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Dietary Manipulation at Pre-Conception and During Development Influence Metabolism and Gut Microbiota in Rats

by

Fatima Chleilat

A THESIS

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Abstract

Background Obesity is a complex disease, modulated by a plethora of factors, including gut microbiota, early post-natal nutrition, parental diet and epigenetics.

Objective This dissertation examines how dietary manipulation during the early post-natal period and during pre-conception alters metabolic, microbial and epigenetic outcomes in rats. Specifically, the objectives were to determine: 1) the impact of human milk oligosaccharide (HMO) supplementation on health status in weanling rats; 2) the impact of a paternal high protein (HP) diet on offspring metabolic health; 3) whether supplementation of a methyl donor cocktail (HF/S+M) before conception attenuates the deleterious metabolic and epigenetic effects of a paternal high fat/ sucrose diet intergenerationally; 4) the impact of paternal prebiotic consumption on microbial and metabolic outcomes in fathers and offspring.

Methods 1) Weanling male and female rats consumed 2'Fucosyllactose and 3'Sialylactose HMO-fortified diet for 8 weeks. Fathers consumed 2) a diet high in protein, 3) a high fat/ sucrose diet fortified with a methyl donor cocktail of betaine, choline, folic acid and vitamin B12, or 4) a high prebiotic fiber diet. All three paternal studies underwent their dietary intervention for 9 weeks. Offspring consumed a control diet for 13 weeks. Anthropometric, glucocentric and gut microbiota outcomes were measured in all four studies.

Results The primary findings include: HMO supplementation improved intestinal permeability, gut barrier function, and gut microbial composition in females while reducing weight gain and inflammatory cytokines in males; 2) Paternal HP diet reduced adiposity and altered epigenetic markers intergenerationally. Offspring had improved insulin sensitivity; 3) Paternal HF/S+M intake improved paternal reproductive outcomes and intergenerational gut microbial, epigenetic and metabolic outcomes; 4) Paternal prebiotic intake improved paternal gut microbiota with lesser effects in offspring.

Conclusion Our results provide evidence of early post-natal HMO supplementation and paternal HP, HF/S+M and prebiotic intake, as important modulators of gut microbial, epigenetic and metabolic outcomes.

Preface

This dissertation is presented in a manuscript-based format.

Chapter 3 has been published in *Nutrients.*

Chleilat F, Klancic T, Ma K, Schick A, Nettleton JE, Reimer RA. (2020). Human milk oligosaccharide supplementation affects intestinal barrier function and microbial composition in the gastrointestinal tract of young Sprague Dawley rats. *Nutrients*. 12(5): 1532.
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Chapter 4 has been submitted for publication in *Proc Natl Acad Sci.*

Chleilat F, Schick A, Deleemans JM, Ma K, Alukic E, Wong J, Noye Tuplin EW, Nettleton JE, Reimer RA. (2020). Paternal high protein diet modulates body composition, insulin sensitivity, epigenetics and gut microbiota intergenerationally.

Chapter 5 has been published in the *International Journal of Molecular Science.*

Chleilat F, Schick A, Deleemans JM, Reimer RA. (2021). Paternal Methyl Donor Supplementation in Rats Improves Fertility, Physiological Outcomes, Gut Microbial Signatures and Epigenetic Markers Altered by High Fat/High Sucrose Diet. *Int J Mol Sci*. 22(2): 689.
DOI: 10.3390/ijms22020689.

Chapter 6 is in preparation for submission to *Nutrients.*

Chleilat F, Schick A, Reimer RA. Microbiota changes in fathers consuming a high prebiotic fiber diet have minimal effects on male and female offspring in rats.

Given the manuscript-based format, there is some redundancy in the methods described and background information between chapters.

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To Grace, I miss you, I love you and thank you for being a guiding light for me through this PhD and every day.

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Finally, to my faithful pup, Eva- thank you for keeping me sane and providing me endless love and cuddles while I wrote this dissertation.

Dedication

I would like to dedicate this thesis to all the awe-inspiring women who are or have been in my life, paving my way to this very moment.

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List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
2'FL	2'-*O*-Fucosyllactose
3'SL	3'Sialyllactose
AMPK	AMP-Kinase
ANOVA	Analysis of Variance
ASV	Amplicon Sequence Variants
BCFA	Branched Chain Fatty Acids
BMC	Bone Mineral Content
BMD	Bone Mineral Density
BMI	Body Mass Index
CpG	Cytosine Phosphate Guanine
DIO	Diet-Induced Obesity
DNMT	DNA methyltransferase
DOHAD	Developmental Origins of Health and Disease
DXA	Dual energy X-ray absorptiometry
FITC	Fluorescein isothiocyanate-dextran-4000
FOS	Fructooligosaccharides
GI	Gastrointestinal
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon-like peptide 1
GLUT	Glucose Transporter
GOS	Galactooligosaccharide
GPR41	G-protein coupled receptor 41
GPR43	G-protein coupled receptor 43
Hcy	Homocysteine
HDL	High-density lipoprotein
HFD	High fat diet
HF/S	High fat/ sucrose
HMO	Human milk oligosaccharide
HOMA-IR	Homeostatic Model Assessment of Insulin Resistance
HP	High Protein
HPLC	High Performance Liquid Chromatography
IL-6	Interleukin-6
IL-10	Interleukin-10
IPT	Intestinal Permeability Test
ITT	Insulin tolerance test
Kcal	Kilocalories
LDL	Low-density lipoprotein
LEfSe	Linear discriminant analysis effect size
LPS	Lipopolysaccharide
MiR	MicroRNA
MiRNA	MicroRNA
MMP2	Matrix metalloproteinase 2
MMP9	Matrix metalloproteinase 3

mRNA	Messenger RNA
mTOR1	Mammalian Target of Rapamycin Complex 1
MUC2	Mucin 2
ncRNA	Non-coding RNA
OGTT	Oral glucose tolerance test
PCoA	Principal Coordinates Analysis
PERMANOVA	Permutational Multivariate Analysis of Variance
PYY	Peptide tyrosine tyrosine
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RT-PCR	Real-time polymerase chain reaction
SCFA	Short chain fatty acid
SD	Sprague Dawley
SEM	Standard error of the mean
T2D	Type 2 Diabetes
TJP	Tight Junction Protein
TNA	Tumour Necrosis Factor
VLDL	Very low-density lipoprotein
ZO-1	Zonula occludens

Epigraph

"If I have seen further, it is by standing on the shoulders of giants"

Isaac newton

CHAPTER ONE: INTRODUCTION

1.1 Background

The Developmental Origins of Health and Disease (DOHaD) hypothesis states that environmental stimuli or perturbations during critical prenatal and postnatal periods influence developmental pathways that induce permanent changes in metabolism and chronic disease susceptibility[1]. Maternal health at pre-conception, during gestation and during lactation has been widely attributed with influencing long-term health status in offspring[2]. A myriad of factors influence maternal health including nutrition, alcohol and drug ingestion, smoking, exercise and pollutant exposures, all of which have been attributed with either lasting health benefits or increased risks for offspring[3]. Notably, maternal nutrition can directly impact postnatal programming by altering the diverse soluble carbohydrate polymers in breast milk known as oligosaccharides. Milk oligosaccharides are the third most abundant component of human milk after lactose and lipids[4] and serve as biological and physiological protective agents[5,6]. These human milk oligosaccharides (HMOs) have the potential to substantially influence the establishment of the developing gut microbiota in the newborn and consequently influence metabolism and disease risk long term[7].

Beyond maternal factors, there is growing recognition for the importance of paternal health at the time of conception to also influence embryo development, fetal growth and offspring health into adulthood. Consumption of a high fat/sucrose (HFS) diet by fathers has been shown to be detrimental for offspring health[8], but the impact of other dietary patterns or constituents are not as well understood at present. One of the chief mechanisms by which maternal and paternal nutrition influence offspring health is via epigenetics[6]. Epigenetics is the study of heritable and observable changes in an organism resultant of alterations in gene expression that occur independent of changes to DNA sequence. Epigenetic changes are frequently occurring manifestations; however, they are also influenced by multiple factors including diet. Systems commonly responsible for initiating and sustaining epigenetic changes throughout cell division include: DNA methylation, histone modification and non-coding RNA. DNA methylation occurs chiefly on the cytosines of a dinucleotide sequence, cytosine phosphate guanine (CpG)[10]. CpG methylation can directly affect transcription by altering the binding of methyl-sensitive-DNA-binding proteins and indirectly by influencing regional chromatin conformation[10]. This epigenetic mechanism is highly influenced by early nutrition in the form

of dietary methyl donors and cofactors, where one-carbon mammalian metabolism may supplement methyl groups to all biological methylation reactions [11]. Histone modification is another form of epigenetic regulation. Histones facilitate DNA packaging into nucleosomes to ultimately form chromatin[12]. Post-translational modifications to histones by acetylation or deacetylation can alter chromatin structure and function, which may also impact transcriptional activity or DNA repair[12]. This involves the addition or removal of an acetyl group on the N-terminus of lysine residues located on histones within a nucleosome [12]. MicroRNAs (miRNAs) are endogenous small (~22 nucleotides long) RNA molecules encoded in the genome that affect gene expression[13]. MiRNAs bind with complete or incomplete complementarity to their target mRNA and downregulate translation or stability[13]. Each miRNA is believed to have multiple targets[14].

The gut microbiota, which is now known to influence host metabolism, is impacted by both nutrition and epigenetic status[15–18]. Bacteria reside on many external and internal locations on the human body, including the skin, oral mucosa, saliva and gastrointestinal tract[19]. The colon is the most densely colonized ecosystem in the human body, which amounts to approximately 3.8×10^{13} microbial cells in a 70 kg *reference man*[20]. At the phylum level, approximately 90% of the large intestine microbes are comprised of Firmicutes (anaerobic, many but not all, spore forming bacteria) and Bacteriodetes (anaerobic non-spore forming bacteria)[21]. Other phyla that are not as abundant in the gut microbiota, but elicit significant effects on human health include: Actinobacteria, Proteobacteria and Verrucomicrobia[22,23]. During the different periods of human development, the human gut microbiome experiences dynamic changes in composition and function[24]. Although a 'healthy' gut microbiota has not been identified and may not exist as a single entity, characteristics of healthy microbiotas include an increase in species richness[25] and prevalence of commensal bacteria belonging to the *Bifidobacterium, Lactobacillus, Akkermansia, Roseburia, Blautia, Faecalibacterium*, *Ruminococcus* genera, among others[24,25]. Notable species increased in diseased states belong to the *Clostridium, Enterocococcus, Enterobacter,* and *Bacteroides* genera [26,27].

The predominant factors that influence gut microbial composition include birth mode[28,29], infant feeding practices[30,31], antibiotic use[32], genetics[33,34], age[35], geography[36] and diet[37]. Perhaps the most impactful factor on the gut microbiota is diet[38], accounting for an estimated 57% of gut microbiota changes, compared to 12% of host genes in

one study[39]. During breastfeeding, the infant is privy to the *microbial fuel,* HMOs, which elicit selective nourishment and growth capabilities in highly specific bifidobacteria strains, yielding long term health benefits[40]. Similarly, ingestion of high protein and more notably high prebiotic fiber diets[41,42] have all yielded alterations in gut microbial communities.

Given the importance of early life in molding future health and disease susceptibility, it is important to better understand the prenatal and postnatal factors that result in positive or negative consequences for offspring. This thesis focuses on two aspects of early life development, namely HMO supplementation in weanling rats and paternal diets high in fat/sucrose, protein and prebiotic fiber as well as supplementation with methyl donors.

1.2 Purpose of Research

The overall purpose of this thesis is to explore the impact of diet before conception and during postnatal development on metabolic outcomes and the gut microbiota. Findings from this research will add to the knowledge of diet-based therapies targeting epigenetic status and the gut microbiota to improve the health status of offspring into adulthood. Additionally, the goal of this research is to ultimately stimulate further research that will help guide health professionals on potential proactive and retroactive therapies to improve heritable metabolic, epigenetic, fertility and gut microbial outcomes.

1.3 Overview of Thesis Chapters

This manuscript-based thesis consists of seven chapters. Chapter one provides a brief introduction to the dissertation. Chapter two is a literature review that discusses the interplay between metabolic outcomes, the gut microbiota, nutrition and epigenetics on health status. Chapter three assesses the impact of human milk oligosaccharide supplementation in weanling rats on intestinal barrier function and gut microbial composition. Chapter four examines how paternal high protein diet modulates body composition, insulin sensitivity, epigenetics and gut microbiota intergenerationally. Chapter five examines how paternal methyl donor supplementation preconceptionally improves fertility, physiological outcomes, gut microbial signatures and epigenetic markers altered by high fat/ high sucrose diet. Chapter six assesses how paternal high prebiotic diet affects metabolic and gut microbial outcomes

intergenerationally. Chapter 7 discusses the significance of this dissertation’s main findings and considers the strengths, limitations, and future directions of this research.

CHAPTER TWO: LITERATURE REVIEW

2.1 Obesity and Metabolic Syndrome

The rising prevalence of obesity and metabolic syndrome is a global health concern impacting adults, adolescents and children. Obesity is defined as excessive fat accumulation, with a body mass index (BMI) ≥30 kg/m^2 in adults and a BMI above the 95th percentile in children, which could increase the risk of chronic disease [43]. The aetiology of obesity is extremely complex, involving a myriad of causes and individual factors, including genetics, metabolism and environment[44]. It is known that weight gain is partially owing to an increase in total energy intake, typified by increased intake of refined carbohydrates, fats and/or processed foods (resulting in an increase in insulin release and fat storage), and reduced physical activity[45]. Maternal obesity during pregnancy is an important driver of obesity in children[46], which can be further exacerbated by exposure to an *obesogenic* environment in childhood, therefore increasing their proclivity to consume energy dense, nutrient-poor foods[47]. Evidence from the Lifeways cross-generational cohort study showed that maternal BMI influenced BMI across three generations[48]. Similarly, the 1958 British Birth Cohort Study showed that paternal BMI also influenced offspring BMI in a way that was independent of, but additive to effects of maternal BMI[49]. In fact, BMI from the maternal and paternal line influenced offspring BMI at 11 years of age, which persisted until the mid-forties in both sexes[50]. In animal models, maternal[51] and paternal[52] high fat diet (HFD)-induced obesity adversely affects body weight and glucose metabolism in offspring.

Metabolic syndrome is a combination of cardiometabolic risk factors, which together increase susceptibility to cardiovascular disease (CVD) and type 2 diabetes. Type 2 diabetes is characterized by hyperglycemia and impaired glycemic response owing to insulin resistance and relative deficiencies in insulin secretion by β-cells[53]. Insulin is an anabolic peptide hormone secreted by pancreatic beta cells and functionally acts through receptors on target cells, primarily the liver, adipose tissue and skeletal muscle[54]. The condition whereby cells are unable to respond well to circulating insulin is known as *insulin resistance.* Insulin is the chief regulator of macronutrient metabolism[55]. It functions to impede gluconeogenesis in the liver; inhibit lipolysis of stored fat in adipose tissue; triggers the translocation of GLUT4 protein to allow glucose to enter cells like myocytes; and finally, it indirectly modulates brain glucose

homeostasis. Therefore, dietary patterns or specific nutrients that influence insulin homeostasis in the body play an important role in determining the metabolic health of an individual.

It has been established that the *in utero* environment influences insulin resistance and metabolic syndrome later in life in offspring[56]. The Thrifty Phenotype, otherwise known as the Barker hypothesis, postulates that an adverse *in utero* environment causes intrauterine growth retardation and reduced birth weight which are risk factors in the development of metabolic syndrome in adulthood[57]. Following birth, the nutritional environment can either attenuate or accentuate the programming that occurred *in utero*[57]. Studies have shown that breastfed infants are generally characterized by more protective metabolic health later in life compared to formula fed infants[58,59]. This phenomenon and the factors that may contribute to this protection will be reviewed in greater detail later on in this review.

In metabolic disease states, the gut microbiota is notably altered in its composition to become more inflammatory in nature and to facilitate increased energy harvest from the diet[60]. Originating with a sterile gut (although this has recently been debated[61]), the neonate's intestinal tract is quickly colonized by bacteria from their surrounding environment during birth[62]. The initial colonizers vary substantially depending on delivery mode, with vaginal birth eliciting a more favorable gut microbial signature for long term metabolic health compared to caesarean section[62]. In early life especially and throughout the lifespan, one of the most dominant contributors to gut microbial composition is diet[60]. Diets rich in dietary fiber that provide abundant fermentative fuel for the microbiota are among the most protective in terms of metabolic health[63]. Diets low in dietary fiber and high in fat and simple sugars predispose individuals to obesity and cause disruptions to the gut microbial community, a condition termed *dysbiosis*[64]. Turnbaugh and colleagues[65] were among the first researchers that identified a link between the *obese* gut microbiota and weight gain. The authors suggested that the bacteria inhabiting the gut have energy-harvesting capabilities which are altered in metabolically diseased states[65]. More specifically, the gut microbiome harbours glycoside hydrolase enzymes, not found within the human genome, which are vital in hydrolysing and fermenting numerous dietary polysaccharides that contribute to host energy[66,67]. The microbial by-products derived from these polysaccharides are known as short chain fatty acids (SCFA). The main SCFAs are acetate, propionate and butyrate, which are crucial in maintaining host health and in the case of butyrate, the main energy source for colonocytes[60].Given the importance of the gut microbiota

to metabolic health, it is important to understand the dietary factors, particularly in early life that shape the gut microbiota and contribute to long-lasting effects on host metabolism.

2.2 Developmental Origins of Health and Disease

2.2.1 Epigenetics

Epigenetics is a fast-growing field of research that is emerging as a tool to understand the influence of the environment on gene expression across a wide range of diseases including obesity, type 2 diabetes and inflammation[68]. One of the chief mechanisms by which parental experiences influence offspring health is through epigenetics. Epigenetics is the study of heritable and observable changes in an organism, resultant of alterations in gene expression[69]. Epigenetic changes are frequently occurring manifestations; however, they are also influenced by multiple factors. Systems commonly responsible for the initiation and sustained epigenetic change include: DNA methylation, histone modification and small non-coding RNA[69].

DNA methylation is the most widely researched epigenetic change[69]. It is a stable, transmissible epigenetic modification that is recalled during sperm maturation, when other epigenetic changes are terminated[70]. DNA methylation is also easy to evaluate experimentally, however, the biological significance is difficult to deduce, since modifications target a multitude of different genes, many of which affect the same phenotype[71]. Despite limitations, several studies have been conducted that demonstrated that key methylation patterns from the paternal germline may influence offspring metabolism[72]. In humans, cord blood from infants born of obese fathers showed significantly lower methylation levels in the human imprinted gene, paternal expressed gene 3 (PEG3)[73]. This gene is involved in fetal growth, suggesting that periconceptional over-nutrition reprograms key imprint marks during gametogenesis and early development[73]. Other studies have observed similar hypomethylation patterns in the sperm of pups born of metabolically compromised fathers[74,75].

The transmission of fetal alcohol syndrome through the paternal germline was among the first models to assess how paternal experiences affect offspring outcomes. Paternal alcohol exposure four weeks prior to mating in a rodent model, increased growth retardation up to one year of age in offspring, reduced glial cell maturation and impaired behaviour[76]. Additionally, research in pubescent rats maintained on an alcohol-liquid diet for 39 days showed impairments in spatial learning performance in their offspring compared to non-alcohol sired offspring[77].

Reports have attributed these offspring outcomes to a reduction in cytosine methyltransferase mRNA levels- a key enzyme that catalyzes methyl group transfer[78]. Changes in this enzyme could affect genomic imprinting by reducing DNA methylation and leading to the expression of paternal alleles that are meant to be silent[78]. Likewise, paternal exposure to the industrial chemical bisphenol A during the perinatal period resulted in impaired glucose tolerance, impairments in insulin secretion, and reductions in β cell mass[79]. These changes were linked with the increased methylation of Igf2 gene in paternal sperm[79]. Still, since functional genes are largely de-methylated following fertilization, it is improbable that DNA methylation alone implicates heritable programming from the paternal lineage to their offspring. It is however possible that another heritable molecular epigenetic factor mediates the methylation patterns in the blastocyst and embryo.

Histone modifications include methylation, phosphorylation, acetylation, ubiquitylation, and sumoylation, all of which modulate chromatin structure[12]. The acetylation of lysine residues in the histone tails of histones H3 and H4 denote transcriptional activation of genes[80]. Trimethylation of lysine 27 on histone 3 (H3K27me3) and trimethylation of lysine 9 on histone 3 (H3K9me3) describe silenced genes[80] denoting epigenetic crosstalk between DNA methylation and histone modifications[80]. However, it remains ambiguous whether these histone modifications are preserved through the germline. What's more is that sperm chromatin and somatic cell chromatin structures are largely distinct. Unlike somatic cells, sperm chromatin is compact. Still, there is evidence in a Xenopus model that chromatin remodelling is retained in developmentally crucial genes in the early embryo[81]. Evidence in animal and human models examining histone modifications in the paternal germline are otherwise limited.

It is postulated that RNA may play a crucial role in epigenetic based evolutionary diversity. Non-coding RNAs (ncRNAs) are classified as long (>200nucleotides) or small (<200 nucleotides). They do not encode proteins but instead operate as small housekeeping or regulatory ncRNA[82]. Sperm is rich in small ncRNAs including piwiRNA, transfer RNAs (tRNA), microRNA among others, all of which function as key regulatory RNAs that affect splicing, transcription, translation and RNA modifications[83]. PiWi-associated RNAs (piRNAs) are associated with germline cells. It is speculated that their primary function is to preserve genome stability[84].

Transfer RNA fragments (tRFs) have recently started to receive recognition in their role influencing offspring development. Transfer RNA fragments are abundant in sperm and represent a paternal epigenetic factor that mediate intergenerational inheritance of metabolic disorders resultant of paternal nutrition[85]. In 2016, Chen *et al.* showed that a high fat diet in rodents alters gene expression of metabolic pathways in early embryos and islets of F1 offspring[86]. These changes in expression were unrelated to DNA methylation[86]. In another paternal nutrition study, protein restriction in male mice showed distinct changes in tRFs in the male reproductive tract as well as in mature sperm[87]. Taken together, these studies show that tRFs may be more sensitive to paternal dietary interventions than other small non-coding RNAs.

Maturing sperm endure significant reorganization of small non-coding RNA in the epididymal tract, likely by interacting with RNA-rich extracellular vesicles[88]. This is characterized by the gain of 115 and loss of 113 microRNAs. There is also evidence that microRNAs are involved in inherited metabolic abnormalities in different metabolic tissues[89–91]. Broadly, microRNAs are involved in various regulatory pathways including insulin signaling, inflammatory pathways, adipokine expression, lipid metabolism and energy intake[92]. For example, the progeny of rat mothers fed a low protein diet had decreased pancreatic β-cell mass and impaired function due to miRNA misexpression in β-cells which has been shown to be transmitted transgenerationally[93]. Human models have similarly shown misexpression of miRNAs in the plasma[94,95] and adipose tissue[96] of individuals with type 2 diabetes. Therefore, it is clear from human and animal models that metabolic abnormalities alter microRNA expression in metabolically vital organs, since they modulate normal physiology and function as mediators of disease onset.

2.2.2 Paternal Programming

There is now an abundance of evidence that adult health is highly dependent on the *in utero* maternal environment[97]. Numerous epidemiological studies and animal studies have corroborated the link between impaired maternal health status during gestation and increased incidence of chronic disease in offspring, including obesity and type 2 diabetes[98]. This forms the basis of the developmental origins of health and disease (DOHaD) hypothesis. Based on the original Barker hypothesis, coined in 2003, DOHaD suggests that the early life environment has widespread consequences for later health[99]. Presently, this hypothesis has evolved to include the full array of parental programming [99]. Recent findings have highlighted multiple

environmentally induced paternal epigenetic effects, periconceptionally and postnatally, that may influence offspring development. The most widely studied to date is paternal body composition and nutrition.

Paternal obesity has been associated with reduced fertility[100,101]. Men with obesity have been shown to have lowered levels of testosterone, partially owing to elevated levels of estrogen, leptin and insulin in circulation, known to impact gonadotrophin secretion that affect spermatogenesis[102]. Increased scrotal adiposity in male obesity decreases sperm motility and increases oxidative stress and DNA damage[101,103]. Obesity and reduced sperm quality have also been linked with fertilization rate and embryo development, wherein, male obesity has been associated with lowered time to conception and fertilization efficacy[104]. The link between male overweight or obesity and impaired fertility has been further corroborated by a large meta-analysis involving 115,158 subjects[105]

Paternal nutritional programming also contributes to the health status of offspring. Human and rodent models have shown protective effects resultant of paternal caloric restriction[106]. Paternal exposure to famine in humans during their slow growth period resulted in a lower incidence of cardiovascular mortality in their sons[106]. Similarly, paternal caloric restriction at different time points prior to conception in mice exhibited a significant reduction in corticosterone and insulin-like growth factor-1 in male offspring, indicative of protective metabolic effects and a potential predictive adaptive response, mediated by sperm epigenetic modifications [107]. Conversely, paternal over-nutrition has been attributed with increased diabetes risk[108] and decreased longevity in male offspring characterized by fasting hyperglycaemia, insulin resistance and glucose intolerance in mice[109].

Formative research utilizing a paternal high fat diet for 10 weeks prior to mating, demonstrated impaired pancreatic β cell function, impaired insulin secretion and increased bodyweight in their offspring[110]. The resultant phenotype increased the risk of obesity in the offspring in adulthood[110]. Similarly, paternal low protein diet (LPD) has been associated with metabolic disturbances in offspring by increasing the expression of genes associated with cholesterol and hepatic lipid biosynthesis, like DNA methylation in peroxisome proliferator-activated receptor (*Ppar*) α, a key lipid regulator[111]. Another paternal LPD study showed increased BMI, adiposity, glucose intolerance and altered gut bacterial profiles in offspring of LPD fathers compared to control fathers[112]. The authors associated these detrimental effects to

sperm-DNA methylation and transfer RNA fragments[112]. Many other paternal dietary patterns have the potential to affect offspring metabolism but have yet to be examined.

2.3 Gut Microbiota

The gastrointestinal tract is home to a complex microbial community. The link between the gut microbiota and host metabolism is regulated in part via the ability of the microbiota to extract energy from dietary compounds that are indigestible by the host, while simultaneously producing metabolites and cytokines that affect host metabolism[44]. Molecular analyses of fecal samples have provided important information regarding the composition of the intestinal microbiota. Of the eight phyla identified in human gut microbiota, five are most common: Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria and Verrucomicrobia. Of these, Bacteroidetes and Firmicutes account for the highest abundance. Species belonging to these phyla are symbiotic in nature and carry out multiple functions involved in metabolism, immunity and overall human health[113]. These include maintaining mucosal integrity, aiding in the breakdown of ingested food, reducing pathogen colonization in the gut, and the age-appropriate maturation of the immune system[113]. Disrupting this gut microbial homeostasis can lead to a dysbiotic state. Dysbiosis is linked with deleterious health effects that may have life-long consequences linked with multiple diseases like obesity and type 2 diabetes[114,115]. Gut dysbiosis is characterized by decreased alpha diversity, an increase in facultative taxa (like Enterobacteria) and a reduction in advantageous metabolites, like short chain fatty acids (SCFA)[116]. Gut dysbiosis that occurs during critical period of development has the potential to increase susceptibility to immune-related diseases, glucose intolerance, increased fat mass and higher risk of obesity in adulthood[117]

The gut microbiota plays a crucial role in energy homeostasis; therefore, it is no surprise that there is a link between gut microbial composition and body weight. Early DNA sequencing studies suggested that the microbiome of individuals with obesity is more efficient at harvesting energy from diet compared to lean controls [65]. This seminal work utilized obese mice compared to a lean *obl*+ mouse, derived from the same litter[65]. They subsequently examined the gut habitat from the mice and validated that the increased ability of the *ob/ob* microbiome for dietary energy harvest, using biochemical assays and transplantation of the cecal microbiotas of lean and obese mice into germ-free wild-type mice[65]. Today, our understanding of the gut

microbiota's contribution to body weight regulation has expanded and is also known to include microbial influence on intestinal permeability, endotoxemia, insulin resistance, hormonal environment, expression of genes regulating lipogenesis, and interaction with bile acids[118]. Animal studies have elucidated key mechanisms underlying the distinctions between microbiota seen in the obese versus lean state, including[119]:

1. Hepatic *de novo* lipogenesis via carbohydrate and sterol response-element binding proteins, resultant of increased caloric intake.
2. Increased cellular uptake of fatty acids and storage of triglycerides in adipocytes.
3. Suppression of skeletal muscle fatty acid oxidation.
4. Interaction of SCFA and G-protein coupled receptor 41, increasing peptide tyrosine tyrosine (PYY).
5. Inducing low-grade inflammation by mediating lipopolysaccharide (LPS) levels.

While early studies indicated that obese mice and humans were characterized by an increase in Firmicutes and a decrease in Bacteroidetes compared to lean, this characterization has now been expanded to include lower bacterial diversity and lower abundance of *Bifidobacterium*, *Christensenellaceae*, *and Akkermansia*[120]. While diet and other factors such as antibiotics can affect gut microbiota composition throughout life, early life alterations in the microbiota appear to be particularly important in shaping individual risk to metabolic and immune dysfunction later. The most well-recognized early life environmental influences include: delivery mode, antibiotic use, and early life nutrition[114]. Maternal milk, as the sole nutrition for the infant and developing gut microbiota in exclusively breastfed infants, is a major early life nutritional influence.

2.4 Gut Microbiota and Breastfeeding

Breast milk consumption during the first year of life is a fundamental determinant of infant microbiota development and can mitigate disease risk later in life[121]. In Canada, less than 17% of infants are exclusively breastfed for the recommended period of 6 months[122]. Here we will review age-appropriate succession in the infant gut of vaginally born infants, with a key focus on compositional and diversity shifts in breast-fed versus formula fed infants.

Bacterial colonization of the neonate's gastrointestinal tract commences during birth when the neonate encounters bacterial communities of the maternal vaginal tract. Infants born by

caesarean section have a gut microbiota colonized by skin bacteria as well as bacteria from their surrounding environment (i.e. the hospital)[28]. Typically, during the first few days of life, healthy infants are colonized by facultative anaerobic bacteria like *Enterobacteriaceae, Staphylococcus* and *Streptococcus* which persist for the first 2 weeks before *Bifidobacterium* spp. start to flourish[123]. Prevalence of *Bifidobacterium* spp. after this period is dependent on feeding patterns.

Human breast milk plays a key role in promoting the growth of *Bifidobacterium* species. The KOALA Birth Cohort Study, a prospective birth cohort based out of the Netherlands, demonstrated that exclusively formula-fed infants at 1 month of age were colonized with increased levels of *Clostridium difficile* as well as *Escherichia coli, Bacteroides fragilis* and *Lactobacillus* species, compared with exclusively breast-fed infants[124]. The presence of these bacteria in early infancy has been attributed with higher body mass later in childhood[125]. Functionally, breastfed infants compared to formula-fed infants carry microbes that contribute to oxidative phosphorylation and vitamin B synthesis[126].

Immune related processes are postulated to drive the majority of the functional effects linked with breast-feeding. The GI mucosal membrane is lined with a local adaptive immune system that develops gradually[127]. Colonization of the gut with commensal bacteria, like bifidobacteria, functions as a key antigenic stimulus for the development of gut-associated lymphoid tissue (GALT)[127]. This antigenic stimulus increases Immunoglobulin A (IgA) producing cells[127]. At four weeks of age, secretory IgA antibodies are detected in the neonate's stool[123]. This is consistent with the maturation of the infant gut, moving from facultative anaerobes at birth, to *Bifidobacterium* spp. Isolated *Bifidobacterium breve*, orally administered in pre-weaned mice showed an increase in FoxP3+ regulatory T cells[128], which are critical in maintaining immune tolerance and immune homeostasis. This demonstrates a robust gradual maturation of the intestine's immunological defense mechanisms and that *Bifidobacterium* species are strong modulators of the immune response[127].

Early studies examining the dynamics and stabilization of human gut in the first year of life found that infants exclusively breastfed in the first four months showed higher levels of *Lactobacillus*, which are essential microbes involved in lactate metabolism[129]. They also observed increased abundance in *Bifidobacterium, Collinsella* and *Veillonella* species[129]. Alpha diversity in these exclusively breast-fed infants was markedly lower[130]. Comparatively,

the Canadian Healthy Infant Longitudinal Development (CHILD) birth cohort study showed that formula-fed infants at four months of age had a higher alpha diversity, with an enhanced abundance of *C. difficile,* relative to exclusively breastfed infants[131].

As previously mentioned, breastmilk increases bifidobacteria in the infant's GI tract, a fact that has been mostly attributed to the presence of Human Milk Oligosaccharides (HMOs)[132]. HMOs are a group of unconjugated glycans that occupy the third most abundant component of breast milk[132]. HMOs reach the colon largely intact, functioning as prebiotics for *Bacteroides*[133] and *Bifidobacterium*[134] species.

2.5 Human Milk Oligosaccharides

Nearly a century ago scientists postulated that oligosaccharides in human milk might serve as a growth factor that enriches healthy *bifidus* gastrointestinal bacteria in breast fed infants[135]. HMO concentrations and composition vary between women and throughout lactation[135]. A few days before and a few days after childbirth, a thick, yellowish fluid, secreted by the mammary gland, known as colostrum contains the most concentrated amount of HMOs, approximately 20-25g/L[136]. Eventually, as milk production matures, HMO concentrations drop to approximately 5-20g/L[137] which reflects a substantial interpersonal difference between mothers. These oligosaccharides are made up of five monosaccharides: glucose, galactose, *N*-acetylglucosamine, fucose and sialic acid[134]. At the reducing end of all HMOs, lactose is present and can be elongated or extended into a myriad of different structures[134]. These structures can then be either fucosylated or sialylated. To date, approximately 200 HMOs have been identified[134]. HMOs synthesized by the mother are dependent on their blood group of which there are four[134]. These milk groups are assigned according to the Le and Secretor (Se) blood group. Gene loci encode for α1-2/4-fucosyltransferase FUT3 (encoded by the Le gene) and α1-2 fucoslyltransferase FUT2 (encoded by the Se gene)[134], the latter are classified as Secretors. The HMO composition of secretor women is abundant in 2'fucosyllactose (2'FL), Lacto-N-fucopentose 1 (LNFP 1) and other fucosyllated HMOs[134]. Milk oligosaccharide quantity and composition are very different between species, for example mice only contain 3'- and 6'- sialyllactose, but none of the fucosyllated oligosaccharides predominately present in human milk[138]. Bovine milk on the other hand possess a fraction of the oligosaccharides present in human milk[139]. This makes it

challenging to investigate oligosaccharides in other species and create milk oligosaccharide fortified formulas for infants with all the encompassing benefits of HMOs. Sela and colleagues[140] sequenced *Bifidobacterium longum* subsp. *infantis* (*B. infantis*), a bacterium known to flourish when HMOs are the only carbohydrate source. They found that *B. infantis* contains entire gene clusters that control the expression of specific glycosidases, sugar transporters and glycan-binding proteins for HMO utilization, suggesting a critical functional benefit of the HMO component of breastmilk [140]. Recently, research has demonstrated that HMOs are more than just a substrate that promote the growth of desired bacteria; HMOs also serve as antiadhesive antimicrobials, immune modulators, and nutrients for brain development[136].

Some HMOs resemble mucosal cell surfaces, thus enabling them to act as antiadhesive antimicrobials by serving as decoy receptors[141]. This prevents pathogen binding to the mucosal surface and reduces the risk of infections [135]. Transmission of human immunodeficiency virus (HIV) between infected mothers and their infants is a key example of the antiadhesive antimicrobial effects of HMOs. HIV glycoprotein 120 (gp120) binds to dendritic cell-specific ICAM3-grabbing non-integrins (DC-SIGN) found on dendritic cells on the mucosal surface[142]. This gp120/DC-SIGN complex is critical in facilitating mother-to-child HIV transmission during breastfeeding[142]. However, DC-SIGN appears to have a higher affinity for Le blood group antigens compared with gp120[143]. The mucosal surfaces of breastfed infants are covered with an extraordinary amount of HMOs, which are rich in Le blood group antigens[143]. This makes HIV transmission from mother-to-child inefficient and further validates the effects of HMOs as robust antiadhesive antimicrobials.

HMOs also appear to directly and indirectly modulate the infant's local (via mucosa-associated lymphoid tissues) and systemic (1% of HMOs are absorbed) immune response. Researchers in Austria postulated that sialylated HMOs affect lymphocyte development and influence a balanced T cell production[144]. LNFP HMOs increase macrophage activity and increase the secretion of TNF, IL-10 and prostaglandins[145]. Cumulatively, HMOs have significant effects on the progression of inflammatory responses by interacting with immune modulators like: selectins, integrins, toll-like receptors and leukocytes[146].

Given that it is difficult to produce human breast milk on a large scale, it has become increasingly important to find a functional equivalent. Oligosaccharides isolated from farm

animal milk are substantially less abundant and structurally less complex compared to HMOs. Therefore, no natural or synthetic source has been able to reproduce the beneficial effects of human breastmilk. Presently, commercially available formula are fortified with non-HMO prebiotics: galactooligosaccharides (GOS) synthesized from lactose, and fructooligosaccharides (FOS) derived from chicory root inulin or synthesized by the transfructosylation of sucrose[134]. Importantly, GOS and FOS are neither fucosyllated or sialylated, however, they do still influence the human infant's gut microbiota, eliciting numerous beneficial effects on host metabolism and immunity[146]. While the benefits of HMOs have been demonstrated in breastfed infants, whether or not supplementing a solid food diet of infants or weanling laboratory animals confers metabolic and microbial benefits is not well understood and forms the basis of the first study of this thesis.

2.6 Effects of dietary protein

Dietary proteins have an insulinotropic effect, promoting insulin secretion, which result in enhanced glucose clearance from circulation[147]. Multiple interventions have verified this effect and highlighted that insulin and glucagon secretion are controlled in part by specific amino acids derived from protein[148,149]. High protein intake from milk-derived whey and casein or soy protein sources have been shown to reduce peak glycaemia and increase plasma insulin compared to high carbohydrate intake in lean, healthy individuals[150]. On the opposite spectrum, low protein consumption has been shown to elicit deleterious metabolic effects, particularly with respect to programming epigenetic alterations that affect pancreatic beta-cell development. The offspring of mice fed a maternal protein-restricted diet resulted in a reduction in beta cell function at birth, increased apoptotic rates, and glucose metabolism alterations that persisted into adulthood[151]. During pre-implantation, a low-protein maternal diet alters fluid composition in a murine model and seems to have an intergenerational effect on growth and cardiovascular health[152]. In this model, the researchers examined mouse uterine luminal fluid, defined as the liquid medium connecting the embryos and the uterus, commonly known as the *amniotic fluid* [152]. Upon examining amino acid composition, they found, at the time of blastocyst formation, significant reductions in BCAAs: leucine, isoleucine and valine both in uterine fluid and plasma[152]. This is significant because these BCAAs aid in metabolic signalling of protein translation and growth via the mTOR pathway known to be underway in

blastocysts[153]. Paternal low protein diet studies showed reduced blastocyst expression of genes associated with metabolism, transcription regulation and protein synthesis[154]. The expression of these genes persisted into late gestation in fetal liver tissues associated with fetal growth[154]. The same lab previously demonstrated that paternal low protein diet programmed rapid foetal growth which increased adult offspring adiposity and impaired glucose metabolism[155].

High protein diets have been shown to elicit detrimental metabolic effects when consumed by mothers during gestation and lactation [41], however, no research to date has investigated the effects of a paternal high protein diet on offspring metabolism. Despite some reports of detrimental effects with maternal high protein diet, numerous other animal and human studies have reported improvements in energy metabolism, satiety and energy intake, and better weight loss or maintenance with high protein diets[156]. Therefore, it is important to investigate whether paternal high protein consumption before conception will yield beneficial or deleterious metabolic outcomes in offspring. This forms the basis of study two in this thesis.

2.7 Effects of methyl donor supplementation

One carbon (1C) metabolism, otherwise known as *methyl transfer*, is a vital component of cellular metabolism, participating in many metabolic reactions, DNA synthesis and methylation reactions that could induce epigenetic changes[157]. One carbon metabolism responds to dietary intake and is modulated by endocrine signals. These dietary constituents include methionine, folate, choline, betaine, and B vitamins like B6 and B12. Intracellular methionine and folate are the most crucial components of one carbon transfer[158]. One carbon metabolism is made up of the interrelation of the methionine and folate cycles that expedite one carbon transfer, required for adequate cellular processing[159]. Methionine metabolism involves the transmethylation cycle and transsulfuration pathway. The transmethylation cycle converts methionine and ATP into S-adenosylmethionine (SAM) using a methionine adenosyltransferase catalyst [158]. SAM is a ubiquitous methyl donor in methyltransferase reactions[160]. A by-product of this reaction is S-adenosylhomocysteine (SAH) which gets reversibly cleaved into homocysteine[158]. Homocysteine is then remethylated into methionine using betaine or via methionine synthase which is dependent on vitamin B12 as a cofactor[158]. The transsulfuration cascade induces the catabolism of methionine, converting homocysteine to cysteine and alpha

ketobutyrate and requires the actions of the cofactor, vitamin B6. This cascade occurs in the liver, pancreas, kidney, intestine and potentially the brain[158].

