n=10; 60% fat, 20% protein, 20% carbohydrates, 5.24 kcal/g, Research Diets Inc. D12492) (Fig. 2.1.1). Mice were housed two per cage in standard mouse cages with free access to water and 150 g of food, in the same room with a constant temperature of 25°C, a 12-hour (hr) light, 12-hr dark cycle and maintained on their respective diets for 5 weeks. Body weights were recorded during the 6-week nutritional intervention. Following 6 weeks on their respective diets, fecal samples were collected from each mouse (identified as before mating (BM)). After 6 weeks of dietary intervention, all mice were housed with a C57BL/6J male and mating was confirmed by the appearance of a vaginal plug. The morning of vaginal plug identification is described as embryonic day 0.5 (E0.5)of gestation. Pregnant females were housed individually and provided with free access to water. Throughout pregnancy, maternal gestational weight was recorded, and maternal fecal samples were collected at E0.5, E10.5, E15.5 and E18.5 and stored at -80°C for future analysis. On E18.5, each mouse was sacrificed by cervical dislocation. Maternal cecal content and gut (all segments) tissue were snap-frozen in liquid nitrogen and stored at -80°C. Maternal blood from the tail was collected using capillary tubes (Fisher Brand, 22-362574, Ottawa,

Canada) and centrifuged at 4°C for 10 minutes (min) at 3000 rotations per minute (rpm), serum was collected and stored at -80°C. All placentas were collected from each mouse: half were snap-frozen in liquid nitrogen and stored at -80°C, and the other half were fixed in 4% paraformaldehyde (MP Biomedicals, 02199983, California, USA) and processed for haematoxylin and eosin staining to measure placental cross-sectional area.

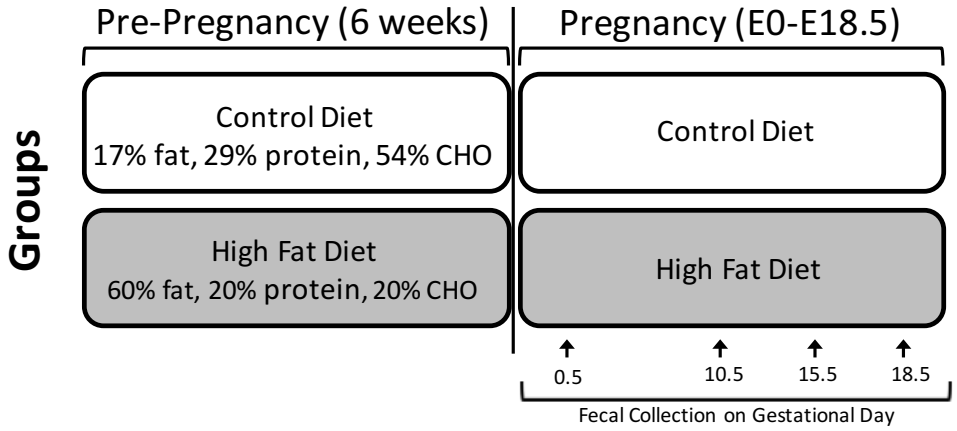

Figure 2.1.1: Schematic of animal model.
Three-week-old female C57BL/6J mice (n =19) were randomly assigned to one of 2 nutritional groups: 1) Control diet (Con; n = 9, 17% fat, 29% protein, 54% CHO, 3 kcal/g) or 2) high fat diet (HF; n = 10; 60% fat, 20% protein, 20% CHO, 4.73 kcal/g), fed for 8 weeks and then mated with viable males. Pregnancy was identified when a vaginal plug was visible and designated as embryonic (E) day 0.5 in both control (n = 9) and high fat (n = 10) groups. Fecal samples were collected from control mice prior to mating (before mating; BM), and then at time points during pregnancy (E0.5, E10.5, E15.5 and 18.5).

2.1.1 Genotyping fetuses

Fetal tails were transferred to a 1.5 ml microfuge tube containing 500 µL of lysis buffer (100 mM Tris-Cl (pH 8-8.5), 5 mM EDTA (pH 8.0), 200 mM NaCl, 0.2% (w/v) sodium dodecyl sulfate (SDS) and 100 g/mL proteinase K) and incubated overnight at 37°C. The following day, the samples were centrifuged at 13,000 rpm for 20 min. The supernatant was removed and transferred to a fresh microfuge tube containing 500 µL isopropanol (100%), and centrifuged at 13,000 rpm for 15 min. Isopropanol was gently decanted from the tubes and 700 µL of ethanol (100%) was added to precipitate the DNA. The DNA was pelleted by centrifugation and washed again in 75% ethanol. Ethanol was removed by pipetting and the DNA pellet was dried for 10 min at room temperature, and then dissolved in 50 µL ultrapure water.

One microliter of dissolved DNA was added to a 20 uL PCR reaction (0.1 U *Taq* DNA polymerase, 10X *Taq* buffer, 2.5 mM $MgCl_2$, 0.02 mM dNTPs, 7.5 µM *Sry* forward primer, 7.5 µM *Sry* Reverse Primer). Primers were for male specific *Sry* gene (5' TTG TCT AGA GAG CAT GGA GGG CCA TGT CAA 3', 5' CCA CTC CTC TGT GAC ACT TTA GCC CTC CGA 3'). BioRad C1000 Touch Thermal Cycler was used with the following program: 1 cycle 95°C 5 min, 30 cycles (95°C 30 sec, 58°C 30 sec, 72°C 1 min), 1 cycle 72°C 5 min. A 10 µL aliquot of each PCR product was loaded on a 1% agarose gel containing RedSafe. Electrophoresis was performed in 1XTAE (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) for 20 min at 110 V. The gel was visualized under UV transillumination using a BioRad ChemiDoc MP System.

2.2 Gut microbiota analysis

2.2.1 Genomic DNA extraction from fecal pellets

Fecal pellets collected from these mice were used to analyze gut microbial communities. Genomic DNA was extracted from fecal pellets as previously described[147] with a few modifications. Briefly, 0.2g of fecal material was mechanically lysed using 800µl 200 mM $NaPO_4$, 100 µl guanidine thiocyanate-ethylenediaminetetraacetic-Sarkosyl, and 0.2g of 2.8mm ceramic beads (Mo Bio). A further mechanical and enzymatic lysis was performed using 0.2 g of 0.1-mm glass beads (Mo Bio), 50 µl lysozyme (100 mg/mL), 50 µl mutanolysin (10 U/µl), and 10 µl of RNase A (10 mg/mL) and incubated for 1 hr at 37°C. A second enzymatic lysis using 25 µl 25% SDS, 25 µl proteinase K, and 62.5 µl 5 M NaCl was further incubated for 1 hr at 65°C. The resulting solution was centrifuged (12,000 x g), supernatant removed, and combined with an equal volume of phenol-chloroform-isoamyl alcohol in a new microcentrifuge tube. This was centrifuged (12,000 x g) and the solution of the lowest density removed and combined with 200 µl DNA binding buffer (Zymo), which was transferred to a DNA column (Zymo), washed, and eluted into sterile H_2O.

PCR amplification of the variable 3 (V3) region of the 16S rRNA gene was subsequently performed on the extracted DNA from each sample independently using methods described previously[147, 148]. 16S rRNA primers for the V3 region were used, modified to include adapter sequences specific to the Illumina technology, and 6-base pair barcodes to allow multiplexing of samples. DNA products of this PCR amplification

were subsequently sequenced using the Illumina MiSeq platform, resulting in 150bp paired-end reads.

2.2.2 Processing and analysis of 16S rRNA gene sequencing data

Resultant FASTQ files were processed using a custom in-house developed pipeline for the processing of 16S rRNA gene sequencing data used previously [147]. In this pipeline, Cutadapt[149], is used to trim any reads exceeding the length of the 16S rRNA V3 region, PANDASeq[150]to align paired-end reads, AbundantOTU+[151] to group reads into Operational Taxonomic Units (OTUs) based on 97% similarity, and the RDP Classifier [152] as implemented in Quantitative Insights Into Microbial Ecology (QIIME) [153] against the Feb 4 2011 release of the Greengenes reference database [154,155] to assign a taxonomy to each OTU. Any OTU not assigned to the Bacterial domain were culled, as was any OTU to which only 1 sequence was assigned. This processing resulted in a total of 11027452 reads (mean 137843 reads per sample; range: 59233-249890) and 9237 OTUs.

2.4 SCFA analysis using Gas Chromatography Mass Spectrometry (GC-MS)

SCFA levels were measured in maternal fecal samples by the McMaster Regional Centre of Mass Spectrometry. Exactly 30 mg of each frozen fecal sample was added to 1.5 mL Eppendordf tube that had been sonicated in methanol for 20 min prior to use. Weight equivalent amount of 3.7% HCl, 10 uL of internal standard and 500 uL of diethyl either were added to each sample, and vortexed for 15 min. After vortexing, 400 uL of diethyl either fecal extract was transferred to clean 1.5 mL Eppendorf tubes. In a

chromatographic vial with insert, 20 μL of N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA) was added, after which 60 uL of diethylether fecal extract was added. The mixture was incubated at room temperature for 1 hr and analyzed using GC-MS (Agilent 6890N GC coupled to Agilent 5973N Mass Selective Detector).

2.4 Maternal intestinal barrier function

The *in vivo* intestinal permeability assay was used to assess intestinal barrier function during pregnancy in WT-Con and WT-HF mice. Tail plasma was collected from pregnant mice on gestational day 18.5 at the start of the assay. Mice were administered 150 μl of 80 mg/ml FITC labeled dextran (4kDa) by oral gavage. After 4-h, tail plasma was again collected and mice were sacrificed to collect plasma from the fetuses. Fluorescence intensity of maternal plasma was collected before and 4-h after gavaging (emission 530 nM with excitation at 485 nM).

2.5 Bacterial fragments (ie. LPS) detection in maternal serum

An in-house colourimetric reporter bioassay was used for the quantification of NF-κB activation by the pattern recognition receptor TLR-4 in response to LPS. This assay was generated by the Bowdish lab at McMaster University. LPS-responsive reporter line was generated by transiently transfecting the pNifty2-SEAP plasmid into a commercially available HEK293 cell line expressing TLR-4, MD2, and CD14 (Invivogen, CA, USA). Cells were seeded at 4×10^3 per well in a 96-well plate in Complete DMEM for 24-h.

Serum samples were diluted 1:5 in phosphate buffer saline (PBS), then 1:1 in sterile water, and heat inactivated at 75 C for 5 min. Complete DMEM media was removed prior to the addition of heat-inactivated plasma in HEK Blue Detection (HBD) Media (Invivogen, CA, USA); 10ul of sample and 190 ul of HBD media was added into the appropriate wells. Readings were performed at 630 nm, 72-h after stimulation, and background levels were subtracted from relative absorbance units. Standard curves constructed using different doses of purified LPS were used to calculate the relative amount of bacterial product in serum.

2.6 Intestinal and placental gene expression analysis

2.6.1 RNA extraction and quantification

Tissue were homogenized using 900 uL of TRIzol (Life Technologies, Canada) and small homogenizing beads, and centrifuged at 12,000 x g for 10 min at 4 °C. Supernatant was removed and added to 300 uL of chloroform. The solution was thoroughly mixed and incubated at room temperature for 3 min. Samples were centrifuged for 10 min at 12,000 x g at 4 °C. Aqueous phase of the sample was removed and added to 500 uL of isopropanol. Samples were mixed thoroughly, incubated for 20 min at room temperature, after which they were centrifuged at 12,000 x g for 10 min at 4 °C. Supernatant was removed and the resulting pellet was washed twice with 75% ethanol. RNA was reconstituted in 20 uL ultrapure water. RNA was quantified using NanoDrop 2000 spectrophotometer (Thermo Scientific, Canada) with the NanoDrop 2000/2000c software (Thermo Scientific, Canada). The A260/A280 and A260/A230 ratio for all samples were

>2.0 and >1.5, respectively. All RNA samples were stored at -80°C until required.

2.6.2 cDNA synthesis

One thousand nanograms of total RNA were used for cDNA synthesis using the Superscript® VILO™ cDNA Synthesis Kit (Life Technologies, Canada) according to the manufacturer's instructions and using a standard thermocycler (Bio-Rad C1000 Touch). A single reaction was prepared per sample, consisting of 4µl of 5X VILO™ Reaction Mix, 2µl of 10X SuperScript® Enzyme Mix, 4 uL of RNA (concentration of 250 ng/uL) and 12 uL of ultrapure water to make the total reaction volume 20µl. Contents of the tube were gently mixed and placed into the thermocycler. The tube was incubated in the thermocycler at 25°C for 10 min, followed by 42 °C for 1-hr, and then 85 °C for 5 min. Samples were then stored at -20 °C until required for analysis.

2.6.3 Primer design and validation

Primer sets (Table 1.0) were designed using Primer-BLAST software available at the National Center for Biotechnology Information Website (blast.ncbi.nlm.nih.gov, NCBI) with the following settings changed from default (the rest remained default): product size must be between 50-150 base pairs; primer melting temperature must be between 58°C-62°C; and all primers must span an exon-exon junction. Primers were manufactured by Invitrogen, Life Technologies. Only primer sets producing a single peak in the dissociation curves were used for quantification of unknown samples.

Table 1.0: *Primer sequences for gene of interest.*

	Forward primer	Reverse primer
Housekeepers		
HPRT	CAGTCCCAGCGTCGTGATTA	TCGAGCAAGTCTTTCAGTCCT
β-actin	ACGCAGCTCAGTAACAGTCC	AGATCAAGATCATTGCTCCCCT
Cyclophillin	CTTCGAGCTGTTTGCAGACAAAAAG	TGGCGTGTAAAGTCACACC
UBC	CAAAGCCCCTCAATCTCTGGA	AGATCTGCATCGTCTCTCTCAC
SCFA receptors		
GPR41	GTCCAATACTCTGCATCTGTGAC	AGTCCCACGAGGAACACCAA
GPR43	ACTTGCCCAAGGAGTTCTGG	AGCTGTCTGCTCTTCACCAC
Tight junction proteins		
Tjp1 (ZO-1)	GAGAGACAAGATGTCCGCCA	CCATTGCTGTGCTCTTAGCG
Occludin	ATGTCCGGCCGATGCTCTC	TTTGGCTGCTCTTGGGTCTGTAT
Mucins		
Muc1	CAAGTTCAGGTCAGGCTCCG	TGACTTCACGTCAGAGGCAC
Muc4	AGATGGCTCTGAACCTAAGTATGC	AGGTGGTAGCCTTTGTAGCC
Inflammatory markers		
TLR4	TCTGGGGAGGCACATCTTCT	TGCTCAGGATTCGAGGCTT
TLR2	ATGTCGTTCAAGGAGGTGCG	ATTTGACGCTTTGTCTGAGGTTT
NFκB	CTGCTCAGGTCCACTGTC	TTGCGGAAGGATGTCTCCAC
IL-6	GGGACTGATGCTGGTGACAA	ACAGGTCTGTTGGGAGTGGT
TNFα	GTAGCCCACGTCGTAGCAAA	TTGAGATCCATGCCGTTGGC
Traf6	TGGTTGTGAAGTCTCAGCGT	GAAGACCCAAGTTTCCGTGC
IL-10	ACTTTAAGGGTTACTTGGGTTGC	CCTGGGGCATCACTTCTACC
Immune cell markers		
CD4	CTCCTTCGGCTTTCTGGGTT	GCCTGGCGCTGTTGGT
CD19	CAAGGTCAGCAGTGTGGCTC	CCACTGGGACTATCCATCCAC
CD11c	ACACTGAGTGATGCCACTGT	TTCGGAGGTCACCTAGTTGGG
CD68	TGTTCAGCTCCAAGCCCAAA	GTACCGTCACAACCTCCCTG
EMR1	TTGTGGTCCTAACTCAGTCTGC	AGACACTCATCAACATCTGCG

Abbreviations: HPRT, hypoxanthine&phosphoribosyltransferase; UBC, ubiquitin C; GPR41, G-protein coupled receptor 41; GPR43, G-protein coupled receptor 43; Tjp1, tight junction protein 1; ZO-1, Zonula occludens protein 1; Muc1, mucin 1; Muc4, mucin 4; TLR4, toll-like receptor 4; TLR2, toll-like receptor 2; NFκB, nuclear factor kappa B; IL-6, interleukin 6; TNFα, tumor necrosis factor α; Traf6, TNF receptor associated factor 6; IL-10, interleukin 10; CD4, cluster of differentiation 4; CD19, cluster of differentiation 19; CD11c, complement component 3 receptor 4 subunit; CD68, cluster of differentiation 68; EMR1, EGF-like module-containing mucin-like hormone receptor-like 1.

2.6.4 Quantification polymerase chain reaction (qPCR) assays

To quantify intestinal and placental gene expression levels, quantitative PCR assays were performed using the LightCycler 480 II (Roche) in 384-well plates (LightCycler 480 Multiwell Plate 384 White; Roche, 04729749001, Canada). For each experiment, a well contained 2.5 µl of cDNA (diluted 1:100), 1 µl of 5 µM Forward Primer, 1 µl of 5 µM Reverse Primer, 0.5 µl of H_2O, and 5 µl of LightCycler 480 SYBR Green 1 Master (Roche, 04887352001, Canada). The following PCR cycling conditions were used for each assay: enzyme activation at 95°C for 5 min (Stage 1); amplification of the gene product through 60 successive cycles of 95°C for 10 sec then 60°C for 10 sec then 72°C (Stage 2); followed by a melting curve starting at 65°C and ending at 95°C (Stage 3). Each plate for a gene of interest contained a standard curve of 10-fold serial dilution was generated using intestinal or placental pooled cDNA, and consisted of at least 5 standard curve points. Each sample and standard curve point was run in triplicate and each standard curve had a negative control (containing water instead of cDNA).

2.6.5 Analysis of qPCR results

The crossing point (Cp) of each well was determined via the second derivative maximum method using LightCyler 480 Software, Release 1.5.1.62 (Roche). An arbitrary concentration was assigned to each well based on the standard curve Cp values by the software. The concentration of each sample was determined by calculating the geometric mean of the triplicate for each sample. This value was calculated for every gene of interest in Table 1.0. The geometric mean of housekeeping genes was determined to use

as reference gene value for each sample. HPRT, β-actin and cyclophillin were used as housekeepers for the intestinal samples; UBC and β-actin were used as housekeepers for the placental samples. The concentration of each sample for a gene of interest was divided by the geometric mean of the selected housekeepers, to determine the relative mRNA levels of the gene in the sample.

2.7 Measuring maternal serum TNFα and IL-6

Maternal circulating TNFα and IL-6 levels were measured using Milliplex® Map Mouse Cytokine Magnetic Bead Panel (EMB Millipore, MCYTOMAG-70K, Darmstadt, Germany), that uses the Luminex xMAP detection method. The protocol was followed as per the manufacturer's instructions.

2.8 Measuring placental cross sectional area

2.8.1 Placental tissue processing and sectioning

Placentae fixed in 4% paraformaldehyde were processed by an automated tissue processor (Leica Biosystems, TP1020, Concord, Canada) with the following incubations: 70% ethanol (2x1 hr), 90% ethanol (2x1 hr), 100% ethanol (3x1 hr), and xylene (51 hr). Processed placentae were paraffin-embedded using an embedding center, dispenser, and hot plate (Leica Biosystems, EG1160, Concord, Canada). Placenta cross sections were cut using HM 325 Rotary Microtome (Thermo Scientific, 902100). The diameter of a mouse placenta is approximately 7 mm. In order to measure cross sectional area from the center of the placenta, 3400 μm of placenta was cut before collecting three 4 μm sections from

the center for cross sectional area analysis. Cut placental sections were placed in a 37°C paraffin section flotation bath (Electrothermal, MH8517, Staffordshire, UK) and placed onto Superfrost™ Plus microscope slides (Fisherbrand™, 12-550-15, Ottawa, Canada). Slides were dried overnight at 37°C, and then stored at room temperature.

2.8.2 Haematoxylin and eosin staining

Haematoxylin and eosin staining was performed on placental sections to analyze their cross sectional area. Sections were deparaffinized in xylene (3x5 min), rehydrated through an ethanol gradient (100% EtOH, 2x2 min; 90% EtOH, 2x2 min; 70% EtOH, 2x2 min; 50% EtOH, 2x2 min) and incubated in PBS for 5 min. Slides were rinsed in cold tap water for 1 min, and placed in filtered Haematoxylin Stain 2-Gill Method (Fisher Chemical, CS-401-1D, Ottawa, Canada) for 3 min. Excess haematoxylin was removed by rinsing slides under cold tap water until water was transparent. Background staining was reduced by differentiating slides in 1% acid alcohol (120 mM HCl in 70% EtOH) followed by rinsing slides in cold tap water for 1 min. Slides were incubated in bluing agent, Scott's Tap Water (42 mM $NaHCO_3$, 167 mM $MgSO_4$, filtered), for 1 min, to enhance of the contrast of the staining. Slides were rinsed again in running cold tap water for 1 min followed by incubation in 0.25% Eosin Y (Fisher Chemical, SE22-500D, Ottawa, Canada) in Sodium Acetate Buffer (200 mM, pH 5.5) for 4 min and briefly rinsed in cold tap water for 10 sec. Placental sections were dehydrated through an ethanol gradient (50% EtOH, 1 dip; 70% EtOH, 1 dips; 90% EtOH, 1 dip; 100% EtOH, 1 dip) followed by incubation in xylene (3x5 min). Sections were cover-slipped using

Permount™ Mounting Medium (Fisher Chemical, SP15-100, Ottawa, Canada), and dried overnight at room temperature.