As previously mentioned, epigenetics refers to heritable alterations in gene expression that are not encoded in the genome. The most vastly studied is DNA methylation. DNA methylation is dependent on methyl groups from SAM, as part of 1C metabolism[161]. Methyl groups attach to DNA, RNA, proteins (histones) and small molecules using SAM-dependent enzymes known as DNA methyltransferase (DNMT). DNMT3a and 3b are responsible for *de novo* methylation during embryogenesis, whereas DNMT1 is responsible for DNA methylation maintenance during mitosis[162].

The relationship between maternal diets rich in methyl donors and their influence on epigenetics, reproductive outcomes[158], and disease risk in offspring[158] is well established. Many of these studies have attributed alterations in carbon metabolism, like homocysteine and regulatory cofactors in relation to health status[158]. The Pune Maternal Nutrition Study in India revealed a correlation between higher risk of insulin resistance in offspring and low maternal plasma vitamin B12 concentrations and high erythrocyte folate during pregnancy[163], indicating that an imbalance in maternal methyl donors like folate and B12 are detrimental to child health.

Impaired 1C metabolism has also been attributed to development of non-alcoholic fatty liver disease (NAFLD), the hepatic manifestation of the metabolic syndrome[164]. Early research on seven genetically diverse strains of mice fed a choline and folate deficient diet uncovered causative effects on liver injury that mimic NAFLD[165]. This was accompanied by changes in miRNA(miR) expression[165]. More specifically, changes in liver pathomorphology changed levels of circulating miR-34a and miR-122 among others, which correlated with NAFLD severity[165]. The strongest correlations in NAFLD-specific liver pathomorphological features was observed for miR-34a, wherein increased levels of plasma miR-34a increased overall liver pathology, as measured by total hepatic lesions and their severity[165]. Multiple human studies have similarly demonstrated the potential of these non-coding RNA as biomarkers for NAFLD[166,167].

The parental periconceptional period also appears to be particularly vulnerable to diet-mediated epigenetic changes in gene regulation[168]. In a sheep model, dietary restriction of methionine and vitamin B12 for 8 weeks prior to conception resulted in increased adiposity,

blood pressure and insulin resistance[169]. This was attributed to altered methylation in 4% of 1400 CpG islands assessed by restriction landmark genome scanning in offspring liver. Similar observations were found in a rat model fed a methionine, folate and choline deficient diet for 3 weeks before conception[170]. Obesity and consumption of a high fat diet has also been shown to alter methyl donor status. An example of this was seen in mice fed a high fat diet for eight weeks; they showed reductions in serum and liver folate compared to mice fed a chow diet[171]. Whether or not supplementing a paternal high fat diet with methyl donors could attenuate some of the metabolic risk for their offspring is not known- this forms the basis of study three in this thesis.

2.8 Effects of dietary prebiotics

Dietary fibers have been long appreciated for their health benefits. Hippocrates, in 430 BC first distinguished that course wheat (containing high amounts of fiber) compared to refined wheat resulted in a laxative effect[172]. In the 1920s, bran was credited with increased stool weight and the prevention of disease[172]. More recently, non-digestible food components, that are selectively utilized by microorganisms and elicit a health benefit to the host have been identified as *prebiotics*[173]. All prebiotics are fiber, but not all fibers are prebiotics [173]. To classify a food ingredient as a prebiotic, it must have the following properties:

1. Able to resist gastric acidity, enzyme hydrolysis and absorption by the upper GI tract;
2. Undergo fermentation by the microbiota in the gut;
3. Selectively fuel the growth and activity of gut bacteria, conferring a health benefit to the host.

Prebiotics have been shown in multiple rodent studies [174–176] and some human studies[177,178] to reduce body weight gain and adiposity. High fat diets in mice have shown an accumulation of large adipocytes and increased PPARγ-activated differentiation factors (a mediator of adipogenesis and inflammation)[179]. High fat diets supplemented with prebiotics for four weeks blunted the deleterious effects of a high fat diet and promoted the proliferation of bifidobacteria in the gut[179]. By acting on PPARγ, prebiotics can lower adiposity by changing the gene expression pattern in white adipose tissue, which initiates a reduction in adipogenesis and an increase in lipolysis[179]. Similar prebiotic supplementation benefits have been observed in some human studies[177,178,180]. Prebiotic supplementation for a year showed reductions in

BMI and fat mass in a young healthy cohort compared with non-prebiotic controls[179]. In children aged 7- 12 years with overweight or obesity, prebiotic supplementation with oligofructose-enriched-inulin for 16 weeks, reduced body weight, % body fat and serum inflammatory markers, and beneficially altered the gut microbiota[178]. In adults with overweight or obesity, prebiotic supplementation with oligofructose, increased weight loss, reduced energy intake, increased satiety hormones, and improved glucose regulation[177].

Animal studies have suggested that dietary prebiotic intake enhances satiety thereby reducing energy intake[181,182]. Notably, oligofructose stimulates the release of glucagon-like peptide-1 (GLP-1) as well as upregulates the gene expression of GLP-1's precursor, proglucagon in the proximal colon of rats[183]. GLP-1 is a satiety-inducing gut hormone. When administered exogenously at physiological doses, GLP-1 has been shown to promote weight loss in humans[184]. The administration of prebiotics in humans has similarly shown increases in satietogenic gut peptides and reduced spontaneous energy consumption[183].

Prebiotic administration is also believed to modulate serum lipids in animal and human models. Apolipoprotein E (apo-E) deficient mice, supplemented with prebiotics, demonstrated significantly lower plasma cholesterol concentrations and triacylglycerol concentrations[185]. These changes in lipid metabolism reduced atherosclerotic plaque formation[185]. In a healthy, human cohort, normolipidemic men supplemented with prebiotics for breakfast over four weeks exhibited reductions in total cholesterol and triacylglycerols[186]. They also demonstrated reductions in facultative anaerobes and a concurrent increase in bifidobacteria, suggesting that the alterations in lipid metabolism with prebiotics in healthy young men may be at least in part attributed to colonic fermentation[186]. Similarly, prebiotic-rich pasta consumed over five weeks in a healthy young male cohort significantly increased HDL cholesterol, and reduced total cholesterol/HDL-cholesterol ratio and triglycerides[187].

Prebiotics also reduce low-grade inflammation induced by a dysbiotic gut microbiota, thus improving intestinal barrier function and promoting the proliferation of beneficial gut bacteria[188]. The term *leaky gut* is derived from the process by which tight junction proteins (claudins, occludens), involved in gut barrier functions of the GI epithelial lining, become compromised[189]. This is normally characterized by concurrent inflammation[189]. In a pig model, prebiotics significantly reduced colonic mRNA expression of pro-inflammatory cytokines like IL-6, IL-17 and IL-1β[190]. Prebiotic supplementation in obese mice resulted in a selective

increase of *Bifidobacterium* spp., lower intestinal permeability noted by reduced plasma lipopolysaccharide (LPS) and cytokines, increased occludin and ZO-1 mRNA levels, decreased hepatic expression of inflammatory and oxidative stress markers, and increased circulating GLP-1 and GLP-2[191]. SCFAs, produced by the fermentation of prebiotic dietary fiber have also been shown to play a role in improving intestinal barrier function[192].

Maternal prebiotic supplementation in rodents, namely during gestation and lactation, has been shown to result in beneficial health effects, including improvements in bodyweight, body composition, colon length[193], reductions in immune-related incidents[194], and improved glucose tolerance, insulin sensitivity and hepatic steatosis in offspring[195]. Moreover, prebiotic oligofructose intake in dams significantly improves the abundance of health-promoting bacteria, like *Bifidobacterium* [174] as well as the production of satiety hormones, thereby reducing energy intake in their progeny[174]. Notably, oligofructose supplementation in dams consuming a high fat/ sucrose diet alters the serum metabolomics signature and reduces the insulin resistance of obese pregnant rats with improved metabolic profiles in their offspring[174]. Although maternal diets high in prebiotic fiber have shown beneficial effects for offspring metabolic outcomes, it is not known if the same benefits are seen with paternal high prebiotic diet. This forms the basis of study four in this thesis.

2.9 Conclusion

It has become apparent that dietary manipulations pre-conceptionally or during important periods of prenatal and postnatal development elicit strong and lasting effects on health status. Pre- and post-natal environments have the potential to affect multiple organs and systems that may impact disease risk inter- and trans-generationally. These include whole-body metabolism, the gut microbiota and epigenetic alterations, all of which may be interconnected.

2.10 Research Objectives and Hypotheses

Overall thesis objective: To determine the impact of dietary manipulations at pre-conception and during early postnatal development on metabolic and gut microbial outcomes in rats.

The specific objectives and hypotheses of this dissertation include:

Objective 1:

To examine whether consumption of human milk oligosaccharides (HMO) by weanling rats affects growth, glucose tolerance, gut permeability and gut microbiota.

Hypothesis: Intake of 2'Fucosyllactose and 3'Sialylactose HMOs will improve gastrointestinal health, including intestinal barrier function and microbial composition in young rats.

Objective 2:

To determine whether a paternal diet high in protein improves body composition, metabolic outcomes, gut microbial signatures and epigenetic outcomes in offspring.

Hypothesis: Paternal high protein diet consumption during pre-conception will reduce adiposity, improve glucose tolerance, insulin sensitivity, satiety hormone response, gut microbiota profile and epigenetic changes in fathers and their offspring.

Objective 3:

To determine whether a paternal high fat/high sucrose diet supplemented with a methyl-donor cocktail will attenuate metabolic dysfunction, epigenetic status and gut dysbiosis induced by a high fat/ high sucrose diet.

Hypothesis: Paternal methyl-donor cocktail supplementation during pre-conception will improve fertility, reduce adiposity, improve metabolic outcomes, gut microbiota signatures and epigenetic markers in fathers and their offspring.

Objective 4:

To determine the effect of a high prebiotic paternal diet on gut microbial outcomes, glycemic control and satiety hormone secretion in offspring.

Hypothesis: Elevated prebiotic intake by fathers at pre-conception will improve health status of fathers and offspring.

CHAPTER THREE: HUMAN MILK OLIGOSACCHARIDE SUPPLEMENTATION AFFECTS INTESTINAL BARRIER FUNCTION AND MICROBIAL COMPOSITION IN THE GASTROINTESTINAL TRACT OF YOUNG SPRAGUE DAWLEY RATS

3.1 ABSTRACT

Background & Aims: Human milk oligosaccharides (HMOs) are chief maternal milk constituents that feed the intestinal microbiota and drive maturation of the infant gut. Our objective was to determine whether supplementing individual HMOs to a weanling diet alters growth and gut health in rats.

Methods: Healthy 3-week-old Sprague Dawley rat pups were randomized to control, 2’-*O*-Fucosyllactose (2’FL)- and 3’Sialyllactose (3’SL)- fortified diets alone or in combination at physiological doses for 8 weeks. Body composition, intestinal permeability, serum cytokines, fecal microbiota composition and mRNA expression in the gastrointestinal tract were assessed.

Results: Males fed a control diet were 10% heavier and displayed elevated IL-18 (p=0.01) in serum compared to all HMO-fortified groups at week 11. No differences in body composition were detected between groups. In females, HMOs did not affect body weight but 2’FL+3’SL significantly increased cecum weight. All female HMO-fortified groups displayed significant reductions in intestinal permeability compared to controls (p=0.02). All HMO-fortified diets altered gut microbiota composition and mRNA expression in the gastrointestinal tract albeit differently according to sex.

Conclusion: Supplementation with a fraction of the HMOs found in breast milk has a complex sex-dependent risk/benefit profile. Further investigation of gut microbial profiles long-term and supplementation with other HMOs during early development is warranted.

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3.2 Introduction

Breast milk consumption during the first year of life is a crucial determinant of gastrointestinal tract and microbiota development[196]. Breast milk can enhance the intestinal barrier and reduce chronic disease risk later in life[197]; however, infant formula has become a commonly accepted alternative to breast milk. The infant formula market is anticipated to reach $98 billion by 2025[198]. Based on trends, 39% of infants younger than 6 months of age were reported to be exclusively breastfed in developing countries between 1995 and 2010 [199] with substantial variability in developed countries with breastfeeding initiation rates of 38-97%[200].

Breast milk, when quantified as g/L is composed of protein (10%), fats (30%) and carbohydrates (60%)[201]. A prominent amount of the carbohydrates present includes important complex structures known as human milk oligosaccharides (HMOs). HMOs encompass a structurally diverse group of over 200 soluble carbohydrates[202], the composition of which varies throughout lactation and between mothers[203]. Oligosaccharides in human milk belong to 3 major classes, neutral fucosylated, neutral non-fucosylated and acidic[204]. Despite their physical characterization, the functional significance of the various HMOs is still not fully understood. Although newborns lack the enzymatic capacity to break down HMOs[205], these oligosaccharides are utilized by commensal gut bacteria and act as prebiotics wherein they serve as a growth factor that promotes a healthy gut microbiota, thus improving host health[206]. HMOs have also been shown to affect microbe interactions in the host by serving as decoy receptors to prevent pathogen binding to intestinal epithelial cells[207]. Notably, G-protein coupled receptors (GPCRs), like GPR41 and GPR43 have an important role in modulating intestinal inflammation[208,209]. Likewise, HMOs affect matrix metalloproteinase genes, like MMP2 and MMP9, which modulate efficient barrier function and tight junction protein integrity, including occludin and zonula occluden (ZO)-1 [210]. Mucus is a major component of the physical intestinal barrier that contributes to intestinal protection and host defense[211]. HMOs are closely linked with intestinal mucins, whereby the mucus contributes to intestinal homeostasis by inhibiting bacterial adhesion to epithelial cells[212]. The mucus layer is constructed in part of MUC2 protein of which the polysaccharide component can serve as an energy source for intestinal bacteria in the absence of fiber[213]. Further, HMOs directly stimulate MUC2-processing through protein disulfide isomerase (PDI) and maintain goblet cell

populations[212]. MMP9, an extracellular proteinase has been shown to regulate MUC2 levels[210].

Given that it is difficult to produce human breast milk on a large scale, it has become increasingly important to find a functional equivalent for infants fed infant formula with all the encompassing benefits of HMOs. Oligosaccharides isolated from bovine milk are substantially less abundant and structurally less complex compared to HMOs[214]. Therefore, no natural or synthetic source has been able to reproduce the beneficial effects of human breast milk. Presently, some commercially available infant formulas are fortified with other prebiotics, most commonly galactooligosaccharides (GOS) and/or fructooligosaccharides (FOS). Importantly, GOS and FOS are neither fucosylated or sialylated, and structurally less complex than fucosylated or sialylated HMOs. Therefore, companies have sought to synthesize and make commercially available milk oligosaccharides that are highly purified and structurally similar to those found in human milk. To mimic the presence of acidic and neutral HMOs found in breast milk, we chose to test the effects of supplementing a weanling diet in rats with two synthesized HMOs; the most abundant fucosylated HMO, 2'fucosyllactose (2'FL)[215] as well as the most predominant sialyllactose that remains stable throughout lactation, 3'sialyllactose (3'SL)[216]. Our objective was to examine the effects of 2'FL and 3'SL alone or in combination on growth and body composition, gut microbiota, intestinal permeability and expression of genes involved in gut health in young female and male rats.

3.3 Materials and Methods

3.3.1 Animal Model and Dietary Treatment

Twenty virgin female Sprague Dawley rats were obtained from Charles River Laboratories (Montreal, QC, Canada) and acclimatized to a temperature and humidity-controlled facility with a 12-h light/dark cycle. After 1 week, females were bred in-house with male Sprague Dawley rats in wire-bottomed cages until a copulation plug was identified. Dams were then housed individually and fed a standard chow diet during gestation and lactation. The day after birth, pups were weighed and litters were culled to 10 pups (5 males; 5 females) to minimize differences in nutrition in litters of different sizes. Cross-fostering from dams with similar parturition dates was used to bring litters up to n=10 pups when litters were <10 or the male:female ratio was imbalanced. At weaning, pups were randomized to one of four

nutritionally complete experimental diets for 8 weeks: 1) Control (AIN-93G), 2) 2’Fucosyllactose fortified AIN-93G (2’FL, 0.625% wt/wt), 3) 3’Sialyllactose fortified AIN-93G (3’SL, 0.625% wt/wt) and 4) 2’FL+3’SL-fortified AIN-93G (0.625% wt/wt each). An n=10 rats of each sex were allocated per diet group. An additional n=10 rats per group of each sex were also included for the intestinal permeability test which had to be performed separately from the other tests. The dose of HMOs was chosen to provide a similar average dose of 2’FL that breastfed infants would obtain[215]. All diets were mixed in house using ingredients from Dyets, Inc. (Bethlehem, PA, USA) and HMOs from Glycom A/S (Hørsholm, Denmark). The composition of the experimental diets is found in Table 3.1. Two animals per treatment (one for intestinal permeability test and one for all other outcomes) were co-housed per cage until week 10. Offspring were weighed weekly for 8 weeks and food intake was measured each week for three consecutive days at a time. This study was approved by the University of Calgary Animal Care Committee (AC14-0080).

Table 3.1 Experimental diet composition from weeks 3-9 and 10-11

	C	3’SL	2’FL	3’SL+2FL	C	3’SL	2’FL	3’SL+2FL
g/kg	Weeks 3-9				Weeks 10-11			
Cornstarch	397.5	397.5	397.5	397.5	465.7	465.7	465.7	465.7
Casein	200	200	200	200	140	140	140	140
Dyetrose	132	132	132	132	155	155	155	155
Sucrose	100	100	100	100	100	100	100	100
Soybean Oil	70	70	70	70	40	40	40	40
Alphacel	50	50	50	50	50	50	50	50
AIN-93M Mineral Mix	35	35	35	35	35	35	35	35
AIN-93 VX Vitamin Mix	10	10	10	10	10	10	10	10
L-cystine	3	3	3	3	1.8	1.8	1.8	1.8
Choline-Bitartrate	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
3’Sialyllactose	-	6.25	-	6.25	-	6.25	-	6.25
2’Fucosyllactose	-	-	6.25	6.25	-	-	6.25	6.25
Energy density (kJ/g)	15.73	15.69	15.69	15.64	15.07	15.04	15.04	14.99
Carbohydrate (% of kcal)	63.9	64.0	64.0	64.1	75.9	76.0	76.0	76.1
Protein (% of kcal)	19.4	19.3	19.3	19.2	14.1	14.1	14.1	14.0
Fat (% of kcal)	16.8	16.7	16.7	16.6	10.0	10.0	10.0	9.9

All diets were mixed in house using ingredients from Dyets Inc. (Bethlehem, PA, USA) and HMOs from Glycom A/S (Hørsholm, Denmark). Diet composition from weeks 3-9 is based on the AIN-93G (C) formulation to support growth while the diet from weeks 10-11 is based on AIN-93M(C) for adult maintenance. The purity of 2’FL is 96.1% (w/w%) and of 3’SL is 97.5% (w/w%).

3.3.2 Oral Glucose Tolerance Test

Five days prior to sacrifice, rats were fasted overnight for 12 hours and blood glucose measured from a tail nick sample using a One Touch Ultra 2 glucose meter (Lifescan, Burnaby, Canada). Rats were gavaged with a 2 g/kg dose of glucose and additional blood glucose measurements made at 15, 30, 60, 90 and 120 minutes post gavage. At the 0, 15, 60 and 120 minute time points additional blood was collected from the tail into chilled tubes containing diprotinin-A (0.034 mg/ml blood; (MP Biomedicals, Irvine, CA), Sigma protease inhibitor (1 mg/ml blood; Sigma Aldrich, Oakville, ON, Canada) and Roche Pefabloc (1mg/ml of blood; Roche, Mississauga, ON, Canada). Samples were centrifuged and the serum stored at -80°C until analysis for insulin.

3.3.3 Insulin Tolerance Test

Eight days prior to sacrifice, rats were fasted for 6 hours and then administered a bolus of insulin (0.75 U/kg) via intraperitoneal injection. Glucose concentrations were measured immediately via tail nick at 0, 15, 30, 60, 90 and 120 minutes after the insulin injection using a One Touch Ultra 2 glucose meter.

3.3.4 Intestinal Permeability Test

Intestinal permeability was assessed using fluorescein isothiocynate-dextran-4000 daltons (FITC), average mol wt 3000-5000 (Sigma-Aldrich, St. Louis, Missouri, USA). Given that the FITC is found in circulation throughout the body after it is administered, this test was performed in a separate set of male and female rats. Following a 6 hour fast, rats received an oral gavage of FITC diluted with saline to 250mg/mL (500mg/kg bodyweight DX-4000-FITC). At 1h post gavage, rats were anesthetized using isoflurane and blood was collected via cardiac puncture of the left ventricle in a tube containing ethylenediaminetetraacetic acid (EDTA) (10uL EDTA/mL of blood), stored on ice and kept in the dark. Samples were centrifuged at 4°C for 3 min (12,000g), plasma was collected and then stored at -80°C until analysis. Rats were subsequently overanesthetized and killed via aortic cut. At the time of analysis, plasma samples were diluted in equal volumes of PBS and 50uL were loaded in duplicate onto a 96-well plate that contained standards made via serial dilution. FITC was measured using a fluorescence reader (FLX 800) at emission wavelength of 535 nm and an excitation wavelength of 485nm.

3.3.5 Final Body Composition, Blood and Tissue Collection

One day prior to sacrifice, animals underwent a Dual Energy X-ray Absorptiometry (DXA) scan (Hologic ODR 4500; Hologic Inc.) under light anaesthetic (isoflurane). Lean mass(g), fat mass(g), body fat %, and bone mineral content/ density (BMD) (g and g/cm^2) were assessed using Hologic QDR software for small animals. Following 12 h feed deprivation, rats were anaesthetized with isoflurane and blood collected from the portal vein. From this sample, a Milliplex Rat Cytokine Array/Chemokine Array (Millipore, St. Charles, MO) was used to measure serum TNF*, IL-1α, IL-1*β, IL-5, IL-10, IL-18 and leptin (Eve Technologies, Calgary, AB, Canada). Rats were subsequently killed by overanesthetization and decapitation. The cecum, colon and jejunum were excised, cleaned, weighed, snap-frozen and stored at -80˚C.

3.3.6 Bacterial DNA Extraction and Microbiota Analysis

Fecal matter was collected at 3, 7 and 11 weeks of age, snap frozen and stored at -80˚C. Microbial profiling was performed based on our previous work[217,218]. Briefly, bacterial DNA was extracted from ~60 mg of stool using the FastDNA spin kit for feces (MP Biomedicals, Lachine, QC, Canada). Half of the extracted sample was brought to a concentration of 4 ng/µl prior to storage at -20°C for qPCR analysis and the other half was used for 16S rRNA sequencing at the Centre for Health Genomics and Informatics at the University of Calgary. Quantitative PCR (qPCR) was carried out as previously described[217,218] with primers that cover the major gut bacterial groups in rodents (Table 3.2).

Bacterial community composition was assessed with Illumina's 16S rRNA amplicon sequencing protocol of the V3 and V4 region on the MiSeq platform (Illumina, San Diego, CA, USA). Sequencing primers were removed using Cutadapt (version 1.16) and sequences were filtered for quality using the dada2 package (version 1.12) in R (version 3.5.3). A table of amplicon sequence variants (ASVs) was generated using dada2 and taxonomically classified using the Silva 132 database. Alpha diversity was calculated using Chao1, Shannon and Simpson indices with the phyloseq package (version 1.24.2). Beta diversity was calculated using a principal coordinates analysis (PCoA) on a Bray-Curtis distance matrix containing ASVs present in at least 5% of the samples. Significance of alpha was set at 0.05.

Table 3.2 Gut microbial group specific primers for qPCR

Microbial Group		Primers (5'→3')
Firmicutes:		
***Clostridium difficile* (cluster I)**	Forward	ATGCAAGTCGAGCGAKG
	Reverse	TATGCGGTATTAATCTYCCTTT
***Clostridium leptum* (cluster IV)**	Forward	GCACAAGCAGTGGAGT
	Reverse	CTTCCTCCGTTTGTCAA
***Clostridium perfringens* (cluster XI)**	Forward	ACGCTACTTGAGGAGGA
	Reverse	GAGCCGTAGCCTTTCACT
***Clostridium coccoides* (cluster XIV)**	Forward	ACTCCTACGGGAGGCAGC
	Reverse	GCTTCTTAGTCARGTACCG
Faecalibacterium prausnitzii	Forward	AACCTTACCAAGTCTTGACATC
	Reverse	TTGCGTAGTAACTGACCATAAG
***Lactobacillus* spp.**	Forward	GAGGCAGCAGTAGGGAATCTTC
	Reverse	GGCCAGTTACTACCTCTATCCTTCTTC
***Roseburia* spp.**	Forward	TACTGCATTGGAAACTGTCG
	Reverse	CGGCACCGAAGAGCAAT
Bacteroidetes:		
Bacteroides/Prevotella	Forward	TCCTACGGGAGGCAGCAGT
	Reverse	CAATCGGAGTTCTTCGTG
Actinobacteria:		
***Bifidobacterium* spp.**	Forward	CGCGTCYGGTGTGAAAG
	Reverse	CCCCACATCCAGCATCCA
Collinsella aerofaciens	Forward	CCCGACGGGAGGGGAT
	Reverse	CTTCTGCAGGTACAGTCTTGAC
Archaea:		
Methanobrevibacter	Forward	CTCACCGTCAGAATCGTTCCAGTC
	Reverse	ACTTGAGATCGGGAGAGGTTAGAGG
Proteobacteria:		
Enterobacteriaceae	Forward	CATTGACGTTACCCGCAGAAGC
	Reverse	CTCTACGAGACTCAAGCTTGC
Verrucomicrobia:		
Akkermansia muciniphila	Forward	TCTTCGGAGGCGTTACACAG
	Reverse	AGTTGATCTGGGCAGTCTCG

3.3.7 Tissue Gene Expression Using Real Time PCR

Total RNA was extracted from the proximal jejunum and proximal colon samples and real-time PCR performed as previously described [220] with primers listed in Table 3.3. Gene expression was calculated using the $2^{-\Delta Ct}$ method. The jejunum was harvested 3cm distal to the duodenojejunal flexure. The harvested proximal colon was composed of the ascending colon, terminating at the hepatic flexure.

Table 3.3 Primer sequences for RT-PCR

Gene	Primers (5'→3')	
Proximal Colon		
MMP2	Forward	CCTGAATACTTTCTATGGCTGC
	Reverse	GTATGTAGTGGAGCACCAGAGC
MMP9	Forward	GCAACGGAGACGGCAAACC
	Reverse	GACGAAGGGGAAGACGCA
MUC2	Forward	CCACCATTACCACCACCTCAG
	Reverse	CGATCACCACCATTGCCATTG
GPR41	Forward	TCCTCAGCACCCTCAACTCT
	Reverse	CTAGCTCGGACACTCCTTGG
GPR43	Forward	CCGTGCAGTACAAGCTCTCC
	Reverse	CTGCTCAGTCGTGTTCAAGTATT
β-Actin	Forward	TATCGGCAATGAGCGGTTCC
	Reverse	AGCACTGTGTTGGLATAGAGG
Jejunum		
ZO-1	Forward	GAGTTTCGGGTCCGAGGAG
	Reverse	CATTGCTGTGCTCTTAGCGG
Occludin	Forward	GAGGACTGGCTCAGGGAATATC
	Reverse	TTGTTGACCTCGTCGAGTTCTG

3.3.8 Statistical Analysis

All data are presented as mean ± SEM. Outcomes with a single time point (e.g. body fat, intestinal permeability, etc.) were assessed using a two-way ANOVA to determine the effects of diet and sex and their interaction. If there was a significant effect of sex, a one-way ANOVA with Tukey's post hoc test was performed in males and females separately to determine differences across groups. Outcomes with multiple time points (e.g. body weight, OGTT, etc.) were analyzed using repeated measures ANOVA where time was used as the *within-subject* factor and diet and sex were the *between-subject* factors. If there was a significant sex effect, male and female data was analyzed separately. When a significant diet × time effect was identified, a one-way ANOVA with Tukey's post hoc was used to determine differences across groups. To assess correlations between a panel of inflammatory cytokines and mRNA expression

of genes that maintain intestinal barrier function, a Pearson's correlation analysis was conducted. Results were considered significant at $p < 0.05$. Statistics were performed with IBM SPSS Statistics version 24.0.

3.4 Results

3.4.1 Bodyweight, Body Composition, Food Intake and Serum Leptin

There was a significant effect of sex (p=0.001) on body weight, with males weighing more than females at every age. Given the significant sex effect in the overall model, subsequent analysis was performed in males and females separately. Within the males, no differences in body weight were seen until week 11 when the control group was heavier than the 3'SL-fortified group (p=0.03) (Figure 3.1A). There were no differences in body weight within females (Figure 3.1B).

There was a significant effect of sex (p=0.0001) for food intake, with males consuming more food than females. Based on the significant sex differences in the overall model, male and female data was subsequently presented separately. Within the males and females, there was a significant main effect of time for food intake with intake increasing as the animals grew as well as a significant interaction between time and diet in both males and females (Figure 3.1C & 1D). Males fed control diet consumed significantly more kcal/day at 4 weeks of age compared to 2'-FL-fortified group (p=0.04). Females fed 3'SL consumed significantly more kcal/day at 4 weeks of age compared to the 3'SL+2'FL group and then eventually by week 10 both 3'SL-fortified groups consumed significantly less energy than controls (p=0.01).

Body composition was significantly affected by sex (p=0.0001 for lean + bone mineral content (BMC), fat mass, % body fat and BMC and p=0.02 for bone mineral density). However, within males and females, there were no differences across diets in lean mass, fat mass, body fat % and bone mineral density measured at 11 weeks of age (Table 3.4). Serum leptin levels were significantly affected by sex (p=0.001). Within males, serum leptin levels were significantly higher in rats fed control diet compared to 3'SL-fortified diet at 11 weeks of age (p=0.03) (Figure 3.2A). While not significant, male fat mass appeared to follow similar trends as serum leptin. To determine whether there was a relationship between fat mass and leptin levels, we conducted a correlation analysis stratified by sex and showed a significant positive association

between male fat mass and serum leptin (r=0.85, p=0.0001) (Figure 3.3A). No significant correlation was observed in females (Figure 3.3B).

3.4.2 Intestinal Weight

Intestinal weight relative to body weight was significantly affected by sex (p=0.0001). Within males, there were no differences across diets in cecum weight expressed per body weight at euthanasia at 11 weeks of age (Figure 3.4A). Female cecum weight, however, was significantly higher in rats fed 3'SL+2'FL-fortified diet compared to controls (p=0.002; Figure 3.4B). There was no difference in male colon weight expressed per body weight, however, female colon weight was lower in the 3'SL+2'FL-fortified group compared to the control group (p=0.03) and 3'SL-fortified group (p=0.02) (Figure 3.4C and 3.4D, respectively).

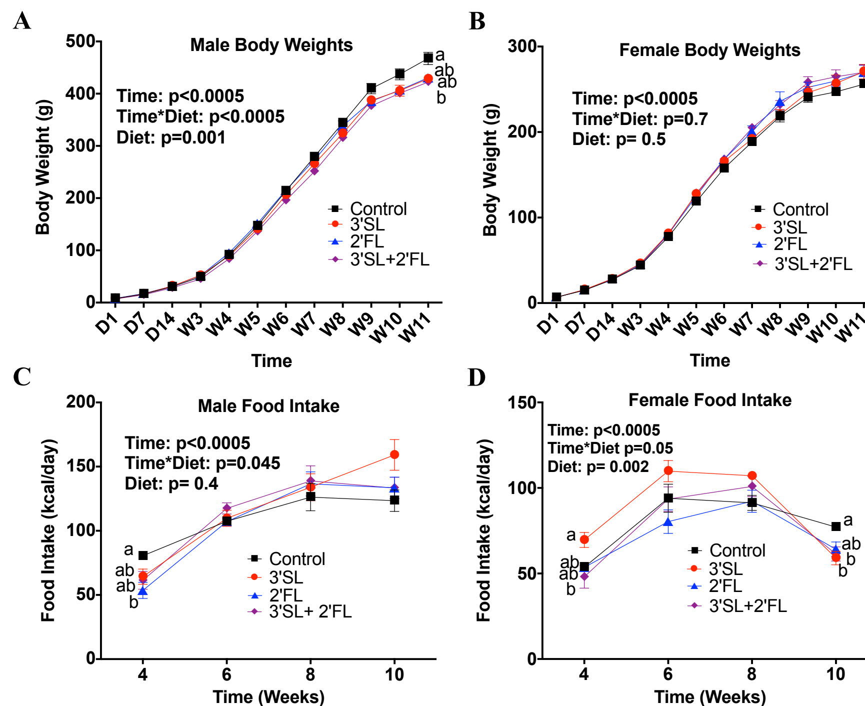

Figure 3.1 Body weight of (A) male and (B) female rats as well as food intake for (C) male and (D) female rats fed AIN-93 diet fortified with 3'SL, 2'FL, both or neither for 8 weeks. Values are means ± SEM, n= 8-10. In the overall model, there was a significant sex effect for body weight (p=0.001) and food intake (p=0.001), therefore subsequent analysis was performed in males and females separately. Within males and females, the superscripts ^{a,b} are used to depict differences between groups where groups without a common superscript differ (p<0.05). Control: AIN-93; 3'SL: AIN-93 + 3'Sialyllactose; 2'FL: AIN-93 + 2'Fucosyllactose; 3'SL+2'FL: AIN-93 + 3'Sialyllactose + 2'Fucosyllactose. D: day; W: week.

Table 3.4 Male and female body composition

	Control	3'SL	2'FL	3'SL+2'FL	p-value
Males					
Total Weight (g)	464.2± 11.2	426.2±11.5	439.1± 10.1	442.6±11.5	0.14
Lean+ BMC (g)	386.9±7.4	366.0±11.8	372.6±8.9	375.9±9.5	0.48
Fat Mass (g)	77.3±5.0	63.7±2.9	66.5±3.8	66.7±3.9	0.11
% Body Fat	16.5±0.8	15.9±0.8	15.1±0.7	15.0±0.7	0.46
Bone Mineral Content (g)	11.0±0.3	10.0±0.3	10.2±0.2	10.5±0.3	0.07
Bone Mineral Density (g/cm^2)	0.145±0.002	0.141±0.002	0.143±0.001	0.143±0.002	0.18
Females					
Total Weight (g)	256.0±4.8	264.1±10.1	269.7±8.8	269.9±8.1	0.59
Lean+ BMC (g)	224.8±5.0	226.9±9.4	235.4±5.7	235.2±6.5	0.60
Fat Mass (g)	31.2±2.7	37.3±4.0	34.8±3.2	33.8±3.7	0.65
% Body Fat	11.5±0.9	14.1±1.4	12.3±0.9	12.8±1.0	0.43
Bone Mineral Content (g)	7.2±0.2	7.7±0.3	7.9±0.2	7.9±0.3	0.17
Bone Mineral Density (g/cm^2)	0.138±0.002	0.139±0.001	0.141±0.001	0.143±0.001	0.09

Values are means ± SEM, n = 8-10. In the overall model, there was a significant sex effect (p=0.0001 for Lean+BMC, Fat Mass, % Body Fat and BMC; p=0.02 for BMD) therefore males and females were analyzed separately. Control: AIN-93; 3'SL: AIN-93 + 3'Sialyllactose; 2'FL: AIN-93 + 2'Fucosyllactose; 3'SL + 2'FL: AIN-93 + 3'Sialyllactose + 2'Fucosyllactose. Significance was set at p<0.05.

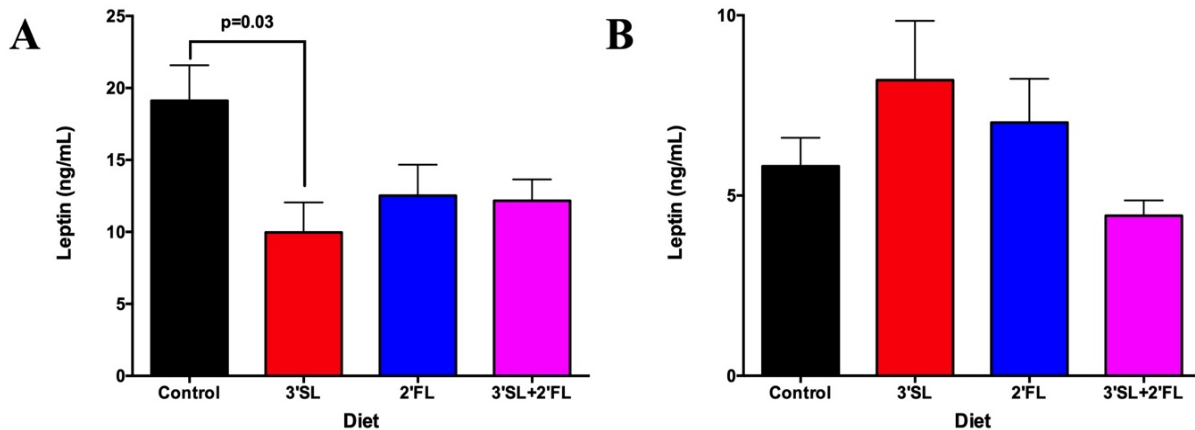

Figure 3.2 Serum fasting leptin levels at 11 weeks of age in (A) male and (B) female rats fed AIN-93 diet fortified with 3'SL, 2'FL, both or neither for 8 weeks. Values are means ± SEM, n = 8-10. In the overall model, there was a significant sex effect (p=0.0001), therefore males and females were analyzed separately. Control: AIN-93; 3'SL: AIN-93 + 3'Sialyllactose; 2'FL: AIN-93 + 2'Fucosyllactose; 3'SL + 2'FL: AIN-93 + 3'Sialyllactose + 2'Fucosyllactose.

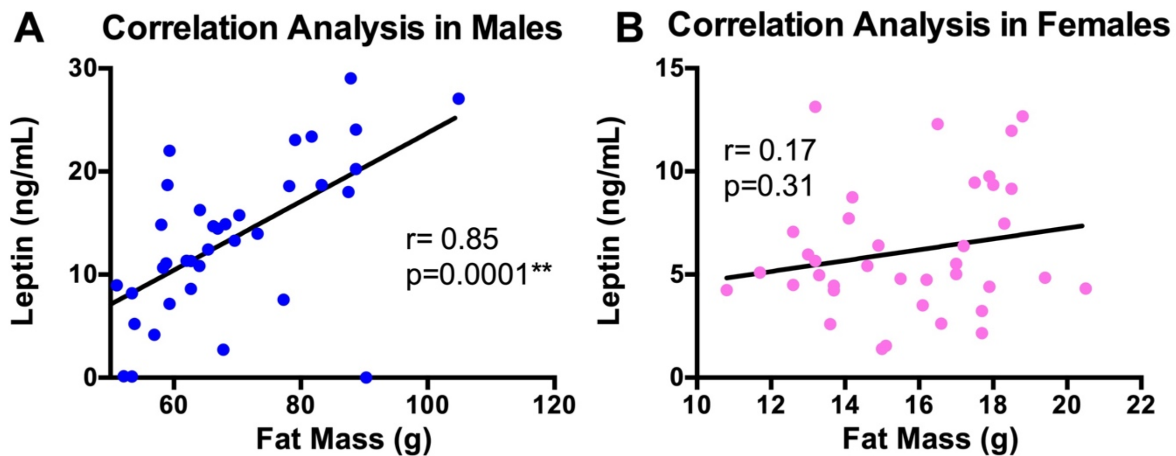

Figure 3.3 Correlation between fat mass and leptin using a Pearson correlation analysis at 11 weeks of age in (A) male and (B) female rats fed AIN-93 diet fortified with 3'SL, 2'FL, both or neither for 8 weeks. n = 38 males, n = 38 females.

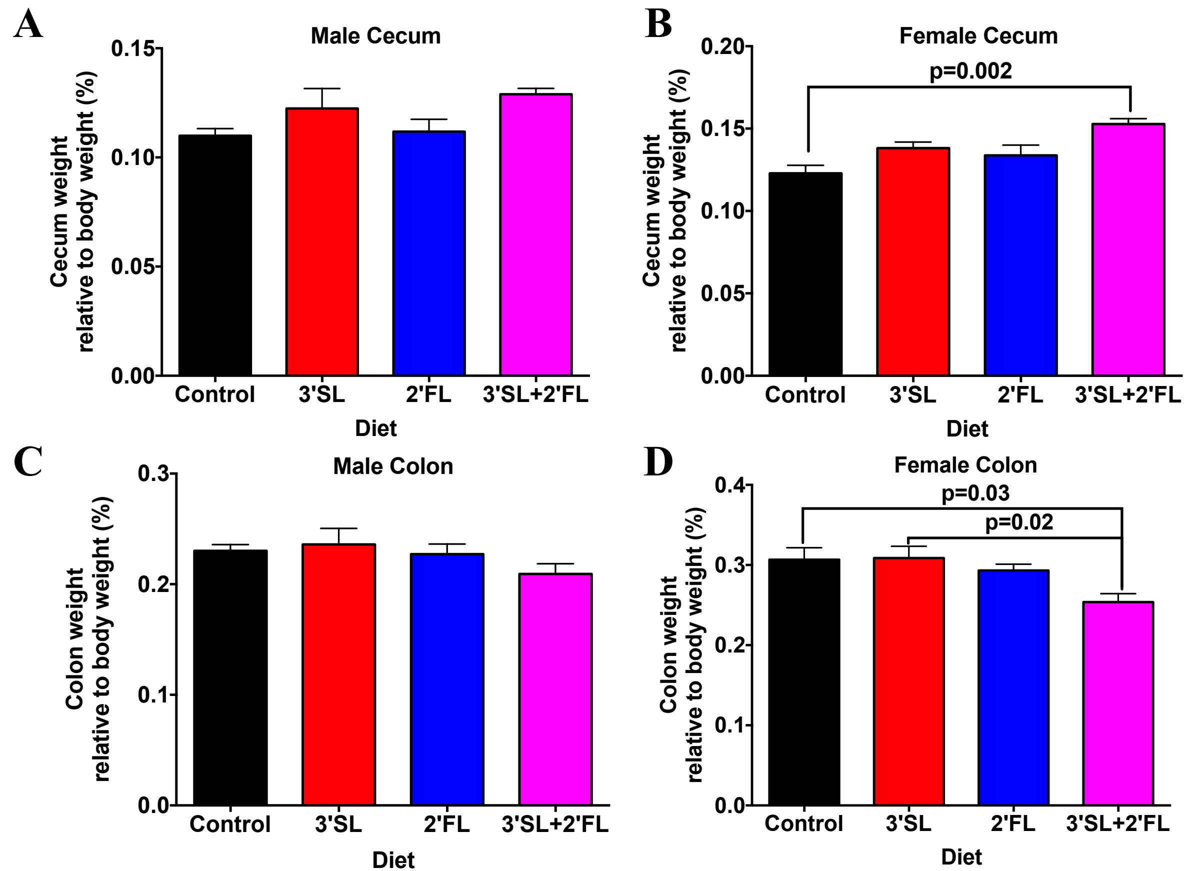

Figure 3.4 Cecum and colon weight relative to body weight respectively in (A, C) male and (B, D) female rats fed AIN-93 diet fortified with 3’SL, 2’FL, both or neither for 8 weeks. Values are means ± SEM, n = 8-10. In the overall model, there was a significant sex effect for cecum and colon (p=0.0001), therefore males and females were analyzed separately. Control: AIN-93; 3’SL: AIN-93 + 3’Sialyllactose; 2’FL: AIN-93 + 2’Fucosyllactose; 3’SL + 2’FL: AIN-93 + 3’Sialyllactose + 2’Fucosyllactose.

3.4.3 Glucose and Insulin Tolerance Tests

Blood glucose concentrations during the OGTT were significantly affected by sex (p=0.0001). Given the significant sex effect in the overall model, subsequent analysis was performed in males and females separately. As expected during an OGTT, there was a significant effect of time on glucose levels in males (Figure 3.5A) and females (Figure 3.5B) at the end of the 8-week feeding period. There was a significant independent effect of diet (p=0.009) in males with 3’SL-fortified group displaying lower glucose over time. No difference in AUC was observed in males (data not shown). In females, there was a trend towards an interaction between time and diet (p=0.05).

During the ITTs, differences in blood glucose were limited to a significant effect of time in both males (Figure 3.5C) and females after log transformation (Figure 3.5D) as well as a significant interaction between time and diet ($p<0.0005$) in males, wherein 3’SL+2’FL showed the greatest insulin sensitivity but significant differences at individual time points could not be detected following Tukey’s post hoc testing.

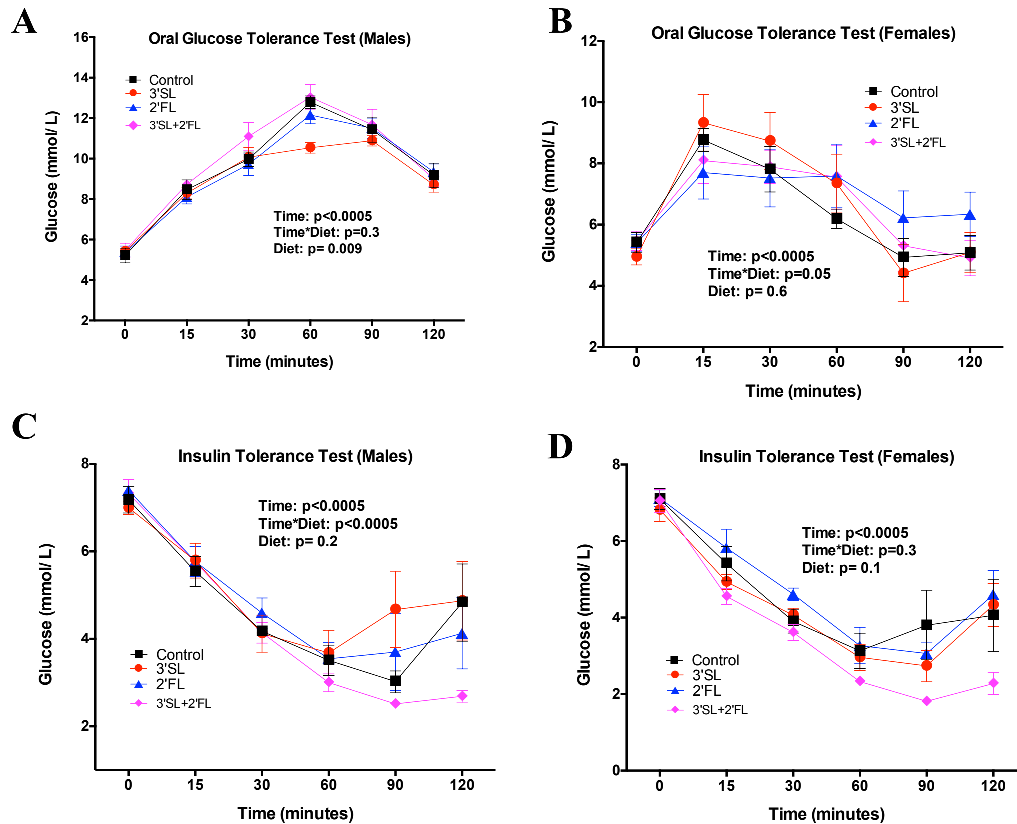

Figure 3.5 Oral Glucose Tolerance Test (OGTT) in (A) male and (B) female rats; **Insulin Tolerance Test (ITT)** in (C) male and (D) female rats fed AIN-93 diet fortified with 3’SL, 2’FL, both or neither for 8 weeks. Values are means ± SEM, *n* = 8-10. In the overall model, there was a significant sex effect for OGTT ($p=0.001$), therefore subsequent analysis was performed in males and females separately. Control: AIN-93; 3’SL: AIN-93 + 3’Sialyllactose; 2’FL: AIN-93 + 2’Fucosyllactose; 3’SL+2’FL: AIN-93 + 3’Sialyllactose + 2’Fucosyllactose. Significance was set at $p<0.05$.

3.4.4 Intestinal Permeability and Inflammatory Biomarkers

In vivo intestinal permeability testing using FITC dextran 4000 (FD4) was significantly affected by sex ($p=0.03$). There were no significant differences across diets in males (Figure 3.6A). In females, however, gut barrier permeability was reduced, as seen in lower plasma FITC

concentrations, after log transformation in rats fed any of the HMO-fortified diets compared to controls (Figure 3.6B).

To assess whether this observed intestinal permeability was associated with changes in markers of systemic inflammation, we then examined a panel of serum inflammatory cytokines (Table 3.5). There were significant sex effects for TNF (p=0.01), IL-5 (p=0.001) and IL-18 (p=0.0001) and a trend for *IL-1*β (p=0.06) and IL-10 (p=0.07) in the overall model, therefore males and females were analyzed separately. In males, the 2'FL- and 2'FL+3'SL-fortified groups

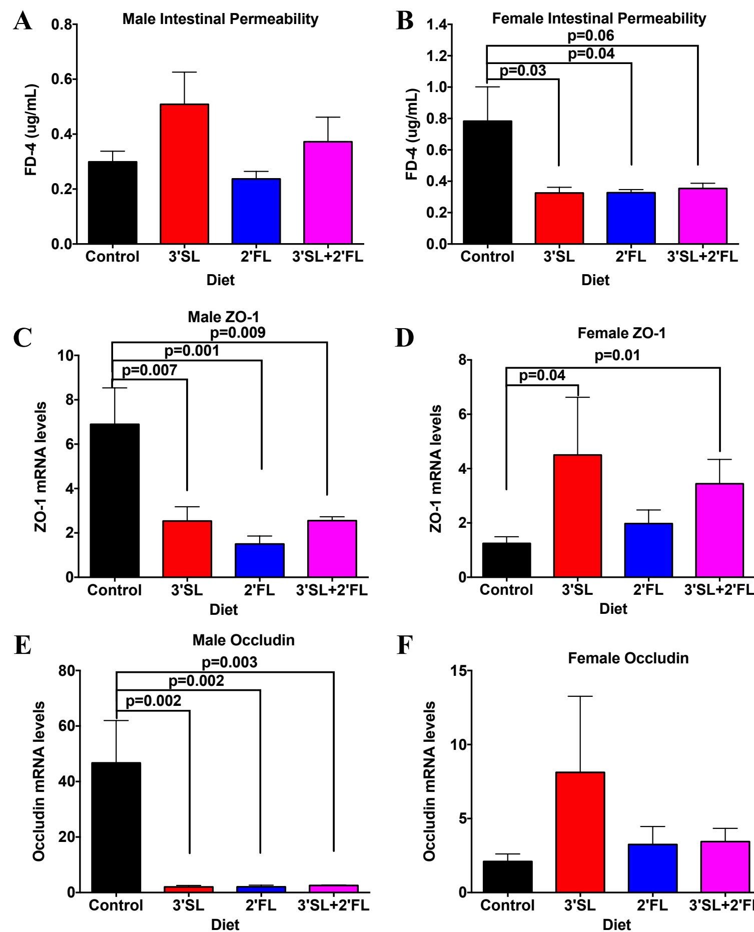

Figure 3.6 Intestinal Permeability and Jejunum mRNA levels. Plasma concentrations of FITC dextran 4000 (FD4) in **(A)** male and **(B)** female rats as well as jejunum mRNA levels in **(C)** male ZO-1, **(D)** female ZO-1, **(E)** male occludin, **(F)** female occludin in rats fed AIN-93 diet fortified with 3'SL, 2'FL, both or neither for 8 weeks. Values are means ± SEM, *n*= 8-10. In the overall model, there was a significant sex effect for IPT (p=0.03), ZO-1 (p=0.001) and occludin (p=0.0002), therefore subsequent analysis was performed in males and females separately. Control: AIN-93; 3'SL: AIN-93+ 3'Sialyllactose; 2'FL: AIN-93+ 2'Fucosyllactose; 3'SL+2'FL: AIN-93+ 3'Sialyllactose + 2'Fucosyllactose. Significance was set at p<0.05.

had lower serum IL-18 concentrations compared to the control group and trends showing reduced TNF (p=0.06) and IL-5 (p=0.08) levels in all HMO-fortified groups compared to control male rats. No differences were observed in females, however a trend (p=0.07) was observed showing an increase in anti-inflammatory IL-10 concentrations in 2'FL-fortified group.

Table 3.5 Male and female serum inflammatory cytokines at 11 weeks of age in rats fed AIN-93 diet fortified diet with 3'SL, 2'FL, both or neither for 8 weeks.

	Control	3'SL	2'FL	3'SL+2'FL	p-value
Males					
TNF	15.4±2.7	13.0±2.2	8.1±1.2	10.4±0.9	0.06
IL-1α	43.1±11.5	32.4±7.2	38.4±7.9	41.1±7.8	0.86
IL-1β	44.5±5.7	53.5±9.0	36.4±5.4	59.8±11.1	0.22
IL-5	113.9±2.2	90.5±11.1	90.4±8.3	85.4±4.9	0.08
IL-10	83.3±16.0	64.9±12.6	81.6±16.6	107.7±23.0	0.42
IL-18	539.6±81.8[a]	428.1±62.9[ab]	258.0±29.8[b]	263.6±20.3[b]	0.001
Females					
TNF	10.8±1.7	6.0±1.6	8.5±1.6	8.7±1.3	0.23
IL-1α	34.4±4.7	33.8±4.7	41.2±10.6	39.1±7.7	0.87
IL-1β	48.9±10.1	36.8±5.8	57.4±10.6	40.3±5.2	0.31
IL-5	87.4±6.9	85.3±5.1	83.55±8.0	81.5±8.5	0.95
IL-10	48.5±8.8	52.8±8.0	84.9±19.0	40.3±7.7	0.07
IL-18	220.8±21.4	239.3±19.4	230.4±26.4	202.1±22.3	0.69

Values are means ± SEM, n = 8-10. In the overall model, there was a significant sex effect for TNF (p=0.01), IL-5 (p=0.001) and IL-18 (p=0.0001) and a trend for IL-1β (p=0.06) and IL-10 (p=0.07), therefore males and females were analyzed separately. Within males and females, the superscripts a,b are used to depict differences between groups where groups without a common superscript differ (P<0.05). Control: AIN-93; 3'SL: AIN-93 + 3'Sialyllactose; 2'FL: AIN-93 + 2'Fucosyllactose; 3'SL+2'FL: AIN-93 + 3'Sialyllactose + 2'Fucosyllactose. All units are pg/mL.

3.4.5 Colon and Jejunum PCR

Based on changes in intestinal permeability, we then examined mRNA levels for select genes involved in gut-barrier function. There was a significant sex effect for certain genes (ZO-1, p=0.001; occluding p=0.0002), therefore males and females were analyzed separately. In the proximal colon, no differences were observed in male MMP2 mRNA levels (Figure 3.7A), however females exhibited significantly reduced MMP2 mRNA levels in 3'SL+2'FL group compared to all other groups (Figure 3.7B).

In males, ZO-1 mRNA levels in the jejunum were significantly reduced in all HMO-fortified groups compared with controls (3'SL: p=0.007; 2'FL: p=0.001; 3'SL+2'FL: p=0.009)

(Figure 3.6C). The opposite was observed in females; ZO-1 mRNA levels were higher in 3'SL (p=0.04) and 3'SL+2'FL (p=0.01) groups, compared with controls (Figure 3.6D). Finally, male occludin gene expression in the jejunum was significantly reduced in all HMO-fortified groups compared with control (3'SL: p=0.002; 2'FL: p=0.002; 3'SL+2FL: p=0.003; Figure 3.6E). No difference was observed in females (Figure 3.6F).

MMP9 mRNA levels in males were higher in 2'FL-fortified group compared to the control and 3'SL+2'FL-fortified groups (p=0.002 and 0.001 respectively; Figure 3.7C). In females, MMP9 mRNA levels were higher in the 3'SL+2'FL-fortified group compared to the 2'FL group (p=0.03; Figure 3.7D). MUC2 gene expression in males was significantly higher in 3'SL+2'FL-fortified group compared to control (p=0.04) (Figure 3.7E). The opposite was true for females, where lower MUC2 mRNA levels were seen in 3'SL+2'FL group compared to the 3'SL-fortified group (p=0.002) while 3'SL-fortified group MUC2 mRNA levels were higher compared to control (p=0.008; Figure 3.7F).

No differences were observed in male GPR41 and GPR43 mRNA levels in the proximal colon (Figure 3.7G and 3.7I respectively), however female GPR41 (Figure 3.7H) and GPR43 (Figure 3.7J) mRNA levels were decreased in 3'SL+2'FL-fortified groups compared with controls and the 3'SL group (GPR41: p=0.02 and 0.01 respectively; GPR43: p=0.002 and 0.009 respectively). The 2'FL- fortified group also showed a marked reduction compared with controls in GPR43 mRNA levels (p=0.047).

To assess whether there was a relationship between inflammatory cytokines and genes associated with barrier function, we conducted a correlation analysis. Males exhibited a significant positive correlation between circulating IL-18 and mRNA levels of tight junction proteins: ZO-1 and occludin (Table 3.6). Females displayed a significant positive correlation between IL-18 and MUC2 mRNA levels (Table 3.7). Knowing that IL-18 has been shown to disrupt tight junctions in gastrointestinal epithelial monolayers[221] we further investigated the seemingly contradictory positive correlation between IL-18 and ZO-1 and occludin mRNA levels in males. Given that male control rats had approximately double the concentration of serum IL-18 as the 2'FL group, we stratified according to group and found a significant negative correlation between IL-18 and ZO-1 mRNA levels in the 2'FL group (r=-0.838; P<0.001) which is consistent with previous findings[221]. No significant correlation was observed among any other dietary intervention.

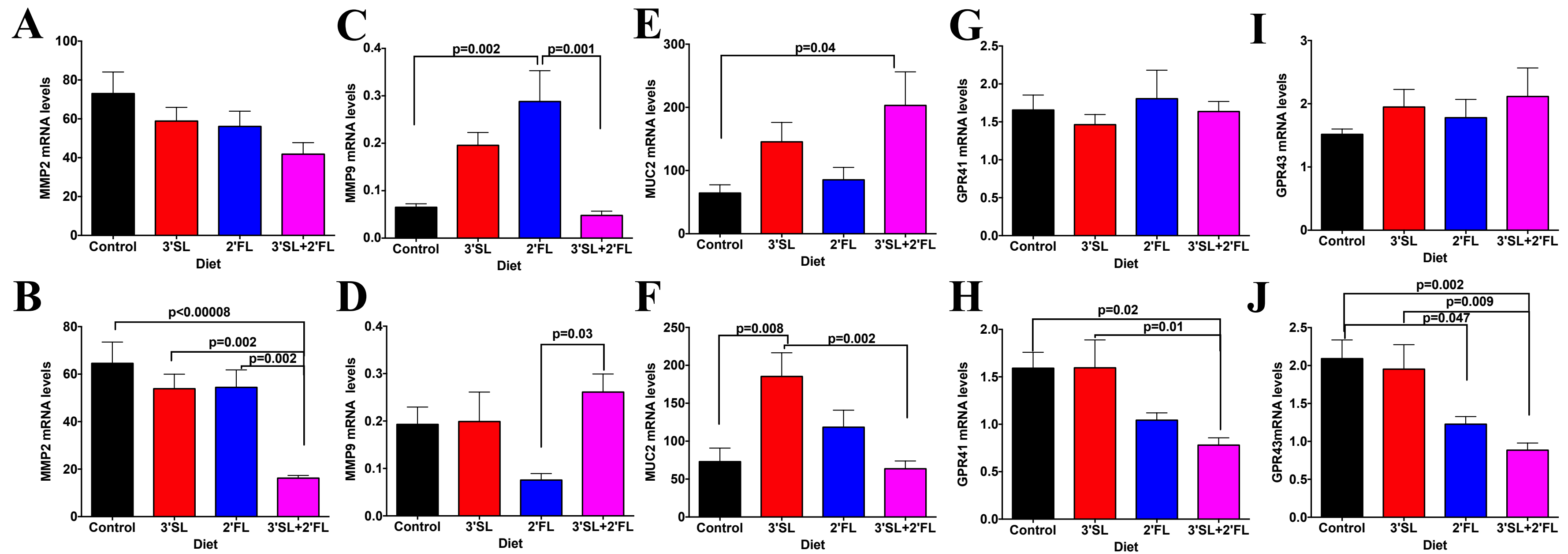

Figure 3.7 Proximal colon mRNA levels of (A) MMP2, (C) MMP9, (E) MUC2, (G) GPR41, (I) GPR43 in male and (B) MMP2, (D) MMP9, (F) MUC2, (H) GPR41 and (J) GPR43 in female rats fed AIN-93 diet fortified with 3'SL, 2'FL, both or neither for 8 weeks. Values are means ± SEM, *n*= 8-10. Control: AIN-93; 3'SL: AIN-93 + 3'Sialyllactose; 2'FL: AIN-93 + 2'Fucosyllactose; 3'SL+2'FL: AIN-93 + 3'Sialyllactose + 2'Fucosyllactose. Significance was set at $p<0.05$.

Table 3.6 Correlation analysis in males between a panel of inflammatory cytokines and genes that maintain intestinal barrier function

	MMP2	**MMP9**	**MUC2**	**GPR41**	**GPR43**	**ZO-1**	**Occludin**
TNF	-0.025	-0.058	0.011	0.248	0.031	0.349	0.219
IL-1α	-0.271	0.214	-0.060	0.234	0.038	-0.177	-0.192
IL-1β	-0.054	-0.066	0.020	0.057	-0.130	-0.188	-0.014
IL-5	0.148	0.244	-0.160	0.188	-0.263	0.183	0.256
IL-10	-0.291	0.038	-0.200	**0.368***	-0.289	-0.020	0.082
IL-18	0.256	-0.036	-0.203	0.220	-0.190	**0.520****	**0.499****

MMP2, Matrix Metallopeptidase 2; MMP9, Matrix Metallopeptidase 9; MUC2, Mucin 2; GPR41, G Protein-Coupled Receptor 41; GPR43, G Protein-Coupled Receptor 43; ZO-1, Zonula occludens. *p<0.05, **p<0.01.

Table 3.7 Correlation analysis in females between a panel of inflammatory cytokines and mRNA expression of genes that maintain intestinal barrier function

	MMP2	**MMP9**	**MUC2**	**GPR41**	**GPR43**	**ZO-1**	**Occludin**
TNF	-0.103	-0.018	-0.092	-0.183	-0.033	0.252	0.296
IL-1α	-0.114	0.003	-0.177	0.064	-0.103	-0.002	0.186
IL-1β	0.172	-0.163	-0.003	0.137	0.056	-0.197	0.096
IL-5	0.118	0.025	0.227	0.137	0.070	0.028	0.271
IL-10	0.151	-0.125	0.048	0.178	0.141	-0.183	0.114
IL-18	0.123	-0.092	**0.358***	0.249	0.039	0.014	0.055

MMP2, Matrix Metallopeptidase 2; MMP9, Matrix Metallopeptidase 9; MUC2, Mucin 2; GPR41, G Protein-Coupled Receptor 41; GPR43, G Protein-Coupled Receptor 43; ZO-1, Zonula occludens. *p<0.05, **p<0.01.

3.4.6 Gut Microbial Profiling: qPCR and 16S rRNA Sequencing

Based on differences in gut epithelial gene expression in HMO-supplemented rats, we proceeded to examine the gut microbial profile of fecal samples right after weaning (start of the diet intervention), at 7 weeks of age (week 4 of the intervention) and at 11 weeks of age (week 8 of the intervention). Due to a significant effect of sex for certain bacterial groups (e.g. *Lactobacillus* spp. p=0.002), males and females were analyzed separately.

Using qPCR, at 7 weeks of age, males showed significantly higher abundance of total bacteria in 3'SL-fortified group compared with controls (p=0.02) (Table 3.8). The relative abundance of *Clostridium* cluster I and *Clostridium* cluster XI was significantly reduced in all HMO-fortified groups compared to controls (p=0.0004 and 0.002 respectively). *Clostridium* cluster IV was reduced in 2'FL-fortified group compared to control (0.04). *Bifidobacterium* spp. was significantly higher in the 2'FL group compared to 3'SL-fortified group (p=0.03). *Akkermansia muciniphila*, after log transformation, showed a significant reduction in 3'SL+2'FL group compared with control (p=0.01).

At 11 weeks of age, qPCR analysis showed that males had an increase in the relative abundance of *Roseburia* spp. in 3'SL-fortified group compared to all other groups (p=0.01; Table 3.9) and a reduction in *Enterobacteriaceae* in 2'FL-fortified group compared to control (p=0.02; Table 3.9).

In female rats at 7 weeks of age, qPCR analysis showed that *Akkermansia muciniphila* spp. was significantly reduced in all HMO-fortified groups compared to control (p=0.04; Table 3.10).

At 11 weeks of age, qPCR analysis showed numerous differences between groups in female rats (Table 3.11). Total bacteria was higher in 2'FL compared with 3'SL+2'FL-fortified group (p=0.018) and showed a trend to be higher than all other groups. *Clostridium* cluster I abundance was increased in 2'FL-fortified group compared to groups fortified with 3'SL, alone or in combination (p=0.004 and p=0.007). *Methanobrevibacter* spp. was significantly reduced in groups fortified with 2'FL, alone or in combination compared to 3'SL group. (p=0.03 and p=0.03 respectively). *Akkermansia muciniphila*, after log transformation was significantly reduced in 3'SL+2'FL-fortified diet group compared to control (p=0.004).

Table 3.8 Relative abundance of fecal microbiota (qPCR) in male rats at 7 weeks of age fed AIN-93 diet fortified with 3'SL, 2'FL, both or neither

Treatment	Control	3'SL	2'FL	3'SL+2'FL	p-value
			Relative abundance (%)		
***Bacteroides/Prevotella* spp.**	1.9±0.3	1.4±0.2	1.7±0.3	1.3±0.2	0.66
***Bifidobacterium* spp.**	0.9±0.2[ab]	0.8±0.2[a]	3.1±1.0[b]	2.3±0.4[ab]	0.03*
Enterobacteriaceae	0.2±0.04	0.2±0.1	0.1±0.02	0.1±0.02	0.31
***Lactobacillus* spp.**	47.1±6.6	57.4±9.2	43.7±9.6	38.2±9.5	0.48
***Clostridium perfringens* (cluster I)**	1.5±0.2[a]	0.7±0.2[b]	0.8±0.2[b]	0.6±0.1[b]	0.0004*
***Clostridium leptum* (cluster IV)**	9.1±1.5[a]	5.1±1.0[ab]	4.7±0.9[b]	7.1±1.2[ab]	0.04*
***Clostridium difficile* (cluster XI)**	0.4±0.1[a]	0.1±0.03[b]	0.1±0.02[b]	0.1±0.01[b]	0.002*
***Clostridium coccoides* (cluster XIV)**	16.3±2.3	10.6±1.4	10.4±2.1	13.5±1.9	0.12
***Roseburia* spp.**	0.003±0.002	0.004±0.002	0.0006±0.0001	0.003±0.001	0.36
***Methanobrevibacter* spp.**	0.005±0.003[a]	0.003±0.0003[b]	0.003±0.0001[b]	0.004±0.001[ab]	0.001*
Akkermansia muciniphila	0.07±0.03[a]	0.04±0.01[ab]	0.02±0.007[ab]	0.003±0.001[b]	0.01*
Faecalibacterium prausnitzii	0.09±0.03	0.03±0.008	0.04±0.008	0.05±0.005	0.20
Collinsella aerofaciens	0.005±0.0004[a]	0.003±0.0003[b]	0.003±0.001[b]	0.004±0.001[ab]	0.002*
Total bacteria (16S rRNA gene copies)	30,736,150± 2,698,774[a]	53,509,340± 6,514,451[b]	49,386,585± 4698662[ab]	38,298,292± 5,529,141[ab]	0.02*

Values are means ± SEM, *n* = 8-10. *Bacteriodes/Prevotella* spp., *Enterobacteriaceae*, *A. muciniphila* and *F. prausnitzii* were log transformed. Total bacteria are represented as 16S rRNA gene copies/20 ng genomic DNA. All other taxa are presented as relative abundance (%) of bacterial taxa per total bacteria (16S rRNA gene copies / total 16S rRNA gene copies). Control: AIN-93; 3'SL: AIN-93 + 3'Sialyllactose; 2'FL: AIN-93 + 2'Fucosyllactose; 3'SL+2'FL: AIN-93 + 3'Sialyllactose + 2'Fucosyllactose. The superscripts [a, b] are used to depict differences between groups where groups without a common superscript differ ($P<0.05$).

Table 3.9 Relative abundance of fecal microbiota (qPCR) in male rats at 11 weeks of age fed AIN-93 diet fortified with 3'SL, 2'SL, both or neither

	Control	**3'SL**	**2'FL**	**3'SL+2'FL**	**p-value**
			Relative abundance (%)		
***Bacteroides/Prevotella* spp.**	2.4±0.7	2.6±0.6	2.4±0.4	3.0±0.5	0.88
***Bifidobacterium* spp.**	0.5±0.1	0.2±0.05	0.3±0.07	0.4±0.1	0.19
Enterobacteriaceae	0.4±0.1[a]	0.2±0.05[ab]	0.1±0.04[b]	0.2±0.04[ab]	0.02*
***Lactobacillus* spp.**	44.9±7.5	57.9±12.5	33.3±11.0	46.9±10.0	0.48
***Clostridium perfringens* (cluster I)**	1.4±0.3	0.7±0.2	1.0±0.2	0.7±0.1	0.11
***Clostridium leptum* (cluster IV)**	14.3±2.2	6.9±1.4	12.0±3.2	8.5±1.9	0.12
***Clostridium difficile* (cluster XI)**	0.34±0.07	0.4±0.06	0.2±0.01	0.2±0.03	0.06
***Clostridium coccoides* (cluster XIV)**	31.1±5.5	23.3±4.2	24.3±4.4	22.3±2.9	0.48
***Roseburia* spp.**	0.002±0.0002[a]	0.01±0.005[b]	0.002±0.0003[a]	0.002±0.0004[a]	0.01*
***Methanobrevibacter* spp.**	0.01±0.002	0.02±0.003	0.02±0.003	0.02±0.002	0.53
Akkermansia muciniphila	0.2±0.07	0.04±0.03	0.08±0.03	0.01±0.006	0.14
Faecalibacterium prausnitzii	0.1±0.03	0.07±0.02	0.07±0.01	0.1±0.03	0.08
Collinsella aerofaciens	0.02±0.002	0.01±0.002	0.01±0.003	0.02±0.004	0.12
Total bacteria (16S rRNA gene copies)	39,654,166± 6,540,301	41,778,495± 5,162,862	42,574,434± 6,551,598	44,569,545± 2,780,744	0.93

Values are means ± SEM, *n* = 8-10. *Methanobrevibacter* spp., *Akkermansia muciniphila, Faecalibacterium prausnitzii* and *Collinsella aerofaciens* were log transformed. Total bacteria are represented as 16S rRNA gene copies/20 ng genomic DNA. All other taxa are presented as relative abundance (%) of bacterial taxa per total bacteria (16S rRNA gene copies / total 16S rRNA gene copies). 3'SL: AIN-93 + 3'Sialyllactose; 2'FL: AIN-93 + 2'Fucosyllactose; 3'SL+2'FL: AIN-93+ 3'Sialyllactose + 2'Fucosyllactose. The superscripts [a, b] are used to depict differences between groups where groups without a common superscript differ ($P<0.05$).

Table 3.10 Relative abundance of fecal microbiota (qPCR) in female rats at 7 weeks of age fed AIN-93 diet fortified with 3'SL, 2'FL, both or neither

Treatment	Control	3'SL	2'FL	3'SL+2'FL	p-value
	Relative abundance (%)				
***Bacteroides/Prevotella* spp.**	1.2±0.1	1.9±0.3	1.8±0.3	1.4±0.04	0.11
***Bifidobacterium* spp.**	0.9±0.4	1.2±0.4	1.7±0.5	1.3±0.3	0.34
Enterobacteriaceae	0.1±0.02	0.2±0.04	0.1±0.02	0.05±0.009	0.06
***Lactobacillus* spp.**	41.7±5.3	42.0±8.1	45.2±5.6	45.7±10.1	0.97
***Clostridium perfringens* (cluster I)**	1.0±0.1	1.3±0.2	1.3±0.2	1.2±0.06	0.60
***Clostridium leptum* (cluster IV)**	6.5±1.1	7.1±1.3	10.7±1.7	6.7±1.2	0.10
***Clostridium difficile* (cluster XI)**	0.1±0.03	0.1±0.03	0.1±0.01	0.08±0.02	0.35
***Clostridium coccoides* (cluster XIV)**	10.9±1.6	11.6±1.5	12.2±1.1	17.6±2.8	0.06
***Roseburia* spp.**	0.001±0.0004	0.0006±0.0001	0.0009±0.0002	0.003±0.001	0.20
***Methanobrevibacter* spp.**	0.004±0.001	0.005±0.001	0.004±0.001	0.006±0.001	0.94
Akkermansia muciniphila	0.1±0.07[a]	0.02±0.006[b]	0.03±0.01[b]	0.009±0.004[b]	0.04*
Faecalibacterium prausnitzii	0.07±0.02	0.05±0.01	0.04 ±0.006	0.05±0.009	0.61
Collinsella aerofaciens	0.003±0.001	0.004±0.001	0.004±0.0004	0.004±0.001	0.36
Total bacteria (16S rRNA gene copies)	47,474,463± 3,993,791	47,238,918± 6,656,759	47,704,183± 2,956,712	33,004,782± 4,106,583	0.10

Values are means ± SEM, *n* = 8-10. *Bacteroides/Prevotella* spp., *Bifidobacterium* spp., *Enterbacteriaceae*, *Roseburia* spp., *Methanobrevibacter* spp. and *F. prausnitzii* abundance was log transformed. Total bacteria are represented as 16S rRNA gene copies/20 ng genomic DNA. All other taxa are presented as relative abundance (%) of bacterial taxa per total bacteria (16S rRNA gene copies / total 16S rRNA gene copies). Control: AIN-93; 3'SL: AIN-93 + 3'Sialyllactose; 2'FL: AIN-93 + 2'Fucosyllactose; 3'SL+2'FL: AIN-93 + 3'Sialyllactose + 2'Fucosyllactose. The superscripts [a, b] are used to depict differences between groups where groups without a common superscript differ ($P<0.05$)

Table 3.11 Relative abundance of fecal microbiota (qPCR) in female rats at 11 weeks of age fed AIN diet fortified with 3'SL, 2'SL, both or neither

Treatment	Control	3'SL	2'FL	3'SL+2'FL	p-value
	Relative abundance (%)				
***Bacteroides/Prevotella* spp.**	3.3±0.7	3.0±0.4	1.5±0.3	2.4±0.5	0.07
***Bifidobacterium* spp.**	0.2±0.1	0.1±0.01	0.2±0.04	0.2±0.04	0.28
Enterobacteriaceae	0.3±0.1	0.2±0.05	0.3±0.1	0.2±0.03	0.42
***Lactobacillus* spp.**	21.9±7.5	34.8±14.6	26.0±9.5	24.4±10.1	0.85
***Clostridium perfringens* (cluster I)**	$1.2±0.2^{ab}$	$0.4±0.1^{a}$	$1.6±0.4^{b}$	$0.6±0.1^{a}$	0.002*
***Clostridium leptum* (cluster IV)**	14.4±1.8	8.8±1.5	8.5±1.8	13.9±2.6	0.19
***Clostridium difficile* (cluster XI)**	0.4±0.1	0.3±0.04	0.3±0.04	0.3±0.04	0.24
***Clostridium coccoides* (cluster XIV)**	30.7±3.1	26.5±4.6	24.5±5.6	16.0±3.7	0.15
***Roseburia* spp.**	0.006±0.002	0.002±0.0004	0.002±0.0004	0.002±0.0003	0.07
***Methanobrevibacter* spp.**	$0.02±0.003^{ab}$	$0.03±0.005^{a}$	$0.01±0.002^{b}$	$0.02±0.003^{b}$	0.002*
Akkermansia muciniphila	$0.2±0.07^{a}$	$0.02±0.007^{ab}$	$0.07±0.03^{ab}$	$0.007±0.003^{b}$	0.009*
Faecalibacterium prausnitzii	0.1±0.03	0.07±0.03	0.1±0.01	0.1±0.02	0.46
Collinsella aerofaciens	0.02±0.003	0.02±0.003	0.02±0.004	0.02±0.003	0.10
Total bacteria (16S rRNA gene copies)	32,776,158± $2,447,995^{ab}$	23,426,482± $903,946^{ab}$	39,659,548± $6,931,952^{a}$	20,657,973± $2,909,108^{b}$	0.02*

Values are means ± SEM, *n* = 8-10. *Bacteroides* spp., *C. leptum, Roseburia* spp., *A. muciniphila* and *C. aerofaciens* were log transformed. Total bacteria are represented as 16S rRNA gene copies/20 ng genomic DNA. All other taxa are presented as relative abundance (%) of bacterial taxa per total bacteria (16S rRNA gene copies / total 16S rRNA gene copies).3'SL: AIN-93+ 3'Sialyllactose; 2'FL: AIN-93 + 2'Fucosyllactose; 3'SL+2'FL: AIN-93 + 3'Sialyllactose + 2'Fucosyllactose. The superscripts [a, b] are used to depict differences between groups where groups without a common superscript differ ($P<0.05$).

Based on 16S rRNA sequencing, HMO-fortified diets showed no effect on alpha diversity according to Shannon or Chao1 indices in both males and females (data not shown). However, the Simpson index in males showed higher alpha diversity in 3’SL+2’FL-fortified group at 11 but not 3 or 7 weeks (p=0.02; Figure 3.8). No difference was seen in females at 3, 7 or 11 weeks … n between groups (Figure 3.9).

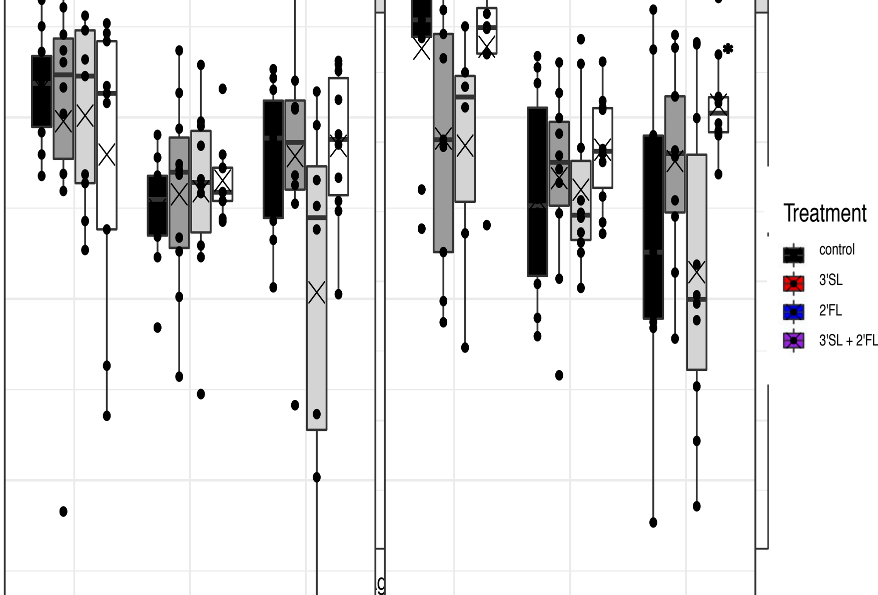

Figure 3.8 Male and female alpha diversity according to the Simpson index using the phyloseq package at 3, 7 and 11 weeks of age. Control: AIN-93; 3’SL: AIN-93 +3’Sialyllactose; 2’FL: AIN-93 + 2’Fucosyllactose; 3’SL + 2’FL: AIN-93 + 3’Sialyllactose + 2’Fucosyllactose. *Significance was set at p<0.05.

Complementary to qPCR data, 16S rRNA sequencing data in males showed *Clostridiaceae_1* relative abundance was reduced in the 3’SL and 3’SL+2’FL groups compared with controls in both males and females (Figure 3.10). *Prevotellaceae* relative abundance was significantly lower in 2’FL group compared to 3’SL and 3’SL+2’FL in both males and females (Figure 3.10). Similarly, *Erysipelotrichaceae and Tannerellaceae* relative abundance in males was higher in groups fortified with 3’SL, alone or in combination. In females, *Bifidobacteriaceae* relative abundance was highest in the 2’FL group compared to 3’SL.