2.8.3 Measuring placental crossing sectional area

The stained placental sections were visualized using the Nikon Eclipse *Ni* microscope and images were captured using the Nikon DS-Qi2 Monochrome Microscope Camera. The area (μm^2) of the labyrinth and junctional zone of the placenta was measured using a NIS-Element AR Software. For each placenta, 3 sections were used to measure the area, and the average of the 3 sections was used to analyze the data.

2.9 NanoString nCounter gene expression system

A custom nCounter Reporter CodeSet designed by Dr. Kjetil Ask (McMaster University) was used to analyze gene expression using NanoString nCounter gene expression system. This CodeSet included 184 genes that were involved in inflammation, immunity, apoptosis, and ER stress. A 100 ng of total RNA was incubated overnight at 65°C with nCounter Reporter CodeSet, Capture ProbeSet and hybridization buffer. The next day, post-hybridization, the samples were processed using the nCounter Prep-station.

The raw NanoString was normalized to six housekeepers: UBC, TUBA1A, HPRT, IPO8, GusB and β-actin, and nSolver Analysis Software 2.5 was used to determine fold change between control and treatment groups.

2.10 Statistical analysis

Maternal gestational weight gain and change in gestational weight were analyzed using two-way repeated measures ANOVA, with maternal diet and gestational age as factors. Bonferroni post-hoc analyses were used for multiple comparisons where appropriate. Gut and placentae relative mRNA levels, intestinal permeability and TLR4 reporter assay data were all analyzed using a two-tailed, heteroscedastic, unpaired student's t-test, which assumed unequal standard deviation using Welsh's correction, where $p<0.05$ was considered statistically significant. For morphological analyses of placentae, sample size represents the number of litters analyzed; a representative male and female placenta was analyzed from each litter. Outliers were removed using the Grubb's test ($\alpha=0.05$). NanoString data were analyzed using a one-tailed Welsh's t-test.

All data are presented as box and whisker plots, where the whiskers indicate the maximum and minimum values, and the box represents 25th and 75th percentiles (the lower and upper quartiles, respectively), within which the 50th percentile (the median) is indicated. All data were analyzed using GraphPad Prism 6.0 (GraphPad Prism 6.0a Macintosh Version by Software Mackiev © Copyright GraphPad Software, Inc. 1994-2013).

Analyses of maternal microbiome data were completed using various open source software. Taxonomic summaries were computed using QIIME [153]. Measures of beta diversity were computed using Phyloseq's implementation of the Bray Curtis distance metric, and tested for whole community differences across groups using vegan's implementation of permutational multivariate analysis of variance (PERMANOVA) in

the adonis command[156]. These results were visualized via Principal Coordinates Analysis (PCoA) ordination[153]. Calculations of genera which differed significantly between mouse groups were computed using DESeq2[154] (considered significant if the p-value was < 0.01 after adjustment for multiple testing via DESeq2's implementation of the Benjamini-Hochberg multiple testing adjustment procedure) and visualized using R's ggplot2 package [157].

3.0 RESULTS

Recently, changes in the gut microbiota over the course of pregnancy have been observed in human and rodent pregnancies. We used a mouse model of HF diet induced obesity to examine how an obesogenic maternal diet affects pregnancy microbiota and maternal intestinal barrier function and to determine whether altered maternal gut adaptations to pregnancy influence placental pro-inflammatory status as an impetus to offspring disease risk.

3.1 Maternal and fetal phenotype

Females fed a high-fat (HF, n=10) diet for 6 weeks prior to mating were significantly heavier than mice fed control diet (Con, n=9) prior to mating. Gestational weight of Con and HF dams steadily increased over the course of gestation, and HF dams remained heavier than Con dams (Fig. 3.1.1A). There was a main effect of maternal diet on maternal weight gain during pregnancy ($p<0.0001$), HF dams remained significantly heavier than Con dams from embryonic (E) day 0.5 to E15.5 ($p<0.05$; Fig. 3.1.1A). The change in weight during pregnancy did not differ between the two groups of mice at E0.5, E10.5, E15.5 and E18.5 (Fig. 3.1.1B). Maternal fasting blood glucose levels at E18.5 were not changed by maternal HF diet (Fig. 3.1.1C). Maternal HF diet did not alter fetal weight at E18.5, litter size or sex ratio of the fetuses (Fig. 3.1.2A, B, C).

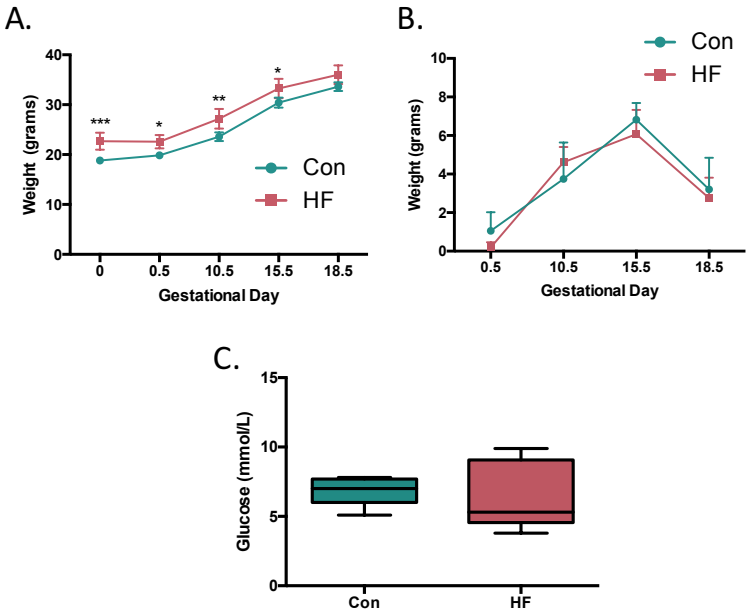

Figure 3.1.1: *Maternal phenotype.*
A. Maternal body weight over pregnancy in Con (n=9) and HF (n=10) dams **B.** Change in gestational weight over the course of pregnancy. **C.** Maternal fasting blood glucose levels on embryonic (E) 18.5.***p<0.001, **p<0.01, *p<0.05, data (A, B) were analyzed using two-way repeated measures ANOVA. Data (C) was analyzed using a heteroscedastic Student's t-test. Con = control, HF= high-fat maternal diet.

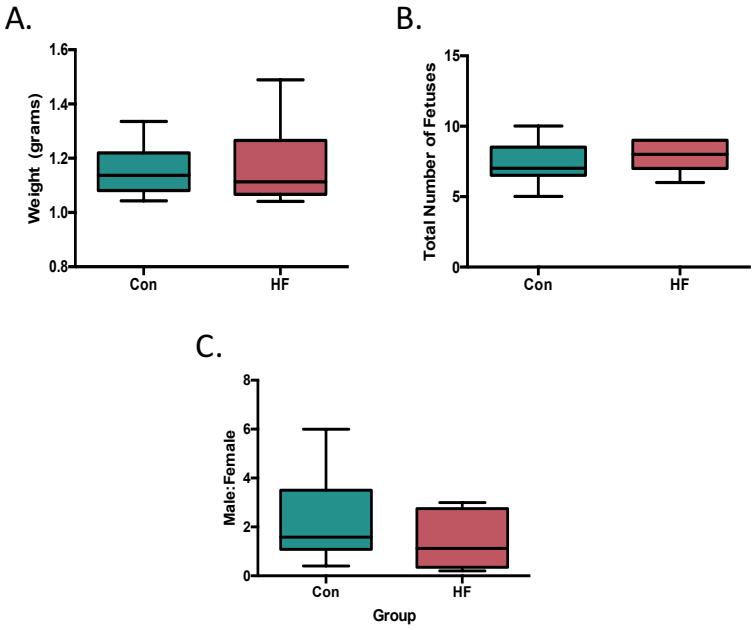

Figure 3.1.2: *Fetal weight, litter size and sex ratio is not affected by maternal HF diet.*
A. Fetal weight at embryonic (E) day 18.5 in Con (n=9) and HF (n=10) pregnancies. **B.** Litter size at E18.5 from Con (n=9) and HF (n=10) pregnancies. **C.** Sex ratio of Males:Females per litter. Data were analyzed using a heteroscedastic Student's t-test. Con = control, HF= high-fat maternal diet.

3.2 Maternal gut microbiota

To investigate pregnancy modulation of the gut microbiota, fecal samples were collected from Con mice before mating (BM), and on E0.5, E10.5, E15.5, and E18.5. The relative abundance of twenty-five most abundant taxa present in the gut microbiota of Con mice is displayed in Fig. 3.2.1A. When tested for whole community differences across different gestational time points using vegan's implementation of permutational multivariate analysis of variance (PERMANOVA) in the adonis command, microbial community in Con mice were found to not change over the course of pregnancy (PERMANOVA, $p=0.4$). We visualized the results using Principle Coordinate Analysis (PCoA) of the Bray Curtis distance and saw no separation between pregnant and non-pregnant Con samples (Fig. 3.2.1B).

Next we examined gut microbiota from HF mice to investigate how preconceptional and pregnancy HF diet effects the maternal gut microbiota. The relative abundance of the twenty-five most abundant taxa present in the gut microbiota of HF mice is displayed in Fig. 3.2.2A. There was a significant change in the maternal gut microbiota over the course of gestation in HF fed mice (PERMANOVA, $p=0.035$) when comparing samples from the 4 different gestational time points. However, when comparing HF non-pregnant samples to all HF pregnant samples, there are no whole community differences. The results were visualized on a PCoA of Bray Curtis distance, and no distinct separation between non-pregnant and pregnant samples was observed (Fig. 3.2.2B).

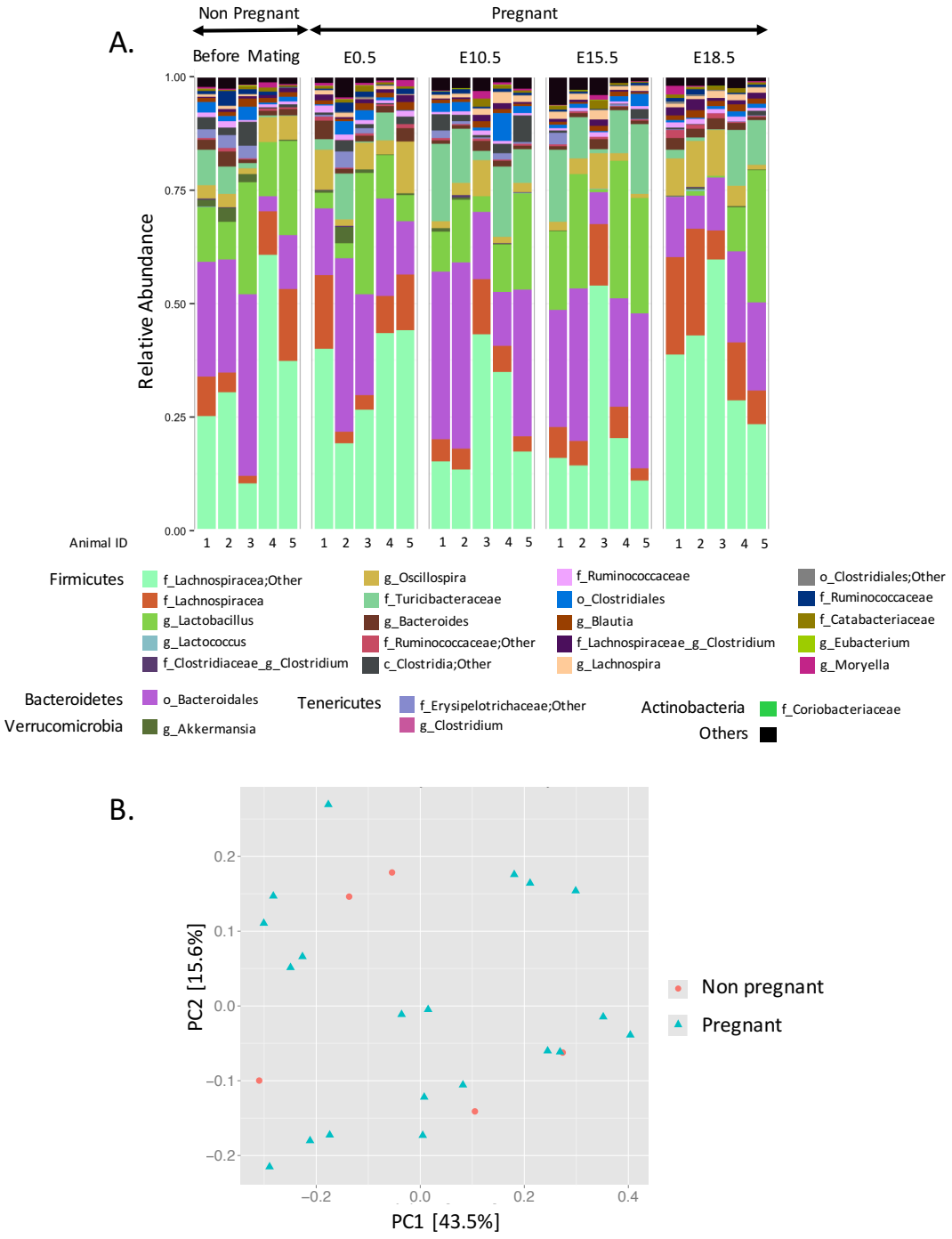

Figure 3.2.1: *Maternal gut microbiota of control fed mice.*
A. Taxonomic summaries of the relative abundance of the top 25 most abundant taxa for each control mouse (n=5) at non-pregnant and pregnant time points. The taxonomic summaries are displayed for five timepoints: before mating (non-pregnant samples), embryonic (E) day 0.5, E10.5, E15.5, and E18.5. The taxonomic classifications are listed in the taxa legend; o=order, f=family, g=genus. **B.** Principle Coordinate Analysis (PCoA) of the Bray Curtis distance shows no separation between pregnant and non-pregnant Con samples.

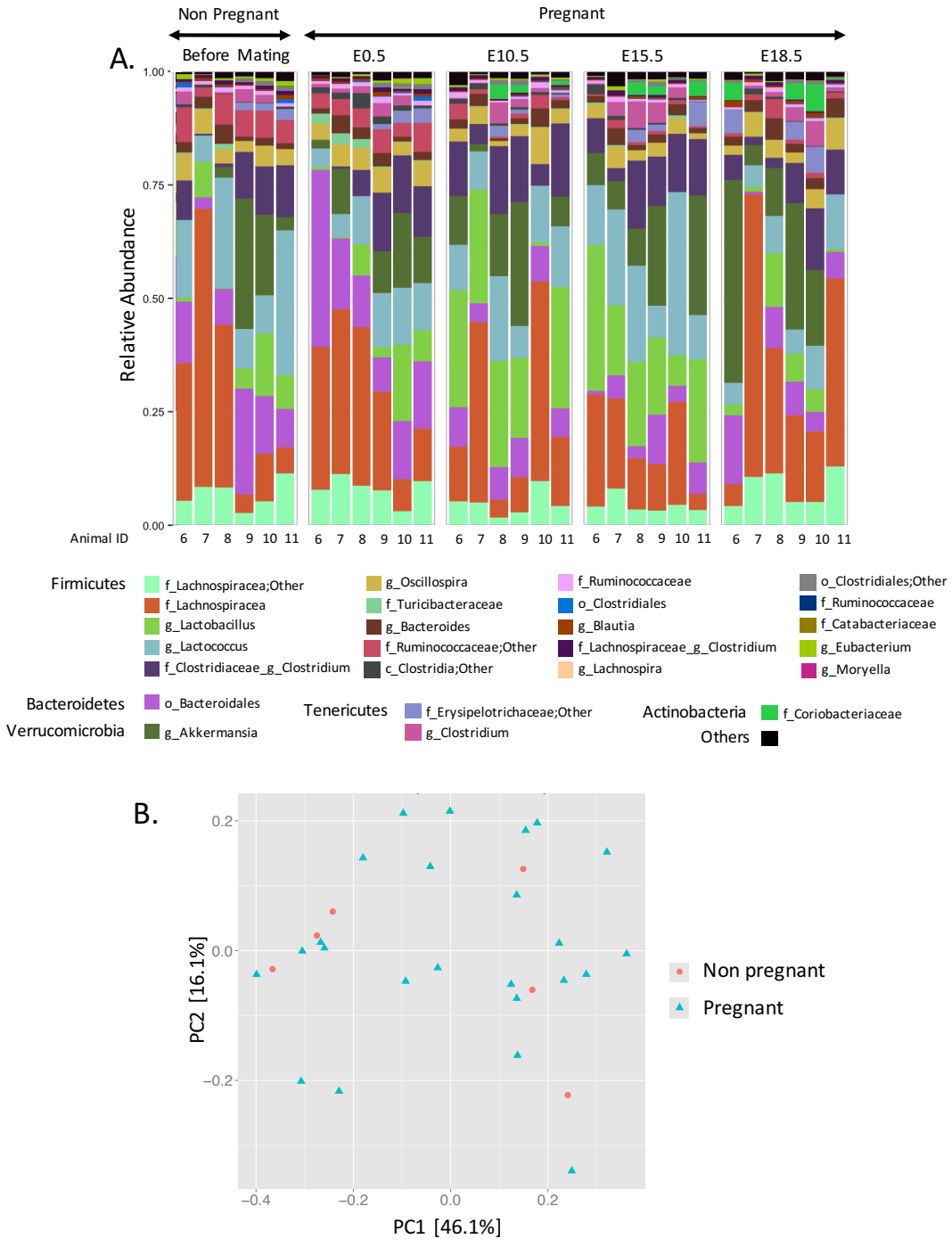

Figure 3.2.2: *Maternal gut microbiota of high fat fed mice.*
A. Taxonomic summaries of the relative abundance of the top 25 most abundant taxa for each high fat mouse (n=6) at non-pregnant and pregnant time points. The taxonomic summaries are displayed for five timepoints: before mating (BM; non-pregnant samples), embryonic (E) day 0.5, E10.5, E15.5, E18.5. The taxonomic classifications are listed in the taxa legend; o=order, f=family, g=genus. **B.** Principle Coordinate Analysis (PCoA) of the Bray Curtis distance shows no separation between pregnant and non-pregnant HF samples.

We compared HF microbiota to Con microbiota to investigate how HF diet intake effects gut microbiota. HF gut microbiota is significantly different from Con gut microbiota before pregnancy and at every gestational time point. The relative abundance of twenty-five most abundant taxa present in the gut microbiota of Con and HF mice at E18.5 is displayed in Fig. 3.2.3A. PCoA of Bray Curtis distance shows distinct separation of Con and HF mice (Fig. 3.2.3B). A more detailed investigation revealed 29 genera were significantly different between Con and HF pregnancies (Table 2.0). Of the genera identified as significant, 9 are increased in relative abundance and 20 are decreased in relative abundance in HF pregnancies (Fig. 3.2.4). In order to understand whether these changes were entirely due to diet or due to pregnancy, we compared the non-pregnant Con to non-pregnant HF samples, and found 17 significantly different genera. Out of the 17 genera different between the non-pregnant Con to non-pregnant HF samples, 16 genera are ones identified to be significantly different between Con and HF pregnant mice. Thus, the remaining 13 genera that are different between Con and HF pregnant mice are due to pregnancy-associated shifts and not diet induced shifts (Fig. 3.2.5).

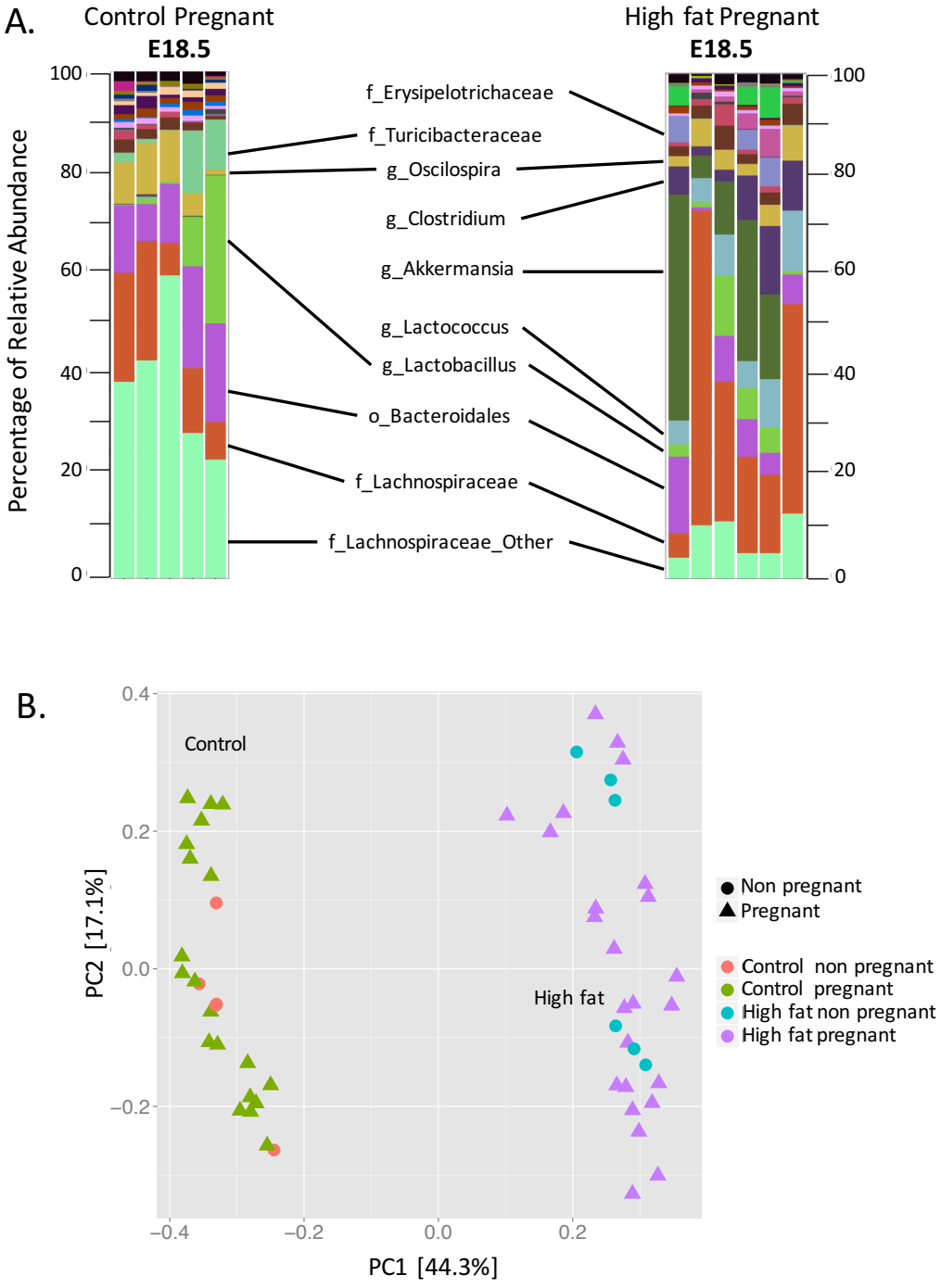

Figure 3.2.3: *Maternal gut microbiota during pregnancy is altered by maternal diet.*
A. Taxonomic summaries of the relative abundance of the top twenty-five most abundant taxa for each Con (n=5) and HF (n=6) mice at E18.5 show significant differences in microbial relative abundances. o=order, f=family, g=genus. **B.** Principle Coordinate Analysis (PCoA) of the Bray Curtis distance shows distinct separation based on maternal diet (PERMNOVA, p=0.001).