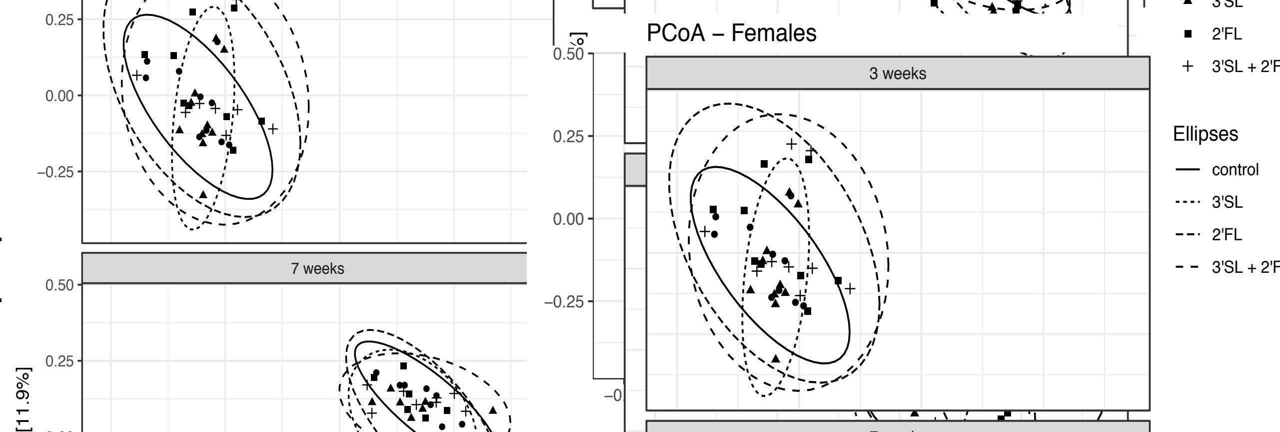

Figure 3.9 Male and female beta diversity at 11 weeks of age calculated with principal coordinates analysis (PCoA) using a Bray-Curtis distance matrix. ASVs are normalized proportionally, by relative abundance. ASVs present in less than 5% of the samples were removed. Control: AIN-93; 3'SL: AIN-93 +3'Sialyllactose; 2'FL: AIN-93 + 2'Fucosyllactose; 3'SL + 2'FL: AIN-93 + 3'Sialyllactose + 2'Fucosyllactose.

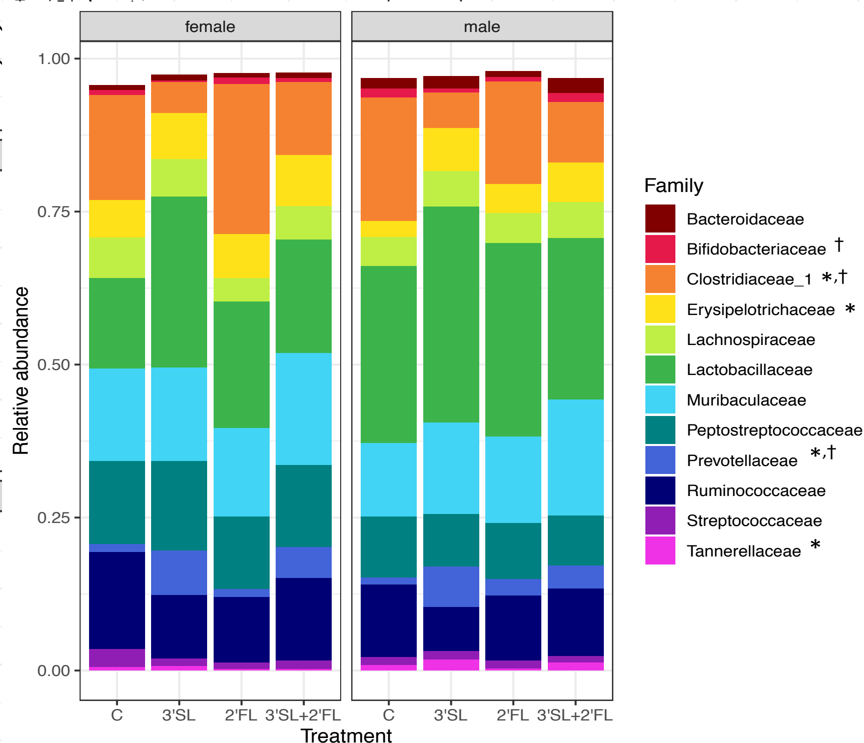

Figure 3.10 Relative abundance plots of bacterial taxa using 16S rRNA sequencing in male and female rat feces at 11 weeks of age. Taxa were identified to the taxonomic level of family using the Silva reference database. Control: AIN-93; 3'SL: AIN-93 + 3'Sialyllactose; 2'FL: AIN-93 + 2'Fucosyllactose; 3'SL+2'FL: AIN-93 + 3'Sialyllactose + 2'Fucosyllactose. * *P*<0.05 (males); † *P*<0.05 (females).

3.5 Discussion

Human milk is a highly evolved, structurally complex, complete biomaterial that nourishes developing infants, while simultaneously acting as a growth factor, prebiotic, modulator of gut microbiota/gut barrier function and immune regulatory factor[222]. Breastfeeding for less than 4-6 months or not at all has been associated with greater incidence of immune-mediated diseases, infectious diseases, overweight, obesity and other metabolic ailments in adulthood[223]. This is in part due to the absence of important HMOs like 3'SL and 2'FL [222]. A complete HMO profile provides a biological advantage within the gastrointestinal tract and throughout the body[222]. To our knowledge, this is the first study looking at the fortification of a weanling diet with the HMOs, 3'SL and 2'FL alone or in combination and how they may impact gut microbial composition, intestinal permeability, inflammatory cytokines and intestinal gene expression in males and females. HMO supplementation in females improved intestinal permeability, mRNA expression of important genes involved in maintaining gut barrier function and gut microbial composition. Males supplemented with HMOs displayed reductions in weight gain at the end of an eight-week intervention, improved pro-inflammatory cytokine profiles and an increased abundance in beneficial gut microbes.

HMOs cannot be digested by the human infant; they are primarily considered prebiotics, denoting their indigestible nature and selective utilization by beneficial gut microbes. Using a chemically defined medium, facilitating vigorous growth of gut-related microbes, researchers found that some strains of *Bifidobacterium* and *Bacteroides* are able to utilize HMOs with high efficiency[40]. A dysbiotic gut at an early age may be predictive of disease later in life. Breast-fed infants harbor a distinct gut microbiota, dominated by bifidobacteria[224]. Our study found that neutral 2'FL compared to 3'SL and control diets enhanced this bifidogenic effect at 7 weeks of age in males and 11 weeks of age in females. This observation might be indicative of the genetic capability of select bacteria co-evolving with HMOs to enable their utilization[222]. Bifidobacteria strains for example utilize varied oligosaccharides as growth substrates[225]. 2'FL as noted in our study in both males and females may be one of them. Other researchers found that *Bifidobacterium infantis* utilize HMOs lacto-*N*-tetraose (LNT) and lacto-*N*-neotetrose (LNnT)[225]. Inefficient metabolism of these HMOs will result in a deleterious shift in *B. longum* subsp. *infantis* physiology, thereby impacting offspring health[225]. Another example is Disialyllactose-N-tetraose (DSLNT), which is the most effective HMO to reduce necrotizing

enterocolitis-like symptoms in a neonatal rat model[226]. *B. longum* ssp. *infantis* ATCC 15697 and B. *infantis* M-63 are the only two microbes known to be able to ferment 3'SL, 6'SL, 2'FL and 3'FL, with the latter able to degrade about 90% of 2'FL[227]. Further, out of all bifidobacteria strains, only *B. infantis* species and *B. breve* ATCC 15700 are able to ferment LNnT, while *L. acidophilus* NCFM, among lactobacilli utilize LNnT most efficiently[227]. It is important to note that while we saw differences in bifidobacteria abundance in male and female Sprague Dawley rats, we are not aware of research in which HMOs utilization by bifidobacteria from the rodent gut has been shown directly.

A growing body of evidence suggests that in the infant gut, there exist a multitude of HMO-adapted microbes like bifidobacteria and *Bacteroides*[40,227]. Therefore, it becomes increasingly vital to determine which of the over 200 identified HMOs are responsible for beneficial effects metabolically, immunologically, cognitively or otherwise. This characterization would be vital in selecting individual HMO's for supplementation purposes. *In vitro* incubations of multiple strains of bifidobacteria using Lacto-N-biose (LNB) or sialyllactose indicate that only a select few species are able to proliferate using isolated HMOs as a carbon source[228,229]. *B. infantis* grows on HMOs as a sole sugar source, whereas *L. gasseri* does not[230].

Akkermansia muciniphila is known for its mucin-utilizing characteristics[231]. Using a comprehensive array of techniques to analyze and differentiate between all the bacteria in the intestinal tract, *A. muciniphila* was uniquely found to reach 100 times its original abundance following prebiotic ingestion, which corresponds with an improved metabolic profile[22]. It is important to note, that this discovery was only observed in genetic or diet-induced obese mice[22]. Our findings showed a 10 to 30-fold reduction in *A. muciniphila* in females fed 3'SL+2'FL-fortified formula at 7 and 11 weeks of age respectively, despite increased expression of select tight junction proteins in the jejunum as well as observed reductions in intestinal permeability in the colon as demonstrated by lower FITC in all HMO-supplemented groups in females. This is likely because unlike previously published findings, showing a 100-fold increase, our rodents were neither genetic nor diet-induced obese animals. In fact, we found no changes in body weight, fat or lean mass or insulin resistance between groups, therefore perhaps, *A. muciniphila* relative abundance only increases in metabolically overweight or obese models. It is possible, *A. muciniphila* is only needed to reverse HFD-induced metabolic disorders and

improve intestinal barrier function. Supporting these findings, we also found a concurrent increase in the relative abundance of the Verrucomicrobia phylum, of which *A. muciniphila* is a member, using 16S rRNA sequencing technology.

Clostridia and *enterococci* have been characterized as non-HMO consumers using a chemically defined medium, ZMB1[40], explaining why our study found significantly reduced abundance of *Enterobacteriaceae* in the 2'FL-fortifed group, alone or in combination with 3'SL and a trend of reduced presence of *C. difficile* in the 2'FL and 3'SL+2'FL-fortified male groups at 11 weeks of age.

Increased intestinal permeability is postulated to be resultant of reduced expression of tight junction proteins[232]. Tight junctions are made up of claudins, occludins and zonula occludens (ZO)- 1, 2, 3, which regulate the paracellular permeability of endothelial and epithelial cells, while also operating as a barrier against bacterial invasion[233]. Importantly, tight junction proteins show sex-dependent expression and modulation. One study examined the expression of estrogen receptor ER-α/β and ZO-1 in male and female gut tissues as well as concurrent inflammatory activation *in vitro*[234]. They found that ZO-1 expression was significantly lower in female compared to male tissue and estrogen treatment decreased ZO-1 mRNA and protein expression, signifying that sex hormones may regulate tight junction proteins in the gut[234]. This differential expression could explain why we saw distinct sex differences in intestinal permeability in males and females. The increased expression of ZO-1 in 3'SL HMO-fortified groups, alone or in combination with 2'FL uniquely reduced intestinal inflammation in females. ZO-1 maintains a selectively permeable epithelial barrier and impedes the translocation of bacterial populations into circulation from the intestinal lumen. HMO-fortified diets, in females at least, appear to maintain these tight junction proteins, and reduce intestinal permeability approximately 3-fold compared to control. A 2009 study using the lactulose/mannitol test found similar changes, where breast-fed infants showed a 2.8-fold reduction in intestinal permeability compared to exclusively formula fed infants[197]. Two early studies in term infants, also examining different feeding types showed reduced intestinal permeability in breast-fed compared to formula-fed infants but at different periods postnatally[235,236]. Rats fed a high fat diet for a prolonged period of time showed increased intestinal permeability as well as a dysbiotic microbiota which has been shown to be prevented with the addition of Bovine Milk Oligosaccharides (BMO) and prebiotic inulin[237]. Inulin, like milk oligosaccharides, is a

prebiotic and a soluble fiber. Supplementation with soluble fibers is known to ameliorate gut dysbiosis and reduce low-grade inflammation, typically linked, at least in part, with decreased intestinal permeability[238,239].

HMOs have been associated with anti-inflammatory effects by affecting cytokine production, the initial change from an intrauterine Th2 prevailing-response to a Th1/ Th2 balanced one[240] and the inhibition of leukocyte rolling and adhesion to endothelial cells under variable conditions [241]. Among these cytokines, IL-18 is traditionally considered a pro-inflammatory cytokine produced by a myriad of structures, including lactating mammary glands and intestinal epithelial cells[242]. In human milk, preterm delivery or complications during pregnancy is associated with higher levels of IL-18 in breast milk[242]. Our findings show reductions in serum IL-18 and a trend toward a decrease in TNF and IL-5 in male groups fortified with 2'FL HMOs, alone or in combination with 3'SL. Validating these findings, a randomized controlled trial found that healthy infants born at term, fed formula fortified with 2'FL had reductions in pro-inflammatory cytokines, as measured *ex vivo* in plasma and mirroring those of breast-fed infants [243]. Further, comparable *in vivo* differences in cytokine levels were found in allergy-prone infants in the first year of life between breast-fed versus formula-fed infants[244]. Pu *et al*. [245] recently suggested a dual function of IL-18, primarily in a colitis model, whereby, IL-18 may have pro- or anti-inflammatory functions. They found that IL-18 treatment at an earlier stage of colitis changed colon length, reduced inflammatory infiltration and increased Muc2 expression[245]. This potentially explains why we saw a positive correlation between IL-18 and MUC2 expression in our young, healthy females, as well as a reduction in intestinal permeability across all HMO-fortified groups.

To further explore a link with inflammation, we examined GPR41 and GPR43 mRNA expression. Short chain fatty acids (SCFA) bind to these receptors to modulate intestinal inflammation, by reducing the secretion of proinflammatory cytokines and chemokines[246], however GPR41 and GPR43 mRNA expression in males and females in our study did not mirror our serum inflammatory cytokine data. As such, it would be important in future studies to examine intestinal histology (infiltration, crypt alterations, erosion, etc.) and expression of inflammatory genes in a sex-dependent manner to determine if the HMOs indeed affect inflammation at the intestinal level. Estrogen has an anti-inflammatory effect due to inhibition of NF-κB activation[247], which may explain the important sex differences in serum inflammatory

cytokines, where females showed a trend toward an increase in anti-inflammatory cytokine IL-10 in the 2’FL- HMO fortified group.

Our study demonstrated that HMO supplementation of 3’SL or 2’FL alone or in combination elicits distinct sex differences which may be positive or negative. Previous reports have combined data from both sexes, failing to distinguish important sex differences in metabolic outcomes. There is also growing evidence showing that sex is an important factor to consider when examining interactions between gut microbiota and environmental factors such as diet and not stratifying by sex can obscure important sex-by-diet interactions[248]. To our knowledge, we are the first group to demonstrate important sex differences after 3’SL and 2’FL HMO supplementation. We established that females experienced improvements in gut morphology and barrier function, as well as overall improvements in gut microbial composition at the family taxonomic level. In males, however, 3’SL and 2’FL HMO supplementation resulted in patterns of mRNA levels in the jejunum and colon, including ZO-1, occludin and MMP9 that are commonly associated with compromised gut permeability although MUC2 mRNA levels showed upregulation with HMOs. In the future it would be important to examine protein levels of these genes and other indices of gut barrier function to fully understand the implications of the changes in gene expression identified. Males also displayed slightly lower weight gain and inflammatory biomarkers during the final week of the intervention compared to control, as well as an increased abundance of beneficial gut microbes at varying taxonomic levels. Still, our study is not without limitations. We found apparent conflicting findings resultant of the supplementation of 3’SL and 2’FL alone or in combination, which may be owing to changes in HMO metabolism within the gut during postnatal development. Based on fecal oligosaccharide profiles, HMO metabolism is postulated to progress through three stages in human infants[249]: 1) From birth to two months: prevalence of neutral or acidic oligosaccharide metabolism (7 day old rat is approximately equivalent to a newborn human infant in terms of central nervous system and reproductive development); 2) At 2-4 months, reduction of HMOs in infant fecal matter and an increase in HMO metabolites (approximately equivalent to rats in the week leading up to weaning); 3) Four months, onwards, when complementary solid foods begin introduction, a substantial reduction of HMOs as well as their metabolites and an increase in oligosaccharides, typically complimenting the introduction of solid foods (approximately equivalent to 21 days and on onwards in rats). Our study introduced HMOs at approximately stage 2 of HMO metabolism

and continued these interventions well past stage 3. In stage 3 we maintained a single standard diet, AIN-93 and HMO-fortification, therefore it is likely that HMOs and their metabolites would have remained consistent in fecal matter throughout the intervention, though perhaps eliciting less than beneficial effects[250]. Alternatively, findings from a randomized controlled trial of healthy term infants given the HMOs 2'FL and LNnT, shifted the gut microbiota towards that of breast-fed infants. We did not supplement our rodents with LNnT HMO which may be why we did not observe similar effects. Finally, we acknowledge we only supplemented a small fraction of these HMOs, which are unlikely to provide the exact benefits conferred from the evolutionary forces perfecting the process of exclusive breast feeding, ensuring the greatest health benefit for the infant[40]. Future studies should investigate whether the addition of *Lactobacillus*, *Bifidobacterium* and *Bacteroides* combined with more HMOs including: 3'SL, 2'FL and LNnP in the formula will elicit similar immunoregulatory and symbiotic gut microbial proliferation as breast-fed infants, stratified by sex.

3.6 Acknowledgments

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CHAPTER FOUR: PATERNAL HIGH PROTEIN DIET MODULATES BODY COMPOSITION, INSULIN SENSITIVITY, EPIGENETICS AND GUT MICROBIOTA INTERGENERATIONALLY IN RATS

4.1 ABSTRACT

BACKGROUND & AIMS: Mounting evidence demonstrates that paternal diet programs offspring metabolism. Our objective was to examine whether paternal high protein (HP) or high fat/high sucrose (HF/S) diets alter body composition, insulin sensitivity, gut microbiota and epigenetic markers intergenerationally.

METHODS: Male Sprague Dawley rats were weaned at 3 weeks of age onto control, HP diet (40% wt/wt) or HF/S diet for 9 weeks until they reached sexual maturity and were mated with virgin female rats. Offspring were weaned onto control diet and followed until 16 weeks of age. Body composition, metabolic markers, gut microbial composition and DNA methyltransferase expression were measured in fathers and offspring at weaning and adulthood.

RESULTS: Compared to control and HF/S, paternal HP diet reduced fat mass ($p=0.002$) and % body fat ($p<0.001$) in fathers and was mirrored in male and female adult offspring ($p<0.05$). HP fathers consumed 27% fewer kcal/day compared to HF/S which was similarly observed in male offspring at weaning. HP offspring at weaning and adulthood had greater insulin sensitivity than HF/S offspring. Paternal and offspring incretin and pancreatic hormone levels further substantiated the distinct differences in energy intake and glycemic control between groups. HP fathers had higher gut microbiota alpha diversity than controls, which was mirrored in female adult offspring. Distinct intergenerational bacterial clustering was observed. Paternal HP diet increased *Bifidobacterium, Akkermansia, Bacteroides* and *Marvinbryantia* in male and/or female adult offspring. DNMT1and 3b expression was altered intergenerationally.

CONCLUSION: Overall, paternal HP diet altered gut microbial composition and epigenetic markers and reduced metabolic dysfunction intergenerationally.

4.2 INTRODUCTION

Metabolic ailments have placed a massive burden on the healthcare system globally, the majority of which stem from complex diseases like obesity and type 2 diabetes. Both obesity and type 2 diabetes have a heritable genetic component, as well as a prominent epigenetic component resultant of environmental exposures, including the preconceptional period [69]. The preconceptional period includes: 1) gamete maturation, 2) fertilization and 3) early embryo development [251]. These various stages involve important epigenetic, cellular and metabolic activities that are tremendously susceptible to environmental factors, either by agitations or adaptive compensatory responses thereby affecting the new organisms' phenotype throughout its lifetime [251]. These environmental factors include nutrition.

High protein diets have been used to promote weight loss [252,253], modify lipid metabolism and increase satiety [254,255]. However, our understanding of how a protein-rich diet may affect the gut microbiota remains limited and even less is known about its effects intergenerationally. It is known that dietary protein increases the availability of nitrogenous compounds, like protein, peptides and amino acids in the large intestine, wherein luminal proteolysis and metabolism by the intestinal microbes leads to production of numerous by-products [256,257]. These microbial metabolites include short-chain fatty acids (SCFA; acetate, propionate, and butyrate) and more notably branched chain fatty acids (BCFA; isobutyrate, isovalerate) [258]. Gut microbes and microbial metabolites have been shown to regulate epigenetic mechanisms, including the process of DNA methylation that is catalyzed by the DNA methyltransferase family of enzymes, as a consequence of diet composition and nutrient availability [259].

Although the majority of research has focused on maternal nutritional exposures, a growing body of evidence is emerging that substantiates the importance of the paternal periconceptional period in the developmental origins of disease (DOHaD) [260]. Sperm contributes more than a single haploid genome complement to offspring [261]. In fact, paternal environments and phenotypes have been occasionally considered better predictors of DOHaD in their F_1 progeny compared to maternal environments and phenotypes. For example, paternal body composition marked by elevated fat mass, a marker of an obese phenotype in rodents, has been associated with delayed development, mitochondrial impairments, altered carbohydrate metabolism [262] as well as cardiovascular impairments [155], reproductive disorders [263] and

obesity [264] in their offspring. Comparable effects have been observed in humans [265]. Three possible mechanisms have been postulated for these effects: 1) direct - alterations to the paternal epigenome, i.e. DNA methylation, histone modifications and small non-coding RNA alterations [261,266,267]; 2) indirect - seminal fluid composition may influence female reproductive tract physiology and conceptus development [262]; 3) indirect - female dynamically modifies her investment in the offspring, based on paternal fitness [268].

More recently, a series of novel studies in murine models have established a link between paternal nutrition and metabolic changes in offspring. These include paternal high fat diet at preconception [264], low protein diet [155] and intermittent fasting [269]. Even more strikingly, *in utero* perturbations, including undernutrition in the third trimester of the grandparent, resulted in low birth weight and impaired glucose tolerance in the second generation that was transmitted via the first generation paternal line [270]. Analogous results were found in human cohorts, most notably the *Dutch Hunger Winter* of 1944-1945, highlighting paternal line multigenerational passage of nutritional status, where paternal undernutrition resulted in higher susceptibility to metabolic ailments in offspring [271]. Taken together, rodent and human paternal nutrition research has elucidated important metabolic outcomes inter- and trans-generationally, where biological parenting starts before conception.

We and others have shown that a maternal high protein diet affects offspring health later in life [41,42,272].To our knowledge, no research has examined whether a paternal high protein diet affects offspring metabolism, the gut microbiota and epigenetic regulation. Therefore, our objective, was to investigate the long-term effects of paternal high protein diet compared to control and high fat/sucrose diets (previously shown to be metabolically detrimental to offspring [264]), in an attempt to elucidate the intergenerational effects on body composition, organ development, metabolic outcomes, gut microbial signatures and epigenetic changes.

4.3 MATERIALS AND METHODS

4.3.1 Animal Model and Dietary Treatment

Sixty male Sprague Dawley rats (aged 3 weeks) were purchased from Charles River Laboratories (Montreal, QC, Canada) and housed in a temperature and humidity-controlled facility with a 12-h light/dark cycle. Rats were allocated to 1 of 3 diets from 3 to 12 of age: 1) control AIN-93G diet (weeks 3-9) and AIN-93M (weeks 10-12); 2) high protein diet (40% wt/wt

as casein); or 3) high fat/ sucrose diet (DYETS# 103915: age 3-9 weeks; DYETS# 102412: weeks 10-12). All diets were purchased from Dyets, Inc. (Bethlehem, PA, USA). Diet composition is provided in Table 4.1. At 12 weeks of age, each male was placed with a virgin female Sprague Dawley rat during the dark cycle until a copulation plug was identified. Male rats were returned to their designated diet during the light cycle to limit epigenetic changes resultant from acute alterations to diet during conception. The pregnant females consumed control AIN-93G diet throughout pregnancy and lactation. Litters were culled to 10 offspring (n=5 males; n=5 females) to minimize differences in nutrition due to differing litter sizes. Small litters were increased to n=10 through cross-fostering from dams within the same treatment of similar birth dates. One male and one female offspring from each litter was assessed at weaning and an additional one male and one female were weaned onto control diet (AIN-93; Dyets) and water *ad libitum* at age 3 weeks and followed until age 16 weeks. In our analysis, all offspring within the same litter were considered as n=1. Body weight was assessed weekly and food intake was quantified every 3 weeks until the termination of the study. This study was approved by the University of Calgary Animal Care Committee (AC18-0074) and conformed to the *Guide to the Care and Use of Laboratory Animals*.

4.3.2 Oral Glucose Tolerance Test

Fasted blood glucose was measured from a tail nick sample using a One Touch Ultra® 2 glucose meter (LifeScan, Burnaby, Canada), following an overnight 12 hour fast, 2 weeks prior to euthanasia in fathers and offspring. Additional blood glucose measurements were made at 15, 30, 60, 90 and 120 minutes following an oral gavage of a 2g/kg glucose solution.

4.3.3 Insulin Tolerance Test

Using an intraperitoneal injection, insulin (0.75 U/kg) was administered to rats following a 6 hour fast, 7 days prior to euthanasia of fathers and offspring. Using a One Touch Ultra® 2 glucose meter (LifeScan, Burnaby, Canada), glucose concentrations were measured via tail nick at 0, 15, 30, 60, 90 and 120 minutes following the insulin injection.

Table 4.1 Experimental diet composition from weeks 3-9 and 10-16

	Control	**HP**	**HF/S**	**Control**	**HP**	**HF/S**
g/kg	**Weeks 3-9**			**Weeks 10-16**		
Cornstarch	397.5	197.5	0	465.7	197.5	0
Casein	200	400	240	140	400	200
Dyetrose	132	132	0	155	132	0
Sucrose	100	100	459.48	100	100	499.48
Soybean Oil	70	70	100	40	70	100
Lard	0	0	100	0	0	100
Alphacel	50	50	50	50	50	50
AIN-93M Mineral Mix	35	35	35	35	35	35
AIN-93 VX Vitamin Mix	10	10	10	10	10	10
L-cystine	3	3	0	1.8	3	0
DL-Methionine	0	0	3	0	0	3
Choline-Bitartrate	2.5	2.5	2.5	2.5	2.5	2.5
Energy density (kJ/g)	15.7	15.7	19.3	15.1	15.7	19.3
Carbohydrate (% of kcal)	63.9	44.7	49.8	75.9	44.7	49.8
Protein (% of kcal)	19.4	38.6	11.1	14.1	38.6	11.1
Fat (% of kcal)	16.8	16.7	39.1	10.0	16.7	39.1

The digestible energy of the control diets were 3.76 kcal/g and 3.6 kcal/g for the 3-9 and 10-16 week formulations respectively. Digestible energy of high protein and high fat/ sucrose diets were 3.76 kcal/g and 4.6 kcal/g respectively. All diets were purchased from Dyets, Inc. (Bethlehem, PA, USA).

4.3.4 Body Composition and Tissue Harvest

To assess body composition, a Dual Energy X-ray Absorptiometry (DXA) scan (Hologic ODR 4500; Hologic Inc.) was performed. Animals were placed under light anaesthetic (isoflurane) to ensure the animal remained still during the scan. Bone mineral content/density (BMC/BMD) (g and g/cm^2), fat mass(g), lean mass(g) and body fat % were quantified using Hologic QDR software for small animals.

Following a 12 h fast, rats were anaesthetized with isoflurane and 1 mL of blood was collected from the portal vein and placed into chilled tubes containing diprotinin-A (0.034 mg/ml blood; MP Biomedicals, Irvine, CA), Sigma protease inhibitor (1 mg/ml blood; Sigma Aldrich, Oakville, ON, Canada) and Roche Pefabloc (1mg/ml of blood; Roche, Mississauga, ON, Canada). Samples were centrifuged and serum was stored at -80°C until analysis. Rats were subsequently killed by decapitation. The heart, liver, kidney, cecum, colon and male testes were excised and weighed.

4.3.5 Blood Analysis

Using serum from the portal vein, a Rat Metabolic Multiplex Array (MRDMET) (Millipore, St. Charles, MO) was used to measure fasting levels of: amylin (active), C-peptide 2,

glucose-dependent insulinotropic polypeptide (GIP) (total), glucagon-like peptide 1 (GLP-1) (active), ghrelin (active), glucagon, insulin, leptin, pancreatic polypeptide (PP) and peptide tyrosine tyrosine (PYY) (Eve Technologies, Calgary, AB, Canada).

4.3.6 Hepatic Triglyceride Analysis

To assess triglyceride content, 25mg of liver was obtained and analyzed according to the manufacturer instructions for the GPO reagent set (Pointe Scientific Inc., Lincoln Park, MI).

4.3.7 Bacterial DNA Extraction and Microbiota 16S rRNA Gene Sequencing

Paternal fecal matter was collected at 3, 9 and 12 weeks of age and offspring fecal matter was collected at 3, 9 and 16 weeks of age, snap frozen and stored at -80°C. Microbial profiling was performed based on our previous work [217,218]. In short, bacterial DNA was extracted using the FastDNA spin kit for feces (MP Biomedicals, Lachine, QC, Canada) from ~60mg stool. The sample was diluted to a concentration of 4 ng/uL and stored at-80°C until further analysis. To quantify global microbial composition, the MiSeq Illumina platform was used to amplify the V3 and V4 region of the 16S rRNA gene (Illumina, San Diego, CA, USA). Samples were processed at the Centre for Health Genomics and Informatics at the University of Calgary (Calgary, AB, Canada).

4.3.8 Short Chain Fatty Acid Analysis using High-Performance Liquid Chromatography

SCFAs were quantified as previously described [273]. Briefly, cecal matter (~150mg) was collected in 1mL of 0.15 mmol/L sulfuric acid containing the internal standard 2-ethyl butyric acid. The sample was homogenized twice for 30 seconds using the Bead Mill 24 Homogenizer (Fisher Scientific, Waltham, MA, USA). Cecal matter was then centrifuged at 14,000g for 15 min at 4°C and 100uL of the supernatant was collected in a pyrex reaction tube. Each sample was mixed with 200 uL 2-nitrophenylhydrazine (adjusted to 20mmol/L in ethanol), 400 uL of 3% pyridine and 1-ethyl-(3-dimethylamineopropyl) carbodiimide (adjusted to 250 mmol/L). The solution reacted at 60°C for 20 min. To stop the reaction, 100uL of potassium hydroxide solution (15% w/v in water) and methanol (80:20 ratio) was mixed in and allowed to react for 15 min at 60°C. After the sample had cooled, 4 mL phosphate buffer mixture was added (phosphate buffer, pH 6.4 and 0.5mol/L HCL at 3.8:0.4 v/v) as well as 3 mL of hexane. After thorough vortexing, the supernatant (hexane layer) was discarded. To extract the SCFAs, 3 mL

of diethylether was mixed into the solution and the upper ether layer was collected and dried in a speedvac concentrator (Savant™ SPD111 SpeedVac™ Kits, Thermo Scientific™). The concentrate was dissolved in 1mL of 50% methanol and centrifuged at 14,000g for 15 minutes. Samples were injected into reverse-phase HPLC on a C18 column with a gradient of acetonitrile with 0.05% trifluoroacetic acid. Flow rate was set at 0.8mL/min for 30 minutes. Elution absorption was set at 230nm.

4.3.9 DNA methyltransferase mRNA quantification using RT-PCR

MicroRNAs were extracted from liver tissue using the miRNeasy mini kit according to the manufacturer instructions for Purification of Total RNA, Including Small RNA, from Animal Tissue; cDNA was subsequently quantified using the miScript II RT kit (Qiagen). Real-time PCR was performed as previously described[220]. MicroRNA primer sequences are listed in Table 4.2. Relative gene expression was calculated using the $2^{-\Delta CT}$ method[195], wherein results were normalized to both SNORD68 and SNORD96A controls genes.

For DNA methyltransferases, we used primers: DNA (cytosine-5)-methyltransferase (DNMT) 1, 3A and 3B (Bio-Rad, CA, USA). The amplicon context sequences are provided in Table 4.3. Total RNA was extracted from retroperitoneal adipose tissue using the RNAeasy Lipid Tissue Mini Kit followed by cDNA synthesis using SuperScript II RT (Qiagen). RT-PCR was performed similar to microRNAs, however, results were normalized to the 18S control gene.

Table 4.2 Universal primer sequences for microRNA in liver tissue

microRNA	Universal Primer
Liver	
Rn_miR-21_2	CTAGCTTATCAGACTGATGTTG
Rn_miR-24_1	TGGCTCAGTTCAGCAGGAAC
Rn_miR-33_2	GTGCATTGTAGTTGCATTGCA
Rn_miR-34a_1	TGGCAGTGTCTTAGCTGGTTG
Rn_miR-103_2	AGCAGCATTGTACAGGGCTATG
Rn_miR-107_2	AGCAGCATTGTACAGGGCTATC
Rn_miR-122a_1	TGGAGTGTGACAATGGTGTTT
Rn_miR-130a_1	CAGTGCAATGTTAAAAGGGC
Rn_miR-143_1	TGAGATGAAGCACTGTAGCT
Hs_SNORD68_11	**TTTGAACCCTTTTCCATCTG**
Hs_SNORD96A_11	**GACATGTCCTGCAATTCTGAA**

Hs_SNORD68_11 and Hs_SNORD96A_11 were used as controls.

Table 4.3 Amplicon Context Sequence for DNMTs in adipose tissue

Gene	Amplicon Context Sequence
Adipose Tissue	
DNMT1	ATCGTCCTTAGCGTCGTCGTAACTTTCTACCTGGCTCACCACAAACTGG GCATGGCGTAGGAGGGAGTCCTCGGTGAACCGGTTCACATTGATGGCA GAAGAGGAA CAGTGGTCTCGATCTTATTGATCAAG
DNMT3a	GAATGATAAGCTGGAGTTGCAAGAGTGTCTGGAACACGGCAGAATAGC CAGTTCAGCAAAGTGAGGACCATTACCACCAGGTCAAACTCCATA
DNMT3b	TTCAGGCAGTAGGAACTTAGAAGCCAGGAGACGCGAGAACAAAAGTCG AGAC GCACAACCATTGACTTTGCCGCTTCTGAGTACTCCACACCCC
18S	Forward (5'→3') TGACTCAACACGGGAAACC Reverse (3'→5') TCGCTCCACCAACTAAGAAC

18S was used as the housekeeping gene.

4.3.10 Statistical and Bioinformatics Analysis

All data is presented as mean ± standard error of the mean (SEM). Paternal outcomes with multiple timepoints (i.e. body weight, OGTT, etc.) were analyzed using repeated measures ANOVA, where time was the *within-subject* factor and the dietary intervention was the *between-subject* factor. If a significant interaction effect was identified, a one-way analysis of variance (ANOVA) with Tukey's *post hoc* was used to determine differences between groups. Offspring outcomes with multiple timepoints were assessed using repeated-measures ANOVA, with time as the *within-subject* factor and diet and sex as the *between-subject* factors. If a significant sex effect was identified, a repeated measures ANOVA and a one-way ANOVA with Tukey's *post hoc* analysis was used. Offspring results with single time-points (i.e. fat mass, satiety hormones, etc.) were analyzed using a two-way ANOVA to determine the effects of diet and sex. If the sex effect was significant, male and female data were analyzed separately using a one-way ANOVA with Tukey's *post hoc* analysis. The 'n' of experimental groups is listed under each table and/or figure. Statistics were performed with IBM® SPSS Statistics version 24.0.

For sequence data processing, raw sequence reads were processed using the R package dada2 (version 1.10.1) [274]. Quality filtering was performed using the filterAndTrim function with the following criteria: 1) Forward and reverse reads were truncated at a length of 240 bp; 2) After truncation, forward and reverse reads with higher expected errors (EE = sum(10^(-Q/10)) than 2 were discarded. A table of ASVs (amplicon sequence variants) was generated using the standard dada2 workflow: generating an error model of the data, inferring sequence variants, merging forward and reverse reads, generating a count table, and removing chimeric sequences. Taxonomic classifications were assigned to ASVs using the assignTaxonomy and assignSpecies functions, using the Silva v132 database as a reference.

For diversity analysis, preliminary analyses were carried out using the R package phyloseq (version 1.24.2) [275]. Alpha diversity was estimated using the Shannon, Simpson, and Chao1 indices of diversity and significance determined using ANOVA and TukeyHSD if significant. Beta diversity was estimated using PCoA (principal coordinates analysis) on a matrix of Bray-Curtis distances. Prior to ordination, ASV counts were normalized proportionally and low abundance ASVs were removed (those present in less than 5% of all samples). To determine

if treatment groups differed significantly, a permutational multivariate analysis of variance (PERMANOVA) was performed.

Differentially abundant features were analyzed using three different methods. 1) A beta-binomial regression using the R package corncob (version 0.1.0), implemented with a Wald significance test and default parameters. 2) A LEfSe analysis [276], using a significance of alpha = 0.05 and default parameters. 3) A DESeq2 analysis (version 1.24.0) [277], using a Wald significance test with alpha = 0.01 and correcting for multiple comparisons using the Benjamini and Hochberg method. All statistical outcomes were considered significant at $p \leq 0.05$, unless otherwise stated.

4.4 RESULTS

We sought to understand whether paternal high protein diet impacts next-generation phenotypes. To do this, we examined body composition, metabolic markers, gut microbial composition and epigenetic changes in the fathers as well as their male and female progeny at weaning and adulthood.

4.4.1 HP consumption alters body weight and adiposity intergenerationally

No difference was observed in paternal bodyweight between groups (Figure 4.1A), however fat mass and % body fat was significantly lower in HP compared to HF/S-fed fathers ($p<0.05$) (Table 4.4).

The presence of a significant sex effect in offspring affected how downstream analysis was conducted. At weaning, no significant sex effect was identified for bodyweight, body composition or organ weight ($p>0.05$), thus sexes were analyzed together. From 5 weeks of age onwards, there was a significant sex effect ($p<0.0005$) for bodyweight, body composition and organ weight, therefore males and females were assessed separately.

At day 7 and day 21 (weaning), HF/S offspring were significantly heavier than control and HP ($p<0.0005$; Figure 4.1B). From 5 weeks to 16 weeks, male and female HF/S offspring were significantly heavier compared to control and HP offspring ($p<0.05$; Figure 4.1C and D).

No differences were observed in weanling body composition (Table 4.4). Both male and female HP adults showed significantly lower fat mass compared to HF/S offspring, even though all offspring consumed the same AIN-93 diet for 13 weeks (Table 4.4). This discrepancy in adiposity amounted to a mean of 40.8g in males and 43.7g in females between HP and HF/S

offspring. Male adult HP offspring similarly had lower % body fat compared to HF/S and controls.

Table 4.4 Body composition of fathers at mating at 12 weeks of age and offspring at weaning and 16 weeks of age

	Sex	Diets			2-Way ANOVA		
		Control	HP	HF/S	Diet	Sex	Diet×Sex
Fathers							
BMC (g)	M	15.0±0.3	14.9±0.3	15.5±0.3	n/a	n/a	n/a
BMD (g/cm^2)	M	0.166±0.001	0.164±0.002	0.165±0.002	n/a	n/a	n/a
Fat Mass (g)	M	85.5±5.8^a	91.4±11.8^a	129.4±7.2^b	n/a	n/a	n/a
Lean+ BMC (g)	M	504.1±14.6	492.2±14.6	498.1±10.8	n/a	n/a	n/a
% Body Fat	M	15.0±0.9^a	13.0±0.7^a	21.0±1.0^b	n/a	n/a	n/a
Weanlings							
BMC (g)	n/a	1.5±0.05	1.5±0.04	1.6±0.06	0.85	0.38	0.48
BMD (g/cm^2)	n/a	0.077±0.001	0.078±0.001	0.079±0.001	0.79	0.54	0.78
Fat Mass (g)	n/a	4.4±0.3	4.3±0.2	4.5±0.5	0.68	0.36	0.17
Lean+ BMC (g)	n/a	64.5±2.4	65.6±1.1	68.5±3.0	0.95	0.20	0.55
% Body Fat	n/a	6.3±0.4	6.1±0.3	6.4±0.6	0.55	0.30	0.30
Adult Offspring							
BMC (g)	M	17.2±0.4	16.5±0.3	17.1±0.2	0.20	0.0001	0.98
	F	11.6±0.3	11.1±0.3	12.0±0.6			
BMD (g/cm^2)	M	0.175±0.002	0.174±0.002	0.174±0.002	0.23	0.0001	0.14
	F	0.168±0.002	0.164±0.001	0.164±0.002			
Fat Mass (g)	M	108.2±7.0^{ab}	94.3±9.0^a	135.1±6.0^b	0.0001	0.02	0.69
	F	78.0±8.1^{ab}	73.3±7.5^a	117.0±19.4^b			
Lean+ BMC (g)	M	519.5±12.0	527.0±10.8	526.2±9.9	0.28	0.0001	0.57
	F	287.5±7.7	292.0±5.2	296.4±7.4			
% Body Fat	M	19.5±1.6^a	14.9±1.1^b	19.9±0.7^a	0.002	0.0001	0.38
	F	21.1±1.7	19.9±1.7	26.7±3.3			

Values are means ± SEM, n = 8-11. Paternal p-values following a 1-way ANOVA are as follows: BMC (p=0.4); BMD (p=0.6); Fat mass (p=0.002); Lean+BMC (p=0.8); % body fat (p=0.000001). In weanling offspring, no sex differences in the majority of the overall models were observed; therefore, subsequent analyses in weanlings were not stratified based on sex. In adult offspring, sex differences in the overall models were observed; therefore, subsequent analysis was performed in males (M) and females (F) separately. The superscripts ^{a,b} are used to depict differences between groups where groups within a sex without a common superscript differ (p<0.05).