Table 2.0: *Summary of the statistically significant genera identified using DESeq2 to between pregnant Con and HF mice.*

Twenty-nine genera were found to have significant differences in their relative abundances between pregnant Con and HF animals. log2 fold changes are positive if the relative abundance of a genera was increased with the high fat diet and negative if the genera decreased with the high fat diet. Raw p-values as well as those adjusted for multiple testing are shown.

Phylum	Class	Order	Family	Genus	log2 Fold Change	p-value	adjusted p-value
Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	Akkermansia	4.155091262	2.84E-11	1.33E-10
Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Lactococcus	8.142986786	8.57E-90	1.74E-88
Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium	6.088297195	7.53E-36	7.66E-35
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillospira	-0.866607049	0.001210897	0.002735729
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Blautia	-1.735425063	7.23E-11	3.15E-10
Tenericutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	Clostridium	5.548838611	1.16E-19	7.87E-19
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Clostridium	-1.460194754	7.56E-10	2.88E-09
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Lachnospira	-10.44132034	3.30E-139	2.01E-137
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Moryella	-5.796526734	3.11E-35	2.71E-34
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ruminococcus	-10.06659583	5.57E-108	1.70E-106
Tenericutes	Mollicutes	Anaeroplasmatales	Anaeroplasmataceae	Anaeroplasma	-10.46193497	6.26E-76	9.54E-75
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Anaerostipes	-6.332599535	4.59E-18	2.55E-17
Firmicutes	Clostridia	Clostridiales	ClostridialesFamilyXIIIIncertaeSedis	Eubacterium	1.641983667	1.85E-07	5.93E-07
Tenericutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	Coprobacillus	2.686532665	5.10E-10	2.07E-09
Actinobacteria	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	Bifidobacterium	-5.575041095	4.75E-14	2.42E-13
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Lachnobacterium	-2.078467003	0.000593099	0.001391502
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Eubacterium	-9.809288291	7.39E-37	9.01E-36
Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Enterococcus	3.562054374	4.49E-19	2.74E-18
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Clostridium	-1.320364223	0.000141804	0.000376089
Actinobacteria	Actinobacteria	Coriobacteriales	Coriobacteriaceae	Adlercreutzia	-1.710778009	0.002788847	0.006075702
Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus	-1.706711373	0.000166257	0.00042257
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Coprococcus	-8.257513656	1.67E-29	1.27E-28
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Dorea	2.854172492	0.000233408	0.000569516
Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Moraxella	-2.621957483	1.00E-08	3.59E-08
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ethanoligenens	-7.219572534	2.90E-07	8.86E-07
Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	Peptostreptococcus	-5.889345017	3.87E-08	1.31E-07
Actinobacteria	Actinobacteria	Coriobacteriales	Coriobacteriaceae	Olsenella	5.579764305	5.01E-05	0.000139042
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Bacteroides	-2.023857178	0.003486314	0.00733328
Firmicutes	Clostridia	Clostridiales	Eubacteriaceae	Anaerofustis	-5.247547877	1.07E-05	3.11E-05

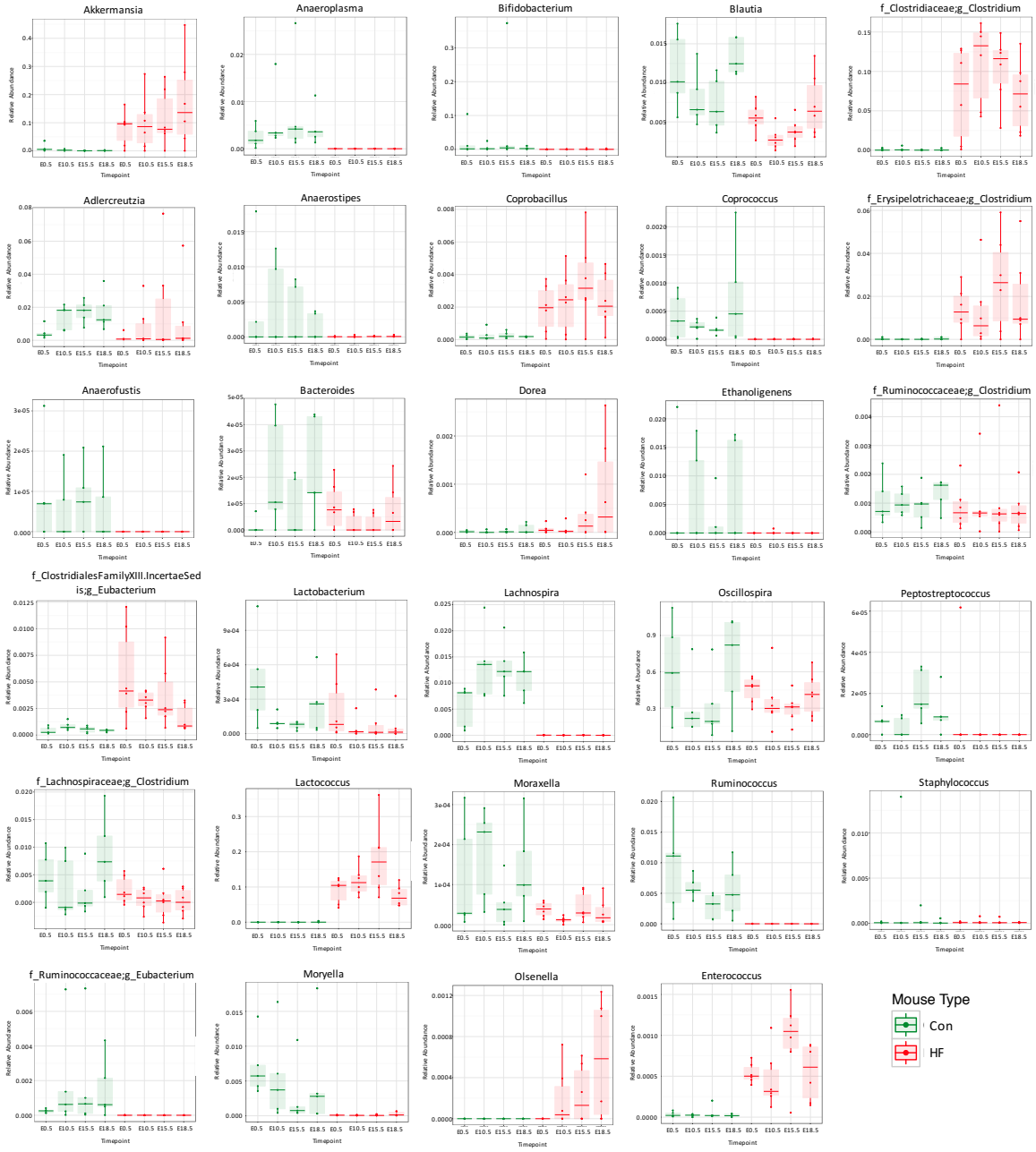

Figure. 3.2.4: *Relative abundance of 29 genera distinguished as significantly different between Con and HF pregnant mice, over the course of gestation.*

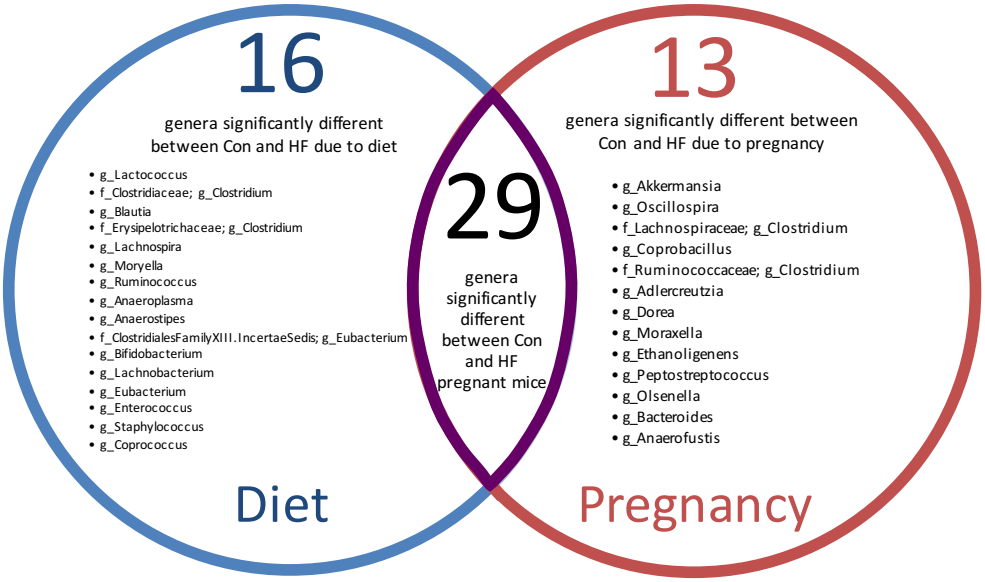

Figure 3.2.5: *Changes in relative abundance of genera between Con and HF pregnancies are due to diet and pregnancy.*
29 significantly different genera were identified between Con and HF pregnancies. Of these, 16 genera were different between Con and HF mice prior to pregnancy, however the relative abundance of 13 genera significantly differs between Con and HF mice only during pregnancy.

3.3 Maternal intestinal SCFA and GPR41, 43 gene expression

3.3.1 Fecal SCFA levels and their receptor expression levels during pregnancy are affected by maternal HF diet

Since SCFA are produced by fermentation of dietary fibers by anaerobic microbes of the gut and shifts in bacterial populations have been shown to impact levels of SCFA, we investigated whether gut microbial shifts due to maternal HF diet would affect SCFA levels. Our preliminary analysis suggests acetate, propionate, butyrate and lactate levels are lower in fecal samples of HF dams compared to Con at E18.5 (Fig. 3.3.1A). GPR43 and GPR41 are G-coupled protein receptors that are activated by binding of specific SCFAs. Maternal HF diet significantly decreased GPR41 mRNA levels in maternal

duodenum and jejunum ($p<0.05$) (Fig. 3.3.1B) but not GPR43 compared to Con mothers (Fig. 3.3.1C).

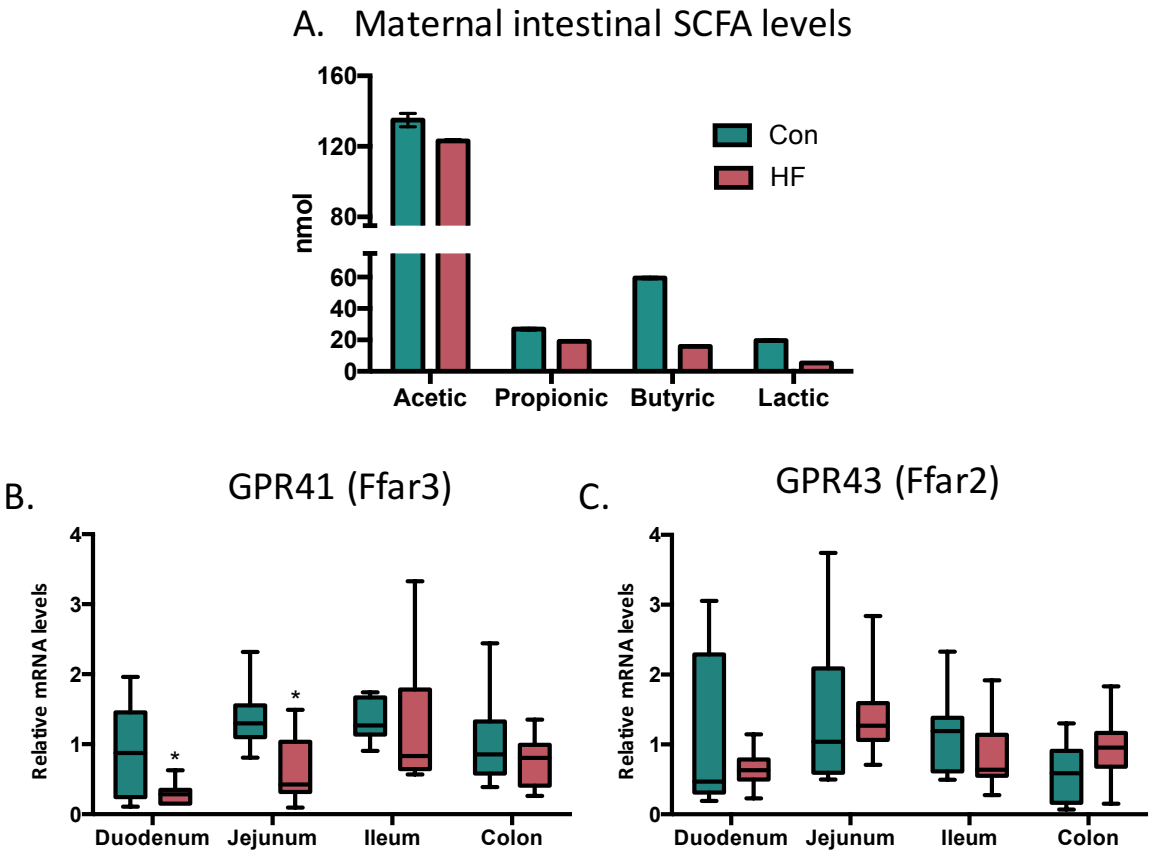

Figure 3.3.1: *Maternal fecal short chain fatty acid (SCFA) levels and SCFA receptor gene expression is altered by maternal HF diet.*
A. Pilot data of SCFA levels in maternal colon measured using Gas Chromatography Mass Spectrometry (GC-MS). Fecal samples from 9 Con and 10 HF dams were pooled; Con samples were analyzed in triplicate and HF samples were analyzed in duplicate. No statistical analysis has been performed. **B.** GPR41 (Ffar3) mRNA levels in Con (n=9) and HF (n=10) maternal gut tissue. **C.** GPR43 (Ffar2) mRNA levels in Con (n=9) and HF (n=10) maternal gut tissue. *$p<0.05$, significantly different from Con dams within the same gut section. Data (B, C) were analyzed using a heteroscedastic Student's t-test within gut sections. Con = control, HF= high-fat maternal diet.

3.4 Maternal intestinal barrier function

3.4.1 Intestinal barrier function is impaired during pregnancy and modulated by diet

We assessed intestinal barrier function towards the end of pregnancy (at E18.5) in three groups of mice: Con non-pregnant, Con pregnant and HF pregnant, to study the effect of pregnancy to determine whether diet impacted on pregnancy associated changes. Intestinal barrier function was significantly decreased with pregnancy (p=0.001) in Con mice (Fig 3.4.1) compared to non pregnant control females. There was a strong tendency for lower barrier function in HF dams compared to Con dams, however this difference did not reach statistical significance (p=0.0553; Figure 3.4.1).

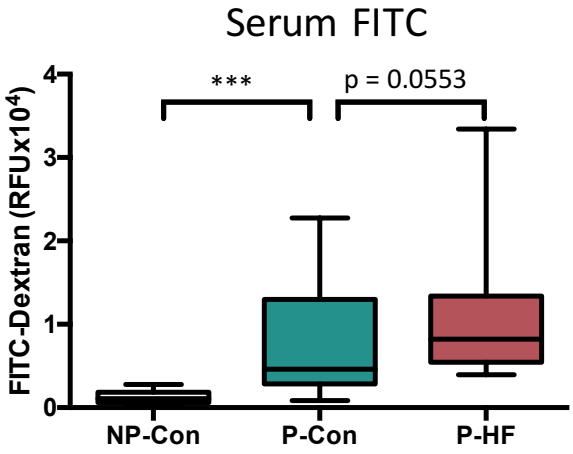

Figure 3.4.1: *Intestinal barrier function during pregnancy is compromised by HF diet.*
Blood FITC as a marker of barrier function. FITC measured in plasma of E18.5 dams four hours after oral gavage with 150 µl of 80 mg/ml FITC labeled dextran. NP-Con=non-pregnant control (n=6), P-Con=pregnant control (n=14), P-HF=pregnant high fat (n=14). ***p<0.001. Data were analyzed using a heteroscedastic Student's t-test. Con = control, HF= high-fat maternal diet.

3.4.2 Bacterial fragment (lipopolysaccharide (LPS) in maternal serum

Serum LPS levels in HF and Con dams was evaluated using a[56] TLR4 reporter bioassay and showed that HF dams demonstrated significantly higher levels of serum LPS compared to Con (p=0.014; Fig. 3.4.2).

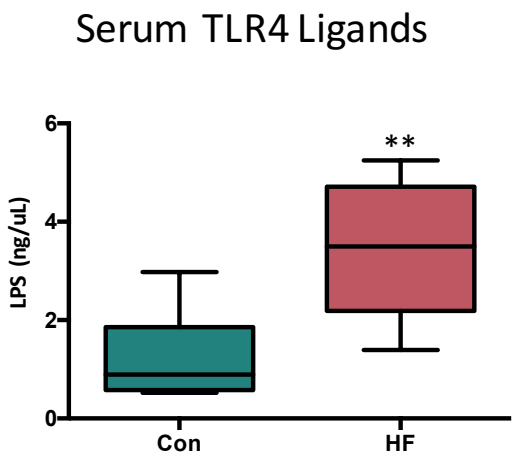

Figure 3.4.2: *TLR4 ligands (ie. LPS) are increased by HF diet during pregnancy*
Maternal serum LPS levels were determined using a colourimetric reporter bioassay based on the activation of TLR4 in Con (n=6) and HF (n=7) at E18.5. **p<0.01. Data were analyzed using a heteroscedastic Student's t-test. Con = control, HF= high-fat maternal diet.

3.4.3 Barrier proteins and mucin production during pregnancy in the context of maternal HF diet

At E18.5, TJP1 (ZO-1) mRNA levels were decreased in the jejunum and ileum of HF dams (p<0.05; Fig. 3.4.3A) compared to Con. Occludin mRNA levels were increased in the duodenum (p<0.001) and decreased in the jejunum (p<0.01; Fig. 3.4.3A).

A major component of the mucus layer covering the intestinal epithelial cells are mucins, secreted by goblet cells. Muc1 and Muc4 mRNA levels were increased in the

duodenum of HF dams compare to Con (Fig. 3.4.3B). The expression of either mucins was not changed in the other three gut segments.