4.4.2 Paternal HP consumption increases relative organ weight

An examination of paternal organ weight between groups showed significantly smaller hearts in HF/S fed fathers compared to control and HP groups (Table 4.5). HP-fed fathers had significantly heavier kidneys and cecum compared to HF/S and control groups (p=0.004 and p=0.00004) as well as heavier testes compared with HF/S. At weaning, HF/S offspring had significantly lower heart, liver and kidney weight compared to control (p<0.05, Table 4.5). They

also had lower cecum weight compared to HP offspring (p=0.03, Table 4.5). In adulthood, HP male offspring had the highest testes weight which was significantly different than control (p=0.005) as well as colon weight that was significantly higher than control (p=0.016) (Table 4.5). Female adult offspring of HF/S fathers had lower colon weight compared to control (p=0.025).

Table 4.5 Relative Organ Weight

	Sex	Diets: Control	Diets: HP	Diets: HF/S	2-Way ANOVA: Diet	2-Way ANOVA: Sex	2-Way ANOVA: Diet×Sex
Fathers							
Body Weight (g)		594.2±19.5^{ab}	581.0±10.1^{a}	651.6±21.2^{b}	n/a	n/a	n/a
Organ weight (% BW)							
Heart	M	0.32±0.01^{a}	0.31±0.01^{a}	0.27±0.01^{b}	n/a	n/a	n/a
Liver	M	3.04±0.07	3.03±0.11	3.00±0.08	n/a	n/a	n/a
Kidney	M	0.27±0.01^{a}	0.33±0.01^{b}	0.29±0.01^{a}	n/a	n/a	n/a
Cecum	M	0.10±0.004^{a}	0.13±0.01^{b}	0.09±0.003^{a}	n/a	n/a	n/a
Colon	M	0.21±0.01	0.22±0.01	0.21±0.01	n/a	n/a	n/a
Testes	M	0.31±0.01^{ab}	0.33±0.01^{a}	0.29±0.01^{b}	n/a	n/a	n/a
Weanlings							
Body Weight (g)		68.9±2.9^{a}	69.9±1.2^{a}	78.3±3.0^{b}	0.12	0.36	0.02
Organ weight (% BW)							
Heart	n/a	0.52±0.01^{a}	0.49±0.01^{ab}	0.48±0.02^{b}	0.93	0.17	0.21
Liver	n/a	3.22±0.05^{a}	3.42±0.05^{ab}	3.17±0.11^{b}	0.03	0.05	0.07
Kidney	n/a	0.60±0.01^{a}	0.60±0.01^{b}	0.55±0.02^{b}	0.004	0.87	0.46
Cecum	n/a	0.82±0.02^{ab}	0.82±0.01^{a}	0.75±0.03^{b}	0.07	0.1	0.19
Colon	n/a	0.41±0.01	0.46±0.01	0.41±0.02	0.01	0.79	0.08
Testes	M	0.33±0.01	0.36±0.02	0.35±0.01	n/a	n/a	n/a
Adult Offspring							
Body Weight (g)	M	630.5±14.4	629.7±15.8	666.9±6.2	0.003	0.0001	0.81
	F	372.6±13.5	361.5±11.3	413.5±21.8			
Organ weight (% BW)							
Heart	M	0.27±0.01	0.27±0.01	0.27±0.01	0.07	0.0001	0.12
	F	0.32±0.01	0.32±0.01	0.30±0.01			
Liver	M	2.69±0.10	2.96±0.15	2.70±0.07	0.19	0.16	0.56
	F	2.65±0.05	2.67±0.06	2.59±0.05			
Kidney	M	0.26±0.01	0.25±0.003	0.25±0.005	0.01	0.42	0.09
	F	0.27±0.01	0.27±0.002	0.26±0.01			
Cecum	M	0.27±0.01	0.30±0.004	0.30±0.01	0.54	0.007	0.98
	F	0.12±0.01	0.13±0.01	0.12±0.01			
Colon	M	0.09±0.002^{a}	0.11±0.01^{b}	0.09±0.003^{ab}	0.21	0.0001	0.16
	F	0.25±0.01^{a}	0.25±0.01^{ab}	0.20±0.01^{b}			
Brain	M	0.35±0.01	0.35±0.01	0.34±0.01	0.50	0.0001	0.61
	F	0.55±0.03	0.55±0.01	0.52±0.03			
Testes	M	0.16±0.01^{a}	0.20±0.01^{b}	0.17±0.01^{b}	n/a	n/a	n/a

Values are means ± SEM, n = 9-13. Paternal liver weight was log transformed for analysis. Paternal p values following a one-way ANOVA are as follows: heart (p=0.0002); liver (p=0.93); kidney (p=0.004); cecum (p=0.00004); colon (p=0.40); testes (p=0.03). In weanlings, no sex differences in the majority of the models for the relative weight organs were observed; therefore, subsequent analyses in weanlings were not stratified based on sex. In adult offspring, an independent-samples Kruskal-Wallis Nonparametric test was used for male testes and female kidneys. Sex differences in the majority of the overall models for the relative weight of organs were observed, therefore, subsequent analysis was performed in males (M) and females (F) separately. The superscripts ^{a,b} are used to depict differences between groups within a sex where groups without a common superscript differ (p<0.05).

4.4.3 HP consumption improves fertility index

Due to the differences in testes weight in fathers and adult male offspring, we sought to examine important reproductive parameters. Fertility index was measured according to: Fertility index= Number of successful pregnancies/ number sperm positive fathers. Sperm positivity is designated once a copulation plug has been identified. We discovered HP-fed fathers exhibited a 100% fertility index, while control fathers exhibited 83% and HF/S exhibited the lowest at 77% (Table 4.6). There were no significant differences in the number of nights cohabited until conception, pup survival, number of stillbirths or percentage of male and female pups.

Table 4.6 Reproductive Performance for Paternal Fertility

	Control	HP	HF/S
Males (n)	12	12	13
Males cohabited	12	12	13
Nights cohabited until conception	4.20±0.51	3.3±0.28	3.00±0.56
Fertility Index (%)	83	100	77
# of pups born alive	13.7±0.6	15.3±0.3	14.7±0.6
# of stillbirths	1	2	3
Pup survival (%)	99.4	98.9	97.8
Relative abundance of male pups (%)	51.4±3.7	53.5±4.3	51.6±4.1
Relative abundance of female pups (%)	48.7±3.7	46.5±4.3	48.4±4.1

Values are means ± SEM. Fertility index= # pregnant/ number sperm positive

4.4.4 HP consumption improves glucose tolerance in fathers and insulin sensitivity in male and female adult offspring

Excess fat is directly related to impairment in insulin function [278]. Due to the observed reduction in fat mass in HP groups compared to HF/S groups, we decided to examine glucose tolerance and insulin sensitivity in fathers and offspring at different timepoints using oral glucose tolerance tests (OGTTs) and insulin tolerance tests (ITTs).

Male and female offspring at weaning did not exhibit significant sex differences in OGTTs or ITT ($p>0.05$), therefore they were not stratified. Adult offspring exhibited a significant sex effect for both the OGTT and ITT ($p<0.05$). Given this sex difference, adult males and females were assessed separately.

As expected, there was a significant main effect of time on glycaemia during the OGTTs in the fathers and offspring as blood glucose rose and fell over the 120 min ($p<0.0005$; Figure

4.1E-G). In fathers, there was a significant diet × time interaction ($p<0.005$) where the HF/S group had higher blood glucose at 15 min and lower glucose at 120 min compared with control at 11 weeks of age (Figure 4.1E). The HP group was intermediate. No such glycaemia during an OGTT was observed in offspring at weaning (Figure 4.2A) or adulthood (Figure 4.1F and 4.1G). During the ITTs, there was a similar expected main effect of time ($p<0.0005$) as blood glucose declined following insulin injection (Figure 4.1H-J). There were no effects of diet or diet × time in fathers, however notably, weanlings and adult offspring showed significant differences. Weanlings had a significant main effect of diet ($p<0.0005$) and diet × time ($p<0.0005$) (Figure 4.2B). HP and control weanlings had significantly lower blood glucose levels compared to HF/S at 15 and 30 min during the ITT with control also lower at 60 min ($p<0.05$, Figure 4.2B). Similarly, there was a significant main effect of diet ($p<0.005$) and diet × time ($p=0.04$) in adult male offspring (Figure 4.1I). Adult male HP offspring had the greatest insulin sensitivity which was demonstrated by significantly lower blood glucose compared to HF/S and control at 0, 15, 60 and 120 min and lower compared to HF/S at 90 min during the ITT (Figure 4.1I). In female adult offspring, there was a significant diet × time interaction ($p=0.001$) whereby HP offspring exhibited lower blood glucose levels at 90 and 120 min following an insulin load compared to HF/S but not control offspring (Figure 4.1J).

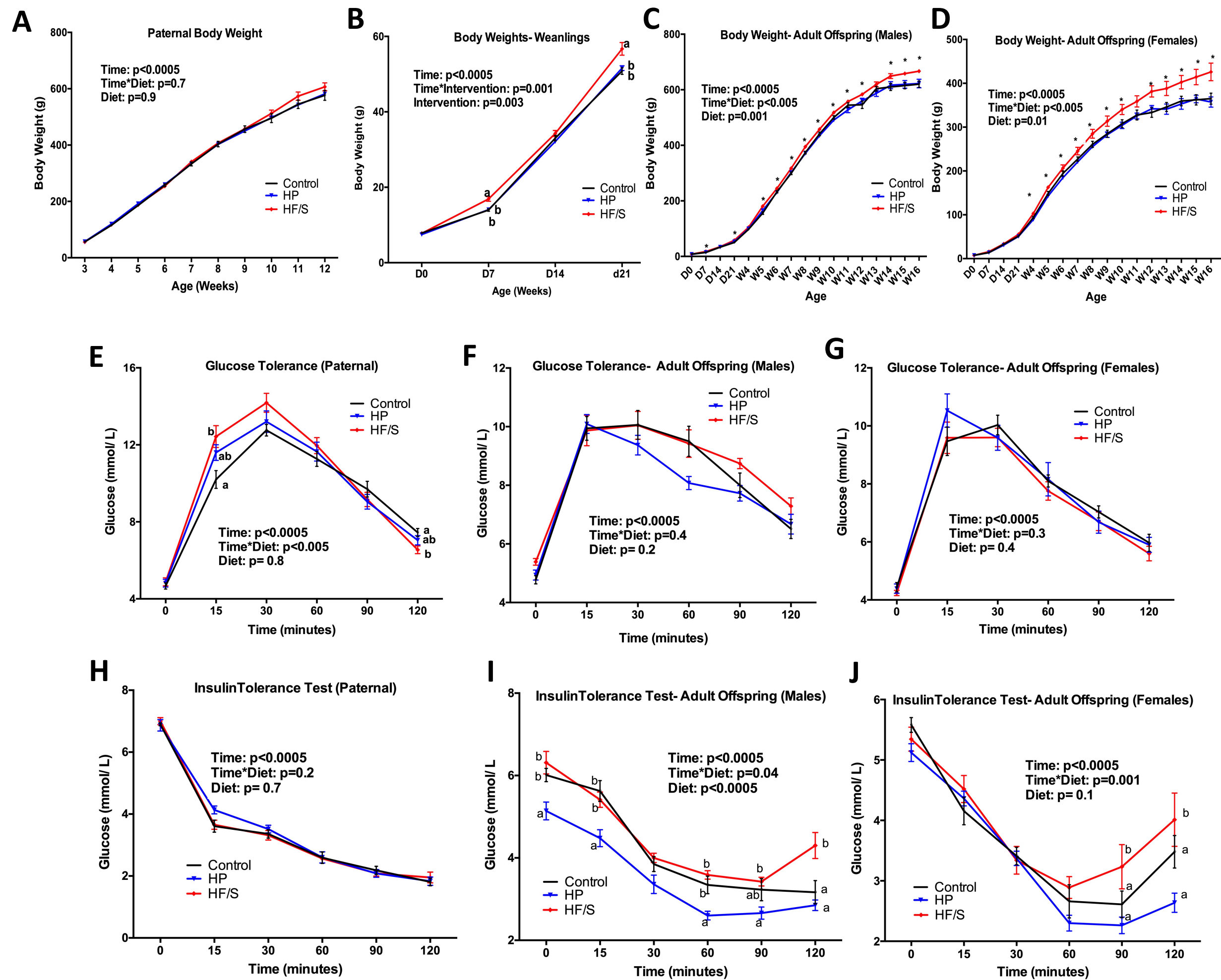


Figure 4.1 Body weight, OGTT and ITT. Body weight of A) Fathers, B) Weanlings, C) Adult male offspring and D) Adult female offspring. OGTT of E) fathers, F) male offspring G) female offspring; ITT of H) fathers, I) male offspring J) female offspring. Values are means ± SEM, n= 8-13. In weanlings, no sex difference in the overall model for bodyweight was observed (p=0.05); therefore, ensuing analyses in weanlings were not stratified based on sex. In adult offspring, there was a significant sex effect in the overall model for bodyweight (p=0.0001); therefore, subsequent analysis was performed in males and females separately. In the overall model, a significant sex effect for OGTT (p=0.0001) and ITT (p=0.0001) was observed; therefore, subsequent analysis was performed in adult male and female offspring separately. The superscripts a,b are used to depict differences between groups where groups without a common superscript differ (p<0.05).

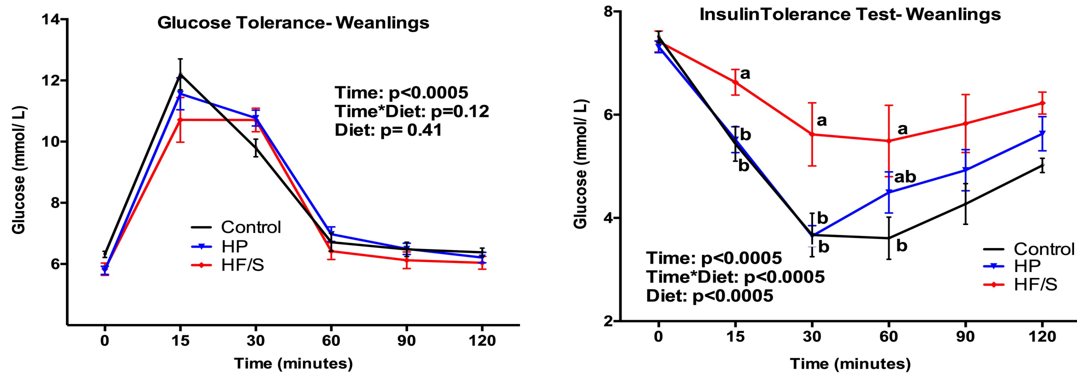


Figure 4.2 Oral glucose tolerance test (OGTT) and Insulin tolerance test (ITT) of weanling offspring. A) OGTT and B) ITT. Values are means ± SEM, *n*= 8-10. In the overall model, no significant sex effects for OGTT or ITT (p<0.05) was observed; therefore, subsequent analysis was performed in males and females together for OGTT and ITT. The superscripts ^{a,b} are used to depict differences between groups where groups without a common superscript differ (*p*<0.05).

4.4.5 Paternal HP consumption modulates energy intake, satiety and metabolic hormones, but not hepatic triglyceride content in offspring

In part, insulin resistance originates in the hypothalamus, wherein the balance of hunger and satiety signals are disrupted and therefore no longer match energy intake to energy expenditure[278]. Therefore, we sought to examine energy intake and the satiety hormones that modulate food intake. There was a significant sex effect among offspring at all food intake time points and among all satiety hormones assessed (p<0.0001). Based on these sex differences, male and female food intake and satiety hormone data were subsequently stratified. There was a significant diet × time interaction (p≤0.005) for food intake in fathers and their male offspring, wherein HP groups consumed fewer kcal/day compared to HF/S (p<0.005) at paternal 3 and 9 weeks of age and male offspring 3 weeks of age (Figure 4.3A and 4.3B respectively). No differences were observed in females (Figure 4.3C).

Based on differences in food intake in fathers and male offspring, we examined serum concentrations of a battery of hormones that affect appetite and food intake. HP fathers showed significantly higher fasting amylin compared to HF/S and control (p=0.008) and a trend (p=0.08) towards higher pancreatic polypeptide (PP) (Figure 4.3D and 4.3E respectively). This persisted in female offspring where serum amylin and PP was significantly higher in the HP group compared to the control and HF/S group (Figure 4.3D and 4.3E). With the HF/S diet, GIP was significantly higher in fathers (p=0.005) and female adult offspring (p=0.001) (Figure 4.3F) in

the HF/S group compared to control and HP. HP female adult offspring also exhibited higher GLP-1 compared to control and higher glucagon compared to HF/S and controls (Figures 4.3G and 4.3H respectively). The significantly lower serum leptin seen in HP fathers ($p=0.002$; Figure 4.3J) was mirrored by a trend ($p=0.076$) for lower leptin in female offspring (Figure 4.3J). C-peptide was significantly higher in HF/S female offspring compared to control and HP (Figure 4.3L). Hormone patterns in males were less likely to mirror those of their fathers with significantly lower PP in HP and HF/S male offspring (Figure 4.3E), lower GLP-1 in HF/S compared to control and HP offspring (Figure 4.3G), higher ghrelin in HF/S compared to HP with control intermediate (Figure 4.3I) and a trend ($p=0.08$) towards lower glucagon in HP (Figure 4.3H). No difference between groups in hepatic triglyceride content was observed in fathers or offspring (data not shown).

4.4.6 Paternal HP consumption results in unique gut microbial signatures in male and female offspring

The gut microbiome influences host metabolism, such as glucose homeostasis [279]. To better understand the metabolic differences we observed in fathers and adult offspring, we sought to examine the gut microbial composition intergenerationally. We used 16S rRNA gene sequencing to ascertain whether the paternal microbiota harboured a unique microbial signature based on their preconception diet and whether these signatures persisted or influenced offspring gut microbiota. By sequencing the V3 and V4 region of 16S rRNA bacterial DNA, we identified 3 paternal microbial signatures aligning with control, HP and HF/S diets. To minimize baseline confounding differences in gut microbiota, we sequenced bacterial DNA from fecal matter of fathers at week 3 of life before dietary interventions were introduced. There were no differences in alpha or beta diversity at this point. Following the consumption of a HP, HF/S or control diet for 6 weeks (9 weeks of age), alpha diversity using Shannon and Simpson indices significantly differed, displaying significantly elevated richness and evenness in HP fathers compared to control and/or HF/S diet groups (Table 4.7). At 12 weeks of age, HF/S groups exhibited higher alpha diversity compared to controls using Shannon and Simpson indices (Table 4.8). Similar to fathers, no differences in alpha diversity were observed between groups at weaning, however, at 9 weeks of age, female offspring exhibited marked differences in alpha diversity according to Chao1, Shannon and Simpson indices ($p<0.0005$) (Table 4.7), where HP and HF/S diets

harboured significantly higher alpha diversity compared to controls. Male offspring at 16 weeks of age, showed significantly higher alpha diversity according to Chao1 and Shannon indices in the HP group compared to control (p<0.01) (Table 4.8).

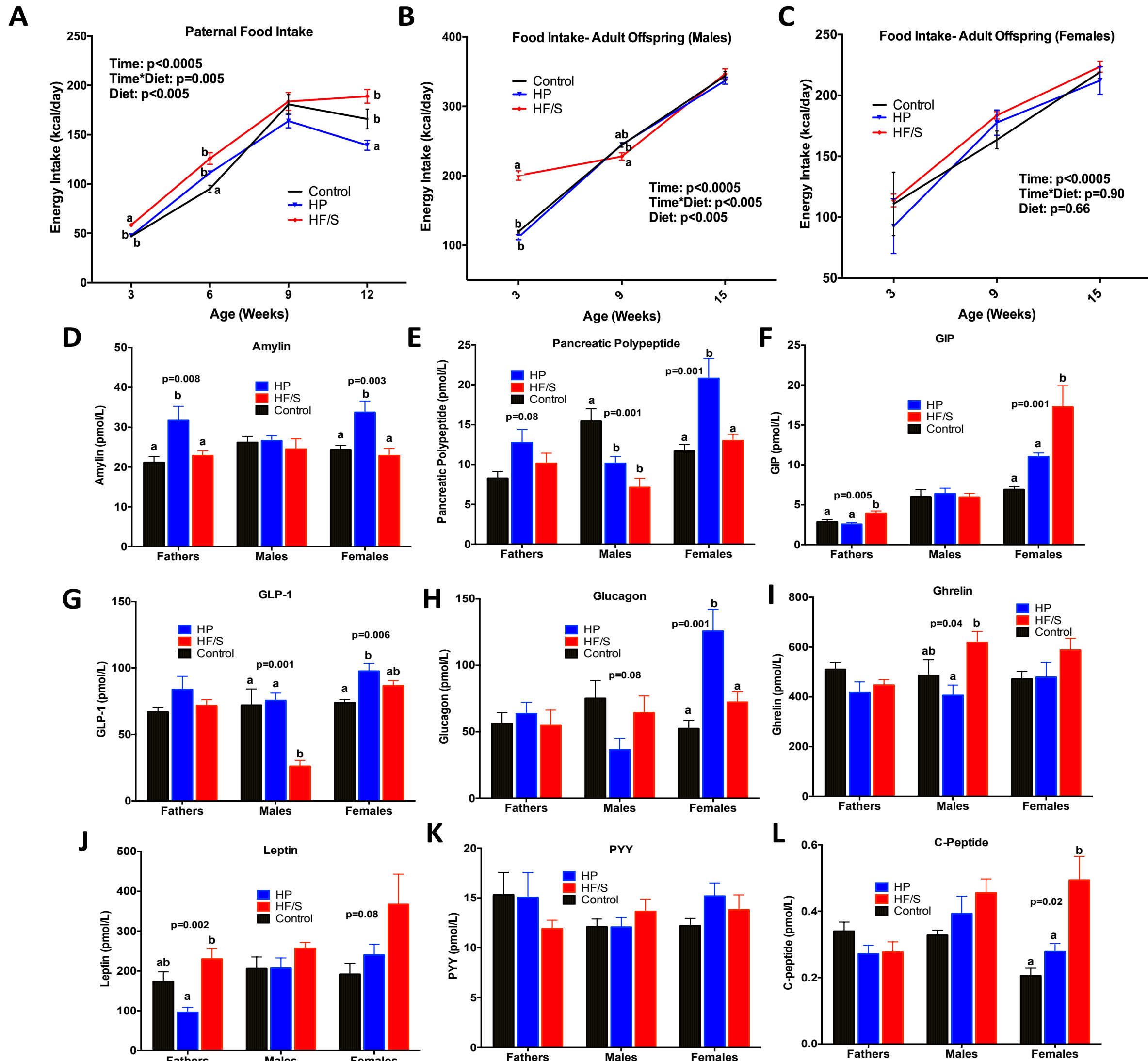


Figure 4.3 Food Intake and Satiety/ Metabolic Hormones. Food intake of A) fathers, B) male offspring C) female offspring. Food intake was analysed using independent samples Kruskal-Wallis tests. In adult offspring, the overall model yielded significant sex differences for food intake (p=0.0001) therefore, subsequent analysis was performed in males and females separately. Satiety and metabolic hormones at 12 weeks of age in fathers and offspring at 16 weeks of age D) amylin, E) pancreatic polypeptide, F) GIP, G) GLP-1, H) glucagon, I) ghrelin, J) leptin, K) PYY, L) C-peptide. In adult offspring, significant sex differences were observed for all satiety and metabolic hormones (p<0.05) therefore, subsequent analysis was performed in males and females separately. Values are means ± SEM, n= 8-13. The superscripts ^{a,b} are used to depict differences between groups where groups without a common superscript differ (p<0.05).

subsequent analysis was performed in males and females separately. Satiety and metabolic hormones at 12 weeks of age in fathers and offspring at 16 weeks of age **D)** amylin, **E)** pancreatic polypeptide, **F)** GIP, **G)**

Table 4.7 Alpha Diversity at 9 weeks of age

	Sex	Control	HP	HF/S	p-value
Fathers					
Chao1		225.34±14.2	222.99±12.11	209.19±8.37	0.57
Shannon		3.75±0.08[a]	4.09±0.05[b]	3.87±0.09[ab]	0.009
Simpson		0.94±0.004[a]	0.96±0.002[b]	0.95±0.004[a]	0.001
Offspring					
Chao1	M	120.01±24.76	153.27±19.4	182.82±28.3	0.22
	F	124.93±11.43[a]	169.32±8.62[b]	192.31±11.22[b]	0.0004
Shannon	M	3.42±0.13	3.63±0.08	3.76±0.08	0.087
	F	3.45±0.08[a]	3.84±0.06[b]	3.99±0.06[b]	0.000008
Simpson	M	0.93±0.009	0.94±0.003	0.95±0.005	0.05
	F	0.93±0.01[a]	0.96±0.002[b]	0.96±0.003[b]	0.000009

Values are means ± SEM, n = 9-11. The superscripts [a,b] are used to depict differences between groups where groups within a sex without a common superscript differ (p<0.05).

Table 4.8 Alpha Diversity at 12 Weeks of age for Fathers and 16 Weeks for offspring

	Control	HP	HF/S	p-value
Paternal				
Chao1	149.09±10.88	157.86±9.77	191.48±23.52	0.17
Shannon	3.76±0.06[a]	3.97±0.04[ab]	4.00±0.10[b]	0.048
Simpson	0.95±0.002[a]	0.96±0.002[ab]	0.96±0.003[b]	0.030
Males				
Chao1	109.62±7.41	141.32±11.69	140.53±8.79	0.047
Shannon	3.45±0.1[a]	3.73±0.09[ab]	3.89±0.09[b]	0.013
Simpson	0.93±0.01	0.95±0.01	0.96±0.004	0.057
Females				
Chao1	156.8±19.58	134.28±12.15	148.47±23.18	0.67
Shannon	3.65±0.13	3.67±0.08	3.64±0.13	0.99
Simpson	0.94±0.01	0.95±0.004	0.95±0.01	0.92

Values are means ± SEM, n = 8-13. While male offspring alpha diversity, using a Chao1 were significant, following a 1-way ANOVA with Tukey's post hoc analysis, they lost this significance.

A principal coordinates analysis (PCoA) identified distinct bacterial clustering based on paternal dietary interventions at 9 weeks of age (Figure 4.4; (R^2=35.8%, p<0.001 by PERMANOVA; Figure 4.4A). Male and female adult offspring also exhibited significant differences in beta diversity at 9 weeks of age (R^2= 15.6%, p=0.005 and R^2=21.9%, 0.001 respectively; Figure 4.4B-C), wherein the dispersion was large. Specifically, male HF/S had a larger dispersion compared to control and HP groups and female dispersion was larger in control groups compared to HP and HF/S groups. Also, important to note, the first principal coordinate

(Axis 1) explains approximately 40% of the variation in the data, explaining why the clusters appear small, yet the PERMANOVA remains significant. To further understand the intergenerational microbi... examine the taxonomic composition between gro...

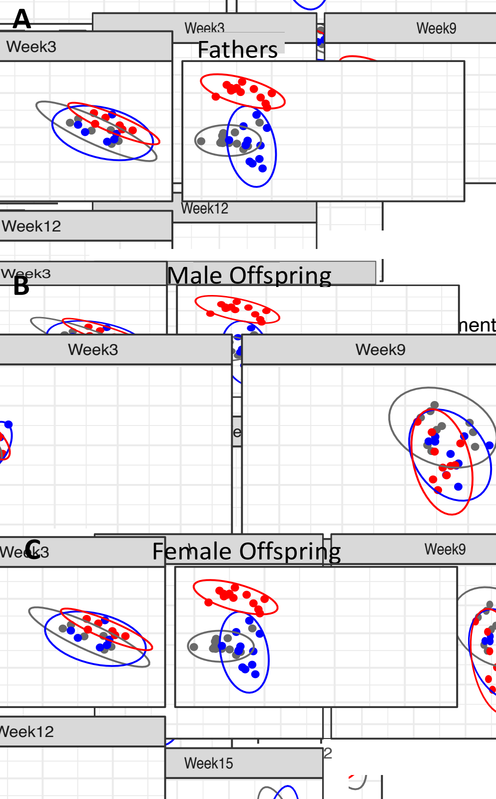


Figure 4.4 Be... spring and C) Female offspring at 9 weeks of age, calculate... CoA) using a Bray-Curtis distance matrix. ASVs are no... dance. ASVs present in less than 5% of the samples were removed. To determine significance, a PERMANOVA was performed. n=8-13. Paternal PERMANOVA, R^2=35.8%, p<0.001; male offspring PERMANOVA, R^2= 15.6%, p=0.005 and female offspring PERMANOVA, R^2=21.9%, p<0.001.

At the phylum level (Figure 4.5A), Firmicutes and Bacteroidetes dominated all samples. At 9 weeks of age, the control group had a higher relative abundance of Firmicutes compared with HP and HF/S groups in fathers as well as male and female offspring. At 12 weeks of age a higher abundance of Firmicutes was found in control and HF/S groups compared to HP in all cohorts (Figure 4.6). The relative abundance of Bacteroidetes was inversely related to Firmicutes within groups at 9 and 12 weeks of age, where a higher relative abundance of Bacteroidetes in HP groups was identified compared to control and HF/S groups. At 9 and 12 weeks of age,

Actinobacteria was most abundant in the control group compared to HP and HF/S. Female offspring at 9 weeks of age had a higher relative abundance of Actinobacteria in the control and HP groups compared with HF/S group. Male HP offspring at 16 weeks of age exhibited higher abundance of Actinobacteria (Figure 4.6A). Proteobacteria was present in higher abundance in paternal HP groups and persisted in female HP offspring at 9 weeks of age.

At the family level (Figure 4.5B), the composition of gut bacteria varied greatly with diet ($p<0.05$). At 12 weeks of age, paternal HP consumption resulted in lower abundance of *Clostridiaceae_1* and *Lactobacillaceae* compared to control and HF/S groups. *Ruminococcaceae* and *Lachnospiraceae* were more abundant in HP groups. *Bacteriodaceae* was more abundant in HF/S diets compared to all other groups. *Muribaculaceae, Prevotellaceae*, *Bacteriodaceae* were found to be more abundant in HP and HF/S female offspring at 9 weeks of age, whereas a lower abundance of *Lactobacillaceae* and *Clostridiaceae_1* was observed (Figure 4.5B). A higher abundance of *Muribaculaceae* was found in HP female offspring at 16 weeks of age (Figure 4.6B) and male offspring at 9 weeks (Figure 4.5B).

At the genus level (Figure 4.5C), higher abundance of *Akkermansia, Romboutsia, Bacteroides, Marvinbryantia* was seen in HP fathers, the latter two displaying similar abundance in HP female offspring at 9 weeks of age. A lower abundance *of NK4A136* (*Lachnospiracaeae* group) was observed in HP fathers at 9 weeks of age, which was differentially observed in female offspring. Lower abundance of *Bifidobacterium* and *Ruminococcus_2* was observed in HF/S fathers at 9 weeks of age, the former persisting to 12 weeks and similarly observed in female HF/S offspring (Figure 4.6C). At 16 weeks of age, female offspring exhibited a higher abundance of *Bifidobacterium* compared to control and HF/S groups. No distinctions in abundance were observed in male offspring (Figure 4.6C).

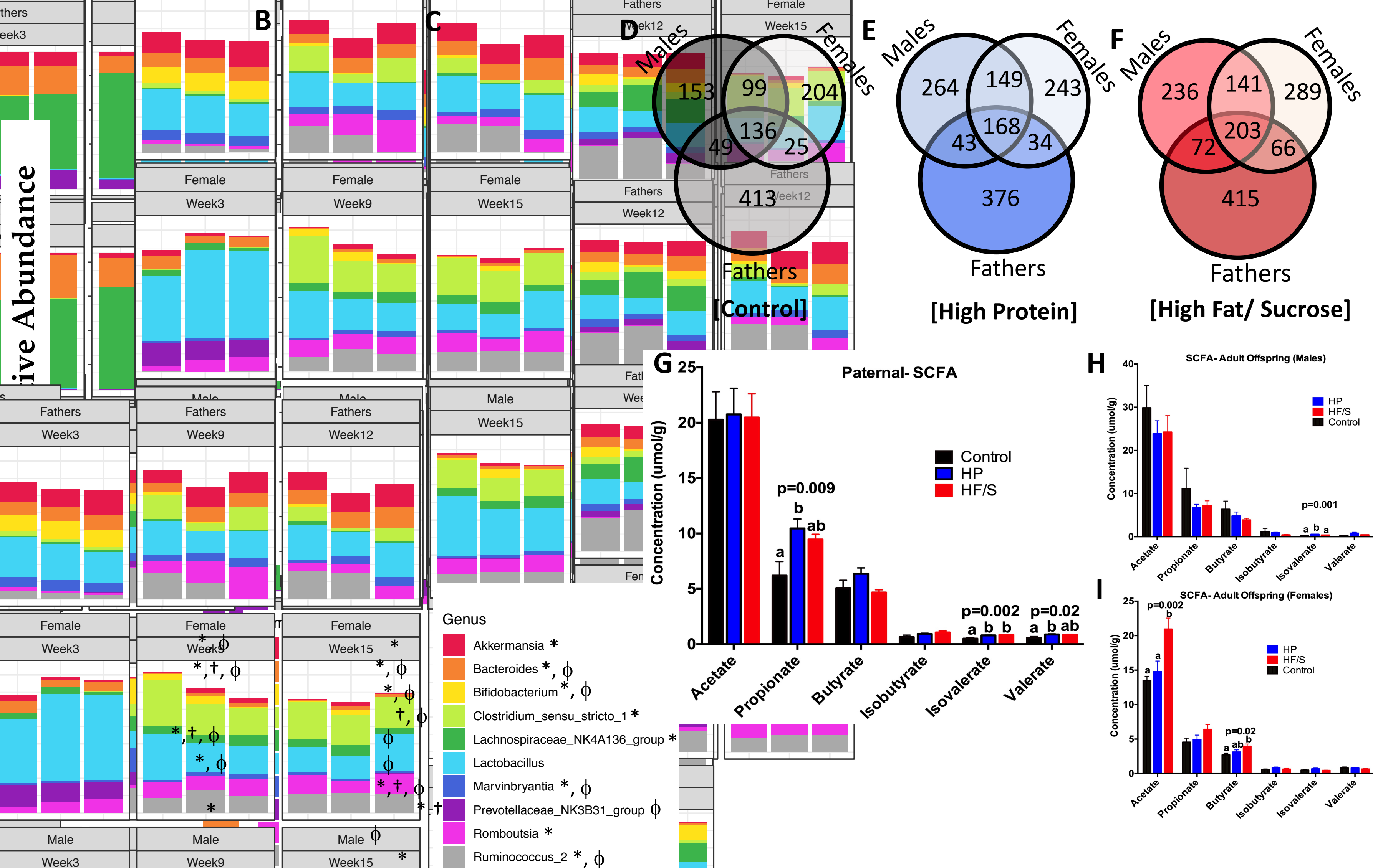
The above data demonstrates that fecal bacteria displayed unique signatures depending on the dietary intervention of father and his offspring. To characterize specific bacteria related to diet, linear discriminant analysis effect size (LEfSe) analysis was conducted on ASVs with a relative abundance >0.1%. Males appeared to exhibit the highest variability at 9 weeks of age (Figure 4.7B). Belonging to the Lactobacillales order, 3 *Enterobacteraceae* and 3 Clostridia, including one belonging to the family *Lachnospriaceae* were more abundant in male HF/S offspring compared to HP offspring. HP male offspring exhibited an overabundance of the order Bacteriodales and Lactobacillales including ASVs belonging to the genus' *Marinifillaceae,*

Odoribacter, Alistipes and *Rikenellaceae* as well as *Lactobacillus reuteri*. Relative abundance data at the phylum, family and genus level were summarized in a heat map, stratified by time of fecal collection (Figure 4.7C-E).

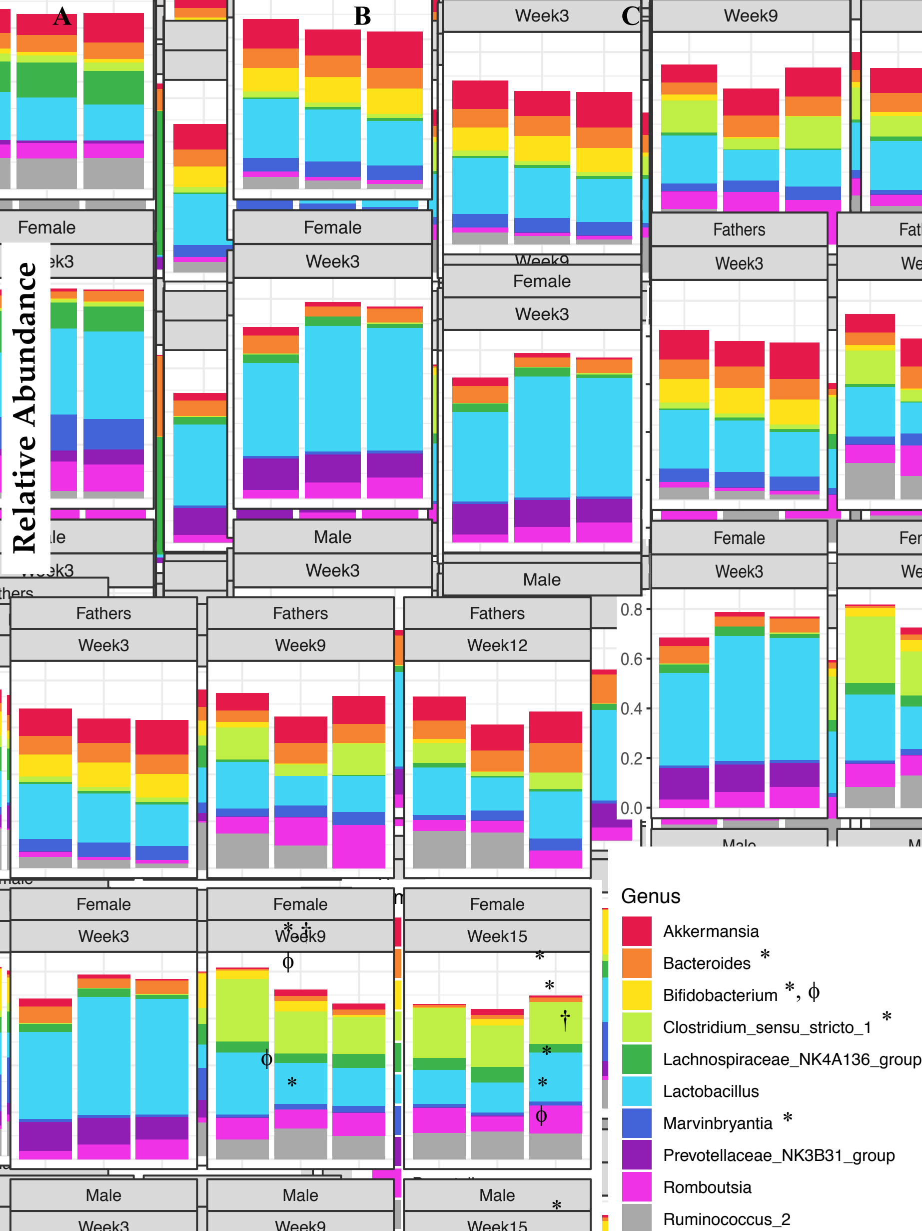
The Venn diagrams highlight that paternal control (Figure 4.5D), HP (Figure 4.5E) and HF/S (Figure 4.5F) groups cumulatively had 623, 621 and 756 amplicon sequence variants (ASVs) at 12 weeks of age respectively. Of those ASVs, 42.3%, 33.8% and 42.2% were shared between fathers and male control, HP and HF/S offspring respectively. Female offspring shared 34.7%, 34.0% and 38.5% ASVs with fathers.