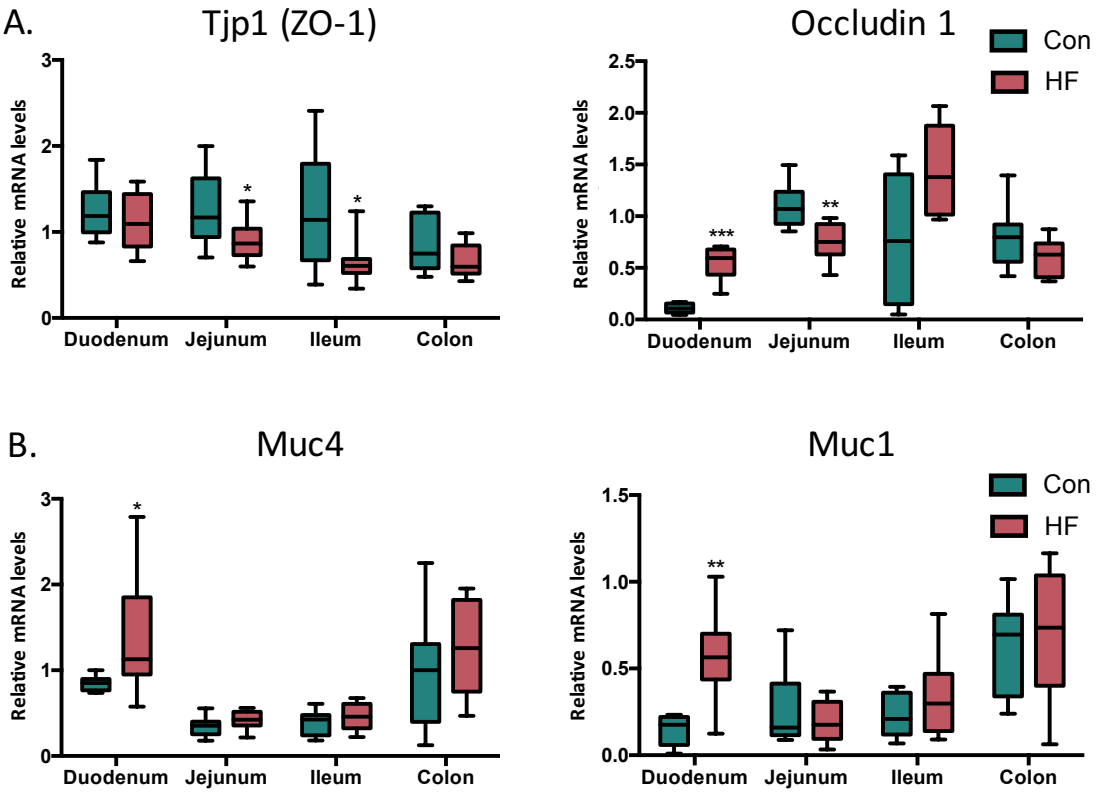

Figure 3.4.3: *Tight junction proteins and mucin gene expression is altered by maternal HF diet.*
A. Tight junction proteins, Tjp1 and occludin 1, mRNA levels in different gut sections of Con (n=9) and HF (n=10) dams. **B.** Mucins, Muc1 and Muc4, mRNA levels in different gut sections of Con (n=9) and HF (n=10) dams. *p<0.05, **p<0.01, ***p<0.001, significantly different from Con dams within the same gut section. Data were analyzed using a heteroscedastic Student's t-test within gut section. Con = control, HF= high-fat maternal diet.

3.5 Maternal gut inflammatory status

3.5.1 Gene expression of inflammatory markers in maternal gut

We measured mRNA levels of inflammation-associated genes in four gut sections of Con and HF dams to investigate whether maternal HF diet induced gut inflammation during pregnancy. TLR4 expression was significantly increased in HF dam colon ($p<0.01$; Fig. 3.5.1A), while TLR2 expression was increased only in the duodenum of HF dams ($p<0.001$; Fig. 3.5.1B). Transcription factor, NFκB, expression was reduced significantly in HF dam jejunum ($p<0.05$) and ileum ($p<0.0001$; Fig. 3.5.1C). TNFα expression was not altered by maternal HF diet in the ileum and colon (Fig. 3.5.1D), and gene expression could not be detected in the duodenum and jejunum. IL-6 expression was increased in the duodenum of HF dams compared to Con ($p<0.001$; Fig. 3.5.1E).

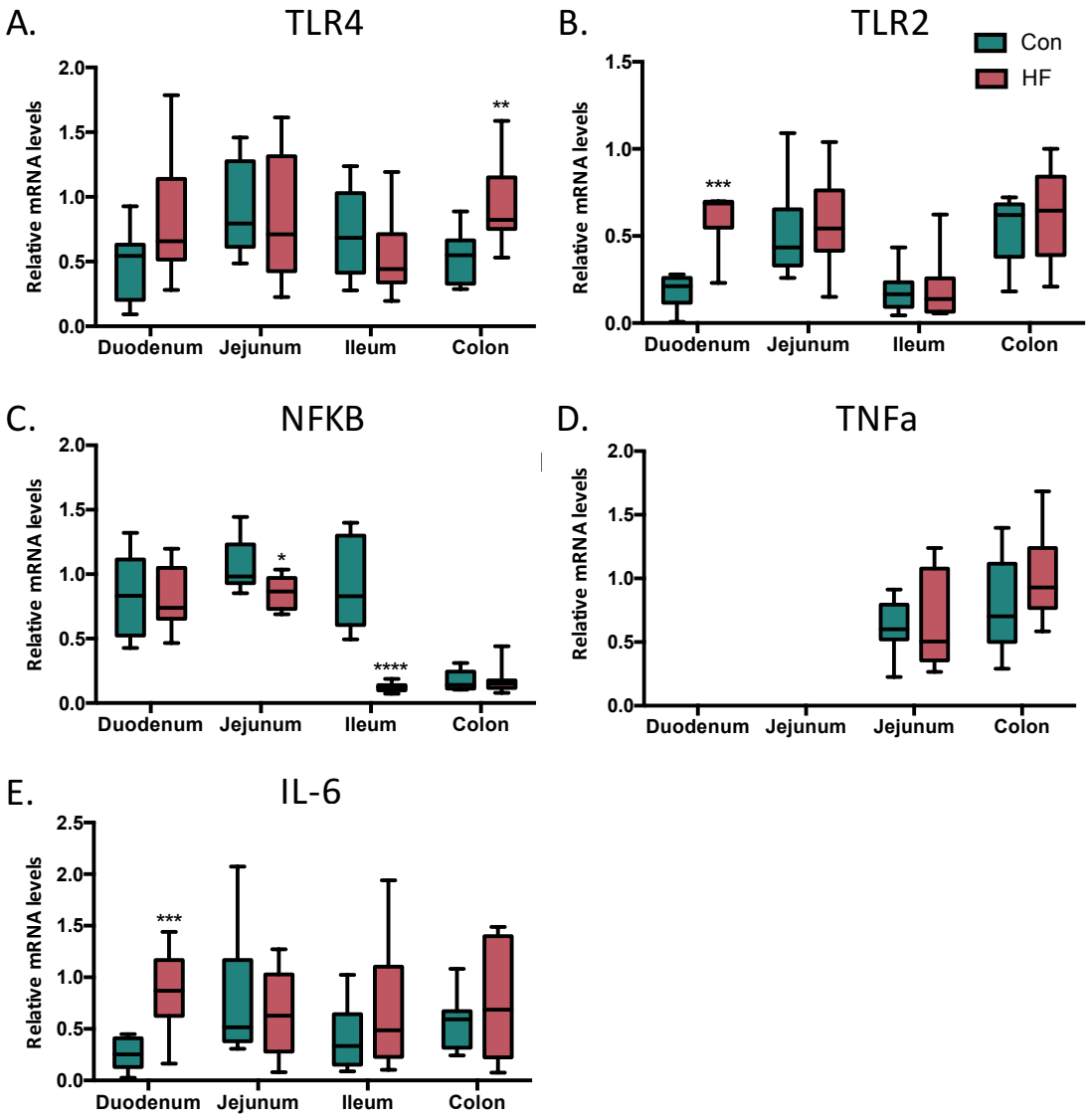

Figure 3.5.1: *Maternal intestinal inflammatory gene levels are altered by a HF diet.*
A. TLR4 mRNA levels in Con (n=9) and HF (n=10) maternal gut sections. **B.** TLR2 mRNA levels in Con (n=9) and HF (n=10) maternal gut sections. **C.** NF-κB mRNA levels in Con (n=9) and HF (n=10) maternal gut sections. **D.** TNFα mRNA levels in Con (n=9) and HF (n=10) maternal gut sections. **E.** IL-6 mRNA levels in Con (n=9) and HF (n=10) maternal gut sections. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, significantly different from Con dams within the same gut section. Data were analyzed using a heteroscedastic Student's t-test within gut section. Con = control, HF= high-fat maternal diet.

3.5.2 Cytokine levels in maternal serum

Despite higher levels of serum levels of TNFα and IL-6 in HF dams, these data showed significant variability and were not statistically difference between groups. (Fig. 3.5.2A, B).

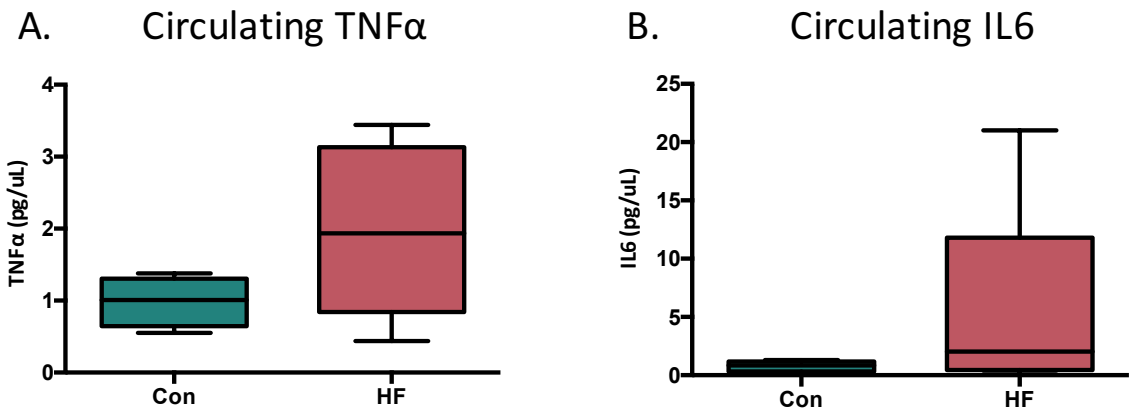

Figure 3.5.2: *Serum concentration of* TNFα and IL-6.
A. Circulating serum levels of TNFα in Con (n=5) and HF (n=8) dams at E18.5. p=0.1243. **C.** Circulating concentration of IL-6 in Con (n=5) and HF (n=8) dams at E18.5. p=0.3308. Data were analyzed using a heteroscedastic Student's t-test. Con = control, HF= high-fat maternal diet.

3.5.3 Gene expression of immune cell markers in maternal intestinal samples

CD4 mRNA levels, a protein found on the surface of T cells, were increased in maternal duodenum, jejunum and ileum of HF dams ($p<0.05$; Fig. 3.5.3A). CD19 mRNA levels, a B cell marker, were markedly increased in the duodenum ($p<0.0001$; Fig. 3.5.3B). CD11c mRNA levels, a dendritic cell marker, were not altered by maternal HF diet (Fig. 3.5.3C). While, macrophage markers EMR1 and CD68, were decreased in the jejunum ($p<0.05$; Fig. 3.5.3D) and increased in the duodenum respectively ($p<0.05$; Fig. 3.5.3E).

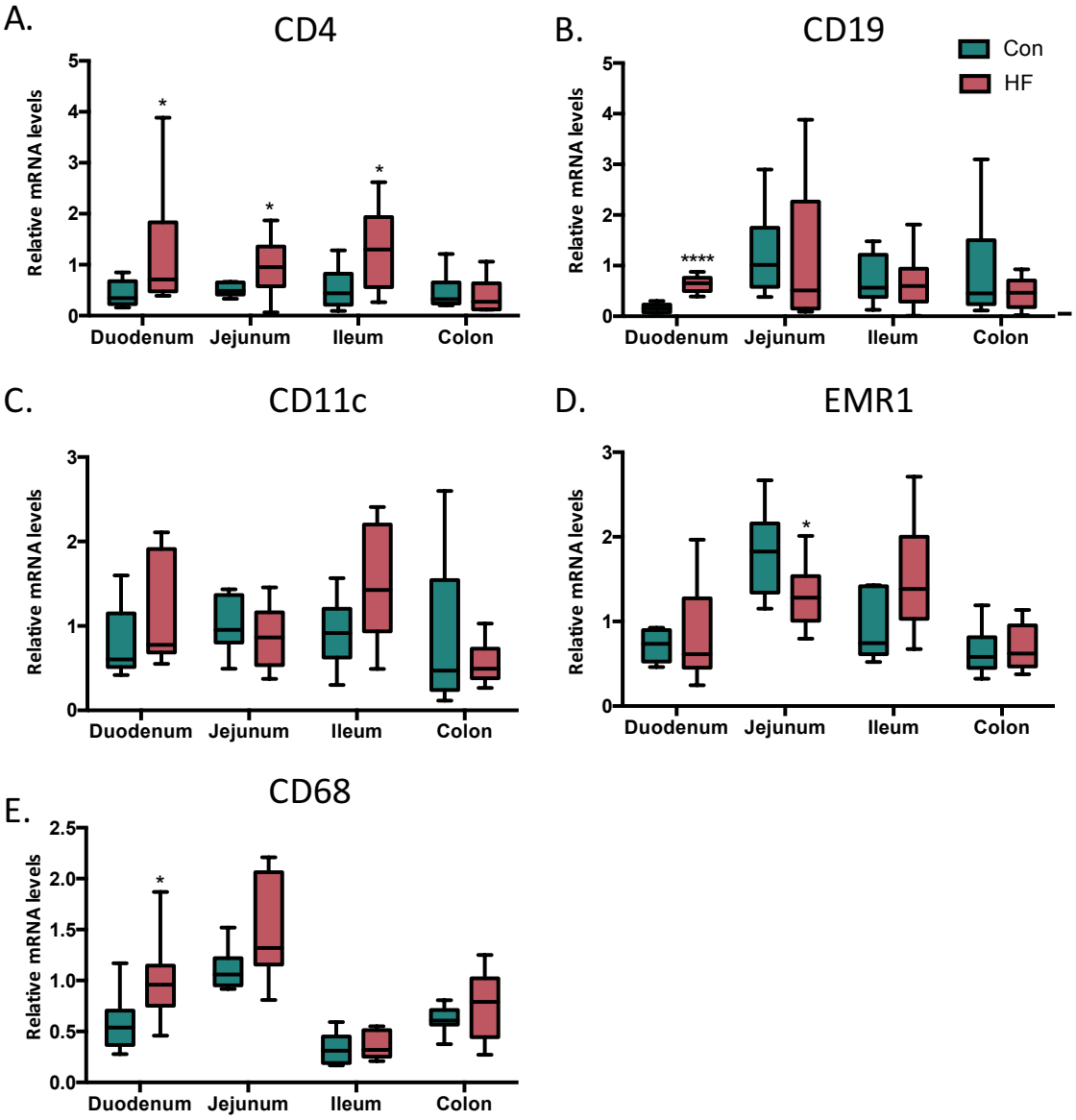

Figure 3.5.3: *Immune cell marker mRNA levels are altered by maternal HF diet.*
A. CD4 mRNA levels in Con (n=9) and HF (n=10) maternal gut sections. **B.** CD19 mRNA levels in Con (n=9) and HF (n=10) maternal gut sections. **C.** CD11c mRNA levels in Con (n=9) and HF (n=10) maternal gut sections. **D.** EMR1 mRNA levels in Con (n=9) and HF (n=10) maternal gut sections. **E.** CD68 mRNA levels in Con (n=9) and HF (n=10) maternal gut sections. *p<0.05, ****p<0.0001, significantly different from Con dams within the same gut section. Data were analyzed using a heteroscedastic Student's t-test within gut section. Con = control, HF= high-fat maternal diet.

3.6 Placental morphology and gene expression

3.6.1 Placental cross-sectional area is reduced by HF intake during pregnancy in a sex-specific manner

Cross-sectional area of the two distinct placental zones, the labyrinth zone and the junctional zone, was measured. Female placentae from HF dams had reduced labyrinth, junctional and total placental surface compared to female placentae from Con dams (Fig. 3.6.1A, B, C). Cross-sectional area of HF male placentae was not changed.

3.6.2 Placental gene expression analysis: NanoString analysis

The NanoString nCounter gene expression system was used to examine the expression of 180 candidate genes in 4 male and 4 female placentas from each group, Con and HF. A total of 16 genes were altered in HF pregnancies, 7 in female placental tissue and 9 in male placental tissue (Table 3.0). Among the changes, HF female placentae had increased mRNA levels of ER stress proteins, calreticulin ($p=0.0199$) and HSP47 ($p=0.0124$) (Fig. 3.6.2A), as well as changes in mRNA levels of apoptosis proteins where Bad mRNA was increased and Bcl2l11 mRNA levels were decreased (Fig. 3.6.2B).

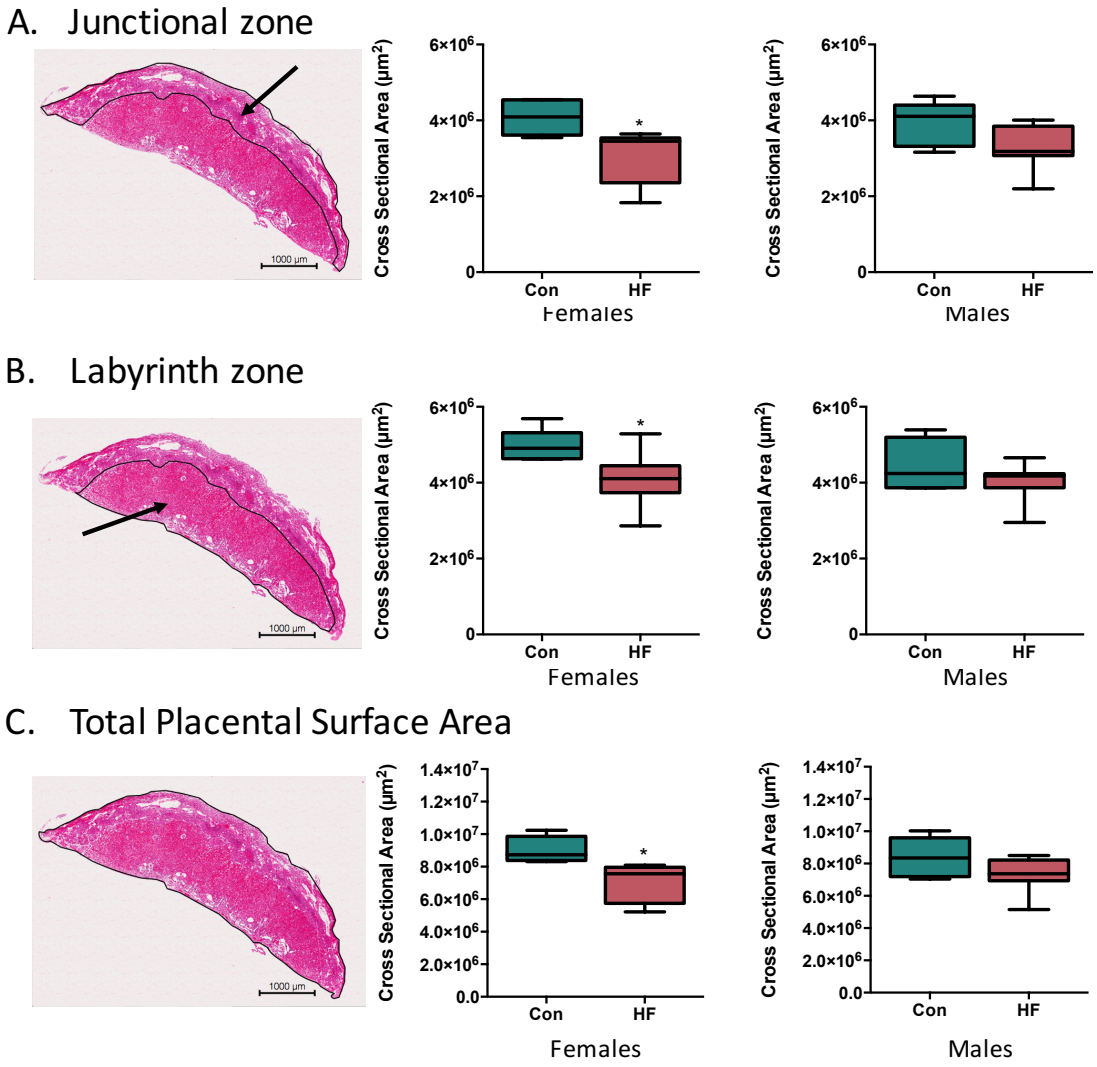

Figure 3.6.1: *Placental cross sectional area is decreased in females of HF dams.*
A. Cross sectional area of junctional zone of male and female placenta from Con and HF dams. **B.** Cross sectional area of labyrinth zone of male and female placenta from Con and HF dams. **C.** Total cross sectional area of male and female placenta from Con and HF dams. Con (n=7 male and n=7 female placentae) and HF (n=5 male and n=5 female placentae), 3 sections per animal were measured. *p<0.05; data were analyzed using a heteroscedastic Student's t-test. Con = control, HF= high-fat maternal diet.

Table 3.0: *NanoString nCounter gene expression results in male and female placentae from Con and HF dams.*
Results for all genes significantly different in males (n= 4 Con, n=4 HF) and females (n= 4 Con, n=4 HF) placentae. *p<0.05, data were analyzed using Welsh's t-test.

	Female Placentae		Male Placentae	
Gene	Fold change	p value	Fold change	p value
ATF4	-1.1	0.3731	-1.29	*0.0297
Bad	1.08	*0.0399	1.01	0.801
Bcl2l11	-1.32	*0.0228	-1.41	0.0679
BMPR1A (also called alk 3)	-1.03	0.7435	-1.09	*0.0388
Calreticulin	1.15	*0.0225	-1.09	0.5638
CD44	-1.18	*0.0342	-1.1	0.4379
HSP27	1.14	*0.0293	1.04	0.5722
HSP47	1.22	*0.0081	1.07	0.5727
IDO1 Indoleamine 2	1.31	*0.0192	-1.01	0.9112
sfrp2	1.14	0.7684	1.59	*0.0249
Traf6	-1.23	0.0694	-1.37	*0.0179
TrkC	1.42	0.2025	2.79	*0.006
VEGF	-1.22	0.0685	-1.38	*0.0487
VPAC1	-1.33	0.4675	-1.53	*0.0431
VPS-15	-1.03	0.3217	-1.14	*0.0184
VPS-34	-1.05	0.2256	-1.18	*0.02

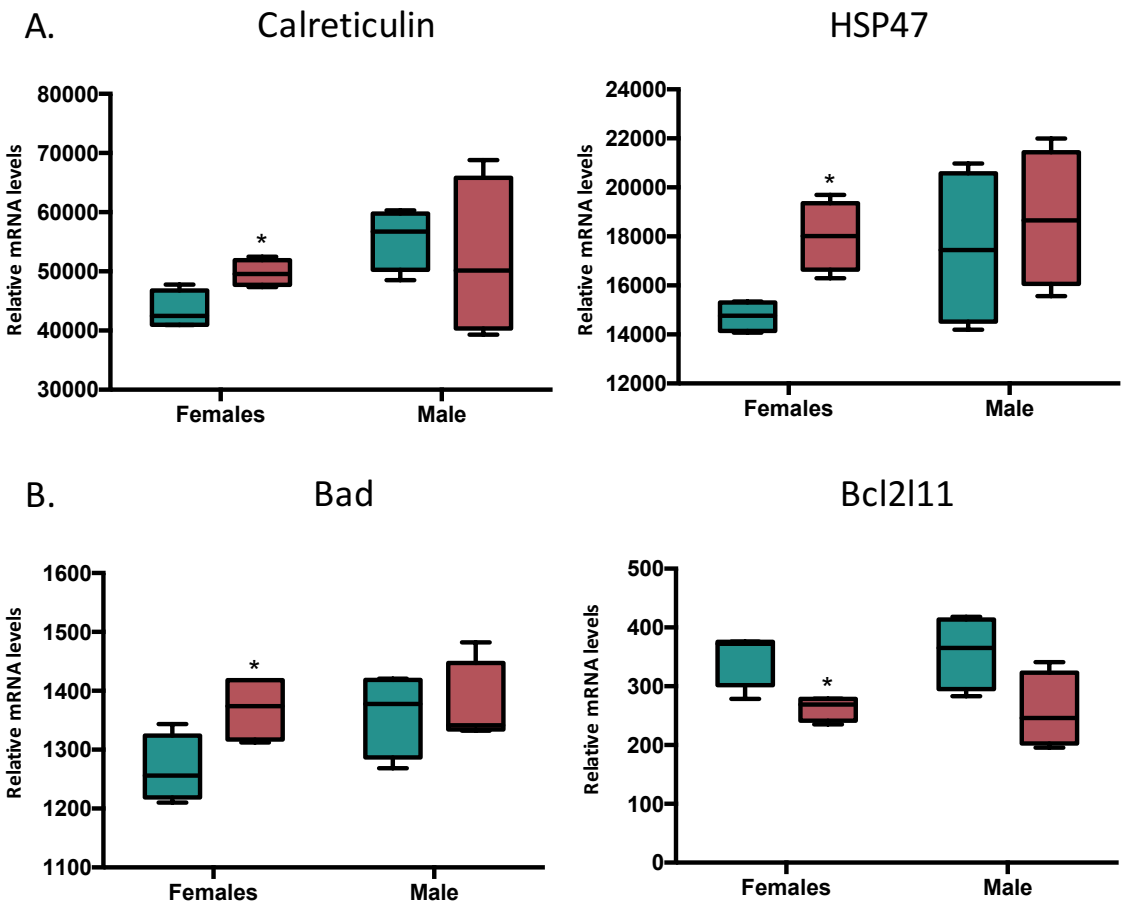

Figure 3.6.2: *Altered gene expression of ER stress and apoptosis related genes in HF female placentae collected on E18.5.*
A. ER stress proteins, calreticulin and HSP47, mRNA levels in male and female placentae from Con and HF dams. **B.** Apoptosis proteins, Bad and Bcl2l11, mRNA levels in levels in male and female placentae from Con and HF dams. C*$p<0.05$, Con (n=4 male and n=4 female placentae) and HF (n=4 male and n=4 female placentae), data were analyzed using a heteroscedastic Student's t-test. Con = control, HF= high-fat maternal diet.

3.6.3 Placental inflammatory and immune cell marker gene expression is altered by HF diet in a sex specific manner

Results for NanoString gene analysis for inflammatory and immune cell markers are presented in Table 4.0. Of the 23 possible targets, 2 were significantly different in female placenta and 1 was different in male placental tissue. Female placentae derived from HF pregnancies showed a modest increase in CD4 ($p=0.0447$) and a 2.36 fold increase in anti-inflammatory cytokine IL-10 ($p=0.0451$), whereas HF male placentae demonstrated lower levels of Traf6.

Follow-up analysis using qPCR of all placental tissue collected from Con (n=7 male and n=7 female placentae) and HF (n=8 male and n=8 female placentae) for female and male placenta was performed on inflammatory and immune cell markers identified in the NanoString analysis. Analysis of all samples in the groups demonstrated that Traf6 mRNA levels are increased in female HF placentae ($p<0.05$; Fig. 3.6.3C) compared to Con, but that IL10 mRNA is similar between groups (Fig. 3.6.3D). CD4 mRNA levels were decreased in HF male placentae ($p<0.05$) (Fig. 3.6.4A), and unchanged in female placentae; while EMR1 mRNA levels were increased in HF female placenta compared to control ($p<0.05$) (Fig 3.6.4C), No difference in mRNA levels for CD68 and CD11c was observed between Con and HF, female and male placentae (Fig. 3.6.4B, D). TLR4 and TLR2 mRNA did not change in male or female placentae due to maternal HF diet (Fig. 3.6.3A, B).

Table 4.0: *Subset of NanoString nCounter gene expression results in male and female placentae from Con and HF dams.*
Results for inflammatory and immune cell marker genes in male (n= 4 Con, n=4 HF) and female (n= 4 Con, n=4 HF) placentae. *p<0.05, data were analyzed using Welsh's t-test.

	Female Placentae		**Male Placentae**	
Gene	Fold change	p value	Fold change	p value
CD4	1.4	*0.0447	1.27	0.2122
CD68	-1.06	0.7209	-1.17	0.3059
Cd11c	-1.2	0.4292	1.11	0.7248
Emr1	1.23	0.4154	1.09	0.501
IFN-gamma	1.01	0.9629	1.61	0.111
IL-10	2.36	*0.0451	2.45	0.1633
IL-13	-1.35	0.2845	1.27	0.3846
IL-15	1.06	0.811	-1.05	0.6888
IL-1B	-1.09	0.706	1.11	0.3367
IL-2	-1.02	0.9009	1.28	0.4837
IL-21	-1.11	0.6495	-1.09	0.7894
IL-22	2.22	0.1908	2.08	0.1779
IL-23	-1.06	0.8337	1.66	0.4252
IL-4	1.3	0.565	1.28	0.2714
IL-6	-1.08	0.7831	1.62	0.2004
MCP1	1.31	0.1531	1.03	0.776
NFKB	-1.07	0.2567	-1.03	0.5761
NOD1	-1.01	0.9241	-1.32	0.078
NOD2	1.01	0.9419	1	0.976
TNFalpha	1.05	0.7268	-1.01	0.8987
Tlr2	-1.07	0.7804	1.01	0.9649
Tlr4	-1.13	0.6369	1.02	0.9124
Traf6	-1.23	0.0694	-1.37	*0.0179

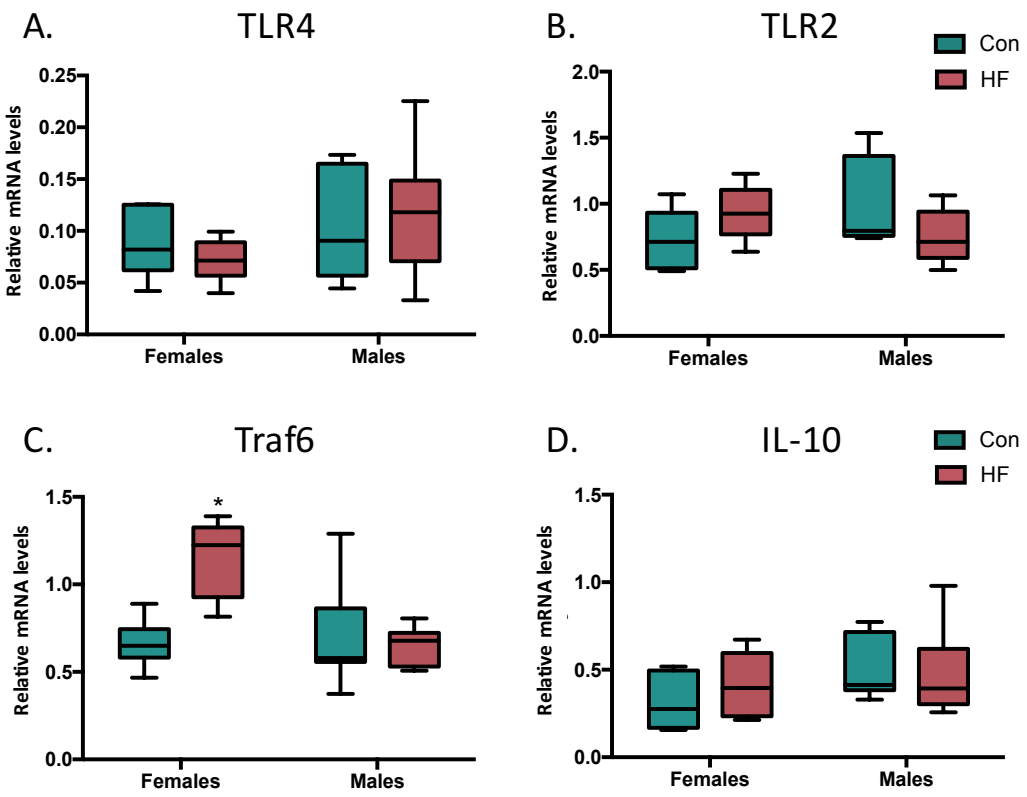

Figure 3.6.3: *Gene expression levels of inflammatory genes in male and female placentas from Con and HF dams.*
A. TLR4 mRNA levels in male and female placentas from Con and HF dams. **B.** TLR2 mRNA levels in male and female placentas from Con and HF dams. **C.** Traf6 mRNA levels mRNA levels in male and female placentas from Con and HF dams. **D.** IL-10 mRNA levels in male and female placentas from Con and HF dams. Con (n=7 male and n=7 female placentae) and HF (n=8 male and n=8 female placentae). *$p<0.05$, significantly different from placenta from Con dams within the same sex. Data were analyzed using a heteroscedastic Student's t-test. Con = control, HF= high-fat maternal diet.

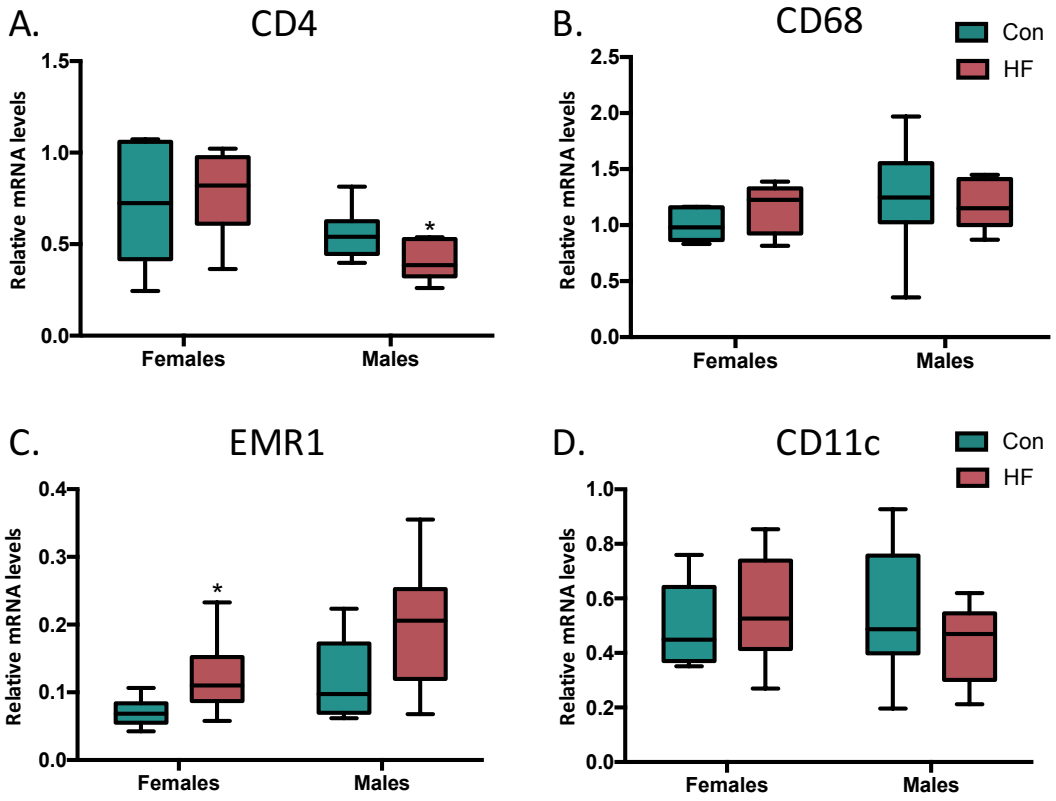

Figure 3.6.4: *Gene expression levels of immune cell markers in male and female placentae from Con and HF dams.*
A. CD4 mRNA levels in male and female placentae from Con and HF dams. **B.** CD68 mRNA levels in male and female placentae from Con and HF dams. **C.** EMR1 mRNA levels mRNA levels in male and female placentae from Con and HF dams. **D.** CD11c mRNA levels in male and female placentae from Con and HF dams. Con (n=7 male and n=7 female placentae) and HF (n=8 male and n=8 female placentae). *$p<0.05$, significantly different from placenta from Con dams within the same sex. Data were analyzed using a heteroscedastic Student's t-test. Con = control, HF= high-fat maternal diet.

4.0 DISCUSSION

Maternal obesity imparts an increased risk of obesity and other metabolic diseases in the offspring[158]. Recently, reports of changes in the gut microbiota of pregnant women have suggested intestinal changes during pregnancy may assist with the maternal metabolic adaptations[122]. But whether these intestinal changes also underpin the relationship between maternal obesity and an adverse intrauterine environment is unknown. This study investigated the impacts of maternal obesity on gut microbiota and maternal intestinal adaptations during pregnancy, and whether these changes are related to placental inflammation.

In the current study, we used a maternal HF diet containing 60% kcals from fat, to induce maternal obesity. As a result, HF-fed dams were heavier than Con dams until the end of pregnancy when the gestational weight of the two groups converged. This is consistent with previous reports of maternal HF diet-induced obesity[159], and suggests that maternal metabolic adaptations to pregnancy are modified with HF diet intake, and result in reduced body weight gain towards the end of pregnancy.

4.1 The maternal gut microbiota is modified by diet and pregnancy

We report distinct differences between the pregnancy microbiota of Con and HF mice, due to both HF diet intake and pregnancy. Although HF diet, independent of pregnancy, altered the maternal gut microbiota, further changes are observed due to the onset of pregnancy. Previously, we have shown pregnancy-induced changes in the gut microbiota and further modulation of pregnancy microbiota by maternal HF diet intake[129]. In the

present study, we observe similar changes between Con and HF pregnant microbiota compared to our previously published results. Of the 29 genera changed during HF pregnancy in the present study, 17 are the same ones that we observed shifted with a 45% kcal from fat diet[129]. It has been proposed that pregnancy-induced changes in maternal gut microbiota may mediate the necessary metabolic changes associated with pregnancy[122]. Early pregnancy is characterized by a maternal anabolic state, and associated with increased lipogenesis[160], glycogenesis[161], and adipocyte hypertrophy[162]. In late pregnancy, the mother is in a catabolic state, where the production of pregnancy hormones help mediate insulin and leptin resistance thus ensuring high levels of glucose are transferred to the placenta and fetus for normal growth and development[162]. Due to the role gut microbiota plays in modulating energy extraction[117] and lipid metabolism[116], it is plausible that gut microbial changes help facilitate pregnancy-associated metabolic adaptations. Further, metabolic adjustments to pregnancy are augmented during an obese pregnancy[163]; where obese pregnancies are accompanied by an increase in maternal basal metabolic rate[164], increased insulin resistance, higher risk of gestational diabetes mellitus (GDM)[165] and exacerbated hyperlipidemia[163].

We observed an increase in the relative abundance of *Akkermansia* in HF microbiota compared to control, but only in a pregnant state. This is the second incidence that we report an increase in *Akkermansia* abundance over the course of pregnancy in HF mice[129]. *Akkermansia muciniphila* (*A. muciniphila*), a mucin degrading bacterium[166], has come forth as a key gut microbe in metabolic regulation and is involved in the maintenance of intestinal barrier function[167]. Multiple studies have reported that the

abundance of *A. muciniphila* is inversely correlated to body weight in humans and rodents[167-169]. *A. muciniphila* abundance has been shown to be significantly reduced in genetically induced (ob/ob) mice, diet-induced obese mice and mice with type 1 diabetes[167,170,171]. Administration of *A. muciniphila* has been shown to improve adverse metabolic phenotypes observed in diet-induced obese mice[167], suggestive of a beneficial role in mitigating obesity. Most studies have shown a decrease in *A. muciniphila* abundance with HF diet or obesity, although recently a few studies have shown an increase in *A. muciniphila* abundance with HF diet[172,173]. The underlying mechanisms between the inverse association of *A. muciniphila* abundance and obesity remain unclear. Notably, all experimental studies that have investigated *A. muciniphila* abundance in the context of obesity and weight gain have used male mice, and gut microbial profiling in females with obesity is not well documented. Since it appears that our observed change in *Akkermansia* abundance is exclusive to pregnancy, we hypothesize that a change in *Akkermansia* abundance may be initiated by pregnancy specific events, such as the hormonal changes that accompany pregnancy. Over the course of pregnancy there is a rise in circulating estrogen[174]; *in vivo* studies have shown that small intestinal epithelial cells have estrogen receptors and are responsive to changes in estrogen levels[175]. However, the relationship between endocrine-mediated versus gut microbial-mediated metabolic changes during pregnancy remains to be elucidated.

Short chain fatty acids (SCFAs) produced by anaerobic bacteria via fermentation of dietary fibers are a source of energy for the host and play a key role in regulating intestinal immunity[176]. We observed a decrease in the abundance of specific anaerobic

microbes, *Lachnospira Bifidobacterium, and Ruminococcus* in HF mice. This change was not exclusive to pregnancy; thus it was likely caused by HF diet-induced shifts. The HF diet used in the present study contains proportionately less dietary fibers compared to the control diet (12.8% in Con vs 6.4% in HF diet); the lower abundance of anaerobic SCFA producing bacteria could be a result of lower levels of dietary fibers present in the diet. Although we presently only have pilot data suggesting that maternal fecal SCFA levels are decreased in HF pregnancies, we did observe a decrease in gene expression levels of the SCFA receptor, GPR41 (Ffar3) in the duodenum and jejunum, while GPR43 (Ffar2) expression was not altered. GPR41 is found on enteroendocrine cells and when activated by SCFAs it promotes the secretion of peptide YY (PYY), which stops gastric emptying and food intake. Studies using GPR41 knockout (KO) mice have suggested loss of GPR41 promotes obesity by increasing body fat mass[177]. Thus, hypothetically if a reduction in SCFA producing anaerobic bacteria reduces SCFA levels, the reduced SCFA exposure to GPR41 may decrease its expression. Future experiments are required to understand the downstream effect of reduced GPR41 expression during obese pregnancies.

4.2 HF diet-induced obesity alters maternal intestinal adaptations to pregnancy

Obesity has been shown to impair intestinal barrier function[112]. When human pregnant microbiota is transplanted in GF mice it results in increased adiposity[122]. Given the fact that pregnancy is often considered an "obesogenic" state due to maternal hyperglycemia, hyperinsulimia and hypertriglyceridemia we investigated whether pregnancy microbiota

was associated with other impacts observed during obesity, such as reduced intestinal barrier function. In control pregnant mice, pregnancy alone increased gut permeability to FITC-Dextran, an indigestible polymer that is used as a marker for paracellular permeability[178]. Previous work in obese male mice, showed that increased gut permeability was associated with excessive diffusion of bacterial components, such as LPS, into the circulation[112]. Furthermore activation of pattern recognition receptors, like TLR4, in adipocytes by LPS resulted in the production of pro-inflammatory mediators, including TNFα, which has been linked to the development of insulin resistance[56,112]. In humans during healthy pregnancy, plasma TNFα levels are increased in late gestation and are inversely correlated with insulin sensitivity[58]. Thus, we hypothesize that reduced barrier function during pregnancy likely plays a role in activating/mediating maternal insulin resistance, which is a key event that occurs during late pregnancy to help maintain high levels of glucose in the maternal circulation.

Pregnant HF-fed mice showed a strong tendency for reduced intestinal barrier to FITC-Dextran, compared to pregnant Con mice. We hypothesize that this suppression of barrier function during HF pregnancy may be attributed to an exacerbated pro-inflammatory state and thus measured LPS activation of NFκB in maternal serum as a functional marker for increased bacterial components entering the maternal circulation due to impaired intestinal barrier function. Maternal serum LPS levels were significantly higher in HF dams, consistent with previous work that linked HF diet included changes in microbial profiles to impaired intestinal barrier permitting increased diffusion of LPS into the circulation[114]., *Bifidobacterium* specifically has been linked to reduced LPS levels in

the circulation and improving gut barrier function[179-181]. Consistent with this, we observed a decrease in *Bifidobacterium* abundance due to HF diet in non-pregnant and pregnant HF animals. Taken together, our results suggest that normal pregnancy is accompanied by decreased in intestinal barrier function, which likely facilitates maternal metabolic adaptations to pregnancy, and that these intestinal adaptations are further modulated by HF diet. Future studies need to compare LPS concentrations in non-pregnant and pregnant Con mice to determine whether in a healthy pregnancy, increased intestinal permeability is associated with increased LPS diffusion.