4.4.7 Paternal HP consumption altered SCFA levels intergenerationally

In addition to the gut microbiota, microbial metabolites, like SCFAs also improve insulin sensitivity and glucose tolerance. Therefore, we examined SCFA from cecal matter. Paternal HP consumption significantly increased cecal propionate, isovalerate and valerate concentrations compared to controls (Figure 4.5G). Adult male HP offspring also had higher isovalerate concentrations compared to controls and HF/S groups (Figure 4.5H). Adult female offspring exhibited higher levels of acetate and butyrate in the HF/S group compared to controls (Figure 4.5I).



/S diets and the intergenerational similarities in male and female offspring. pring and female offspring at 9 weeks of age. Taxa were identified to the Genus using the Silva reference database. Venn diagram comparison of ASVs that overlap between fathers g, stratified by sex and D) control diet E) HP diet and F) HF/S diet. Short Chain Fatty Acids in: G) paternal er and I) adult female offspring cecal matter at euthanasia. Values are means ± SEM, n= 8-13. For relative abundance plots of bacterial taxa, differences are identified as follows: * $P<0.05$ (Fathers); † $P<0.05$ (Males); ϕ $P<0.05$ (Females). For SCFA. the superscripts [a,b] are used to depict differences between groups where groups without a common superscript differ ($p<0.05$).



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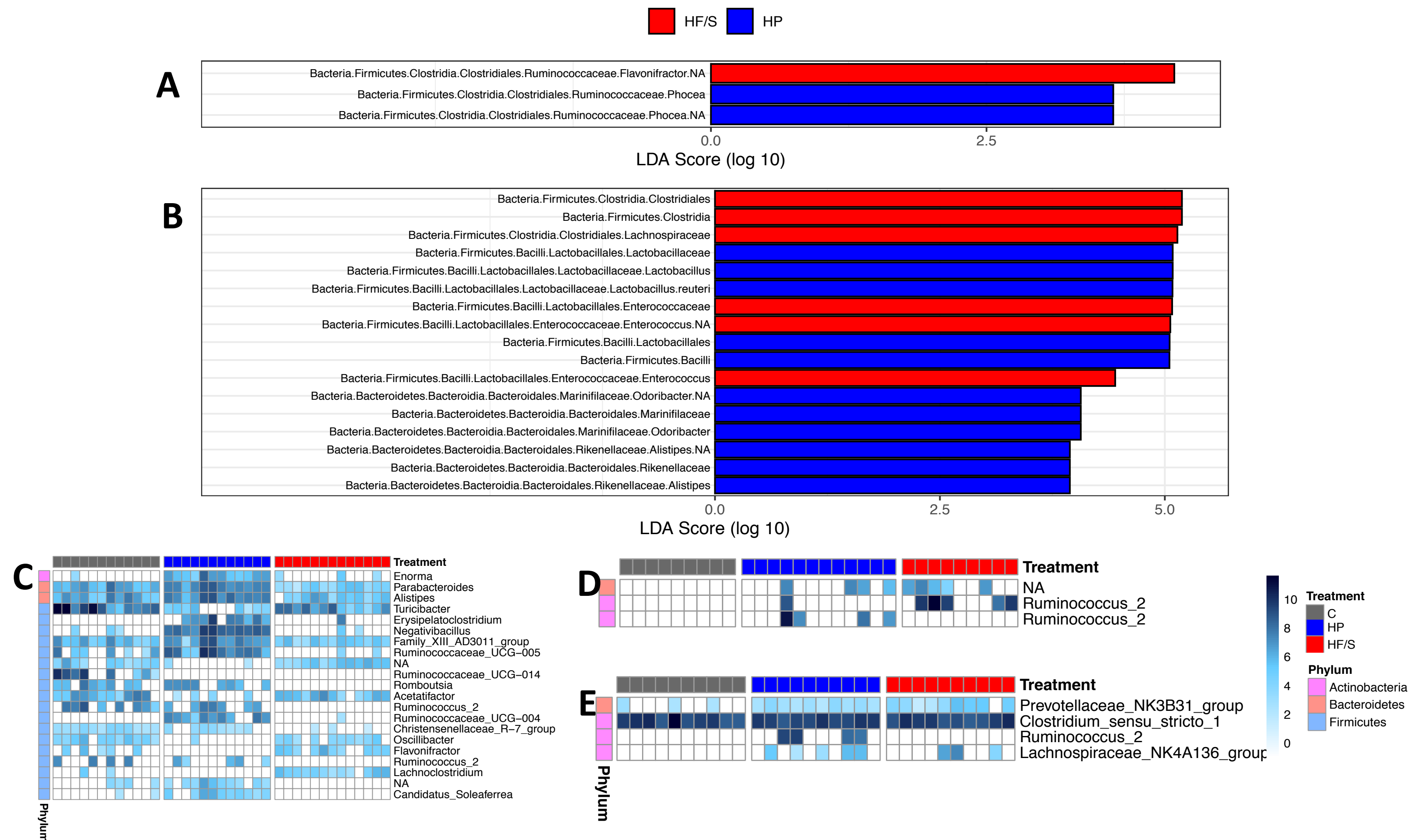

Figure 4.7 Comparison of microbiota using LEfSe from A) paternal fecal matter and B) male offspring fecal matter at 9 weeks of age. Female offspring did not exhibit any differences at 9 weeks of age using LEfSe. Heatmaps of top gut bacterial phyla, families, genera between diets in C) fathers, D) male offspring and E) female offspring at 9 weeks of age.

4.4.8 Paternal HP intake modulates DNA methyltransferase expression in adipose tissue and hepatic small non-coding RNA

To further explain our findings, we sought to look at key epigenetic markers, since it has been postulated that epigenetics is among the chief mechanisms by which paternal experiences influence offspring health. One crucial epigenetic regulator of gene expression is DNA methylation catalyzed by DNA-methyltransferases (DNMTs) [280]. In mammalian systems, DNMT1 is mostly involved in maintaining DNA methylation during cell division, while DNMT3s are associated with *de novo* cystine re-methylation and maintenance in somatic and embryonic cells [281].

Adipose tissue DNMT1 mRNA expression was significantly downregulated in HP fathers compared to HF/S group (Figure 4.8A). We followed this with a Pearson correlation analysis and learned that DNMT1 was positively correlated with % body fat (r=0.455, p=0.04) and isovalerate (r=0.517, p=0.02). This persisted in adult male offspring, wherein HF/S DNMT1 expression was significantly higher compared to controls (Figure 4.8B). Similar findings were observed in DNMT3a expression in adult males (Figure 4.8B). DNMT3b was significantly higher in the HP

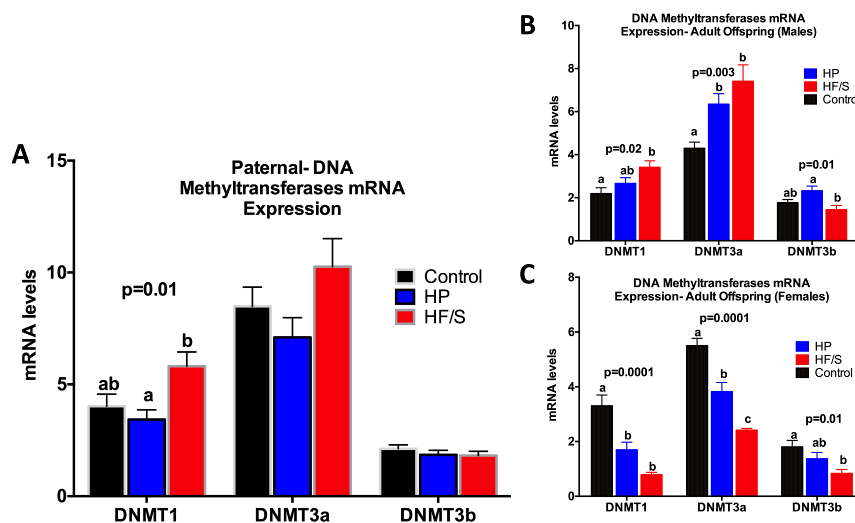

Figure 4.8 Adipose tissue messenger mRNA levels of DNA Methyl Transferase in: A) fathers, B) adult male offspring and C) adult female offspring. Values are means ± SEM, n= 8-13. Sex differences in all of the overall models for DNMT1 (p=0.0001), DMNT3a (p=0.0001), DNMT3b (p=0.009) were observed; therefore, subsequent analysis was performed in males and females separately. The superscripts [a,b] are used to depict differences between groups where groups without a common superscript differ (p<0.05).

group compared to HF/S in adult male offspring (Figure 4.8B). Female adult offspring exhibited lower DNMT1, 3a and 3b in HF/S group compared to controls (Figure 4.8C).

MicroRNAs make up a large proportion of small, non-coding RNAs that modulate post-transcriptional expression of target genes [282]. Paternal microRNA examination in liver tissue yielded differences in miR-34a, wherein HP levels were significantly reduced compared to controls (Table 4.9). Adult male offspring exhibited significantly reduced miR-122a levels in the HP group compared to controls and HF/S group (Table 4.10).

Table 4.9 Paternal microRNA Expression in Liver Tissue

	Control	**HP**	**HF/S**	**p-value**
miR-21	670.9±143.1	1089.0±433.2	472.0±151.2	0.25
miR-24	0.03±0.004	0.04±0.009	0.05±0.007	0.20
miR-33	0.0001±0.00003	0.0001±0.00003	0.0002±0.00002	0.50
miR-34a	1.7±0.4[a]	0.7±0.1[b]	1.0±0.2[ab]	0.02
miR-103	18.2±2.4	17.7±2.2	20.3±1.9	0.67
miR-107	0.3±0.02	0.3±0.05	0.4±0.04	0.26
miR-122a	7889.5±1987.6	11347.1±3134.5	5966.1±1122.8	0.23
miR-130a	0.01±0.002	0.02±0.003	0.02±0.002	0.77
miR-143	0.03±0.01	0.03±0.01	0.04±0.01	0.54
miR-let-7c	3.9±1.1	11.1±4.7	3.8±0.9	0.13

Values are means ± SEM, n = 10-13. The superscripts [a,b] are used to depict differences between groups where groups without a common superscript differ (p<0.05).

Table 4.10 Adult Offspring microRNA Expression in Liver Tissue

	Sex	Diets			2-Way ANOVA p-values		
		Control	HP	HF/S	Diet	Sex	Diet x Sex
miR-21	M	675.6±272.6	879.3±386.9	1591.2±722.1	0.57	0.009	0.57
	F	686.12±283.42	914.29±434.25	910.2±221.59			
miR-24	M	0.1±0.02	0.1±0.02	0.1±0.02	0.15	0.0001	0.15
	F	0.09±0.01	0.09±0.02	0.07±0.02			
miR-33	M	0.0003±0.0001	0.0003±0.0001	0.0003±0.00004	0.83	0.0001	0.83
	F	0.0002±0.00003	0.0004±0.0001	0.0002±0.0001			
miR-34a	M	0.9±0.1	0.8±0.1	0.7±0.2	0.75	0.0001	0.75
	F	1.01±0.18	1.18±0.56	1.07±0.35			
miR-103	M	16.3±3.0	14.5±2.5	17.8±2.7	0.64	0.0001	0.64
	F	17.61±3.43	14.84±3.57	9.86±1.93			
miR-107	M	0.4±0.05	0.5±0.1	0.3±0.05	0.07	0.0001	0.07
	F	0.58±0.10	0.55±0.11	0.57±0.11			
miR-122a	M	7895.9±1786.3[a]	2695.8±295.4[b]	8370.1±1788.1[a]	0.20	0.001	0.048
	F	8682.96±2495.63	16419.94±6804.82	12872.52±4519.43			
miR-130a	M	0.02±0.002	0.02±0.002	0.02±0.004	0.26	0.0001	0.26
	F	0.01±0.004	0.01±0.003	0.01±0.002			
miR-143	M	0.04±0.01	0.04±0.01	0.03±0.01	0.48	0.0001	0.48
	F	0.01±0.01	0.03±0.01	0.02±0.01			

Values are means ± SEM, n = 9-11. Male miR-21 and miR-103 were log transformed. Female miR-34a was assessed using an independent samples Kruskal-Wallis test. The superscripts [a,b] are used to depict differences between groups within a sex where groups without a common superscript differ (p<0.05).

4.5 DISCUSSION

There is an increasing body of evidence ranging from worms to humans highlighting that parental lifestyle affects offspring phenotype [283]. Of note, the paternal environment has been shown to transmit information intergenerationally via epigenetic molecules in the sperm [283]. Still, the mechanism of this inheritance remains unclear. Our study demonstrated that paternal HP diet at preconception elicits protective effects on male and female offspring that persist into adulthood. These protective effects derived from a paternal HP diet reduced paternal adiposity, influencing offspring bodyweight and adiposity, thereby improving insulin sensitivity and gut microbial/ epigenetic signatures intergenerationally (Figure 4.9).

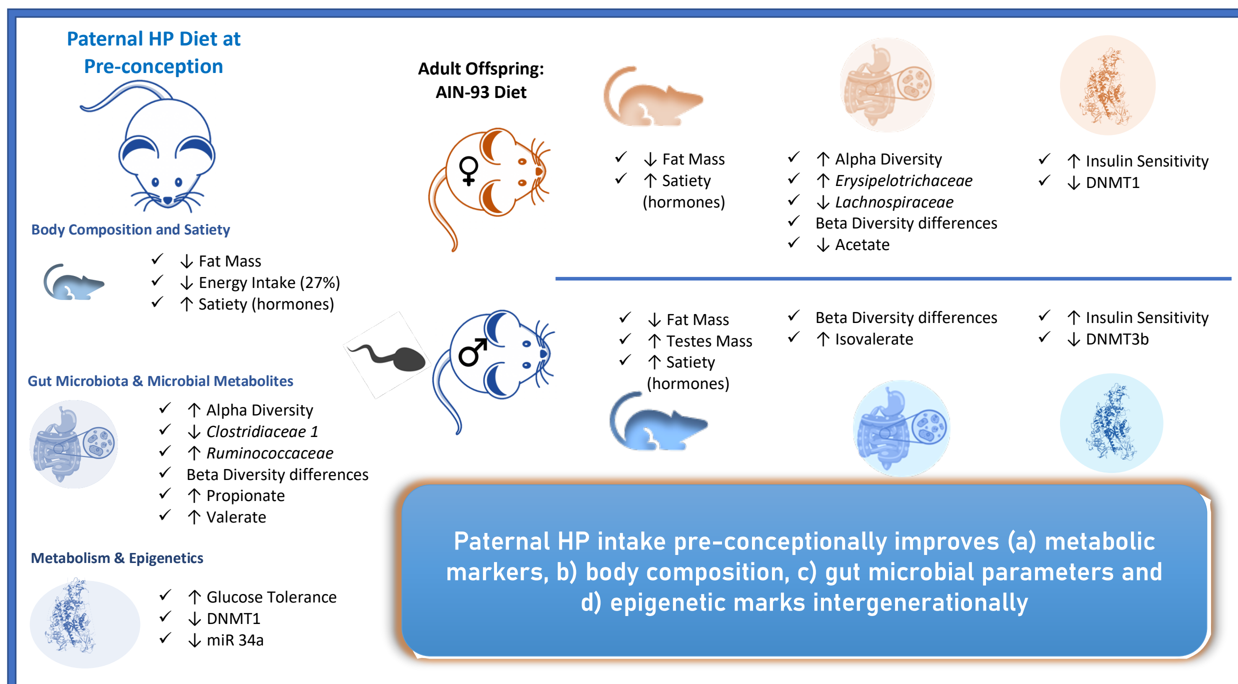

Figure 4.9 Overall summary of the major findings in fathers and adult offspring following a HP diet. All data are compared to a nutritionally poor diet (HF/S).

According to the *thrifty gene hypothesis,* mammals exhibit important adaptive mechanisms, which exist to optimize vital organ growth in response to peripheral organ development[284]. This adaptation could explain why fathers consuming a HP diet, exhibited distinct and beneficial differences in vital organ weight compared to a cohort consuming a nutritionally poor diet (HF/S). Specifically, heart, kidney and cecum as a ratio of body weight were larger in HP fathers compared to HF/S and control groups. Similar observations were found

in prepubertal female rabbits consuming a high fat diet, wherein there was a decrease in heart/body-weight ratio[285]. They further investigated biochemical and ultrastructural cardiac alterations and deduced that the evolution of body-mass, surpasses that of the heart[285]. Wang *et al.* suggested that increased body weight that was not followed with concurrent cardiac growth was indicative of substantial visceral fat accumulation, which was a predictor of cardiac disease[286].

Yet another predictor of metabolic disease is insulin resistance (IR). IR is a multi-factorial disturbance of the internal processes of a target cell, modulated by the hormone insulin and eventually resulting in inflammation[278]. This inflammation can be increased or decreased depending on dietary fatty acid composition[278]. High protein diets in rodent models revealed important metabolic adaptations, like the downregulation of lipogenesis and increased hepatic gluconeogenesis as well as glycogenesis[287,288]. Further, dietary proteins are known to have an insulinotropic effect which could explain why we saw augmented insulin secretion and improved glucose clearance from circulation in offspring and fathers respectively during the ITT and OGTT. Insulin functions to modulate important energy functions, including glucose and lipid metabolism. Popular weight loss diets like the Atkins or the Zone diet, characteristically high in protein and low in carbohydrates have shown positive effects on body composition and weight[289,290]. Complimentary to this research, we found intergenerational reductions in adiposity in both fathers and adult offspring among our casein-specific, HP fed groups. This is an extension of epidemiological studies indicating that high protein consumption derived from dairy sources protects against obesity[291,292]. Our findings similarly deduced that HP paternal protein consumption from dairy sources protects their offspring against obesity. Important to note, we did not see any intergenerational difference in triglyceride content in liver tissue, suggesting no ectopic fat deposition in the liver.

Amino acids are involved in core biological process, like protein synthesis as well as regulation of metabolism. The aforementioned impact on metabolism can be directly linked to dietary intake. For example, dietary consumption of tryptophan or phenylalanine affects appetite regulation; arginine consumption impacts nitric oxide production; branched chain amino acids (BCAAs) activate the mammalian target of rapamycin complex 1 (mTOR1). Newgard *et al.* [293] found that supplementing a high fat diet with BCAAs in rats attenuated the obese phenotype. Further, the satiety-inducing effect of high protein diet contributes to weight loss and

improved body composition[294]. As such, it was not surprising to find fathers consuming the HP diet, concurrently consumed less energy. Similar results were observed in males at weaning in terms of food intake, wherein males belonging to the HP group consumed less energy. Gastrointestinal anorexigenic hormone, pancreatic polypeptide (PP), showed the most striking outcome. PP is known to cause sustained satiety and decreased food intake[295]. HP fathers showed a trend toward increased plasma PP, and a much more pronounced increase in PP in female adult offspring compared to HF/S offspring, further explaining our reduced energy intake. Peptide hormone amylin regulates the in-flow of glucose into circulation by delaying nutrient delivery and is co-secreted with insulin from pancreatic β-cells and concurrently inhibits glucagon secretion post-prandially[296]. Decreased leptin in HP fathers was reflective of decreased fat mass. Similar trends in leptin were observed in adult female offspring imitating reductions in fat mass, signifying improved metabolic health in fathers and a sex-specific intergenerational inheritance of improved metabolic health status in female offspring.

The adipoinsular axis is a dual hormonal feedback loop involving hormones produced by pancreatic β-cell and adipose tissue hormones, insulin and leptin respectively[297]. As a growth hormone, insulin is adipogenic and also simulates the production and secretion of leptin[297]. Leptin acts centrally to ensure satiety is achieved and reduces food intake[297]. Leptin and insulin have an inverse correlation, where leptin acts centrally and directly on β-cells to suppress insulin secretion[297]. Further, an increase in adiposity, increases plasma leptin levels thus, reducing insulin production, which exacerbates adiposity[297]. We postulate, HP diet directly maintains the proper functioning of the adipoinsular axis, thus maintaining nutrient balance, as observed by our reduction in adiposity in fathers and offspring, as well as improved insulin sensitivity. Conversely, paternal HF/S diet at preconception seemingly causes the dysregulation of this axis intergenerationally, as demonstrated by our data. Furthermore, leptin is directly involved in energy and glucose homeostasis as well a plethora of gastrointestinal functions, like the secretion of GLP-1[298]. GLP-1 is an incretin, meaning it stimulates insulin secretion and it inhibits glucagon secretion[299]. The gastrointestinal bacteria independently produce metabolites that interact with host cells and facilitate inter-organ communication, further regulating whole body metabolism.

Speculation exists as to whether the paternal microbiota signatures may be transmitted to the offspring. This study asserts that it does, however likely not in the same way the maternal

microbiota does. The intergenerational transmission of microbial communities has been largely attributed to physical contact between the neonate and the dam. This colonization commences at birth, following contact of dam's microbial community during and after passage through the birth canal, during suckling and maternal care and contact with the neonate's environment[300]. Similarly, crosstalk between the microbiota and the innate immune system exists to facilitate host-mediated tolerance and maintenance of the gastrointestinal system's microbial communities[300]. Previous studies have hypothesized that the seminal fluid microbiota is influenced by paternal health and the gut microbiota indirectly. Therefore, during the time of copulation, the female reproductive tract physiology is altered[69] and/or, it is possible seminal fluid metabolites or epigenetic markers influence maternal reproductive health, thereby influencing offspring health and the gut microbiota indirectly. In any event, we sought to examine the offspring microbiota in an effort to substantiate whether paternal HP intake may have an impact.

We found that paternal HP consumption had significant effects on gut morphology in both fathers and offspring. HP fathers had greater cecum size, likely reflecting fermentative activity of the microbiota. HP male adult offspring had larger relative colon mass compared to controls, whereas HF/S female adult offspring had the smallest colons signifying differential microbial fermentation between groups resulting in a plausibly healthier internal colonic environment in HP fathers and offspring and an unhealthy colonic environment in HF/S offspring. The bacterial phyla that dominated in fathers and their offspring included Firmicutes, Bacteroidetes, Actinobacteria and Verrucomicrobia. We observed 33.8%- 42.3% shared ASVs between paternal gut microbiota and male and female offspring gut microbiota, suggesting possible intergenerational transmission, however future metagenomic research should be undertaken to assess whether fathers and offspring shared identical bacterial genomes.

The gut microbiome influences host metabolism, however, whether changes in the gut microbiota are linked with casein-specific anti-obesogenic effects remain unknown. Previous reports have found that several strains of *Bifidobacterium* exhibit anti-obesogenic properties in rodents[301]. In contrast, increased abundance of *Lactobacillus* has been found in high-fat diet groups [302]. Yet, some lactobacilli species like *Lactobacillus plantarum* have been associated with weight loss in humans[303] and reduced adipose tissue mass in diet-induced obese mice[304]. This pattern fits with the high *Bifidobacterium* shown in our study in fathers fed HP

diet that also persisted in female offspring. Similarly, low *Lactobacillaceae* in female offspring sired by HP fathers was observed. Isokpehi *et al.* [303] examined protein function to understand bacterial determinants influencing obesity development, specifically, mechanisms involving energy extraction from diet in the human GI system. They found that *Lactobacillus plantarum* strains possess a two-gene operon that encodes a universal stress protein involved in stress responses and the membrane translocator protein (TSPO), which are involved in mitochondrial fatty acid oxidation in humans[303]. Moreover, authors uncovered a three-gene operon in *Akkermansia muciniphila* possessing a gene whose mitochondrial homolog is linked with fat distribution[303], potentially explaining the inherited reduction in adiposity in male and female adult HP offspring.

Additionally, the gut microbiota impacts metabolism by influencing glucose homeostasis and insulin resistance in vital metabolic organs (i.e. liver, skeletal muscle, fat tissue) as well as the production and secretion of gut hormones that modulate these important metabolic processes. For example, a strain of *Bifidobacterium* can increase glycogen synthesis and reduce gluconeogenesis-related gene expression in the liver[305]. Similarly, some *Bifidobacterium* strains improved the translocation of GLUT4 as well as insulin-stimulated glucose uptake[305]. *Lactobacillus* strains have been shown to increase GLUT-4 expression in muscle tissue[306]. *Lactobacillus plantarum* and *Akkermansia muciniphila* reduce the expression of an important enzyme involved in xenobiotic metabolism[307], whose knockdown is known to prevent hyperglycemia and hyperlipidemia in insulin resistant mice[308]. Such findings help explain the differences we saw in paternal glucose metabolism and offspring hypolipidemia, whom also exhibited high *Bifidobacterium* abundance and unique differences in *Lactobacillaceae*. In addition to the microbiota, gut microbial products, like SCFA also improve insulin resistance and glucose tolerance[307]. For example, butyrate can act as a ligand for G-protein coupled receptors, GPCR41 and GPCR43 promoting the release of gut hormones, GLP-1 and PYY[309]. A dysbiotic gut reportedly increases acetate production in rodents, thereby activating the parasympathetic nervous system and prompting insulin and ghrelin over-secretion with concurrent hyperphagia and obesity[310]. This was consistent with our findings in adult HF/S female offspring, whom exhibited a concurrent increase in butyrate, which acts as another obesity and hyperinsulinemia regulator[311]. Early work in Gordon's lab found similar results following a comparative metagenomic analysis in cecal matter from a genetically obese rodent

model (*ob/ob)*[312]. They found that this obese model had higher concentrations of butyrate and acetate[312] postulating that this was owing to the increased prevalence of Firmicutes bacterium, which are known butyrate producers[313,314]. Finally, HP diets have been shown to modify branched chain fatty acid profile, increasing the cumulative production of isovalerate [258], which was consistent with our findings. Paternal isovalerate was increased in HP groups and persisted in adult male HP groups.

Both male and female offspring displayed significantly greater abundance of *Muribaculaceae* in adult HP offspring compared to controls. While *Muribaculaceae* was reduced in paternal HP group, the opposite was true in adult offspring. We postulate this occurred because the gut microbiota functionally operates in part to maintain energy homeostasis and facilitate a survival advantage to its host during periods of nutrient scarcity or abundance. Alternatively, previous work showed that *Muribaculaceae* is negatively associated with fat mass[315], providing another potential microbial explanation for the observed reduction in fat mass in adult male and female HP offspring, accompanied with high relative abundance of *Muribaculaceae.*

Interestingly, female offspring appeared to elicit the greatest differences at higher taxonomic levels, whereas males exhibited minimal differences until examined lower taxonomic levels, including notable abundances in Lactobacillales and Bacteriodales in HP offspring, including groups belonging to *Enterobacteriaceae, Marinifillaceae, Odoribacter, Alistipes* and *Rikenellaceae*. Whether these differences in the abundance of microbes were of physiological relevance remains unclear.

The co-evolution of the gut microbiota and the mammalian host has been taking place over millions of year[18]. Ongoing research has been directed towards elucidating the molecular mechanisms by which the microbiota maintains host metabolic homeostasis[18]. Among these molecular mechanisms include the considerably under-researched area of epigenetics and epigenetic changes influenced by diet. Even fewer studies exist pertaining to epigenetic changes caused by paternal nutrition. DNA methylation and small non-coding RNA are epigenetic markers regulated partly by enzymes like methylases, their activity depend on host and microbial-derived metabolites[18]. Epigenetic factors modulate tissue-specific expression as well as X-chromosome inactivation[316]. For this reason, we chose to analyze for a sex-effect in metabolically relevant tissue like adipose and liver, before stratifying the sexes. We observed

significant differences between the sexes in regards to DNA methyltransferase and microRNA transcriptomics.

DNA methylation has recently emerged as an important predictor of obesity and metabolic syndrome[317]. Most notably, changes in nutrition is implicated as a key regulator of epigenetic modifications transgenerationally. Previous reports have indicated that DNA methylation changes are dynamic or may remain stable and inherited by subsequent generations. Our study found that a paternal nutritionally poor diet (HF/S) increased methylation as observed by a methylation catalyst, DNMT1. This intergenerational inheritance of DNA methylation is important to consider when discussing the prevalence of obesity, since DNA methylation may contribute to obesity pathogenesis and its co-morbidities.

Other crucial epigenetic modulators include microRNAs (miRNA or miR). MicroRNAs are branded as important regulatory factors involved in lipid metabolism[318], yet still, the lipid deposition mechanism remains unclear. MicroRNA-34a modulates inflammatory pathways, wherein increased miRNA levels have been observed in obesity or type 2 diabetes models[318]. Our study found reduced levels of miR-34a in paternal groups, as well as a reduction in fat mass in HP groups compared to controls and HF/S groups. This did not persist in adult offspring. We believe this is because paternal miRNAs influence the maternal environment directly and differences in offspring miRNAs depend on maternal miRNA processing machinery. Another possible explanation is that miR-34a regulates DNMTs and thus affects methylation status on several genes involved in intergenerational lipogenesis and insulin resistance. Since their detection in 1993 [319], microRNAs have emerged as important regulators of lipid and glucose metabolism by affecting the status and function of multiple organs, therein, initiating pathologies, like obesity[320]. However, the mechanistic action of these microRNAs remains unclear, since they can affect many pathways or gene networks simultaneously. Future work should investigate the comprehensive function of microRNAs in tissue metabolism and energy homeostasis and their potential cross-talk between the gut microbiota and intergenerational inheritance.

In conclusion, paternal high protein diet, during the preconceptional period is postulated to contribute to phenotypic, metabolic, gut microbial and epigenetic alterations in their progeny. We found that a HP paternal diet results in an intergenerational reduction in adiposity, thereby improving insulin sensitivity and gut hormone profiles with a concurrent alteration in gut

microbial ecosystems. Further investigation into the full spectrum of mechanisms directing this metabolic programming is warranted.

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CHAPTER FIVE: PATERNAL METHYL DONOR SUPPLEMENTATION IN RATS IMPROVES FERTILITY, PHYSIOLOGICAL OUTCOMES, GUT MICROBIAL SIGNATURES AND EPIGENETIC MARKERS ALTERED BY HIGH FAT/ HIGH SUCROSE DIET

5.1 ABSTRACT

BACKGROUNDS & AIMS: Increased consumption of high fat/sucrose (HF/S) diets has contributed to rising rates of obesity and its co-morbidities globally and negatively impacted male reproductive health. Our objective was to examine whether adding a methyl donor cocktail to paternal HF/S diet (HF/S+M) improves health status in fathers and offspring.

METHODS: From 3-12 weeks of age, male Sprague Dawley rats consumed a HF/S or HF/S+M diet. Offspring were followed until 16 weeks of age. Body composition, metabolic markers, gut microbiota, DNA methyltransferase (DNMT) and microRNA expression were measured in fathers and offspring.

RESULTS: Compared to HF/S, paternal HF/S+M diet reduced fat mass in offspring ($p<0.005$). HF/S+M fathers consumed 16% fewer kcal/day which persisted in HF/S+M female offspring, explained by changes in serum GLP-1 and PYY levels. Compared to HF/S, HF/S+M fathers had a 33% improvement in days until conception and 300% fewer stillbirths. In fathers, adipose tissue DNMT3a and hepatic miR-34a expression were reduced with HF/S+M. Adult male offspring had upregulated miR-24, -33, -122a and -143 expression while females had downregulated miR-33 expression. Fathers and offspring showed differences in gut microbial signatures.

CONCLUSION: Supplementing a paternal HF/S diet with methyl-donors improved fertility, physiological outcomes, epigenetic and gut microbial signatures.

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5.2 Introduction

Growing evidence suggests that the decline in male reproductive fitness globally, is in part due to the growing obesity epidemic[321]. Human[322,323] and animal studies[324] have shown that high fat diets, which often reflect poor nutritional status and contribute to increased body mass index (BMI), have been associated with compromised sperm quality, embryo development and fetal growth. Obesity is characterized by excess adipose tissue as well as deposition of fat in ectopic locations[325], increased plasma fatty acids and an augmented occurrence of insulin resistance[326]. In murine models, high fat diet-induced hyperglycemia, hyperinsulinemia and hypercholesterolemia are associated with alterations in testicular morphology [327,328]. The relationship between male reproductive health and obesity is predictable based on the fact that cholesterol is the chief metabolic precursor involved in testosterone synthesis[329] and the principal spermatogenesis regulator[330], both of which are modulated in part by epigenetics.

Epigenetics refers to modifications to DNA that affect gene expression profiles of a cell but occur in the absence of changes to the DNA sequence[331]. In comparison to the genome, there is considerable plasticity at the cellular epigenetic level. Among the most widely studied epigenetic mechanisms are DNA methylation and non-coding RNA expression [82].

Non-coding RNAs (ncRNAs) are classified as long (>200nucleotides) or small (<200 nucleotides) and function as small housekeeping or regulatory ncRNA, the latter including microRNAs[82]. MicroRNAs (19-25 bp in length) function as mRNA translation suppressors or inducers of mRNA breakdown in mammalian cells[332]. In the liver, miRNAs are postulated to coordinate cell lineage differentiation during organ development, playing a crucial role in lipid metabolism and disease modulation, making them valuable prognostic and therapeutic biomarkers clinically[333].

DNA methylation is largely associated with gene regulation and cell differentiation. The primary targets of methylation are CpG dinucleotides in non-coding regions, like promotors[82]. DNA methylation involves the careful coordination of methylating enzymes known as DNA methyl transferases (DNMTs). DNMTs transfer a methyl group from S-adenosylmethionine (SAM) to a cytosine residue, ultimately forming 5-methylcytosine[82]. The varying types of DNMTs include DNMT3a and 3b which control *de novo* DNA methylation activity, and DNMT1, which maintains methylation profiles during DNA replication and cell division[281]. A

by-product of the methyltransferase reaction is homocysteine (Hcy)[334]. DNA methylation machinery operate on a myriad of different tissues, most notably, adipocytes, modulating important adipocyte metabolic functions[335]. Emerging evidence has postulated that DNA methylation is involved in the transgenerational inheritance of obesity and metabolic syndrome[336].

One carbon metabolism is comprised of the interconnected folate and methionine cycles that facilitate the transfer of 1C moieties required for cellular processes[159]. The key dietary constituents that mediate one carbon metabolism are folate, other B vitamins (B2, B6, and B12), methionine, choline and betaine. Amino acid methionine levels in the body are dependent on dietary intake, protein catabolism and the re-methylation of Hcy[337]. Methionine can be converted into SAM, which functions as a universal methyl donor in most methyltransferase reactions[160]. Vitamin B12 is an important co-factor for methionine synthase, the rate-limiting enzyme that converts Hcy to methionine[338]. It is via Hcy remethylation that the folate cycle and methionine cycle are linked[337]. The primary role of folate is to donate or accept one-carbon units which it primarily does via tetrahydrofolate[339]. Betaine, which is derived from the oxidation of choline, can also be used as a methyl donor to recycle Hcy to methionine[340]. The availability of methyl groups originating from diet (derived from methyl-folate, methionine or choline/ betaine) directly affect DNA and histone methylases via their actions as precursors to SAM, and thereby influence epigenetic modifications [341–345]. Importantly, growing evidence has also emerged for the role of dietary methyl donors to impact gut microbiota composition[337,346,347].

While there is substantial evidence for maternal diet via its effects on one carbon transfer to influence long-term metabolism and disease risk in offspring[158], less is known about paternal diet. A nutritionally poor diet in fathers has been shown to affect cellular one-carbon metabolism by increasing levels of serum and tissue Hcy, which negatively affects male fertility[348]. Moreover, alterations in DNA methylation have been assessed in the sperm of infertile males in human[349] and animal[350] studies, where both showed impaired spermatogenesis, with the rodent model observing this impairment among F3 progeny. Interestingly, in sub-fertile men, folate supplementation can improve sperm quality[351].

Previous work showed that maternal diets supplemented with methyl donors attenuates adverse phenotypes in offspring associated with maternal high fat diet intake[352]. To our

knowledge, this is the first study to examine whether a paternal diet supplemented with a methyl donor cocktail of betaine, choline, folic acid and vitamin B12 attenuates the adverse metabolic outcomes, epigenetic changes and gut microbial effects of a paternal high fat/sucrose diet in offspring.

5.3 Materials and Methods

5.3.1 Animal Model and Dietary Treatment

Maintained in a temperature and humidity-controlled facility, twenty-four male Sprague Dawley rats (Charles River Laboratories, Montreal, QC, Canada) were randomized to one of two dietary interventions: 1) high fat/ high sucrose (HF/S) or 2) high fat/ high sucrose supplemented with a methyl donor cocktail (HF/S+M) [betaine (5 g/kg diet), choline (5.37 g/kg diet), folic acid (5.5 mg/kg diet), vitamin B12 (0.5 mg g/kg diet); Sigma Aldrich, Oakville, ON, Canada] according to previous work that showed distinct differences in individual metabolic outcomes as well as DNA methylation patterns in offspring following dam exposure during lactation[353–355]. HF/S diets were purchased from Dyets Inc. (Bethlehem, PA, USA) (DYETS# 103915: age 3-9 weeks; DYETS# 102412: weeks 10-12). Diet composition is provided in Table 5.1. At 12 weeks of age, a virgin female Sprague Dawley rat was co-housed with a male rat from one of the dietary interventions during the dark cycle for as many consecutive nights until a copulation plug was identified. During the light cycle, females were given AIN-93G diet and males were returned to their designated dietary intervention *ad libitum.* During pregnancy and lactation, dams consumed AIN-93G diet. In an effort to limit differences in energy intake due to variances in litter size, one day after birth, litters were culled to 10 offspring (n=5 males; n=5 females). Litters that were less than n=10 were increased via cross-fostering with offspring from another litter belonging to the same treatment group. At 3 weeks of age, one male and one female from each litter (considered as n=1) were weaned onto AIN-93G diet (weeks 3-9) and AIN-93M (weeks 10-12) and water *ad libitum* for 13 weeks. This study was approved by the University of Calgary Animal Care Committee (AC18-0074) and conformed to the *Guide to the Care and Use of Laboratory Animals*.

Table 5.1 Experimental diet composition from weeks 3-9 and 10-16

	HF/S	HF/S+M	HF/S	HF/S+M
g/kg	**Weeks 3-9**		**Weeks 10-16**	
Cornstarch	0	0	0	0
Casein	240	237.5	200	197.9
Sucrose	459.5	454.7	499.5	494.3
Soybean Oil	100	99	100	99
Lard	100	99	100	99
Alphacel	50	49.5	50	49.5
AIN-93M Mineral Mix	35	34.6	35	34.6
AIN-93 VX Vitamin Mix	10	9.9	10	9.9
DL-Methionine	3	2.97	3	2.97
Choline-Bitartrate	2.5	2.47	2.5	2.47
Betaine	0	5	0	5
Choline (CDP choline)	0	5.37	0	5.37
Folic Acid	0	0.0055	0	0.0055
Vitamin B12	0	0.0005	0	0.0005
Energy density (kJ/g)	19.3	19.1	19.3	19.1
Carbohydrate (% of kcal)	49.8	49.3	49.8	49.3
Protein (% of kcal)	11.1	11.0	11.1	11.0
Fat (% of kcal)	39.1	38.7	39.1	38.7

The digestible energy of high fat/ sucrose diets was 4.58 kcal/g and 4.6 kcal/g for the 3-9 and 10-16 week formulations respectively. Diets were purchased from Dyets, Inc. (Bethlehem, PA, USA). Methyl donors (Betaine, Choline, Folic Acid and Vitamin B12) were purchased from Sigma Aldrich (Oakville, ON, Canada).

5.3.2 Body weight, Food Intake and Body composition

Throughout the duration of the study, paternal and offspring bodyweights were measured weekly; food intake was measured every 3 weeks. A Dual Energy X-ray Absorptiometry (DXA) scan (Hologic ODR 4500; Hologic Inc.) was used to assess body composition 1 day prior to sacrifice. To ensure animals remained still during the scan, animals were lightly anaesthetized using isoflurane. Using QDR software for small animals, bone mineral content/density (BMC/BMD) (g and g/cm^2), fat mass(g), lean mass(g) and body fat % were quantified.

5.3.3 Oral Glucose Tolerance Test (OGTT) and Insulin Tolerance Test (ITT)

At 10 and 14 weeks of age, in fathers and offspring respectively, rats underwent a 12 hour fast and a blood glucose measurement was obtained via tail nick and a One Touch Ultra ® 2 glucose meter (Lifespan, Burnaby, Canada), accounting for the 0 min timepoint. Additional blood glucose measurements were collected at 15, 30, 60, 90 and 120 minutes after a 2g/kg glucose solution administered via oral gavage.

In fathers and offspring at 11 and 15 weeks of age respectively, rats were fasted for 6 hours and blood glucose measured via tail nick using a One Touch Ultra ® 2 glucose meter (Lifespan, Burnaby, Canada) accounting for the 0 min timepoint. Additional blood glucose measurements were collected at 15, 30, 60, 90 and 120 minutes after an intraperitoneal injection of insulin (0.75U/kg).