Analyzing gene expression levels of tight junction proteins has provided us with some insight into which gut sections may have impaired tight junction integrity. We observed decreased expression of Tjp1 in jejunum and ileum, and decreased expression of occludin only in the jejunum, thus suggesting that the jejunum and ileum may be contributing to increased intestinal permeability. In contrast however, occludin mRNA levels were increased in the duodenum of HF dams. The role of occludin in maintaining tight junction integrity remains unclear, as there are conflicting reports suggesting occludin expression is both necessary for maintaining tight junction integrity and may also be associated with enhanced flux of molecules through the paracellular pathway[182]. McCarthy et *al.* reported that induction of occludin expression in Madin-Darby Canine Kidney Epithelial (MDCK) cells, that were transfected with with inducible chick occludin, resulted in increased transepithelial electrical resistance (TER)[183], a measure of epithelial monolayer integrity. This observation suggests that induced expression of occludin made the paracellular barrier tighter. In contrast, when mannitol flux from the

basolateral to the apical compartment on the monolayers was measured, increased induction of occludin resulted in increased TER and increased mannitol flux[183]. Therefore, although increased expression of occludin has been suggested to strengthens tight junction integrity, it may also facilitate diffusion of certain molecules. Occludin has also been reported to play a role in transducing cytokine mediated signaling pathways, that may increase or decrease intestinal permeability[184]. Exposure of pro-inflammatory cytokines TNFα and IFNγ to MDCK cells, results in elevated paracellular permeability[184,185]. It is apparent that the role of occludin is regulating tight junction function is complex, thus measuring gene expression is not sufficient to conclude whether it is contributing to changes in intestinal barrier function in HF dams, further studies are required to elucidate the role of occludin in the gut during HF pregnancies.

The intestinal epithelial cells are covered with a thick mucus layer that serves as a physical barrier protecting the epithelium from damage[186]. The mucus layer is composed of a number of high molecular weight, heavily glycosylated proteins that are capable of producing gel, also called mucins. There are 20 mucins that have been identified so far; membrane bound mucins, Muc1 and Muc4 are best characterized for their in the mucosa[187]. In the present study HF diet increased mRNA levels of Muc1 and Muc4 in the duodenum of pregnant mice. Muc1 plays a critical role in mucosal defense[188], where infection by *Campylobacter jejuni* induces a rapid upregulation of Muc1 whereas Muc1 knockout (KO) mice rapidly succumb to infection[188]. Muc1 upregulation in the duodenum of HF dams could be an attempt to increase mucosal defense in response to changes in gut microbial composition and reduced intestinal barrier function. Muc4 has been associated

with intestinal inflammation[189]. Dextran sodium sulfate (DSS) induced colitis in Muc4 KO mice resulted in lower levels of intestinal pro-inflammatory cytokines, (TNFα and IL-1β), and reduced infiltration of macrophages and CD3+ lymphocytes in the mucosa compared to conventional mice administered DSS[189]. Thus, mice lacking Muc4 were more resistant colitis-associated inflammation. We hypothesize that increased Muc4 expression levels in the duodenum of HF dams may be associated with duodenal inflammation. This notion is consistent with our observations that TLR2 and IL-6 gene expression levels are higher in the duodenum of HF pregnancies, but not in any other gut section.

Our hypothesis that maternal intestinal inflammation is present in HF pregnancies is supported by our observation that TLR4 mRNA levels were upregulated in the colon, but is in contrast, to a decrease in the small intestine of the transcription factor NFκB, which is activated by TLR4 and initiates the transcription of several pro-inflammatory cytokines. Although HF diet induced obesity has been associated with increased intestinal inflammation, particularly an increase in ileal TNFα and NFκB activation[115] these effects are found after 16 weeks of HF diet intake. In the present study dams that were fed a HF diet for a maximum of 9 weeks[115]. Interestingly, early stages of HF diet-induced metabolic disease have been associated with reduced intestinal inflammation, marked by a reduction in Th17 cells[190]. Chronic long-term HF diet intake resulted in an increase in pro-inflammatory intestinal immune cells, such as IFNγ-producing Th1 cells and CD8+ T cells, and inflammation in the small and large intestine[191]. We show an increase in CD4 mRNA levels in the duodenum, jejunum and ileum; CD4 is a glycoprotein found on the

surface of many immune cells including T helper cells, monocytes, macrophages, and dendritic cells, all of which are found in the gut[192]. CD4+ T cells may differentiate into number of cell types, including Th1, Th2, Th9, Th17, Th22, and regulatory T cells, each of which produce a select set of cytokines and chemokines and thus play specific roles in maintaining intestinal adaptive immune responses [192]. Despite the increased mRNA levels of CD4 in HF mothers, its ubiquitous expression does not allow specific conclusions on which of the T cell populations are upregulated during HF pregnancies in the gut.

We also observed an increase in CD19 gene expression in the duodenum of HF pregnant mice. CD19 is a protein found on the surface of B cells. Rosser et *al*. showed in a mouse model of arthritis, that altered gut microbiota induced macrophages and dendritic cells to increase their expression of IL-1β and IL-6, which promoted the activation of regulatory B cells to assist in controlling excessive inflammation[193]. We hypothesize increased IL-6 mRNA levels in the HF pregnant duodenum could also be counteracting inflammation by initiating a similar pathway, since we observe an increase in levels of B cell marker CD19 and macrophage marker CD68 in the duodenum. However, the exact nature of the inflammatory milieu of the HF diet pregnant intestine requires future experiments of immune cell profiling using flow cytometry. Furthermore, a more thorough profiling of pro- and anti-inflammatory cytokines and chemokine levels needs to be conducted in order, to correlate changes in immune cell populations with the inflammatory response they may be initiating.

4.3 Maternal HF diet alters placental growth and gene expression in a sex-specific manner

The rodent placenta is comprised of two morphologically distinct zones, the labyrinth and junctional zones. The junctional zone is in contact with the maternal uterine decidual tissue, while the labyrinth zone is in contact with the developing embryo[194]. The junctional zone is composed of spongiotrophoblasts, glycogen trophoblasts and a discontinuous layer of trophoblast giant cells (TGCs)[194]. TGCs are the main endocrine cells in the placenta, and they produce a number of hormones necessary for placental development and progression of pregnancy[194,195]. The exchange between maternal and fetal circulation occurs in the labyrinth[196], a highly vascularized portion of the placenta. We observed that female placentae from HF pregnancies had smaller junctional and labyrinth zones, and overall a smaller placental cross sectional area. This is not dissimilar to observations made by Mark et *al*. who showed a reduction in the junctional zone weight in rat placentae from HF dams[197] and is consistent with other models of nutrient excess showing that female placental weight is reduced in high fructose fed pregnant rats[198]. Others have reported conflicting results; Kim et *al.* observed decreased labyrinth zone thickness of placentae from HF dams, without sex-specific effects[199]. We observed increase mRNA levels of key genes involved in apoptosis and endoplasmic reticulum (ER) stress in HF female placentae; consistent with the notion that, a decrease in female placental size in HF pregnancies may be due to increased apoptosis and ER stress. The murine placenta is completely developed and optimally functional between E12.5-E14.5, after which the placenta is continuously remodeled until the end of gestation[200].

Apoptosis is elevated during late pregnancy in rat and human placentae[201] and elevated placental apoptosis has been associated with changes in placental size and adverse fetal outcomes[202]. Thus, further investigation into apoptotic pathways in the placenta is warranted. TUNEL analysis of placental tissues to examine DNA fragmentation *in situ* or immunohistological staining for activated Caspase-3 would provide more comprehensive understanding of where and to what extent apoptosis is occurring in HF female placentae[203].

Since we observed maternal intestinal adaptations during HF diet-induced obesity, we examined whether increased LPS diffusion into the maternal circulation during HF pregnancies was related to elevated placental gene expression of pro-inflammatory cytokines and immune cell markers. An increase in CD4 and IL-10 was observed in HF female placentae using NanoString nCounter gene expression system. Increase in IL-10 in placentae from HF dams has also been reported by Kim and colleagues[199]. They observed a decrease in IL-10 in placentae at mid gestation, and an increase in late gestation[199]. The upregulation of IL-10 in late gestation was suggested to be a protective mechanism in response to obesity-induced inflammation. Furthermore, IL-10 administration has been shown to attenuate LPS induced fetal death and growth restriction in rats[204]. We predict this may indicate an increase in IL-10 producing T regulatory cells in HF female placentae. Further investigation into inflammatory gene expression in the placenta is required to determine whether IL-10 is upregulated in response to a pro-inflammatory state in the placenta. Since a small sample size was used for NanoString analysis, we followed up these findings by quantifying inflammatory and immune cell marker gene

expression using qPCR with a greater sample size. We show increased TNF receptor associated factor (Traf)6 and macrophage marker, EMR1, mRNA levels in HF female placentae. Traf6 is involved in the TLR4 signaling pathway that activates transcription factor NFκB[205]; where Traf6 activates IκB kinase, which phosphorylates IκBα (NFκB inhibitor), causing it to release NFκB, which is then able to move into the nucleus and initiate transcription of several cytokines[205]. Although we observe an increase in Traf6, this was not accompanied by changes in TLR4, thus we cannot conclude whether there is increased activation of NFκB in HF female placentae. In the future, employing a TLR4 reporter assay with placental homogenates, which would measure NFκB activation by TLR4 ligands present in placental tissue would provide key insight into how maternal obesity alters the placental immune response.

The prevailing hypothesis underpinning the link between maternal and offspring obesity is that maternal obesity induces placental inflammation, and thus the fetus develops in a pro-inflammatory environment[37,70,203]. Our results do not conclusively support the notion that maternal HF diet induces placental inflammation. Heightened placental inflammation has not been observed in every model of maternal obesity. Very recently, Crew et *al.* reported that maternal obesity induced by 'cafeteria' diet containing 41% kcal from fat did not results in maternal, placental or fetal inflammation in late pregnancy in rats, despite an increase in total percentage body fat[206]. Rather, they reported reduced expression of TLR4 and EMR1 in maternal adipose tissue, and reduced expression of TLR4 in the placenta[206]. This is in contrast to studies that have shown increased maternal and placental inflammation in humans and animal studies. Systemic

maternal inflammation in obese pregnant women has been associated with activation of placental inflammatory pathways. Additional, placentas from obese women have been reported to have increased infiltration of macrophages, further suggesting maternal obesity results in a milieu in which the fetus develops. Thus, further studies are warranted to understand in which situations maternal obesity results in maternal, placental and fetal inflammation.

The placenta plays a crucial role in fetal programming, as it mediates signals between the mother and the fetus[207]. Changes in maternal physiology must first be transmitted to the placenta before affecting the fetus. Thus, the gene expression changes we observe in placenta from HF dams may be indicative of changes in the intrauterine environment that could have downstream affects on fetal development. In the future, fetal tissues must be examined in order to elucidate whether changes in maternal and placental tissues are also associated with changes in fetal physiology.

4.4 Future directions

We show that in a model of maternal HF diet-induced obesity, maternal gut microbiota is modulated by diet and pregnancy, and these changes are associated with modified maternal intestinal adaptations to pregnancy. The role of gut microbiota over the course of pregnancy is very weakly defined and only broad changes in microbial populations have been identified due to pregnancy. In order to understand how these alterations in gut microbiota influence maternal metabolic adaptations, pregnancy must be studied in GF mice. GF mice provide an ideal model to study interaction between

microbiota and the physiological processes it plays a role in. Comparing prenatal physiology between GF and conventionally raised wildtype mice would allow us to investigate how gut microbiota modulates maternal, placental and fetal development during pregnancy. Furthermore, studying HF diet intake before and during pregnancy in GF animals would allow us to delineate the extent to which gut microbiota plays a role in altering intestinal function in a model of maternal obesity.

5.0 LIMITATIONS

Owing to the study design, a few limitations were encountered when analyzing the data. In the present study, we used a HF diet containing 60% kcal from fat. Due to the high percentage of fat in the diet, the food pellets were very malleable and would easily crumble due to any physical agitation. This made it difficult to track maternal diet consumption as it was not possible to accurately measure how much food was consumed by the mice as apposed to how much was lost in the cage.

Maternal fasting glucose was measured at the end of pregnancy at E18.5. However, subjecting the mice to a glucose tolerance test early and late in pregnancy would have provided more insight regarding when insulin resistance in occurring during pregnancy, and whether this is changed by maternal HF diet.

It is also important to note that although we report decreased barrier function in a pregnant state by virtue od using FITC-dextran, makes it impossible to ascertain which specific section of the gut contributes to this loss of barrier, or by what mechanism. In future experiments, a more thorough analysis of intestinal barrier function using Ussing chambers should be employed to allow the measurement of ions, nutrients and other molecule transport across selected intestinal epithelial tissue[208]. The intestinal barrier is a complex and highly regulated system, thus measuring the transport of one molecule, FITC-Dextran, does not adequately describe intestinal permeability.

Finally, mucin mRNA levels were measured using qPCR. However, histological analysis of mucosal thickness would have provided a better understanding of how the mucus layer is modified during obese pregnancies.

6.0 CONCLUSION

Maternal diet-induced obesity perturbs pregnancy microbiota, and modifies intestinal adaptations to pregnancy. How modified gut microbiota facilitates changes in metabolic adaptations that occur during obese pregnancies remains unclear. Maternal obesity reduces maternal intestinal barrier function, possibly by impairing tight junction integrity in the small intestine. Increased perfusion of LPS into the maternal circulation during obese pregnancy supports the notion that maternal intestinal barrier is impaired. Maternal obesity increased inflammatory and immune cell marker gene expression in certain gut sections, although further work is required to understand the inflammatory signaling pathways that are initiated in response to altered microbiota and impaired intestinal barrier function. Maternal obesity did not result in augmented placental inflammation, but was associated with smaller female placentae, which may be due to increased placental apoptosis.

In conclusion, we show changes in pregnancy microbiota and intestinal adaptations in a mouse model of maternal obesity. Due to the significant role gut microbiota and intestinal barrier function play in mediating obesity, it is possible that maternal intestinal changes are involved in modulating adverse metabolic adaptations that occur during obese pregnancy, and impart increased risk of obesity in the offspring.

7.0 REFERENCES

1 Organization, W. H. *Obesity*, <http://www.who.int/gho/ncd/risk_factors/obesity_text/en/> (2013).
2 WHO. *Obesity*, <http://www.who.int/gho/ncd/risk_factors/obesity_text/en/> (2013).
3 Andreasen, C. H. & Andersen, G. Gene–environment interactions and obesity—Further aspects of genomewide association studies. *Nutrition* **25**, 998-1003, doi:http://dx.doi.org/10.1016/j.nut.2009.06.001 (2009).
4 Gluckman, P. D. & Hanson, M. A. Developmental plasticity and human disease: research directions. *Journal of Internal Medicine* **261**, 461-471, doi:doi:10.1111/j.1365-2796.2007.01802.x (2007).
5 Tan, E. K. & Tan, E. L. Alterations in physiology and anatomy during pregnancy. *Best Pract Res Clin Obstet Gynaecol* **27**, 791-802, doi:10.1016/j.bpobgyn.2013.08.001 (2013).
6 Ravelli, G. P., Stein, Z. A. & Susser, M. W. Obesity in young men after famine exposure in utero and early infancy. *N Engl J Med* **295**, 349-353, doi:10.1056/NEJM197608122950701 (1976).
7 Patro, B. *et al.* Maternal and paternal body mass index and offspring obesity: a systematic review. *Annals of nutrition & metabolism* **63**, 32-41, doi:10.1159/000350313 (2013).
8 Kristensen, J., Vestergaard, M., Wisborg, K., Kesmodel, U. & Secher, N. J. Pre-pregnancy weight and the risk of stillbirth and neonatal death. *BJOG : an international journal of obstetrics and gynaecology* **112**, 403-408, doi:10.1111/j.1471-0528.2005.00437.x (2005).
9 Triunfo, S. & Lanzone, A. Impact of overweight and obesity on obstetric outcomes. *Journal of endocrinological investigation*, doi:10.1007/s40618-014-0058-9 (2014).
10 Wallace, J. M., Horgan, G. W. & Bhattacharya, S. Placental weight and efficiency in relation to maternal body mass index and the risk of pregnancy complications in women delivering singleton babies. *Placenta* **33**, 611-618 (2012).
11 Priyadarshini, M. *et al.* Maternal short-chain fatty acids are associated with metabolic parameters in mothers and newborns. *Translational research : the journal of laboratory and clinical medicine*, doi:10.1016/j.trsl.2014.01.012 (2014).
12 Aye, I. L. *et al.* Increasing Maternal Body Mass Index Is Associated with Systemic Inflammation in the Mother and the Activation of Distinct Placental Inflammatory Pathways. *Biology of reproduction*, doi:10.1095/biolreprod.113.116186 (2014).
13 Aune, D., Saugstad, O. D., Henriksen, T. & Tonstad, S. Maternal body mass index and the risk of fetal death, stillbirth, and infant death: a systematic review and meta-analysis. *JAMA : the journal of the American Medical Association* **311**, 1536-1546, doi:10.1001/jama.2014.2269 (2014).

14 Ray, J. G., Wyatt, P. R., Vermeulen, M. J., Meier, C. & Cole, D. E. Greater maternal weight and the ongoing risk of neural tube defects after folic acid flour fortification. *Obstet Gynecol* **105**, 261-265, doi:10.1097/01.AOG.0000151988.84346.3e (2005).

15 Stothard, K. J., Tennant, P. W., Bell, R. & Rankin, J. Maternal overweight and obesity and the risk of congenital anomalies: a systematic review and meta-analysis. *JAMA : the journal of the American Medical Association* **301**, 636-650, doi:10.1001/jama.2009.113 (2009).

16 Grayson, B. E. *et al.* Changes in melanocortin expression and inflammatory pathways in fetal offspring of nonhuman primates fed a high-fat diet. *Endocrinology* **151**, 1622-1632, doi:10.1210/en.2009-1019 (2010).

17 Sewell, M. F., Huston-Presley, L., Amini, S. B. & Catalano, P. M. Body mass index: a true indicator of body fat in obese gravidas. *J Reprod Med* **52**, 907-911 (2007).

18 Sewell, M. F., Huston-Presley, L., Super, D. M. & Catalano, P. Increased neonatal fat mass, not lean body mass, is associated with maternal obesity. *American journal of obstetrics and gynecology* **195**, 1100-1103, doi:10.1016/j.ajog.2006.06.014 (2006).

19 Catalano, P. M., Presley, L., Minium, J. & Hauguel-de Mouzon, S. Fetuses of Obese Mothers Develop Insulin Resistance in Utero. *Diabetes Care* **32**, 1076-1080, doi:10.2337/dc08-2077 (2009).

20 Sridhar, S. B. *et al.* Maternal gestational weight gain and offspring risk for childhood overweight or obesity. *American journal of obstetrics and gynecology*, doi:10.1016/j.ajog.2014.02.030 (2014).

21 Sparano, S. *et al.* Being macrosomic at birth is an independent predictor of overweight in children: results from the IDEFICS study. *Maternal and child health journal* **17**, 1373-1381, doi:10.1007/s10995-012-1136-2 (2013).

22 Aiken, C. E. & Ozanne, S. E. Transgenerational developmental programming. *Human Reproduction Update* **20**, 63-75, doi:10.1093/humupd/dmt043 (2014).

23 Reynolds, R. M. *et al.* Maternal obesity during pregnancy and premature mortality from cardiovascular event in adult offspring: follow-up of 1 323 275 person years. *BMJ* **347**, doi:10.1136/bmj.f4539 (2013).

24 Howie, G., Sloboda, D., Kamal, T. & Vickers, M. Maternal nutritional history predicts obesity in adult offspring independent of postnatal diet. *The Journal of Physiology* **587**, 905-915 (2009).

25 Morris, M. J. & Chen, H. Established maternal obesity in the rat reprograms hypothalamic appetite regulators and leptin signaling at birth. *International journal of obesity* **33**, 115-122, doi:10.1038/ijo.2008.213 (2009).

26 Elahi, M. M. *et al.* Long-term maternal high-fat feeding from weaning through pregnancy and lactation predisposes offspring to hypertension, raised plasma lipids and fatty liver in mice. *The British journal of nutrition* **102**, 514-519, doi:10.1017/S000711450820749X (2009).

27 Li, M., Reynolds, C. M., Sloboda, D. M., Gray, C. & Vickers, M. H. Effects of Taurine Supplementation on Hepatic Markers of Inflammation and Lipid

Metabolism in Mothers and Offspring in the Setting of Maternal Obesity. *PLoS ONE* **8**, e76961, doi:10.1371/journal.pone.0076961 (2013).

28 Gabory, A., Roseboom, T. J., Moore, T., Moore, L. G. & Junien, C. Placental contribution to the origins of sexual dimorphism in health and diseases: sex chromosomes and epigenetics. *Biology of sex differences* **4**, 5, doi:10.1186/2042-6410-4-5 (2013).

29 Dahlhoff, M. *et al.* Peri-conceptional obesogenic exposure induces sex-specific programming of disease susceptibilities in adult mouse offspring. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease* **1842**, 304-317, doi:http://dx.doi.org/10.1016/j.bbadis.2013.11.021 (2014).

30 Gabory, A. *et al.* Maternal Diets Trigger Sex-Specific Divergent Trajectories of Gene Expression and Epigenetic Systems in Mouse Placenta. *PLoS ONE* **7**, e47986, doi:10.1371/journal.pone.0047986 (2012).

31 Walker, S. P., Ugoni, A. M., Lim, R. & Lappas, M. Inverse relationship between gestational weight gain and glucose uptake in human placenta from female foetuses. *Pediatric obesity*, doi:10.1111/j.2047-6310.2013.00206.x (2013).

32 Lager, S. *et al.* Diet-induced obesity in mice reduces placental efficiency and inhibits placental mTOR signaling. *Physiological reports* **2**, e00242, doi:10.1002/phy2.242 (2014).

33 Jones, H. N. *et al.* High-fat diet before and during pregnancy causes marked up-regulation of placental nutrient transport and fetal overgrowth in C57/BL6 mice. *FASEB J.* **23**, 271-278, doi:10.1096/fj.08-116889 (2009).

34 Zhang, L. *et al.* Maternal obesity in ewes results in reduced fetal pancreatic beta-cell numbers in late gestation and decreased circulating insulin concentration at term. *Domestic animal endocrinology* **40**, 30-39, doi:10.1016/j.domaniend.2010.08.004 (2011).

35 Yan, X. *et al.* Maternal obesity-impaired insulin signaling in sheep and induced lipid accumulation and fibrosis in skeletal muscle of offspring. *Biology of reproduction* **85**, 172-178, doi:10.1095/biolreprod.110.089649 (2011).

36 Zhu, M. J., Ma, Y., Long, N. M., Du, M. & Ford, S. P. Maternal obesity markedly increases placental fatty acid transporter expression and fetal blood triglycerides at midgestation in the ewe. *American journal of physiology. Regulatory, integrative and comparative physiology* **299**, R1224-1231, doi:10.1152/ajpregu.00309.2010 (2010).

37 Zhu, M. J., Du, M., Nathanielsz, P. W. & Ford, S. P. Maternal obesity up-regulates inflammatory signaling pathways and enhances cytokine expression in the mid-gestation sheep placenta. *Placenta* **31**, 387-391, doi:10.1016/j.placenta.2010.02.002 (2010).

38 Frias, A. E. *et al.* Maternal high-fat diet disturbs uteroplacental hemodynamics and increases the frequency of stillbirth in a nonhuman primate model of excess nutrition. *Endocrinology* **152**, 2456-2464, doi:10.1210/en.2010-1332 (2011).

39 Gregor, M. F. & Hotamisligil, G. S. Inflammatory mechanisms in obesity. *Annu Rev Immunol* **29**, 415-445, doi:10.1146/annurev-immunol-031210-101322 (2011).

40 Kanda, H. *et al.* MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. *J Clin Invest* **116**, 1494-1505, doi:10.1172/JCI26498 (2006).
41 Gordon, S. & Taylor, P. R. Monocyte and macrophage heterogeneity. *Nat Rev Immunol* **5**, 953-964, doi:10.1038/nri1733 (2005).
42 Gordon, S. Alternative activation of macrophages. *Nat Rev Immunol* **3**, 23-35, doi:10.1038/nri978 (2003).
43 Lumeng, C. N., Bodzin, J. L. & Saltiel, A. R. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *J Clin Invest* **117**, 175-184, doi:10.1172/JCI29881 (2007).
44 Reinhard, G., Noll, A., Schlebusch, H., Mallmann, P. & Ruecker, A. V. Shifts in the TH1/TH2 balance during human pregnancy correlate with apoptotic changes. *Biochem Biophys Res Commun* **245**, 933-938, doi:10.1006/bbrc.1998.8549 (1998).
45 Challis, J. R. *et al.* Inflammation and pregnancy. *Reprod Sci* **16**, 206-215, doi:10.1177/1933719108329095 (2009).
46 Madan, J. C. *et al.* Maternal obesity and markers of inflammation in pregnancy. *Cytokine* **47**, 61-64, doi:10.1016/j.cyto.2009.05.004 (2009).
47 Segovia, S. A., Vickers, M. H., Gray, C. & Reynolds, C. M. Maternal obesity, inflammation, and developmental programming. *Biomed Res Int* **2014**, 418975, doi:10.1155/2014/418975 (2014).
48 Gude, N. M., Roberts, C. T., Kalionis, B. & King, R. G. Growth and function of the normal human placenta. *Thromb Res* **114**, 397-407, doi:10.1016/j.thromres.2004.06.038 (2004).
49 Fowden, A. L., Forhead, A. J., Coan, P. M. & Burton, G. J. The placenta and intrauterine programming. *J Neuroendocrinol* **20**, 439-450, doi:10.1111/j.1365-2826.2008.01663.x (2008).
50 Baur, R. Morphometry of the placental exchange area. *Adv Anat Embryol Cell Biol* **53**, 3-65 (1977).
51 Lederman, S. A. & Rosso, P. Effects of food restriction on fetal and placental growth and maternal body composition. *Growth* **44**, 77-88 (1980).
52 Ishikawa, H., Seki, R., Yokonishi, S., Yamauchi, T. & Yokoyama, K. Relationship between fetal weight, placental growth and litter size in mice from mid- to late-gestation. *Reprod Toxicol* **21**, 267-270, doi:10.1016/j.reprotox.2005.08.002 (2006).
53 Panti, A. A., Ekele, B. A., Nwobodo, E. I. & Yakubu, A. The relationship between the weight of the placenta and birth weight of the neonate in a Nigerian Hospital. *Niger Med J* **53**, 80-84, doi:10.4103/0300-1652.103547 (2012).
54 Hay, W. W., Jr. Placental-fetal glucose exchange and fetal glucose metabolism. *Trans Am Clin Climatol Assoc* **117**, 321-339; discussion 339-340 (2006).
55 Ryan, E. A. & Enns, L. Role of gestational hormones in the induction of insulin resistance. *J Clin Endocrinol Metab* **67**, 341-347, doi:10.1210/jcem-67-2-341 (1988).

56 Hotamisligil, G. S., Shargill, N. S. & Spiegelman, B. M. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. *Science* **259**, 87-91 (1993).
57 Barbour, L. A. *et al.* Cellular mechanisms for insulin resistance in normal pregnancy and gestational diabetes. *Diabetes care* **30**, S112-S119 (2007).
58 Kirwan, J. P. *et al.* TNF-alpha is a predictor of insulin resistance in human pregnancy. *Diabetes* **51**, 2207-2213 (2002).
59 Baumann, M. U., Deborde, S. & Illsley, N. P. Placental glucose transfer and fetal growth. *Endocrine* **19**, 13-22, doi:10.1385/ENDO:19:1:13 (2002).
60 Brown, K., Heller, D. S., Zamudio, S. & Illsley, N. P. Glucose transporter 3 (GLUT3) protein expression in human placenta across gestation. *Placenta* **32**, 1041-1049, doi:10.1016/j.placenta.2011.09.014 (2011).
61 Lager, S. & Powell, T. L. Regulation of nutrient transport across the placenta. *Journal of pregnancy* **2012** (2012).
62 Broer, S. Adaptation of plasma membrane amino acid transport mechanisms to physiological demands. *Pflugers Arch* **444**, 457-466, doi:10.1007/s00424-002-0840-y (2002).
63 Cetin, I. *et al.* Fetal amino acids in normal pregnancies and in pregnancies complicated by intrauterine growth retardation. *Early Hum Dev* **29**, 183-186 (1992).
64 Jansson, T. Amino acid transporters in the human placenta. *Pediatr Res* **49**, 141-147, doi:10.1203/00006450-200102000-00003 (2001).
65 Verrey, F. System L: heteromeric exchangers of large, neutral amino acids involved in directional transport. *Pflugers Arch* **445**, 529-533, doi:10.1007/s00424-002-0973-z (2003).
66 Farley, D. M. *et al.* Placental amino acid transport and placental leptin resistance in pregnancies complicated by maternal obesity. *Placenta* **31**, 718-724, doi:S0143-4004(10)00223-7 [pii]
10.1016/j.placenta.2010.06.006 (2010).
67 Aye, I. L., Jansson, T. & Powell, T. L. Interleukin-1beta inhibits insulin signaling and prevents insulin-stimulated system A amino acid transport in primary human trophoblasts. *Molecular and cellular endocrinology* **381**, 46-55, doi:10.1016/j.mce.2013.07.013 (2013).
68 Malti, N. *et al.* Oxidative stress and maternal obesity: Feto-placental unit interaction. *Placenta*, doi:10.1016/j.placenta.2014.03.010 (2014).
69 Dube, E. *et al.* Modulation of fatty acid transport and metabolism by maternal obesity in the human full-term placenta. *Biology of reproduction* **87**, 14, 11-11, doi:10.1095/biolreprod.111.098095 (2012).
70 Challier, J. C. *et al.* Obesity in pregnancy stimulates macrophage accumulation and inflammation in the placenta. *Placenta* **29**, 274-281, doi:S0143-4004(08)00004-0 [pii]
10.1016/j.placenta.2007.12.010 (2008).

71 Holmlund, U. *et al.* Expression and regulation of the pattern recognition receptors Toll-like receptor-2 and Toll-like receptor-4 in the human placenta. *Immunology* **107**, 145-151 (2002).

72 Herrera, E. *et al.* Relationship between plasma fatty acid profile and antioxidant vitamins during normal pregnancy. *European journal of clinical nutrition* **58**, 1231-1238, doi:10.1038/sj.ejcn.1601954 (2004).

73 Harmon, K. A. *et al.* Continuous glucose profiles in obese and normal-weight pregnant women on a controlled diet: metabolic determinants of fetal growth. *Diabetes Care* **34**, 2198-2204, doi:10.2337/dc11-0723 (2011).

74 Thomas, L. V., Ockhuizen, T. & Suzuki, K. Exploring the influence of the gut microbiota and probiotics on health: a symposium report. *British Journal of Nutrition* **112**, S1-S18, doi:doi:10.1017/S0007114514001275 (2014).

75 Whitman, W. B., Coleman, D. C. & Wiebe, W. J. Prokaryotes: The unseen majority. *Proceedings of the National Academy of Sciences* **95**, 6578-6583 (1998).

76 Chow, J., Lee, S. M., Shen, Y., Khosravi, A. & Mazmanian, S. K. in *Advances in immunology* Vol. Volume 107 (eds Fagarasan Sidonia & Cerutti Andrea) 243-274 (Academic Press, 2010).

77 Chow, J., Lee, S. M., Shen, Y., Khosravi, A. & Mazmanian, S. K. Host-bacterial symbiosis in health and disease. *Advances in immunology* **107**, 243-274, doi:10.1016/B978-0-12-381300-8.00008-3 (2010).

78 Hooper, L. V. & Macpherson, A. J. Immune adaptations that maintain homeostasis with the intestinal microbiota. *Nat Rev Immunol* **10**, 159-169, doi:10.1038/nri2710 (2010).

79 Turner, J. R. Intestinal mucosal barrier function in health and disease. *Nat Rev Immunol* **9**, 799-809 (2009).

80 Mason, K. L., Huffnagle, G. B., Noverr, M. C. & Kao, J. Y. Overview of gut immunology. *Adv Exp Med Biol* **635**, 1-14, doi:10.1007/978-0-387-09550-9_1 (2008).

81 Macpherson, A. J. & Harris, N. L. Interactions between commensal intestinal bacteria and the immune system. *Nat Rev Immunol* **4**, 478-485, doi:10.1038/nri1373 (2004).

82 Ostman, S., Rask, C., Wold, A. E., Hultkrantz, S. & Telemo, E. Impaired regulatory T cell function in germ-free mice. *Eur J Immunol* **36**, 2336-2346, doi:10.1002/eji.200535244 (2006).

83 Luckheeram, R. V., Zhou, R., Verma, A. D. & Xia, B. CD4(+)T cells: differentiation and functions. *Clin Dev Immunol* **2012**, 925135, doi:10.1155/2012/925135 (2012).

84 Mazmanian, S. K., Liu, C. H., Tzianabos, A. O. & Kasper, D. L. An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. *Cell* **122**, 107-118, doi:10.1016/j.cell.2005.05.007 (2005).

85 Bischoff, S. C. *et al.* Intestinal permeability--a new target for disease prevention and therapy. *BMC Gastroenterol* **14**, 189, doi:10.1186/s12876-014-0189-7 (2014).

86 Kamada, N., Chen, G. Y., Inohara, N. & Nunez, G. Control of pathogens and pathobionts by the gut microbiota. *Nat Immunol* **14**, 685-690, doi:10.1038/ni.2608 (2013).
87 Marteyn, B. *et al.* Modulation of Shigella virulence in response to available oxygen in vivo. *Nature* **465**, 355-358, doi:10.1038/nature08970 (2010).
88 Ulluwishewa, D. *et al.* Regulation of tight junction permeability by intestinal bacteria and dietary components. *J Nutr* **141**, 769-776, doi:10.3945/jn.110.135657 (2011).
89 Fanning, A. S., Jameson, B. J., Jesaitis, L. A. & Anderson, J. M. The tight junction protein ZO-1 establishes a link between the transmembrane protein occludin and the actin cytoskeleton. *J Biol Chem* **273**, 29745-29753 (1998).
90 Madsen, K. *et al.* Probiotic bacteria enhance murine and human intestinal epithelial barrier function. *Gastroenterology* **121**, 580-591 (2001).
91 Jauregi-Miguel, A. *et al.* Alteration of tight junction gene expression in celiac disease. *J Pediatr Gastroenterol Nutr* **58**, 762-767, doi:10.1097/MPG.0000000000000338 (2014).
92 Zeissig, S. *et al.* Changes in expression and distribution of claudin 2, 5 and 8 lead to discontinuous tight junctions and barrier dysfunction in active Crohn's disease. *Gut* **56**, 61-72, doi:10.1136/gut.2006.094375 (2007).
93 Schmitz, H. *et al.* Altered tight junction structure contributes to the impaired epithelial barrier function in ulcerative colitis. *Gastroenterology* **116**, 301-309 (1999).
94 Cani, P. D. *et al.* Changes in gut microbiota control inflammation in obese mice through a mechanism involving GLP-2-driven improvement of gut permeability. *Gut* **58**, 1091-1103 (2009).
95 Sekirov, I., Russell, S. L., Antunes, L. C. & Finlay, B. B. Gut microbiota in health and disease. *Physiological reviews* **90**, 859-904, doi:10.1152/physrev.00045.2009 (2010).
96 Marcobal, A. *et al.* A metabolomic view of how the human gut microbiota impacts the host metabolome using humanized and gnotobiotic mice. *ISME J* **7**, 1933-1943, doi:10.1038/ismej.2013.89 (2013).
97 Rabot, S. *et al.* Germ-free C57BL/6J mice are resistant to high-fat-diet-induced insulin resistance and have altered cholesterol metabolism. *FASEB J* **24**, 4948-4959, doi:10.1096/fj.10-164921 (2010).
98 Backhed, F. *et al.* The gut microbiota as an environmental factor that regulates fat storage. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 15718-15723, doi:10.1073/pnas.0407076101 (2004).
99 Kasubuchi, M., Hasegawa, S., Hiramatsu, T., Ichimura, A. & Kimura, I. Dietary gut microbial metabolites, short-chain fatty acids, and host metabolic regulation. *Nutrients* **7**, 2839-2849, doi:10.3390/nu7042839 (2015).
100 Brown, A. J. *et al.* The Orphan G protein-coupled receptors GPR41 and GPR43 are activated by propionate and other short chain carboxylic acids. *J Biol Chem* **278**, 11312-11319, doi:10.1074/jbc.M211609200 (2003).

101 Tolhurst, G. *et al.* Short-chain fatty acids stimulate glucagon-like peptide-1 secretion via the G-protein-coupled receptor FFAR2. *Diabetes* **61**, 364-371, doi:10.2337/db11-1019 (2012).
102 MacDonald, P. E. *et al.* The multiple actions of GLP-1 on the process of glucose-stimulated insulin secretion. *Diabetes* **51 Suppl 3**, S434-442 (2002).
103 Kimura, I. *et al.* The gut microbiota suppresses insulin-mediated fat accumulation via the short-chain fatty acid receptor GPR43. *Nat Commun* **4**, 1829, doi:10.1038/ncomms2852 (2013).
104 Kimura, I. *et al.* Short-chain fatty acids and ketones directly regulate sympathetic nervous system via G protein-coupled receptor 41 (GPR41). *Proceedings of the National Academy of Sciences of the United States of America* **108**, 8030-8035, doi:10.1073/pnas.1016088108 (2011).
105 Turnbaugh, P. J. *et al.* An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* **444**, 1027-1031, doi:10.1038/nature05414 (2006).
106 Ley, R. E., Turnbaugh, P. J., Klein, S. & Gordon, J. I. Microbial ecology: human gut microbes associated with obesity. *Nature* **444**, 1022-1023 (2006).
107 Ley, R. E. *et al.* Obesity alters gut microbial ecology. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 11070-11075, doi:10.1073/pnas.0504978102 (2005).
108 Turnbaugh, P. J. & Gordon, J. I. The core gut microbiome, energy balance and obesity. *The Journal of Physiology* **587**, 4153-4158, doi:10.1113/jphysiol.2009.174136 (2009).
109 Payne, A. N. *et al.* The metabolic activity of gut microbiota in obese children is increased compared with normal-weight children and exhibits more exhaustive substrate utilization. *Nutrition and Diabetes* **1**, e12, doi:http://www.nature.com/nutd/journal/v1/n7/suppinfo/nutd20118s1.html (2011).
110 Bervoets, L. *et al.* Differences in gut microbiota composition between obese and lean children: a cross-sectional study. *Gut pathogens* **5**, 10, doi:10.1186/1757-4749-5-10 (2013).
111 Turnbaugh, P. J. *et al.* A core gut microbiome in obese and lean twins. *Nature* **457**, 480-484, doi:10.1038/nature07540 (2009).
112 Cani, P. D. *et al.* Metabolic endotoxemia initiates obesity and insulin resistance. *Diabetes* **56**, 1761-1772, doi:10.2337/db06-1491 (2007).
113 Palsson-McDermott, E. M. & O'Neill, L. A. Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4. *Immunology* **113**, 153-162, doi:10.1111/j.1365-2567.2004.01976.x (2004).
114 Cani, P. D. *et al.* Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. *Diabetes* **57**, 1470-1481, doi:10.2337/db07-1403 (2008).
115 Ding, S. *et al.* High-fat diet: bacteria interactions promote intestinal inflammation which precedes and correlates with obesity and insulin resistance in mouse. *PLoS One* **5**, e12191, doi:10.1371/journal.pone.0012191 (2010).

116 Harris, K. *et al.* Is the Gut Microbiota a New Factor Contributing to Obesity and Its Metabolic Disorders? *Journal of Obesity* **2012**, 14, doi:10.1155/2012/879151 (2012).
117 Turnbaugh, P. *et al.* An obesity associated gut microbiome with increased capacity for energy harvest. *Nature* **444**, 1027-1031 (2006).
118 Kim, K.-A., Gu, W., Lee, I.-A., Joh, E.-H. & Kim, D.-H. High Fat Diet-Induced Gut Microbiota Exacerbates Inflammation and Obesity in Mice via the TLR4 Signaling Pathway. *PLoS ONE* **7**, e47713, doi:10.1371/journal.pone.0047713 (2012).
119 Turnbaugh, P. J., Bäckhed, F., Fulton, L. & Gordon, J. I. Diet-Induced Obesity Is Linked to Marked but Reversible Alterations in the Mouse Distal Gut Microbiome. *Cell Host & Microbe* **3**, 213-223, doi:http://dx.doi.org/10.1016/j.chom.2008.02.015 (2008).
120 Zhang C Fau - Zhang, M. *et al.* Interactions between gut microbiota, host genetics and diet relevant to development of metabolic syndromes in mice. *ISME J* **4**, 232 (2010).
121 Collado, M. C., Isolauri, E., Laitinen, K. & Salminen, S. Distinct composition of gut microbiota during pregnancy in overweight and normal-weight women. *The American Journal of Clinical Nutrition* **88**, 894-899 (2008).
122 Koren, O. *et al.* Host remodeling of the gut microbiome and metabolic changes during pregnancy. *Cell* **150**, 470-480, doi:10.1016/j.cell.2012.07.008 (2012).
123 Mukhopadhya, I., Hansen, R., El-Omar, E. M. & Hold, G. L. IBD-what role do Proteobacteria play? *Nature reviews. Gastroenterology & hepatology* **9**, 219-230, doi:10.1038/nrgastro.2012.14 (2012).
124 Jost, T., Lacroix, C., Braegger, C. & Chassard, C. Stability of the maternal gut microbiota during late pregnancy and early lactation. *Current microbiology* **68**, 419-427, doi:10.1007/s00284-013-0491-6 (2014).
125 Fuller, M. *et al.* The short-chain fatty acid receptor, FFA2, contributes to gestational glucose homeostasis. *American Journal of Physiology-Endocrinology and Metabolism* **309**, E840-E851 (2015).
126 Hildebrand, F. *et al.* Inflammation-associated enterotypes, host genotype, cage and inter-individual effects drive gut microbiota variation in common laboratory mice. *Genome Biol* **14**, R4 (2013).
127 Kim, S. J., Sung, M.-K., Park, M.-Y. & Kim, S.-E. Dietary fat intake and age influence gut microbiota and colonic inflammation in C57BL/6J mice. *The FASEB Journal* **29**, 601.605 (2015).
128 Santacruz, A. *et al.* Gut microbiota composition is associated with body weight, weight gain and biochemical parameters in pregnant women. *The British journal of nutrition* **104**, 83-92, doi:10.1017/S0007114510000176 (2010).
129 Gohir, W. *et al.* Pregnancy-related changes in the maternal gut microbiota are dependent upon the mother's periconceptional diet. *Gut Microbes* **6**, 310-320, doi:10.1080/19490976.2015.1086056 (2015).
130 Puertollano, E., Kolida, S. & Yaqoob, P. Biological significance of short-chain fatty acid metabolism by the intestinal microbiome. *Current opinion in clinical*

nutrition and metabolic care **17**, 139-144, doi:10.1097/mco.0000000000000025 (2014).