5.3.4 Tissue Harvest and Blood Insulin, GLP-1, PYY and HOMA-IR

Animals were anesthetized using isofluroane and denied access to food overnight for a 12 hour fast; 1 mL of blood was collected from the portal vein in a chilled tube containing diprotinin-A (0.034 mg/ml blood; MP Biomedicals, Irvine, CA), Sigma protease inhibitor (1 mg/ml blood; Sigma Aldrich, Oakville, ON, Canada) and Roche Pefabloc (1mg/ml of blood; Roche, Mississauga, ON, Canada). Plasma was collected after centrifugation and stored in -80°C until insulin, peptide tyrosine tyrosine (PYY) and glucagon-like peptide 1 (GLP-1) were measured using a Rat Metabolic Multiplex Array (MRDMET) (Millipore, St. Charles, MO) (Eve Technologies, Calgary, AB, Canada). Animals were henceforth euthanized via decapitation and heart, liver, kidney, cecum, colon and male testes were weighed and stored in -80°C until analysis. Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) was used to estimate insulin resistance, validated in rats[356], using the following formula:

HOMA-IR= [glucose (mmol/L) x insulin (mIU/mL)] / 22.5[357]

5.3.5 Hepatic Triglyceride Analysis

Triglyceride concentrations were assessed from a starting amount of 25mg of liver derived from the right lobe, using the GPO reagent set, according to manufacturer's instructions (Pointe Scientific Inc., Lincoln Park, MI)

5.3.6 Gut microbiota 16S rRNA Gene Sequencing

Baseline fecal matter was collected at 3 weeks of age in fathers and offspring. Additional fecal matter was collected at 9 and 12 weeks of age in fathers and 9 and 15 weeks of age in offspring. All fecal matter was snap frozen and stored at -80°C until analysis. Gut microbial 16S rRNA gene sequencing was performed according to our previous work[217,218]. Briefly, a FastDNA spin kit for feces (MP Biomedicals, Lachine, QC, Canada) was used to extract bacterial DNA according to manufacturer's guidelines. Bacterial DNA concentrations

were diluted to 4 ng/uL, The MiSeq Illumina platform was utilized to amplify the V3 and V4 region of the 16S rRNA gene (Illumina, San Diego, Ca, USA) at the Centre for Health Genomics and Informatics (University of Calgary, Calgary, AB, Canada).

5.3.7 Cecal Short Chain Fatty Acids

SCFAs were extracted from cecal matter and assessed using reverse-phase HPLC on a c18 column as previously described[273].

5.3.8 RT-PCR of DNA methyltransferase mRNA and microRNAs

Total RNA was extracted from retroperitoneal adipose tissue using the RNAeasy Lipid Tissue Mini Kit (Qiagen) and then reverse transcribed into cDNA using the SuperScript II RT (Qiagen). RT-PCR was performed as previously described[220]. The mRNA expression of DNA methyltransferase (DNMT) 1, 3a and 3b of all samples were analyzed relative to 18S housekeeping control gene using the $2^{-\Delta CT}$ method[195]. The amplicon context sequences are provided in Table 5.2.

Table 5.2 Amplicon Context Sequence for DNMTs in adipose tissue

Gene	Amplicon Context Sequence
	Adipose Tissue
DNMT1	ATCGTCCTTAGCGTCGTCGTAACTTTCTACCTGGCTCACCACAAACTG GGCATGGCGTAGGAGGGAGTCCTCGGTGAACCGGTTCACATTGATGG CAGAAGAGGAA CAGTGGTCTCGATCTTATTGATCAAG
DNMT3a	GAATGATAAGCTGGAGTTGCAAGAGTGTCTGGAACACGGCAGAATAG CCAGTTCAGCAAAGTGAGGACCATTACCACCAGGTCAAACTCCATA
DNMT3b	TTCAGGCAGTAGGAACTTAGAAGCCAGGAGACGCGAGAACAAAAGTC GAGAC GCACAACCATTGACTTTGCCGCTTCTGAGTACTCCACACCCC
18S	**Forward (5’→3’) TGACTCAACACGGGAAACC**
	Reverse (3’→5’) TCGCTCCACCAACTAAGAAC

18S was used as the housekeeping gene

Using the miRNeasy mini kit (Qiagen), following the manufacturer's instructions for Purification of Total RNA, including Small RNA from animal tissue, microRNAs were isolated from liver tissue and then reverse transcribed into cDNA using the miScript II RT kit (Qiagen). RT-PCR was conducted as previously described[220], wherein all microRNAs were analyzed relative to SNORD68 and SNORD96A controls genes using the $2^{-\Delta CT}$ method[195]. MicroRNA primer sequences are listed in Table 5.3.

Table 5.3 Universal primer sequences for microRNA in liver tissue

microRNA	Universal Primer
Liver	
Rn_miR-21_2	CTAGCTTATCAGACTGATGTTG
Rn_miR-24_1	TGGCTCAGTTCAGCAGGAAC
Rn_miR-33_2	GTGCATTGTAGTTGCATTGCA
Rn_miR-34a_1	TGGCAGTGTCTTAGCTGGTTG
Rn_miR-103_2	AGCAGCATTGTACAGGGCTATG
Rn_miR-107_2	AGCAGCATTGTACAGGGCTATC
Rn_miR-122a_1	TGGAGTGTGACAATGGTGTTT
Rn_miR-130a_1	CAGTGCAATGTTAAAAGGGC
Rn_miR-143_1	TGAGATGAAGCACTGTAGCT
Hs_SNORD68_11	**TTTGAACCCTTTTCCATCTG**
Hs_SNORD96A_11	**GACATGTCCTGCAATTCTGAA**

Hs_SNORD68_11 and Hs_SNORD96A_11 were used as controls.

5.3.9 Statistical and Bioinformatics Analysis

Statistical comparisons for all outcomes, except 16S rRNA sequencing data, were performed using IBM® SPSS Statistics, version 24.0. Using a multivariate, general linear model (GLM), a sex effect between male and female offspring was assessed. If a significant sex effect was identified, males and females were assessed separately. Measures with multiple time points were assessed using a repeated measures GLM, where diet was the *between-subject* factor and *time* was the *within-subject* factor. When a significant interaction for diet and time among single

time point outcomes or multiple time point outcomes was identified, an independent samples t-test was used to distinguish significance between dietary groups.

Sequence data was first quality filtered using the filterAndTrim, assignTaxonomy and assignSpecies functions with the R package dada2 (version 1.10.1)[274]. Diversity analysis was conducted using R package phyloseq (version 1.24.2)[275], where alpha diversity was determined using ANOVA and TukeyHSD if significant. Beta diversity was assessed using PCoA (principal coordinates analysis) on a matrix of Bray-Curtis distances. A permutational multivariate analysis of variance (PERMANOVA) was performed to determine significant differences between dietary interventions. Differentially abundant features were assessed using a LEfSe analysis [276], using a significance of alpha = 0.05 and default parameters. Significance was set at $p \leq 0.05$, unless stated otherwise.

5.4 Results

5.4.1 Paternal HF/S+M decreases adiposity in adult male and female offspring

In fathers, no difference in body weight (Figure 5.1A), body composition (Table 5.4) or relative organ weights were observed (Table 5.5) between HF/S and HF/S+M groups. From 4 weeks of age until euthanasia, offspring exhibited a significant sex effect ($p<0.05$) for body weight, body composition and relative organ weight, therefore sexes were analyzed separately. No difference in body weight was observed between diets in male (Figure 5.1B) or female (Figure 5.1C) offspring, however, we observed important differences in body composition, wherein male and female HF/S+M offspring had significantly lower fat mass compared to HF/S offspring, even though both groups consumed the same, nutritionally complete AIN-93 diet for 13 weeks (Table 5.4). Male bone mineral content was significantly reduced in HF/S+M compared to HF/S (Table 5.4). No differences were observed in organ weight as a percentage of body weight for heart, liver, kidney, cecum or colon in male and female adult offspring or testes in male offspring (Table 5.5).

Energy intake was reduced in HF/S+M fathers compared to HF/S at 12 weeks of age (Figure 5.1D), which was similarly seen in female offspring at 9 weeks of age (Figure 5.1F). To examine hormonal influences on food intake, we quantified fasting serum concentrations of the glucagon-like peptide-1 (GLP-1) and peptide tyrosine tyrosine (PYY), both known to reduce food intake. GLP-1 was increased in adult HF/S+M male offspring compared to HF/S ($p=0.03$)

(Figure 5.1G). PYY was significantly increased in fathers consuming the HF/S+M diet (p=0.02) (Figure 5.1H). Higher PYY was similarly seen in adult female offspring (p=0.01) (Figure 5.1H).

Table 5.4 Body Composition of fathers at mating at 12 weeks of age and offspring at 16 weeks of age

	HF/S	HF/S+M	p-value
Fathers			
BMC (g)	15.82±0.45	15.39±0.43	0.85
BMD (g/cm^2)	0.17±0.002	0.17±0.002	0.88
Fat Mass (g)	143.82.4±11.53	123.0±11.81	0.86
Lean+ BMC (g)	507.76±13.84	491.72±12.81	0.92
% Body Fat	21.82±1.22	19.63±1.37	0.68
Male Offspring			
BMC (g)	17.07±0.18	16.67±0.49	0.02
BMD (g/cm^2)	0.18±0.002	0.17±0.002	0.34
Fat Mass (g)	135.11±5.95	116.14±1.77	0.005
Lean+ BMC (g)	526.17±9.88	546.88±14.48	0.12
% Body Fat	19.93±0.73	16.83±0.84	0.56
Female Offspring			
BMC (g)	11.78±0.53	11.74±0.41	0.19
BMD (g/cm^2)	0.163±0.002	0.164±0.003	0.96
Fat Mass (g)	100.84±18.78	95.53±5.69	0.005
Lean+ BMC (g)	304.36±9.29	293.33±10.26	0.86
% Body Fat	23.54±3.40	24.48±0.88	0.10

Values are means ± SEM, *n* = 8-11. In adult offspring, sex differences in the overall models for BMC (p=0.0001), BMD (p=0.0001), fat weight (p=0.05), Lean+BMC (p=0.0001) and % body fat (p=0.03) were observed; therefore, subsequent analysis was performed in males and females separately.

Table 5.5 Organ Weights of Fathers and Adult Offspring at euthanasia

	HF/S	HF/S+M	p-value
Fathers			
Body Weight (g)	651.6±21.2	614.7±21.9	0.20
Organ weight (% BW)			
Heart	0.27±0.01	0.28±0.01	0.38
Liver	3.00±0.08	2.99±0.08	0.75
Kidney	0.29±0.01	0.29±0.01	0.26
Cecum	0.09±0.003	0.10±0.004	0.70
Colon	0.21±0.01	0.22±0.01	0.78
Testes	0.29±0.01	0.29±0.01	0.86
Male Offspring			
Body Weight (g)	666.9±6.2	672±22.7	0.20
Organ weight (% BW)			
Heart	0.28±0.01	0.27±0.01	0.30
Liver	2.70±0.07	2.67±0.04	0.90
Kidney	0.252±0.005	0.251±0.008	0.07
Cecum	0.09±0.003	0.11±0.004	0.09
Colon	0.17±0.01	0.19±0.004	0.91
Testes	0.30±0.01	0.28±0.01	0.58
Female Offspring			
Body Weight (g)	405.2±21.2	388.8±14.3	0.40
Organ weight (% BW)			
Heart	0.30±0.01	0.30±0.01	0.29
Liver	2.59±0.05	2.79±0.12	0.07
Kidney	0.26±0.01	0.26±0.01	0.47
Cecum	0.12±0.01	0.11±0.01	0.75
Colon	0.20±0.01	0.13±0.01	0.41

Values are means ± SEM, n = 8-13. Male offspring heart and female kidney were log transformed for statistical analysis.

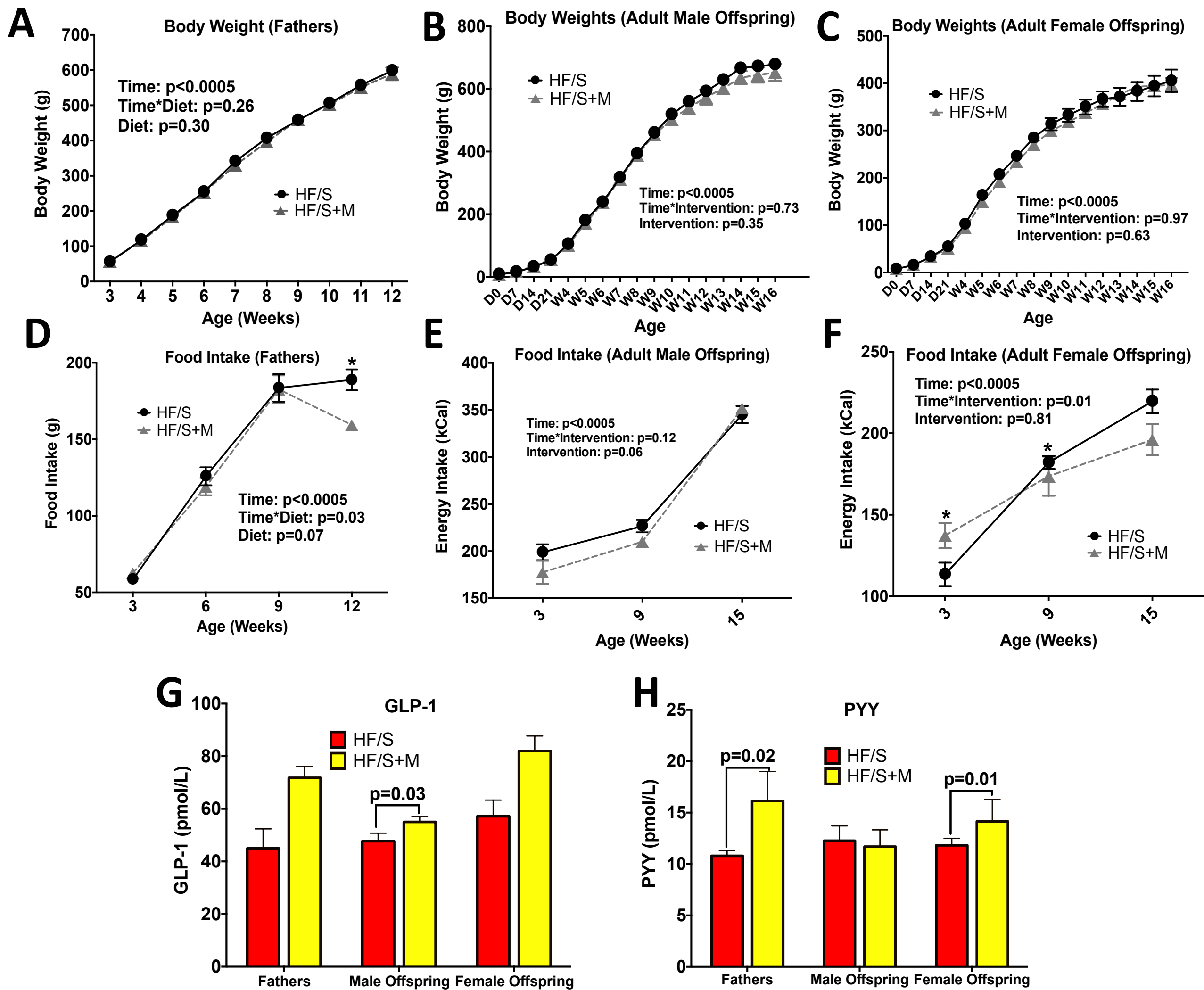

Figure 5.1 Body weight, Food Intake and Gastrointestinal Peptides. Body weight of A) Fathers, B) Adult male offspring and C) Adult female offspring. Food intake of D) fathers, E) male offspring F) female offspring; G) GLP-1 and H) PYY. Values are means ± SEM, n= 8-13. In adult offspring, there was a significant sex effect in the overall model for bodyweight (p=0.0001), food intake (p=0.0001), GLP-1 (p=0.002); therefore, subsequent analysis was performed in males and females separately. * represents a significant difference between groups, p<0.05.

5.4.2 Paternal HF/S+M reduces fasting insulin and insulin resistance

Next, we examined the possible influence of paternal methyl donor diet supplementation on offspring metabolic parameters. Blood glucose concentrations during the oral glucose tolerance test (OGTT) and insulin tolerance test (ITT) in fathers (Figure 5.2A and 5.2D), male (Figure 5.2B and 5.2E) and female (Figure 5.2C and 5.2F) offspring were not independently affected by diet or the interaction of diet and time. Prior to said investigations, a potential sex effect was assessed for glycemia during the OGTT and ITT. Both tests yielded significant sex effects ($p<0.05$), therefore sexes were analyzed separately. Although no differences were seen in glycemia, we did identify a significant decrease in serum insulin (Figure 5.2G) and insulin resistance as assessed by HOMA-IR (Figure 5.2H) in HF/S+M fathers compared to HF/S. This was not evident in offspring. We did not find any differences in hepatic triglyceride concentrations in fathers or offspring, although, we did observe a 40% reduction in hepatic triglyceride concentration in HF/S+M fathers (41.0±1.7 ug triglycerides/mg of liver tissue) compared to HFS fathers (68.7±3.5 ug/mg) (Figure 5.2I).

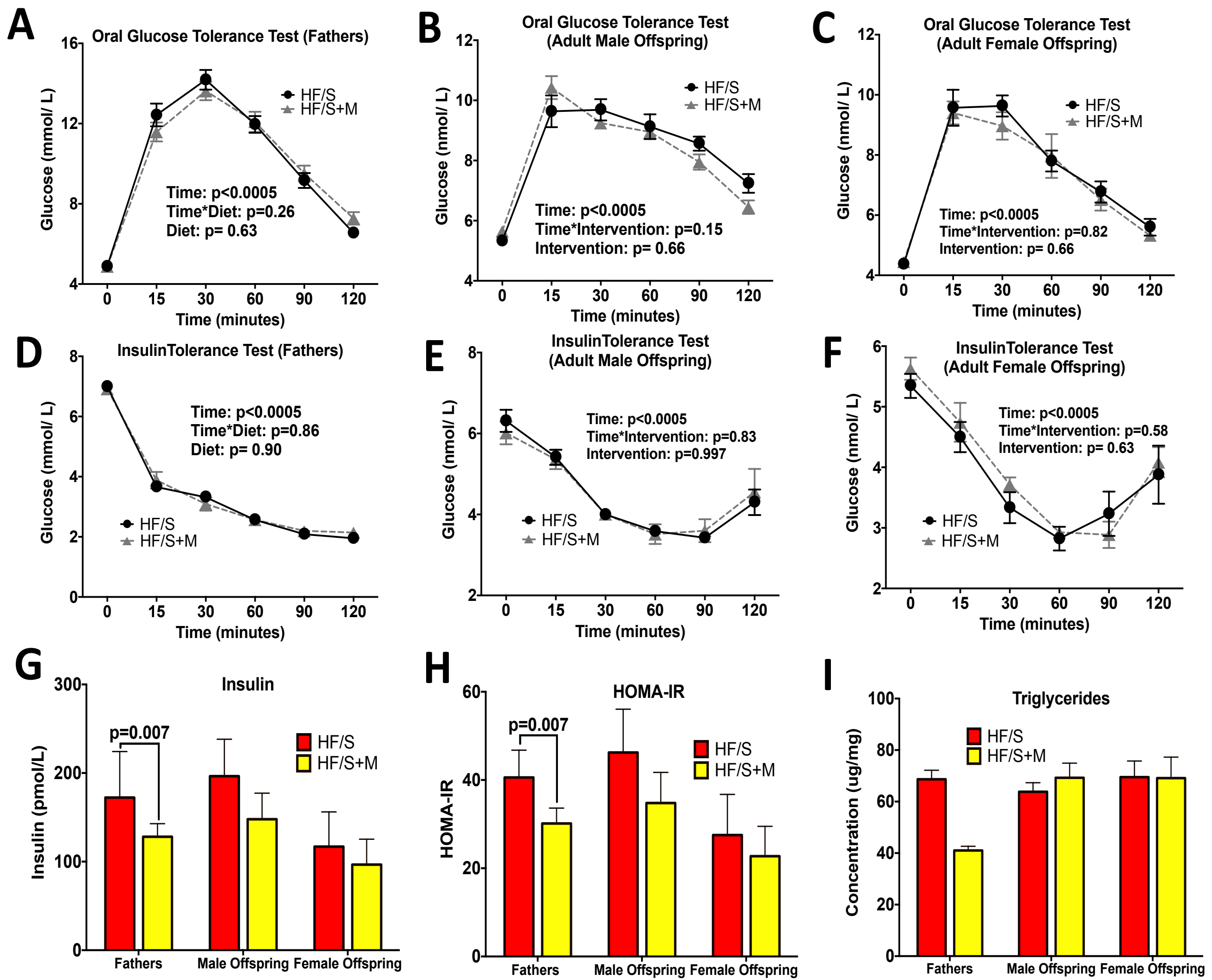

Figure 5.2 OGTT, ITT, Fasted Insulin, HOMA-IR and Hepatic Triglyceride Concentrations. OGTT of A) Fathers, B) Adult male offspring and C) Adult female offspring. ITT of E) fathers, F) male offspring, G) female offspring. H) Insulin levels, I) HOMA-IR, J) Triglyceride content in hepatic tissue. Values are means ± SEM, *n*= 8-13. In adult offspring, there was a significant sex effect in the overall model for OGTT (p=0.0001), ITT (p=0.003), insulin (p=0.0001), HOMA-IR (p=0.0001). Triglyceride sex effect was not significant. Since most assessments in adult offspring had a significant sex effect, subsequent analysis was performed in males and females separately.

5.4.3 HF/S+M improved markers of reproductive performance in fathers

Since the effectiveness of maternal methyl supplementation on reproduction and pregnancy has been demonstrated[358], we examined whether similar effects may be observed following paternal methyl supplementation. We found no difference in fertility index between groups, calculated as number of successful pregnancies divided by number of sperm positive

fathers (Table 5.6). Notably, we found a significant difference in *nights cohabited until conception*, wherein, HF/S+M resulted in fewer days to conception (Table 5.6). Similarly, we observed 300% fewer stillbirths in the HF/S+M group compared to HF/S group (Table 5.6).

Table 5.6 Reproductive Markers for Paternal Fertility

	HF/S	HF/S+M
Males (n)	13	12
Males cohabited	13	12
Nights cohabited until conception	3.00±0.56	2.00±0.33*
# of successful pregnancies	10	9
Fertility Index (%)	77	75
# of pups born alive (per father)	14.7±0.6	13.3±0.9
# of stillbirth pups	3	1
Pup survival (%)	97.8	99.9
Relative abundance of male pups (%)	51.2±4.1	49.2±1.0
Relative abundance of female pups (%)	48.8±4.1	46.98±2.0

Values are means ± SEM. Fertility index= # pregnant/ number sperm positive.
* represents a significant difference between groups, $p<0.05$.

5.4.4 Paternal HF/S+M consumption improved epigenetic markers in fathers and offspring

Due to the fact that DNA methylation is a key epigenetic regulator of adipose tissue development and gene regulation[335], we examined epigenetic markers like, DNA methyltransferases 1, 3a and 3b, important catalysts of DNA methylation[280]. Using RT-PCR, we found reduced expression of DNMT3a in retroperitoneal adipose tissue in fathers in the HF/S+M group (Figure 5.3A). Adult offspring DNMTs were significantly affected by sex ($p<0.001$), therefore male and female offspring were assessed separately. In adult female offspring, we saw significantly elevated retroperitoneal adipose tissue expression of DNMT1, DNMT3a and DNMT3b in the HF/S+M group (Figure 5.3C).

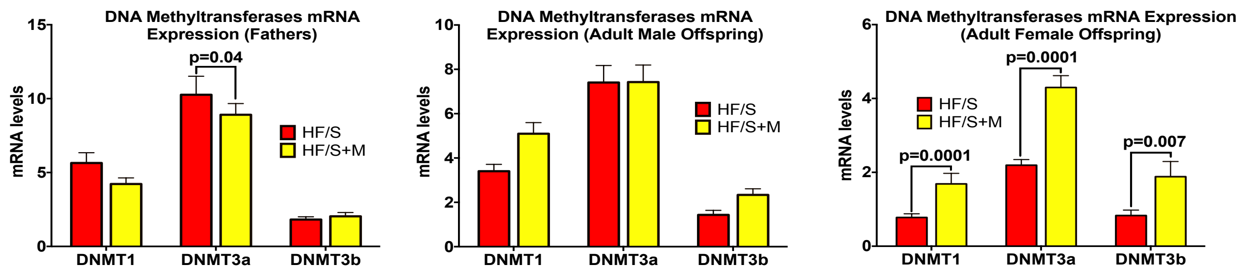

Figure 5.3 Adipose tissue mRNA levels of DNA Methyltransferases (DNMTs) in A) fathers, B) adult male offspring and C) adult female offspring. Values are means ± SEM, *n*= 8-13. Sex differences were observed in DNMT1 (p=0.0001), DNM3a (p=0.0001) and DNMT3b (p=0.03); therefore, subsequent analysis was performed in males and females separately.

Given that the differential expression of several miRNAs in the liver has been associated with obesity and insulin resistance[359], we assessed a panel of 10 microRNAs in liver tissue. We identified 4 out of 10 microRNAs that were differentially expressed in HF/S+M fathers; 3 were upregulated (miR-33, miR-103 and miR-107) and 1 was downregulated (miR-34a) (Table 5.7). In adult males, 4 microRNAs were differentially expressed; miR-24, miR-33, miR-122a, miR-143 were all upregulated in HF/S+M offspring (Table 5.7). In females, miR-33 was downregulated in HF/S+M versus HF/S offspring (Table 5.7).

Table 5.7 MicroRNA Expression in Liver Tissue

	HF/S	HF/S+M	p-value
Fathers			
miR-21	472.0±151.2	701.5±244.9	0.78
miR-24	0.05±0.01	0.05±0.01	0.67
miR-33	0.00017±0.00002	0.0002±0.00006	0.03
miR-34a	1.02±0.17	0.56±0.06	0.002
miR-103	20.35±1.91	21.65±3.94	0.03
miR-107	0.32±0.01	0.42±0.01	0.04
miR-122a	5966.08±1122.78	5822.70±1310.41	0.51
miR-130a	0.02±0.01	0.01±0.01	0.10
miR-143	0.04±0.01	0.03±0.01	0.70
miR-let-7c	3.82±0.89	5.05±0.95	0.90
Male Offspring			
miR-21	1591.20±722.13	1539.55±476.72	0.47
miR-24	0.12±0.02	0.15±0.04	0.03
miR-33	0.00027±0.00004	0.00034±0.0009	0.006
miR-34a	0.66±0.15	0.57±0.10	0.36
miR-103	17.78±2.70	17.57±3.29	0.44
miR-107	0.34±0.05	0.33±0.03	0.16
miR-122a	8370.09±1788.12	18602.11±5733.86	0.001
miR-130a	0.02±0.004	0.01±0.003	0.16
miR-143	0.03±0.01	0.04±0.002	0.009
miR-let-7c	6.15±2.79	7.00±3.02	0.60
Female Offspring			
miR-21	910.20±221.59	723.52±468.95	0.14
miR-24	0.07±0.02	0.07±0.01	0.42
miR-33	0.0002±0.0001	0.0001±0.00001	0.0001
miR-34a	1.07±0.35	0.99±0.16	0.09
miR-103	11.65±1.00	15.47±2.16	0.09
miR-107	0.57±0.11	0.58±0.02	0.11
miR-122a	12872.52±4519.43	8144.62±1220.41	0.21
miR-130a	0.007±0.002	0.01±0.003	0.16
miR-143	0.02±0.006	0.02±0.002	0.16
miR-let-7c	12.86±3.90	6.2±0.64	0.06

Values are means ± SEM, n = 9-11. In fathers, miR-21 and miR-122a were log transformed for analysis. In males, miR-21, miR-107 and miR-let-7c were log transformed for analysis.

5.4.5 Gut microbiota and short chain fatty acids are impacted by paternal HF/S+M consumption intergenerationally

Paternal methyl donor supplementation with HF/S diet impacted offspring fecal microbiota as seen by 16S rRNA sequencing of the V3 and V4 region. There were no differences in alpha diversity at weaning in fathers or offspring (Table 5.8), however, at 9 weeks of age,

fathers supplemented with methyl donors showed significantly higher alpha diversity compared to the HF/S group as seen by Chao1 (p=0.049) (Table 5.9). Interestingly, the opposite was observed in female adult offspring at 9 weeks of age, where HF/S+M group displayed reduced alpha diversity compared to HF/S group across all three alpha diversity indices (p<0.01) (Table 5.9). None of these differences persisted to 12 or 15 weeks of age in fathers or offspring (Table 5.10). Only a trend towards a difference in beta diversity in fathers at 12 weeks of age were observed (R^2=9.1%, p=0.055) (Figure 5.4A) while no differences were found in offspring at any age (Figure 5.4B-C). Linear discriminant analysis effect size (LEfSe) showed that fathers fed a HF/S+M had increased relative abundance of Actinobacteria, Adlercreutzia, Coriobacteriales, and Eggerthellaceae at 12 weeks of age compared to HF/S (Figure 5.4D). Adult HF/S male offspring at 15 weeks of age, showed an increased abundance of Clostridiales compared to HF/S+M (Figure 5.4E). HF/S+M males showed an increased abundance of Defluviitalaceae compared to HF/S (Figure 5.4E). Adult HF/S+M females exhibited an increased abundance of Butyrivibrio (Figure 5.4F).

Table 5.8 Alpha Diversity at 3 Weeks of Age

	HF/S	HF/S+M	p-value
Paternal			
Chao1	269.77±11.47	257.04±13.85	0.49
Shannon	3.96±0.07	3.85±0.12	0.46
Simpson	0.95±0.004	0.94±0.01	0.47
Male Offspring			
Chao1	123.75±9.16	150.53±19.24	0.25
Shannon	3.77±0.05	3.83±0.08	0.50
Simpson	0.96±0.003	0.96±0.004	0.86
Female Offspring			
Chao1	179.27±20.31	173.33±11.55	0.80
Shannon	3.78±0.06	3.72±0.06	0.56
Simpson	0.95±0.003	0.95±0.004	0.29

Values are means ± SEM, *n* = 8-13.

Table 5.9 Alpha Diversity for fathers and offspring at 9 weeks of age

	HF/S	HF/S+M	p-value
Paternal			
Chao1	209.19±8.37	265.78±26.87	0.049
Shannon	3.87±0.09	3.95±0.13	0.61
Simpson	0.95±0.004	0.95±0.01	0.99
Male Offspring			
Chao1	182.82±28.3	168.67±7.09	0.63
Shannon	3.76±0.08	3.83±0.08	0.54
Simpson	0.95±0.01	0.95±0.01	0.55
Female Offspring			
Chao1	192.31±11.22	139.44±11.48	0.004
Shannon	3.99±0.06	3.67±0.07	0.002
Simpson	0.96±0.003	0.95±0.004	0.004

Values are means ± SEM, *n* = 8-13.

Table 5.10 Alpha Diversity- Week 12 for Fathers and Week 15 for offspring

	HF/S	HF/S+M	p-value
Paternal			
Chao1	191.48±23.52	153.09±21.42	0.24
Shannon	4.00±0.1	3.77±0.11	0.14
Simpson	0.96±0.003	0.95±0.004	0.11
Male Offspring			
Chao1	140.53±8.79	153.39±16.61	0.52
Shannon	3.89±0.09	3.73±0.14	0.36
Simpson	0.96±0.004	0.94±0.01	0.20
Female Offspring			
Chao1	148.47±23.18	154.35±16.67	0.86
Shannon	3.64±0.13	3.64±0.17	0.98
Simpson	0.95±0.01	0.94±0.01	0.55

Values are means ± SEM, *n* = 8-13.

The Venn diagrams show that paternal HF/S (Figure 5.4G) and HF/S+M (Figure 5.4H) groups have a total of 756 and 638 amplicon sequence variants (ASVs) respectively at 12 and 15 weeks of age in fathers and offspring. Of those ASVs, offspring shared 45% with fathers in the HF/S group and 41% among HF/S+M.

Paternal HF/S intake supplemented with methyl donors altered cecal short chain fatty acids (SCFA) concentrations in fathers and offspring. Paternal HF/S+M significantly increased cecal butyrate (Figure 5.4I) which persisted in adult male offspring (Figure 5.4J). Adult female

offspring showed significantly reduced isobutyrate and elevated isovalerate in the HF/S+M

group c

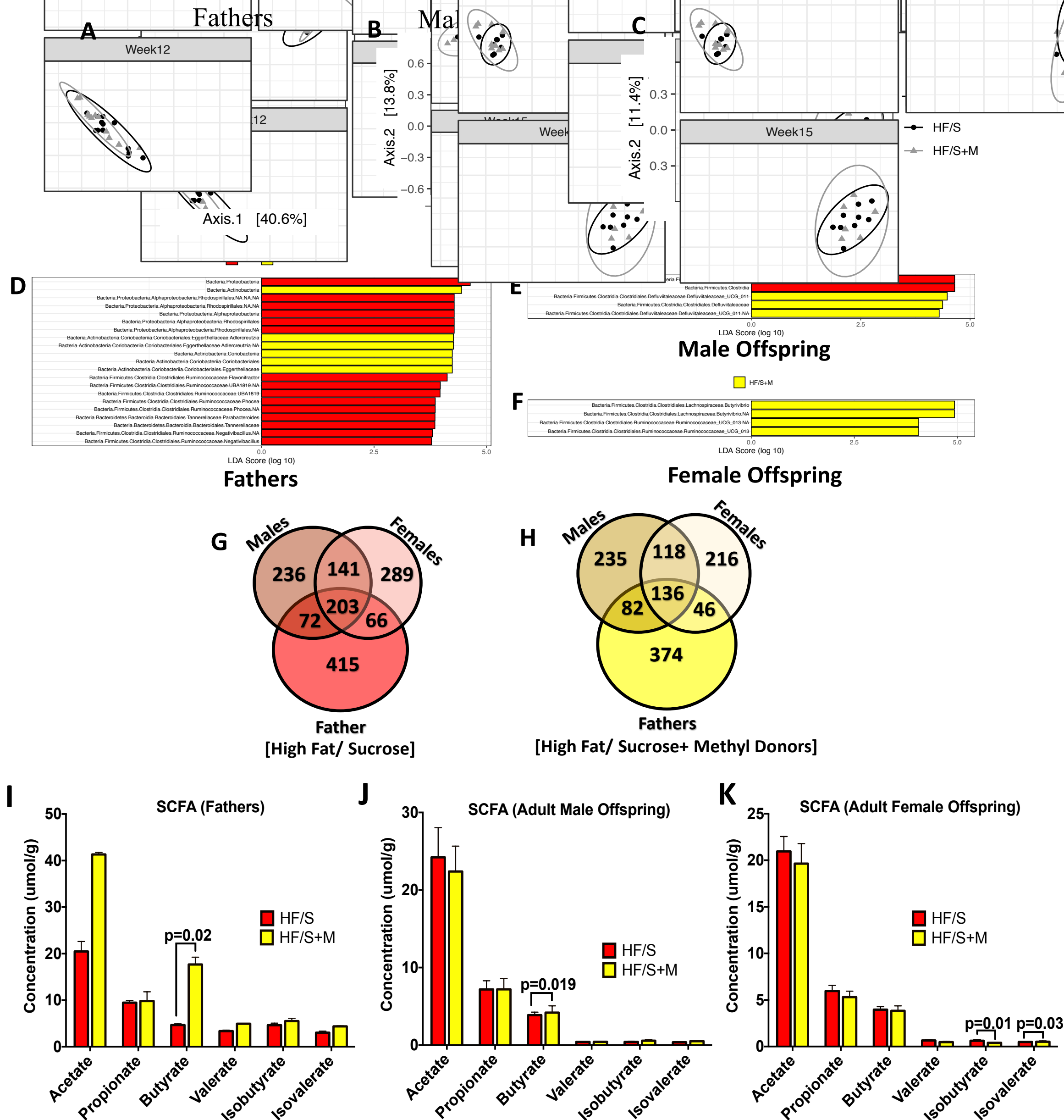

Figure 5.4 Fecal microbiota comparisons of fathers fed HF/S and HF/S+M and the intergenerational similarities in male and female offspring. Beta Diversity of A) Paternal at 12 weeks of age, B) Male offspring and C) Female offspring at 15 weeks of age, calculated with principal coordinates analysis (PCoA) using a Bray-Curtis distance matrix. LefSe comparison of D) Fathers at 12 weeks of age, E) Male offspring and F) Female offspring at 15 weeks of age. Venn diagram comparison of ASVs that overlap between fathers and offspring and those only present in offspring, stratified by sex and G) HF/S diet H) HF/S+M diet. Cecal Short Chain Fatty Acids in: I) paternal, J) adult male offspring and K) adult female offspring at euthanasia. Values are means ± SEM, *n*= 8-13 (p<0.05).

5.5 Discussion

We present novel evidence that supplementing a paternal HF/S diet with a methyl-donor cocktail of betaine, choline, folic acid and vitamin B12 before conception reduced energy intake, fasting serum insulin and insulin resistance alongside alterations in gut microbial signatures, epigenetic markers modulating metabolism, and reproductive outcomes in fathers. Paternal HF/S+M also appears to reduce fat mass and alter microRNA and gut microbial signatures in adult male and female offspring compared to HF/S intake alone (see Figure 5.5 for a summative schematic).

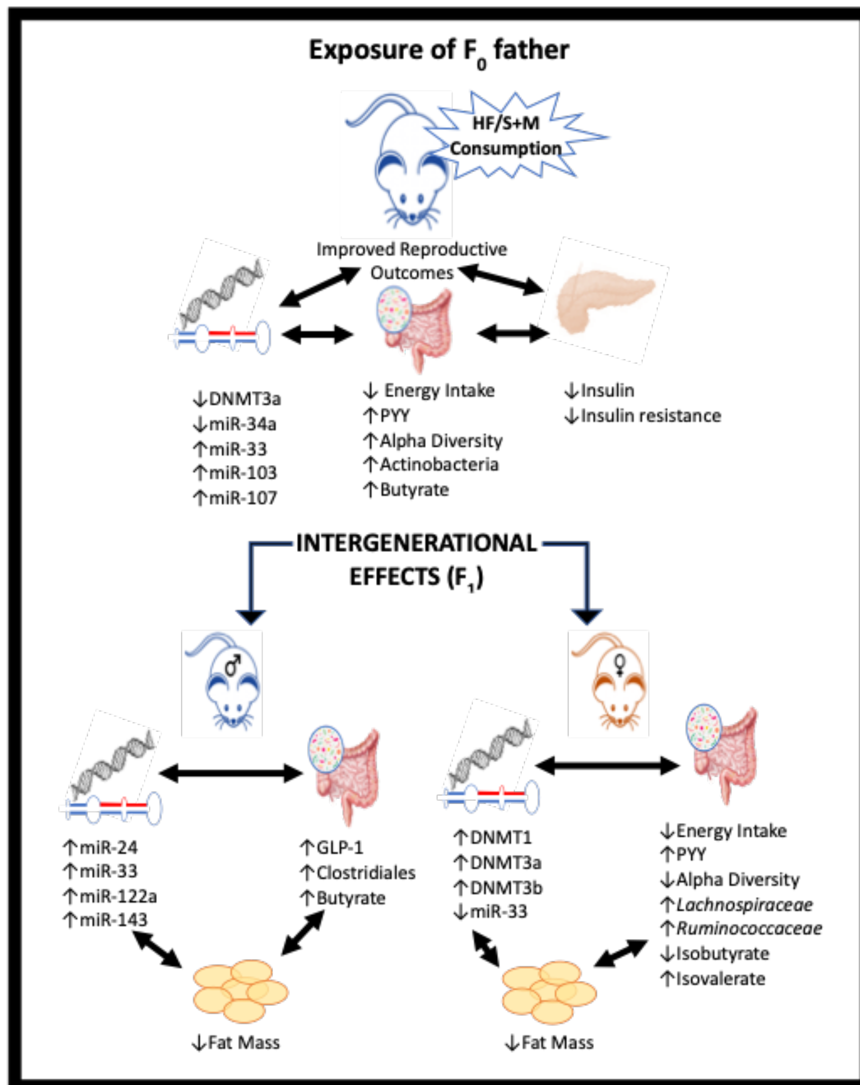

Figure 5.5 Overall summary of the major findings in fathers and adult offspring following a HFS diet, supplemented with a methyl donor cocktail of betaine, choline, folic acid and vitamin B12. All data are compared to a control HF/S diet.