131 Laitinen, K., Poussa, T., Isolauri, E., Nutrition, A. M. I. & Intestinal Microbiota, G. Probiotics and dietary counselling contribute to glucose regulation during and after pregnancy: a randomised controlled trial. *The British journal of nutrition* **101**, 1679-1687, doi:10.1017/S0007114508111461 (2009).

132 Luoto, R., Laitinen, K., Nermes, M. & Isolauri, E. Impact of maternal probiotic-supplemented dietary counselling on pregnancy outcome and prenatal and postnatal growth: a double-blind, placebo-controlled study. *The British journal of nutrition* **103**, 1792-1799, doi:10.1017/s0007114509993898 (2010).

133 Koren, O. *et al.* Host Remodeling of the Gut Microbiome and Metabolic Changes during Pregnancy. *Cell* **150**, 470-480 (2012).

134 Collado, M. C., Isolauri, E., Laitinen, K. & Salminen, S. Effect of mother's weight on infant's microbiota acquisition, composition, and activity during early infancy: a prospective follow-up study initiated in early pregnancy. *The American Journal of Clinical Nutrition* **92**, 1023-1030, doi:10.3945/ajcn.2010.29877 (2010).

135 Flint, H. J., Scott, K. P., Louis, P. & Duncan, S. H. The role of the gut microbiota in nutrition and health. *Nature reviews. Gastroenterology & hepatology* **9**, 577-589, doi:10.1038/nrgastro.2012.156 (2012).

136 Harris, K., Kassis, A., Major, G. & Chou, C. J. Is the gut microbiota a new factor contributing to obesity and its metabolic disorders? *J Obes* **2012**, 879151, doi:10.1155/2012/879151 (2012).

137 Kim, K. A., Gu, W., Lee, I. A., Joh, E. H. & Kim, D. H. High fat diet-induced gut microbiota exacerbates inflammation and obesity in mice via the TLR4 signaling pathway. *PLoS One* **7**, e47713, doi:10.1371/journal.pone.0047713 (2012).

138 Reyes, H. *et al.* Is a leaky gut involved in the pathogenesis of intrahepatic cholestasis of pregnancy? *Hepatology* **43**, 715-722, doi:10.1002/hep.21099 (2006).

139 Aagaard, K. *et al.* The Placenta Harbors a Unique Microbiome. *Science Translational Medicine* **6**, 237ra265, doi:10.1126/scitranslmed.3008599 (2014).

140 Stout, M. J. *et al.* Identification of intracellular bacteria in the basal plate of the human placenta in term and preterm gestations. *American journal of obstetrics and gynecology* **208**, 226.e221-226.e227, doi:http://dx.doi.org/10.1016/j.ajog.2013.01.018 (2013).

141 Hanson, M. A. & Gluckman, P. D. Developmental origins of health and disease: new insights. *Basic Clin Pharmacol Toxicol* **102**, 90-93, doi:10.1111/j.1742-7843.2007.00186.x (2008).

142 Alfaradhi, M. Z. & Ozanne, S. E. Developmental programming in response to maternal overnutrition. *Front Genet* **2**, 27, doi:10.3389/fgene.2011.00027 (2011).

143 Tjepkema, M. *Adult obesity in Canada: Measured height and weight*, <http://www.statcan.gc.ca/pub/82-620-m/2005001/article/adults-adultes/8060-eng.htm> (2005).

144 Willms, J. D., Tremblay, M. S. & Katzmarzyk, P. T. Geographic and demographic variation in the prevalence of overweight Canadian children. *Obes Res* **11**, 668-673, doi:10.1038/oby.2003.95 (2003).
145 Holmes, E., Li, J. V., Marchesi, J. R. & Nicholson, J. K. Gut microbiota composition and activity in relation to host metabolic phenotype and disease risk. *Cell metabolism* **16**, 559-564, doi:10.1016/j.cmet.2012.10.007 (2012).
146 Frazier, T. H., DiBaise, J. K. & McClain, C. J. Gut microbiota, intestinal permeability, obesity-induced inflammation, and liver injury. *JPEN. Journal of parenteral and enteral nutrition* **35**, 14S-20S, doi:10.1177/0148607111413772 (2011).
147 Whelan, F. J. *et al.* The loss of topography in the microbial communities of the upper respiratory tract in the elderly. *Annals of the American Thoracic Society* **11**, 513-521, doi:10.1513/AnnalsATS.201310-351OC (2014).
148 Bartram, A. K., Lynch, M. D., Stearns, J. C., Moreno-Hagelsieb, G. & Neufeld, J. D. Generation of multimillion-sequence 16S rRNA gene libraries from complex microbial communities by assembling paired-end illumina reads. *Appl Environ Microbiol* **77**, 3846-3852, doi:10.1128/AEM.02772-10 (2011).
149 Martin, M. Cutadapt removes adapter sequences from high-throughput sequencing reads. *2011* **17**, doi:10.14806/ej.17.1.200
pp. 10-12 (2011).
150 Masella, A. P., Bartram, A. K., Truszkowski, J. M., Brown, D. G. & Neufeld, J. D. PANDAseq: paired-end assembler for illumina sequences. *BMC bioinformatics* **13**, 31, doi:10.1186/1471-2105-13-31 (2012).
151 Ye, Y. Identification and Quantification of Abundant Species from Pyrosequences of 16S rRNA by Consensus Alignment. *Proceedings. IEEE International Conference on Bioinformatics and Biomedicine* **2010**, 153-157, doi:10.1109/BIBM.2010.5706555 (2011).
152 Wang, Q., Garrity, G. M., Tiedje, J. M. & Cole, J. R. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* **73**, 5261-5267, doi:10.1128/AEM.00062-07 (2007).
153 Caporaso, J. G. *et al.* QIIME allows analysis of high-throughput community sequencing data. *Nature methods* **7**, 335-336, doi:10.1038/nmeth.f.303 (2010).
154 McMurdie, P. J. & Holmes, S. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One* **8**, e61217, doi:10.1371/journal.pone.0061217 (2013).
155 DeSantis, T. Z. *et al.* Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl Environ Microbiol* **72**, 5069-5072, doi:10.1128/AEM.03006-05 (2006).
156 vegan: Community Ecology Package v. R package version 2.0-10. (2013).
157 Wickham, H. *ggplot2: Elegant Graphics for Data Analysis*. (Springer Publishing Company, Incorporated, 2009).
158 Durand, E. F., Logan, C. & Carruth, A. Association of maternal obesity and childhood obesity: implications for healthcare providers. *J Community Health Nurs* **24**, 167-176, doi:10.1080/07370010701429579 (2007).

159 Samuelsson, A. M. *et al.* Diet-induced obesity in female mice leads to offspring hyperphagia, adiposity, hypertension, and insulin resistance: a novel murine model of developmental programming. *Hypertension* **51**, 383-392, doi:10.1161/HYPERTENSIONAHA.107.101477 (2008).
160 Herrera, E. Lipid metabolism in pregnancy and its consequences in the fetus and newborn. *Endocrine* **19**, 43-55, doi:10.1385/ENDO:19:1:43 (2002).
161 Kalhan, S., Rossi, K., Gruca, L., Burkett, E. & O'Brien, A. Glucose turnover and gluconeogenesis in human pregnancy. *J Clin Invest* **100**, 1775-1781, doi:10.1172/JCI119704 (1997).
162 Butte, N. Carbohydrate and lipid metabolism in pregnancy: normal compared with gestational diabetes mellitus. *American Journal of Clinical Nutrition* **71**, 1256S (2000).
163 Iva, S. K. Metabolic Adaptations in Pregnancy in Lean and Obese Women–A Literature Review. *Research in Obstetrics and Gynecology* **2**, 37-47 (2013).
164 Bronstein, M. N., Mak, R. P. & King, J. C. Unexpected relationship between fat mass and basal metabolic rate in pregnant women. *British Journal of Nutrition* **75**, 659-668 (1996).
165 Weiss, J. L. *et al.* Obesity, obstetric complications and cesarean delivery rate--a population-based screening study. *American journal of obstetrics and gynecology* **190**, 1091-1097, doi:10.1016/j.ajog.2003.09.058 (2004).
166 Derrien, M., Vaughan, E. E., Plugge, C. M. & de Vos, W. M. Akkermansia muciniphila gen. nov., sp. nov., a human intestinal mucin-degrading bacterium. *Int J Syst Evol Microbiol* **54**, 1469-1476, doi:10.1099/ijs.0.02873-0 (2004).
167 Everard, A. *et al.* Cross-talk between Akkermansia muciniphila and intestinal epithelium controls diet-induced obesity. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 9066-9071, doi:10.1073/pnas.1219451110 (2013).
168 Dao, M. C. *et al.* Akkermansia muciniphila and improved metabolic health during a dietary intervention in obesity: relationship with gut microbiome richness and ecology. *Gut*, doi:10.1136/gutjnl-2014-308778 (2015).
169 Everard, A. *et al.* Responses of gut microbiota and glucose and lipid metabolism to prebiotics in genetic obese and diet-induced leptin-resistant mice. *Diabetes* **60**, 2775-2786, doi:10.2337/db11-0227 (2011).
170 Everard, A. *et al.* Microbiome of prebiotic-treated mice reveals novel targets involved in host response during obesity. *ISME J* **8**, 2116-2130, doi:10.1038/ismej.2014.45 (2014).
171 Hansen, C. H. *et al.* Early life treatment with vancomycin propagates Akkermansia muciniphila and reduces diabetes incidence in the NOD mouse. *Diabetologia* **55**, 2285-2294, doi:10.1007/s00125-012-2564-7 (2012).
172 Carmody, R. N. *et al.* Diet dominates host genotype in shaping the murine gut microbiota. *Cell Host Microbe* **17**, 72-84, doi:10.1016/j.chom.2014.11.010 (2015).
173 Hamilton, M. K., Boudry, G., Lemay, D. G. & Raybould, H. E. Changes in intestinal barrier function and gut microbiota in high-fat diet-fed rats are dynamic

and region dependent. *Am J Physiol Gastrointest Liver Physiol* **308**, G840-851, doi:10.1152/ajpgi.00029.2015 (2015).

174 King, J. C. Physiology of pregnancy and nutrient metabolism. *The American journal of clinical nutrition* **71**, 1218s-1225s (2000).

175 Thomas, M. L., Xu, X., Norfleet, A. M. & Watson, C. S. The presence of functional estrogen receptors in intestinal epithelial cells. *Endocrinology* **132**, 426-430, doi:10.1210/endo.132.1.8419141 (1993).

176 Smith, P. M. *et al.* The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. *Science* **341**, 569-573, doi:10.1126/science.1241165 (2013).

177 Bellahcene, M. *et al.* Male mice that lack the G-protein-coupled receptor GPR41 have low energy expenditure and increased body fat content. *The British journal of nutrition* **109**, 1755-1764, doi:10.1017/S0007114512003923 (2013).

178 Poste, G. & Crooke, S. T. *New insights into cell and membrane transport processes*. (Springer Science & Business Media, 2013).

179 Wang, Z. *et al.* The role of bifidobacteria in gut barrier function after thermal injury in rats. *J Trauma* **61**, 650-657, doi:10.1097/01.ta.0000196574.70614.27 (2006).

180 Griffiths, E. A. *et al.* In vivo effects of bifidobacteria and lactoferrin on gut endotoxin concentration and mucosal immunity in Balb/c mice. *Dig Dis Sci* **49**, 579-589 (2004).

181 Moratalla, A. *et al.* Protective effect of Bifidobacterium pseudocatenulatum CECT7765 against induced bacterial antigen translocation in experimental cirrhosis. *Liver Int* **34**, 850-858, doi:10.1111/liv.12380 (2014).

182 Krug, S. M., Schulzke, J. D. & Fromm, M. Tight junction, selective permeability, and related diseases. *Semin Cell Dev Biol* **36**, 166-176, doi:10.1016/j.semcdb.2014.09.002 (2014).

183 McCarthy, K. M. *et al.* Occludin is a functional component of the tight junction. *J Cell Sci* **109 (Pt 9)**, 2287-2298 (1996).

184 Van Itallie, C. M., Fanning, A. S., Holmes, J. & Anderson, J. M. Occludin is required for cytokine-induced regulation of tight junction barriers. *J Cell Sci* **123**, 2844-2852, doi:10.1242/jcs.065581 (2010).

185 Patrick, D. M., Leone, A. K., Shellenberger, J. J., Dudowicz, K. A. & King, J. M. Proinflammatory cytokines tumor necrosis factor-alpha and interferon-gamma modulate epithelial barrier function in Madin-Darby canine kidney cells through mitogen activated protein kinase signaling. *BMC Physiol* **6**, 2, doi:10.1186/1472-6793-6-2 (2006).

186 Kim, Y. S. & Ho, S. B. Intestinal goblet cells and mucins in health and disease: recent insights and progress. *Curr Gastroenterol Rep* **12**, 319-330, doi:10.1007/s11894-010-0131-2 (2010).

187 Jonckheere, N. & Van Seuningen, I. The membrane-bound mucins: how large O-glycoproteins play key roles in epithelial cancers and hold promise as biological tools for gene-based and immunotherapies. *Crit Rev Oncog* **14**, 177-196 (2008).

188 McAuley, J. L. *et al.* MUC1 cell surface mucin is a critical element of the mucosal barrier to infection. *J Clin Invest* **117**, 2313-2324, doi:10.1172/JCI26705 (2007).
189 Das, S. *et al.* Mice deficient in Muc4 are resistant to experimental colitis and colitis-associated colorectal cancer. *Oncogene*, doi:10.1038/onc.2015.327 (2015).
190 Garidou, L. *et al.* The Gut Microbiota Regulates Intestinal CD4 T Cells Expressing RORgammat and Controls Metabolic Disease. *Cell Metab* **22**, 100-112, doi:10.1016/j.cmet.2015.06.001 (2015).
191 Luck, H. *et al.* Regulation of obesity-related insulin resistance with gut anti-inflammatory agents. *Cell Metab* **21**, 527-542, doi:10.1016/j.cmet.2015.03.001 (2015).
192 Brucklacher-Waldert, V., Carr, E. J., Linterman, M. A. & Veldhoen, M. Cellular Plasticity of CD4+ T Cells in the Intestine. *Front Immunol* **5**, 488, doi:10.3389/fimmu.2014.00488 (2014).
193 Rosser, E. C. *et al.* Regulatory B cells are induced by gut microbiota-driven interleukin-1beta and interleukin-6 production. *Nat Med* **20**, 1334-1339, doi:10.1038/nm.3680 (2014).
194 Malassine, A., Frendo, J. L. & Evain-Brion, D. A comparison of placental development and endocrine functions between the human and mouse model. *Hum Reprod Update* **9**, 531-539 (2003).
195 Linzer, D. I. & Fisher, S. J. The placenta and the prolactin family of hormones: regulation of the physiology of pregnancy. *Mol Endocrinol* **13**, 837-840, doi:10.1210/mend.13.6.0286 (1999).
196 Soares, M. J. Developmental changes in the intraplacental distribution of placental lactogen and alkaline phosphatase in the rat. *J Reprod Fertil* **79**, 93-98 (1987).
197 Mark, P. *et al.* A maternal high-fat diet in rat pregnancy reduces growth of the fetus and the placental junctional zone, but not placental labyrinth zone growth. *Journal of Developmental Origins of Health and Disease* **2**, 63-70 (2011).
198 Vickers, M. H., Clayton, Z. E., Yap, C. & Sloboda, D. M. Maternal fructose intake during pregnancy and lactation alters placental growth and leads to sex-specific changes in fetal and neonatal endocrine function. *Endocrinology* **152**, 1378-1387, doi:10.1210/en.2010-1093 (2011).
199 Kim, D. W., Young, S. L., Grattan, D. R. & Jasoni, C. L. Obesity during pregnancy disrupts placental morphology, cell proliferation, and inflammation in a sex-specific manner across gestation in the mouse. *Biology of reproduction* **90**, 130, doi:10.1095/biolreprod.113.117259 (2014).
200 Rossant, J. & Cross, J. C. Placental development: lessons from mouse mutants. *Nat Rev Genet* **2**, 538-548, doi:10.1038/35080570 (2001).
201 Smith, S. C., Baker, P. N. & Symonds, E. M. Placental apoptosis in normal human pregnancy. *American journal of obstetrics and gynecology* **177**, 57-65 (1997).
202 Smith, S. C., Baker, P. N. & Symonds, E. M. Increased placental apoptosis in intrauterine growth restriction. *American journal of obstetrics and gynecology* **177**, 1395-1401 (1997).

203 Ramsay, J. E. *et al.* Maternal obesity is associated with dysregulation of metabolic, vascular, and inflammatory pathways. *J Clin Endocrinol Metab* **87**, 4231-4237, doi:10.1210/jc.2002-020311 (2002).
204 Rivera, D. L. *et al.* Interleukin-10 attenuates experimental fetal growth restriction and demise. *FASEB J* **12**, 189-197 (1998).
205 Wu, H. & Arron, J. R. TRAF6, a molecular bridge spanning adaptive immunity, innate immunity and osteoimmunology. *Bioessays* **25**, 1096-1105 (2003).
206 Crew, R. C., Waddell, B. J. & Mark, P. J. Maternal obesity induced by a 'cafeteria'diet in the rat does not increase inflammation in maternal, placental or fetal tissues in late gestation. *Placenta* **39**, 33-40 (2016).
207 Jansson, T. & Powell, T. L. Role of the placenta in fetal programming: underlying mechanisms and potential interventional approaches. *Clin Sci (Lond)* **113**, 1-13, doi:10.1042/CS20060339 (2007).
208 Clarke, L. L. A guide to Ussing chamber studies of mouse intestine. *Am J Physiol Gastrointest Liver Physiol* **296**, G1151-1166, doi:10.1152/ajpgi.90649.2008 (2009).

8.0 APPENDIX I

8.1 Awards

FHS Research Plenary: award for outstanding oral presentation
McMaster University, Hamilton, ON, May 2015

Faculty of Health Sciences Graduate Programs Outstanding Achievement Award
McMaster University, Hamilton, ON, May 2015

CIBC Graduate Bursaries in Breast Cancer
McMaster University, Hamilton, ON, April 2015

8.2 Publications

Wallace JG, Gohir W, Sloboda DM."The impact of early life gut colonization on metabolic and obesogenic outcomes: what have animal models shown us?" J Dev Orig Health Dis. 2015 Sep 24:1-10.

Gohir W, Whelan FJ, Surette MG, Moore C, Schertzer JD and Sloboda DM. "Pregnancy-related changes in the maternal gut microbiota are dependent upon the mother's periconceptional diet." Gut Microbes. 2015 Sep 3;6(5):310-20.

Chan KA, Bernal AB, Vickers MH, Gohir W, Petrik JJ and Sloboda DM. "Early life exposure to undernutrition induces ER stress, apoptosis, and reduced vascularization in ovaries of adult rat offspring." Biol Reprod. 2015 Apr;92(4):110.

Gohir W, Ratcliffe EM and Sloboda DM. "Of the bugs that shape us: maternal obesity, the gut microbiome, and long-term disease risk." Pediatr Res. 2015 Jan;77(1-2):196-204.

8.3 Conference Presentations

Delivered an oral presentation titled "Maternal obesity alters placental gene expression in a sex specific manner", at the 9th World Congress of Developmental Origins of Health and Disease held November 8th -11th, 2015 in Cape Town, South Africa.

Delivered an oral presentation titled "Pregnancy-related changes in the maternal gut microbiota and impacts of a periconceptional high fat diet", at the Faculty of Health Sciences Research Plenary 2016 held May 5th - 7th at McMaster University, Hamiltion.

Delivered an oral presentation titled "Maternal diet induced changes in the gut microbiota during pregnancy in a mouse model", at the 43rd Annual Southern Ontario Reproductive Biology Meeting held on May 1st, 2015.

Delivered an oral presentation titled "Pregnancy-related changes in the maternal gut microbiota and impacts of a periconceptional high fat diet", at the the 2nd Canadian National Perinatal Research Meeting held February 24th – 27th , at Le Chateau Montebello, Montebello, Quebec, Canada

Delivered an oral presentation titled "Pregnancy-related changes in the maternal gut microbiota and impacts of a periconceptional high fat diet in a mouse model" at the 40th Annual R.T. Weaver Scientific Day, McMaster University, Hamilton, ON. November 2014.

Presented a poster at the 42nd Annual Southern Ontario Reproductive Biology Meeting held in April 2013 at the Toronto Zoo, Toronto, ON.

Presented a poster at the 36th Annual Perinatal Investigators Meeting held at University of Toronto, Toronto ON. November 2012.