It has been postulated that epigenetic changes owing to malnutrition *in utero* have a substantial impact on transgenerational metabolic abnormalities[360]. Etiological studies have demonstrated that paternal BMI affected offspring BMI in a way that was independent of, but additive to, the BMI of the mother[50,361]. These findings influenced the work of a seminal study by Masuyama and colleagues in 2016[8]. They examined whether high-fat diet-induced-obesity in fathers before conception would impact the metabolic status of offspring, as seen by the epigenetic status of the *adiponectin* and *leptin* gene promotors in a mouse model[8]. They also investigated whether a normal, control diet would reverse the epigenetic changes in subsequent generations[8]. In the F1 generation, epigenetic changes were diminished, whereas in the F2 generation, epigenetic changes caused by a paternal high fat diet, were completely absent in male offspring[8]. Based on the reversal seen with a control diet, we sought to examine whether a HF/S diet supplemented with methyl donors could attenuate some of the detrimental metabolic outcomes caused by a pre-conception paternal HF/S diet in the F1 progeny. Our model uniquely shows that a paternal high fat diet supplemented with methyl-donors attenuates the accumulation of fat mass in adult male and female offspring. This was accompanied by changes in the expression of DNMTs and miRNAs, albeit differently according to sex.

Previous work has demonstrated that epigenetic changes, including DNA methylation play an important role in modulating gene expression[12,362] and have key roles in obesity-associated gene expression by governing transcriptional dysregulation[363,364]. One such transcriptional dysregulation occurs in the expression of genes involved in fat metabolism causing the decreased expression of adiponectin in adipose tissue of high-fat diet-induced obese models(DIOs) [365]. Given that the gut microbiota participates in epigenetic processes through its metabolites, such as folate and SCFAs, Yao et al[365] sought to determine if altering the gut microbiota with antibiotics affected the transcriptional expression of obesity-related genes such as adiponectin through epigenetic regulation. They showed that antibiotics given to DIO animals upregulated the expression of adiponectin in adipose tissue which was accompanied by a reduction in DNA methylation of the adiponectin promoter and downregulation of DNMT1 and 3a[365]. Previous work from the same lab found that SCFA supplementation in a DIO model reversed the transcriptional alterations in adiponectin in adipose tissue, which was again mediated by reduced expression of DNMTs including DNMT3a[366]. This is consistent with our

findings, wherein methyl-donor supplemented HF/S fathers showed reduced expression of DNMT3a in adipose tissue. Although, this was not accompanied by reductions in adiposity directly in the fathers, we did see reductions in offspring fat mass, suggesting a potential heritable influence of reduced DNMT3a expression in fathers and a subsequent reduction in adiposity in offspring. Although both male and female offspring had reduced fat mass, it is interesting that female HF/S+M offspring showed increased expression of DNMTs including: 1, 3a and 3b, suggesting a sex-specific effect of one carbon metabolism. This might be explained by sex-specific disparities in epigenetic machinery like DNA methylation and histone that has been previously reported[367,368]. Alternatively, the sex difference observed may be due to the established discrepancy in fat distribution and homeostasis between males and females[369], which may also alter the epigenetic machinery and function in adipose tissue, including DNA methylation.

The gut microbiota produces SCFA which interact with the host epigenetic machinery, including DNA and histones, which are able to influence the host's epigenetic state and function[370,371] including lipid metabolism and ultimately weight regulation. The gut microbiota synthesize choline, thiamin (vitamin B1), vitamin B2, nicotinic acid (vitamin B3), pantothenic acid (vitamin B5), pyridoxine (vitamin B6), biotin (vitamin B7), folate, and B12[372,373]. Whether or not dietary supplementation with these methyl donors has the ability to correct some of the microbial dysbiosis observed with HF/S consumption is not well understood, particularly with regards to intergenerational effects. In fathers, we saw increased relative abundance of *Adlercreutzia,* Coriobacteriales, and *Eggerthellaceae*. Coriobacteriales exert saccharolytic activity (fermentation of carbohydrates) in the gut[374], which could reflect the higher butyrate concentrations we detected in the cecal matter of fathers and male offspring. There has been a suggestion that members of the order Coriobacteriales may be indicators of a healthy gut microbiota community[375]. Of interest, supplementing a HF/S diet with polyphenol rich cranberry powder increased the relative abundance of both Coriobacteriales and *Eggerthellaceae* in mice[376]. *Eggerthellaceae*, which has been linked to positive effects in lipid metabolism, was also inversely correlated with body weight gain in mice[376]. Although relatively little is known about the genera *Adlercreutzia*, it has been shown in pubertal human subjects to be positively associated with testosterone[377]. *Adlercreutzia* have also been shown

to metabolize phytoestrogens[378] and it is therefore possible that this bacteria could be affected by sex hormones, although this warrants further investigation.

Offspring gut microbiota did not differ as demonstrated by a PCoA beta diversity assessment and little difference was observed at lower taxonomic levels as seen by LEfSe analysis. This might be indicative of the diminished metabolic influence of a paternal HF/S diet in the F1 progeny, as discussed in previous work[8]. Interestingly, however, males showed increased abundance of *Defluviitaleaceae*, which is reported to increase the statin efficacy of Rosuvastatin, a blood lipid-lowering agent in hyperlipidemia in humans[379]. Therefore, although very little is currently known about this bacteria, it is possible that the increased *Defluviitaleaceae* in male offspring could have contributed to altered lipid metabolism and reduced body fat mass.

MicroRNAs (miRNAs) are important post-transcriptional regulators of gene expression that have been implicated in pathways underpinning metabolic disease in multiple organs including the pancreas, liver, adipose tissue, and skeletal muscle[380]. Here, HF/S+M fathers exhibited decreased hepatic expression of miR-34a and increased miR-103, miR-107 and miR-33. HF/S+M female offspring similarly showed a trend towards decreased miR-34a in liver tissue (p=0.09). MiR-34a is an important mediator in lipid homeostasis in the liver[381]. MiR-34 is characteristically elevated in the liver of DIO mice[382]. For instance, mice treated with anti-sense oligonucleotides that target miR-34a, experienced improvements in glucose tolerance and insulin resistance, suggesting miR-34 to be a crucial target to mitigate insulin resistance. We showed that oral supplementation of a methyl-donor cocktail in our DIO rat model elicited similar results, wherein our HF/S+M displayed improvements in insulin resistance. Additionally, Tryndyak and colleagues[165] showed that circulating miR-34a is the strongest predictor of non-alcoholic fatty liver disease-specific liver pathomorphology, wherein increased levels of plasma miR-34a increase overall liver pathology, as measured by total hepatic lesions and severity[165]. Although not reaching significance, we did observe a 40% decrease in triglyceride concentrations in the livers of HF/S+M fathers compared to HF/S.

MiR-103 and -107 have been previously identified as negative regulators of insulin sensitivity[383], and increased hepatic expression has been observed in both humans and murine models of metabolic disease and/or high fat diet consumption[383]. However, there are also studies suggesting that increasing miR-103 and miR-107 expression is beneficial. For example,

obesity induced by a high-fat, high-cholesterol diet in mice decreased the expression of hepatic miR-103 and -107, while simultaneously increasing fatty acid synthetase protein (FASN), a modulator of fatty acid synthesis [382]. FASN is a putative miR-107 target. Furthermore, Zhang et al[382] showed that overexpression of miR-103 in mice fed a diet high in fructose and sucrose alleviated hepatic lipid accumulation and suppressed lipogenesis in the liver. We postulate that the increased expression of HF/S+M paternal hepatic miR-107 in our study could have reduced FASN, contributing to the observed reduction in fat mass in male and female adult offspring.

MiR-33 is yet another important regulator of lipid metabolism [383]. Inhibiting miR-33 function *in vivo* increases circulating high-density lipoprotein concentrations and lowers very-low-density lipoprotein and triglycerides by increasing the expression of fundamental enzymes involved in fatty acid oxidation[386]. Adult female offspring of HF/S+M fathers exhibited reduced expression of miR-33, potentially explaining the concurrent reduction in adiposity in females. Additionally, miR-33 plays an important role in regulating insulin signaling by targeting insulin receptor substrate 2, a vital component of insulin signaling in the liver[387]. Conversely, miR-33-knockout in mice showed deleterious outcomes, including increased obesity, insulin resistance and food intake[388]. This study elicited a miR-33 conundrum, which was similarly observed in our study. Methyl donor supplementation increased hepatic miR-33 expression in fathers and adult male offspring but reduced it in females. In fathers, reduced miR-33 expression occurred alongside reduced insulin resistance while in offspring the disparate expression was associated with reduced adiposity in both male and female HF/S+M offspring. We also observed reduced energy intake in HF/S+M fathers at 12 weeks of age and 9 weeks of age in female offspring, which could be attributed in part to increased levels of appetite regulating gut hormones like PYY. It is worth noting, with the exception of miR-33 in fathers and adult male offspring, we observed differentially expressed microRNAs intergenerationally. This may be due to the fact that the epididymis, containing mature sperm, facilitates altered microRNA transfer enacted by epididymosomes[389]. Future research should assess this epididymis-specific microRNA alteration *in vivo.*

In male HF/S+M offspring, we saw a substantial increase in miR-122a. Benatti *et al.*[390] showed that maternal high-fat diet consumption modulates hepatic lipid metabolism and microRNA expression in offspring, most notably showing reductions in miR-122a in DIO mice. They concluded that maternal high fat diet impairs offspring lipid metabolism and miRNA

expression which may have lasting metabolic impairments in adulthood[390]. We found that paternal HF/S supplemented with methyl donors ameliorated these effects and in turn increased miR-122a in male offspring.

To our knowledge, no human studies have been conducted that examine whether paternal methyl-donor supplementation could mitigate some of the detrimental reproductive and metabolic effects of a paternal HF/S diet. Given the increasing recognition of the importance of paternal health and nutritional intake on programming metabolism in offspring, future work is warranted to determine the potential for individual methyl donors or cocktails such as we have used here to positively affect male fertility and pregnancy outcomes in humans.

5.6 Acknowledgments

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CHAPTER SIX: MICROBIOTA CHANGES IN FATHERS CONSUMING A HIGH PREBIOTIC FIBER DIET HAVE MINIMAL EFFECTS ON MALE AND FEMALE OFFSPRING IN RATS

6.1 ABSTRACT

Background: The ingestion of a diet high in prebiotic fiber has been associated with improved metabolic and gut microbial parameters intergenerationally; although studies have been limited to maternal prebiotic diet with no studies examining this effect in a paternal model.

Method: Male Sprague Dawley rats were allocated to either 1) control or 2) oligofructose-rich diet for 9 weeks and then mated. Offspring consumed a control diet until 16 weeks of age. Bodyweight, body composition, glycemia, hepatic triglycerides, gastrointestinal peptides and gut microbiota composition were measured in fathers and offspring.

Results: Paternal energy intake was reduced, while satiety inducing PYY gut peptide was increased in prebiotic versus control. Increased serum PYY persisted in female prebiotic adult offspring. Hepatic triglycerides were decreased in prebiotic fathers with a similar trend ($p=0.07$) seen in female offspring. Gut microbial composition showed significantly reduced alpha diversity in prebiotic fathers at 9 and 12 weeks of age ($p<0.001$), as well as concurrent differences in beta diversity ($p<0.001$), characterized by differences in *Bifidobacteriaceae, Lactobacillaceae* and *Erysipelotrichaceae*, and particularly *Bifidiobacterium animalis*. Female prebiotic offspring showed significantly higher alpha diversity at 3 and 9 weeks of age ($p<0.002$) and significant differences in beta diversity at 15 weeks of age ($p=0.04$). Notable differences in Bacteroidetes at 9 weeks of age in female offspring were observed.

Conclusions: Although paternal prebiotic intake before conception improves metabolic and microbiota outcomes in fathers, effects on offspring were limited with increased serum satiety hormone levels in male and female offspring and increased microbiota diversity in females.

6.2 Introduction

Substantial evidence shows that maternal, fetal and neonatal microbiota elicit transient and long lasting impacts on health that is based on the presence of the microbes themselves as well as the metabolites they produce [391–393]. Prebiotics, defined as a substrate that is selectively utilized by host microorganisms conferring a health benefit[173], have been used to beneficially modulate the gut microbiota in animal and human studies. For example, prebiotic supplementation in rodents during gestation and lactation has resulted in improvements in bodyweight, body composition, and colon length in offspring[193], reductions in immune-related incidents[194], and improved glucose tolerance, insulin sensitivity and hepatic steatosis in offspring[195]. Importantly, maternal intake of prebiotic oligofructose has been shown to selectively alter obese maternal gut microbial composition and significantly enhance the abundance of the health-promoting genera *Bifidobacterium* [174]. Maternal prebiotic intake resulted in increased satiety hormone levels and a serum metabolomics signature that suggested prebiotic supplementation of a maternal high fat/sucrose diet could reduce the insulin resistance of obese pregnant rats with benefits for their offspring[174].

Whether or not a paternal diet high in prebiotics could similarly benefit offspring health is not known but there is mounting evidence for the impact of paternal environment, including metabolism, physiology, body composition and diet on sperm quality, fetal development and offspring health into adulthood[394]. In fact, growing animal and human research from epidemiological studies have deduced that the period before conception is vital in influencing the development of health of prospective generations[394]. Mirroring female reproductive fitness, male reproductive health been associated with multiple environmental factors, including nutrition[394]. Paternal high fat diet consumption for 10 weeks before conception was shown to affect pancreatic β-cell function and impair insulin secretion and glucose homeostasis in fathers and offspring, although the detrimental effects could be attenuated if offspring consumed a control diet[264]. Furthermore, paternal low protein diet in a mouse model perturbed the expression of genes modulating hepatic lipid and cholesterol biosynthesis in offspring[395]. Still, while our understanding of the impact of paternal diet on offspring health is increasing, there remains much to be investigated. To our knowledge, no studies have examined whether paternal prebiotic supplementation with oligofructose, before conception impacts offspring health. Our

objective was to examine if a paternal prebiotic-rich diet during pre-conception affects the microbial and metabolic status of the fathers and their offspring.

6.3 Materials and Methods

6.3.1 Animal Model and Dietary Treatment

Twenty-four male Sprague Dawley rats (Charles River Laboratories, Montreal, QC, Canada) were housed in a temperature and humidity-controlled facility with a 12 hour light/ dark cycle. One day following arrival, animals were randomized to 1 of 2 nutritionally complete experimental diets: 1) control AIN-93G diet (from 3-9 weeks of age) and AIN-93M (from 10-12 weeks of age) (Dyets Inc, Bethleham, PA, USA) or 2) prebiotic (10% wt/wt oligofructose; Orafti P95, Beneo-Orafti, Mannheim, Germany). Experimental diet composition can be found in Table 6.1. At 12 of age, fathers were co-housed with a virgin female Sprague Dawley rat during the active dark cycle, with AIN-93G diet given *ad libitum*. Once a copulation plug was identified, dams were moved to a single-housed cage throughout their pregnancy and during lactation. Dams were given a control, AIN-93G diet and water *ad libitum*. Within 24 hours of birth, litters were culled to n=5 females and n=5 males. Cross-fostering with litters from the same dietary intervention group took place if litters were less than n=10. Male and female offspring from the same litter were designated n=1. Due to their young age during the study (from 3 weeks of age), fathers and offspring were co-housed with an age-matched rat in the same treatment group except when breeding took place. Offspring from 3-16 weeks of age consumed a control diet (AIN-93G and/or AIN-93M). Food intake was measured every 3 weeks, for 3 consecutive days each time. This study was approved by the University of Calgary Animal Care Committee (AC18-0074) and conformed to the *Guide to the Care and Use of Laboratory Animals*.

6.3.2 Body weight and composition

Fathers and offspring were weighed weekly. One day before euthanasia, body composition was determined using a dual energy X-ray absorptiometry (DXA) scan and software for small animals (Hologic ODR 4500, Hologic, Bedford, MA, USA). Animals were lightly anaesthetized using isoflurane to ensure stillness. Recorded measurements included: bone mineral content/density (BMC/BMD) (g and g/cm^2), fat mass(g), lean mass(g) and body fat %.

6.3.3 Oral Glucose and Insulin Tolerance Tests

Following an overnight fast, fathers (at 10 weeks of age) and offspring (at 14 weeks of age) underwent an oral glucose tolerance test (OGTT). OGTTs were performed as previously described, using a 2g/kg glucose load via oral gavage[8]. In preparation for the insulin tolerance test (ITT), fathers and offspring were fasted for 6 hours at 11 and 15 weeks of age respectively, and then given a 0.75U/kg insulin load via intraperitoneal injection. Blood glucose measurements for both OGTT and ITTs were collected at baseline and 15, 30, 60, 90 and 120 minutes following the glucose or insulin load, using a One Touch Ultra ® 2 glucose meter (Lifespan, Burnaby, Canada).

Table 6.1 Experimental diet composition from 3-9 and 10-16 weeks of age

	Control	**Prebiotic**	**Control**	**Prebiotic**
g/kg	**Weeks 3-9**		**Weeks 10-16**	
Cornstarch	397.5	357.8	465.7	419.13
Casein	200	180	140	126.0
Dyetrose	132	118.8	155	139.5
Sucrose	100	90	100	90
Soybean Oil	70	63	40	36
Alphacel	50	45	50	45
AIN-93M Mineral Mix	35	31.5	35	31.5
AIN-93 VX Vitamin Mix	10	9	10	9
L-cystine	3	2.7	1.8	1.62
Choline-Bitartrate	2.5	2.25	2.5	2.25
Oligofructose	0	100	0	100
Energy density (kJ/g)	15.7	14.8	15.1	14.2
Carbohydrate (% of kcal)	63.9	65.4	75.9	76.9
Protein (% of kcal)	19.4	18.6	14.1	13.5
Fat (% of kcal)	16.8	16.0	10.0	9.6

Control diets were AIN-93G and AIN-93M respectively for weeks 3-9 and weeks 10-16. Diets were purchased from Dyets, Inc. (Bethlehem, PA, USA). Prebiotic diets were mixed in-house by combining 900 g of control diet with 100 g of oligofructose (Orafti P95, Beneo, Mannheim, Germany).

6.3.4 Tissue and blood collection

At 12 and 16 weeks of age, fathers and offspring respectively, underwent 12 hours of food deprivation before euthanization. Animals were euthanized via overanesthetization with isofluorane, followed by decapitation. Blood was collected from the portal vein in a chilled tube containing diprotinin-A (0.034 mg/ml blood; MP Biomedicals, Irvine, CA), Sigma protease inhibitor (1 mg/ml blood; Sigma Aldrich, Oakville, ON, Canada) and Roche Pefabloc (1mg/ml of blood; Roche, Mississauga, ON, Canada). Samples were centrifuged and serum was collected

and stored in -80°C until insulin, peptide tyrosine tyrosine (PYY) and glucagon-like peptide 1 (GLP-1) analysis, using a Rat Metabolic Multiplex Array (MRDMET) (Millipore, St. Charles, MO) (Eve Technologies, Calgary, AB, Canada). To estimate insulin resistance, we used the formula HOMA-IR= [glucose (mmol/L) x insulin (mIU/mL)] / 22.5[357]. Heart, liver, kidney, cecum, colon and testes (in males) were excised and weighed to determine organ weight, relative to body weight. A liver sample from the right lobe was collected and stored in in -80°C until triglyceride assessment.

6.3.5 Triglyceride Concentration

Using 25 mg of liver, triglyceride concentrations were quantified in ug per mg of liver tissue using enzyme glycerol phosphate oxidase (GPO) reagents, according to manufacturer's guidelines (Pointe Scientific Inc., Lincoln Park, MI).

6.3.6 Gut Microbiota 16S rRNA Gene Sequencing

Paternal fecal matter was collected at baseline (3 weeks of age, prior to dietary interventions), 9 and 12 weeks of age. Offspring fecal matter was collected at weaning (3 weeks of age), 9 and 15 weeks of age. All fecal matter was stored at -80°C until analysis. Bacterial DNA was extracted using the FastDNA spin kit for feces (MP Biomedicals, Lachine, QC, Canada) and brought to a concentration of 4ng/uL. 16S rRNA gene sequencing of the V3 and V4 region took place at the Centre for Health Genomics and Informatics (University of Calgary, Calgary, AB, Canada) using the MiSeq Illumina platform (Illumina, San Diego, Ca, USA) as previously described[175,217].

6.3.7 Cecal Short Chain Fatty Acids

SCFA were measured in cecal matter collected at euthanasia from fathers and offspring as previously described[273]. Reverse-phase High Performance Liquid Chromatography (HPLC) using a c18 column containing a column guard was used to quantify the SCFA. An elution gradient of acetonitrile containing 0.05% trifluoracetic acid (8-100%) and a flow rate of 0.8mL/min over 30 minutes was maintained.

6.3.8 Statistical Analysis and Taxonomy Profiling

Statistical analysis was conducted using IBM® SPSS Statistics, version 24.0 except for 16S rRNA sequencing data. A multivariate, general linear model (GLM) was used to determine a sex effect between male and female offspring. If a sex effect was identified, males and females were analyzed separately using an independent samples t-test. Outcomes with multiple time points were analyzed using a repeated measures GLM, wherein diet was the *between-subject* factor and time was the *within-subject* factor. Identification of a significant interaction between diet and time was followed with an independent samples t-test to determine differences between dietary groups. All data was presented as mean± standard error of the mean (SEM). Significance was set at $p \leq 0.05$.

16S rRNA sequencing data was analyzed using the R statistical software as previously described[396]. Initially, data was quality filtered using the filterAndTrim, assignTaxonomy and assignSpecies functions using dada2 (version 1.10.1)[274]. The phyloseq package (version 1.24.2)[275] was used to determine diversity between groups. Alpha diversity was assessed using Chao1, Shannon and Simpson indices, where differences were analyzed using an independent T Test. Beta diversity was assessed using a principal coordinates analysis (PCoA) using a Bray-Curtis distance matrix, where a PERMANOVA was used to classify significance. LEfSe was used to determine differentially abundant features [276]. Significance was set at $p \leq 0.05$.

6.4 Results

6.4.1 No difference was observed in bodyweights or body composition intergenerationally

To assess whether there was a sex effect between male and female offspring, we conducted a multivariate GLM for all parameters. At every age and parameter (bodyweight, body composition, organ weight), a sex difference was observed, therefore males and females were assessed separately. No difference between control and prebiotic was observed for body weight in fathers (Figure 6.1A), male (Figure 6.1B) or female (Figure 6.1C) offspring. Fathers showed significantly higher bone mineral content in the prebiotic group compared to control ($p=0.02$), with a trend towards increased lean+BMC mass in fathers ($p=0.052$) and adult male offspring ($p=0.08$)(Table 6.2).

6.4.2 Larger distal gut seen with prebiotic diet

Paternal cecum and colon mass were significantly higher in the prebiotic compared to control group (Table 6.3). Adult male offspring exhibited a trend (p=0.08) towards increased testes weight in the prebiotic group (Table 6.3). Adult female prebiotic offspring showed significantly higher brain mass compared to control offspring (Table 6.3).

6.4.3 Gastrointestinal peptides were increased in fathers and male offspring

As expected, there was a significant main effect of time for food intake with intake increasing as the fathers, male offspring and female offspring aged (p<0.0005; Figure 6.1). There was also a significant interaction between time and diet among fathers, with prebiotic fathers consuming significantly fewer kcal/day at 12 weeks of age (p<0.0005; Figure 6.1D). No difference in food intake was observed in male or female offspring (Figure 6.1E and 1F).

We examined serum concentrations of glucagon-like peptide-1 (GLP-1) and peptide tyrosine tyrosine (PYY), gastrointestinal peptides associated with satiety. GLP-1 was increased in adult prebiotic male offspring compared to controls (p=0.04; Figure 6.1G). PYY was significantly increased in fathers consuming the prebiotic-rich diet (p=0.002; Figure 6.1H). Higher PYY was similarly seen in adult prebiotic female offspring (p=0.02; Figure 6.1H).

Table 6.2 Body Composition of Fathers at 12 weeks of age and offspring at 16 weeks of age

	Control	Prebiotic	p-value
Fathers			
BMC (g)	15.0±0.3	15.6±0.2	0.02
BMD (g/cm^2)	0.166±0.001	0.167±0.002	0.44
Fat Weight (g)	85.5±5.8	87.3±6.9	0.57
Lean+ BMC (g)	504.1±14.6	516.5±8.7	0.05
% Body Fat	15.0±0.9	14.3±0.9	0.97
Males			
BMC (g)	17.2±0.4	17.4±0.3	0.28
BMD (g/cm^2)	0.175±0.002	0.172±0.001	0.68
Fat Weight (g)	108.2±7.0	144.4±11.2	0.11
Lean+ BMC (g)	519.5±12.0	521.4±6.3	0.08
% Body Fat	19.5±1.6	21.0±1.4	0.60
Females			
BMC (g)	11.6±0.3	11.3±0.3	0.70
BMD (g/cm^2)	0.168±0.002	0.166±0.002	0.40
Fat Weight (g)	85.1±10.1	76.9±9.8	0.74
Lean+ BMC (g)	287.5±7.7	286.3±10.0	0.49
% Body Fat	22.4±2.0	20.5±2.2	0.96

Values are means ± SEM, *n* = 9-13. BMC, bone mineral content; BMD, bone mineral density. Male BMC was log transformed.

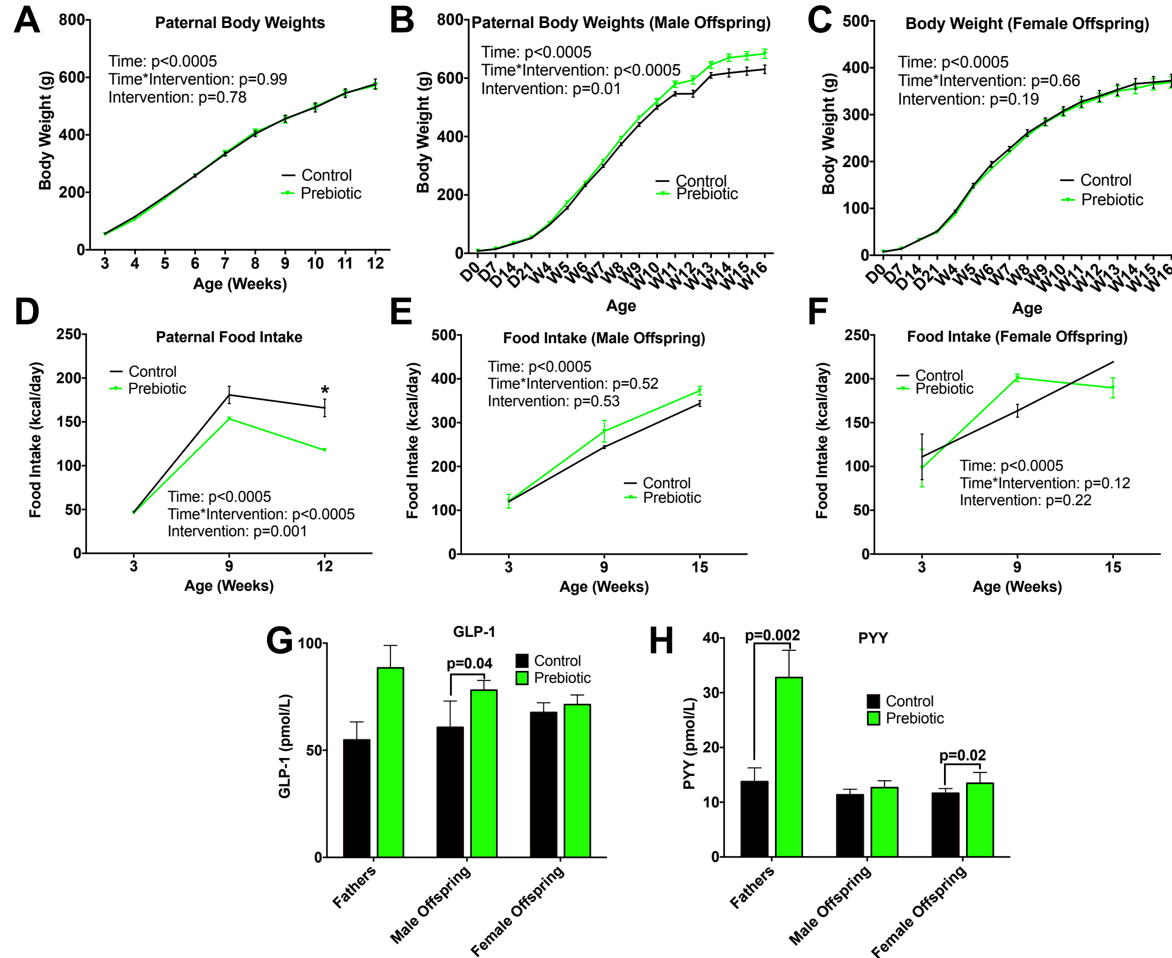

Figure 6.1 Body weight, Food Intake and Gastrointestinal Peptides. Body weight of A) fathers, B) adult male offspring and C) adult female offspring; food intake of D) fathers, E) male offspring F) female offspring; G) GLP-1 and H) PYY. Food intake was analysed using independent samples Kruskal-Wallis tests. Values are means ± SEM, *n*= 8-13. In adult offspring, there was a significant sex effect in the overall model for bodyweight (p=0.0001), food intake (p=0.0001), GLP-1 (p=0.002); therefore, subsequent analysis was performed in males and females separately. * represents a significant difference between groups, *p*<0.05.

Table 6.3 Organ weight of fathers at 12 weeks of age and offspring at 16 weeks of age

	Control	Prebiotic	p-value
Fathers			
Body Weight (g)	594.2±19.5	611.3±12.3	0.09
Organ weight (% BW)			
Heart	0.32±0.01	0.28±0.01	0.43
Liver	3.04±0.07	2.86±0.06	0.31
Kidney	0.27±0.01	0.28±0.004	0.11
Cecum	0.10±0.004	0.29±0.01	0.03
Colon	0.21±0.01	0.23±0.01	0.002
Testes	0.31±0.01	0.31±0.01	0.15
Male Offspring			
Body Weight (g)	630.5±14.4	683.6±15.9	0.02
Organ weight (% BW)			
Heart	0.27±0.01	0.26±0.01	0.40
Liver	2.69±0.01	2.72±0.01	0.87
Kidney	0.26±0.01	0.25±0.01	0.29
Cecum	0.09±0.002	0.10±0.003	0.82
Colon	0.27±0.01	0.30±0.01	0.10
Testes	0.16±0.01	0.17±0.01	0.08
Brain	0.35±0.01	0.33±0.01	0.60
Female Offspring			
Body Weight (g)	372.6±13.5	368.8±12.2	0.20
Organ weight (% BW)			
Heart	0.32±0.01	0.32±0.01	0.20
Liver	2.65±0.05	2.97±0.06	0.85
Kidney	0.27±0.01	0.28±0.01	0.71
Cecum	0.12±0.01	0.13±0.01	0.48
Colon	0.25±0.01	0.28±0.01	0.14
Brain	0.55±0.03	0.57±0.02	0.02

Values are means ± SEM, *n* = 9-11. Paternal colon weight and female kidney weight was log transformed for analysis.

6.4.4 Paternal prebiotic intake did not affect glucose or insulin homeostasis

To determine whether paternal prebiotic intake affected metabolic outcomes intergenerationally, we conducted insulin and glucose tolerance tests. There was a significant interaction between time and diet for glucose tolerance in fathers (Figure 6.2A). Due to the significant sex effect seen within both the OGTT and ITT ($p<0.0001$), male and female adult offspring were assessed separately. No differences were observed in glycemia or insulin sensitivity in male and female offspring (Figure 6.2B, 6.2C, 6.2E, 6.2F). Similarly, no difference was seen in insulin resistance in fathers or adult offspring as determined by HOMA-IR (Figure 6.2G). We did, however, find intergenerational differences in hepatic triglyceride concentrations.

Prebiotic fathers showed reduced hepatic triglyceride concentration compared to control (p=0.001), which persisted in adult female offspring as a trend towards a decrease (p=0.07) (Figure 6.2H).

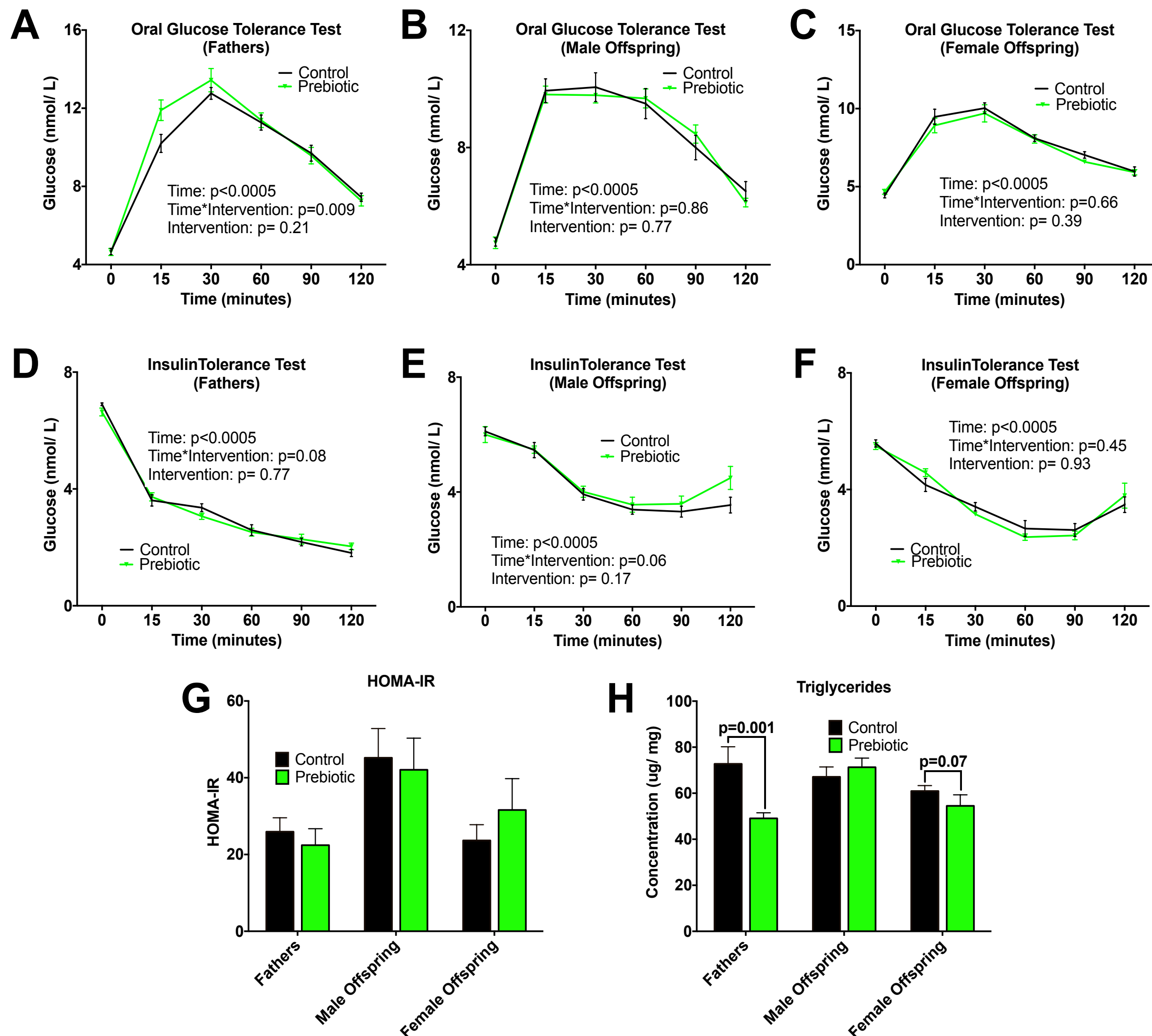

Figure 6.2 OGTT, ITT, HOMA-IR and Hepatic Triglyceride Concentrations. OGTT of A) fathers, B) adult male offspring and C) adult female offspring; ITT of D) fathers, E) male offspring, F) female offspring; G) HOMA-IR; H) triglyceride concentrations in hepatic tissue. Values are means ± SEM, n= 8-13. In adult offspring, there was a significant sex effect in the overall model for OGTT (p<0.0001), ITT (p<0.003), HOMA-IR (p=0.04), triglyceride (p=0.03), therefore subsequent analysis was performed in males and females separately, $p<0.05$.

6.4.5 Paternal high prebiotic consumption alters gut microbial signatures intergenerationally

Using 16S rRNA gene amplicon sequencing, we detected microbial community changes intergenerationally. At 3 weeks of age, prior to dietary intervention, there were no differences in paternal alpha diversity (Table 6.4), however at 9 weeks of age onwards, prebiotic fathers showed a significant reduction in alpha diversity ($p<0.001$, Table 6.4). Male prebiotic offspring at weaning showed reductions in alpha diversity as measured by the Chao1 index, however, this was not observed using Shannon or Simpson indices (Table 6.4). Female prebiotic offspring showed significantly higher alpha diversity compared to controls, as measured by Chao1, Shannon and Simpson indices at weaning. This increased alpha diversity persisted until 9 weeks of age ($p<0.002$, Table 6.4).

To assess bacterial clustering based on paternal dietary interventions, we ran a principal coordinates analysis (PCoA). As expected, fathers showed significant differences in beta diversity between dietary interventions at 9 and 12 weeks of age (R^2=72.7% and R^2=68.4% respectively, $p<0.001$, Figure 6.3A). Female offspring also showed significant differences in beta diversity at 15 weeks of age, showing larger dispersion in the prebiotic group compared to controls (R^2=11.6%, $p=0.04$, Figure 6.3C). Male offspring did not exhibit any differences in beta diversity at any age (Figure 6.3B). To further elucidate the microbial differences between dietary interventions, we used a linear discriminant analysis effect size (LEfSe) tool.

At the phylum level, Actinobacteria were the only significantly increased bacteria in fathers at 9 and 12 weeks of age (Table 6.5). This increase was owing to the increased abundance of the *Bifidobacterium* genera 9 and 12 weeks of age, showing a notable increase in *Bifidobacterium animalis* species. Furthermore, *Lactobacillaceae* and *Erysipelotrichaceae* were increased, with two prominent generas within each family of bacteria- *Pediococcus* and *Streptococcus* in the *Lactobacillaceae* family and *Faecalicoccus* and *Facecalitalea* belonging to the Erysipelotrichaceae family. At 12 weeks of age *Enorma* was also significantly increased in prebiotic fathers (Table 6.5).

Female offspring at weaning showed an increase in the Tenericutes phylum driven almost entirely by the genus *Anaeroplasma*, which was also increased in prebiotic females at weaning. Female offspring showed significant increases in Bacteroidetes phylum at 9 weeks of age (Table

6.5). No differences based on paternal diet were seen in male or female offspring at 15 weeks of age. (Table 6.5)

Table 6.4 Alpha Diversity at 3 different age groups in fathers and male and female offspring

	Control	Prebiotic	p-value
3 Weeks of Age			
Fathers			
Chao1	294.05±24.13	282.82±27.96	0.75
Shannon	3.93±0.08	3.85±0.11	0.65
Simpson	0.94±0.01	0.94±0.01	0.52
Male Offspring			
Chao1	181.01±21.85	111.38±15.29	0.02
Shannon	3.97±0.07	3.77±0.08	0.06
Simpson	0.96±0.003	0.96±0.003	0.68
Female Offspring			
Chao1	210.13±37.54	335.67±30.16	0.002
Shannon	3.94±0.09	4.22±0.06	0.0005
Simpson	0.96±0.003	0.96±0.002	0.022
9 Weeks of Age			
Fathers			
Chao1	225.34±14.2	44.64±3.51	0.001
Shannon	3.75±0.08	2.27±0.08	0.001
Simpson	0.94±0.004	0.82±0.01	0.001
Male Offspring			
Chao1	120.01±24.76	138.22±16.55	0.18
Shannon	3.42±0.13	3.62±0.06	0.069
Simpson	0.93±0.01	0.94±0.003	0.070
Female Offspring			
Chao1	124.93±11.43	166.25±15.58	0.002
Shannon	3.45±0.08	3.72±0.08	0.000001
Simpson	0.93±0.006	0.95±0.004	0.000001
12 & 15 Weeks of Age			
Fathers			
Chao1	149.09±10.88	39.36±2.23	0.000001
Shannon	3.76±0.06	2.51±0.04	0.000001
Simpson	0.95±0.002	0.87±0.01	0.000001
Male Offspring			
Chao1	109.62±7.41	107.44±15.85	0.10
Shannon	3.45±0.10	3.58±0.17	0.059
Simpson	0.93±0.01	0.94±0.01	0.13
Female Offspring			
Chao1	156.80±19.58	168.50±29.49	0.85
Shannon	3.65±0.13	3.76±0.13	0.78
Simpson	0.94±0.01	0.95±0.01	0.79

Values are means ± SEM, n = 9-11.

6.4.6 Paternal prebiotic intake affects offspring SCFA in cecal matter

No differences were seen in cecal concentrations of acetate, propionate, butyrate, isobutyrate, isovalerate and valerate between dietary groups in fathers at 12 weeks of age (Figure 6.3A). Male prebiotic offspring showed a significant increase in isovalerate at 16 weeks of age

acetate at

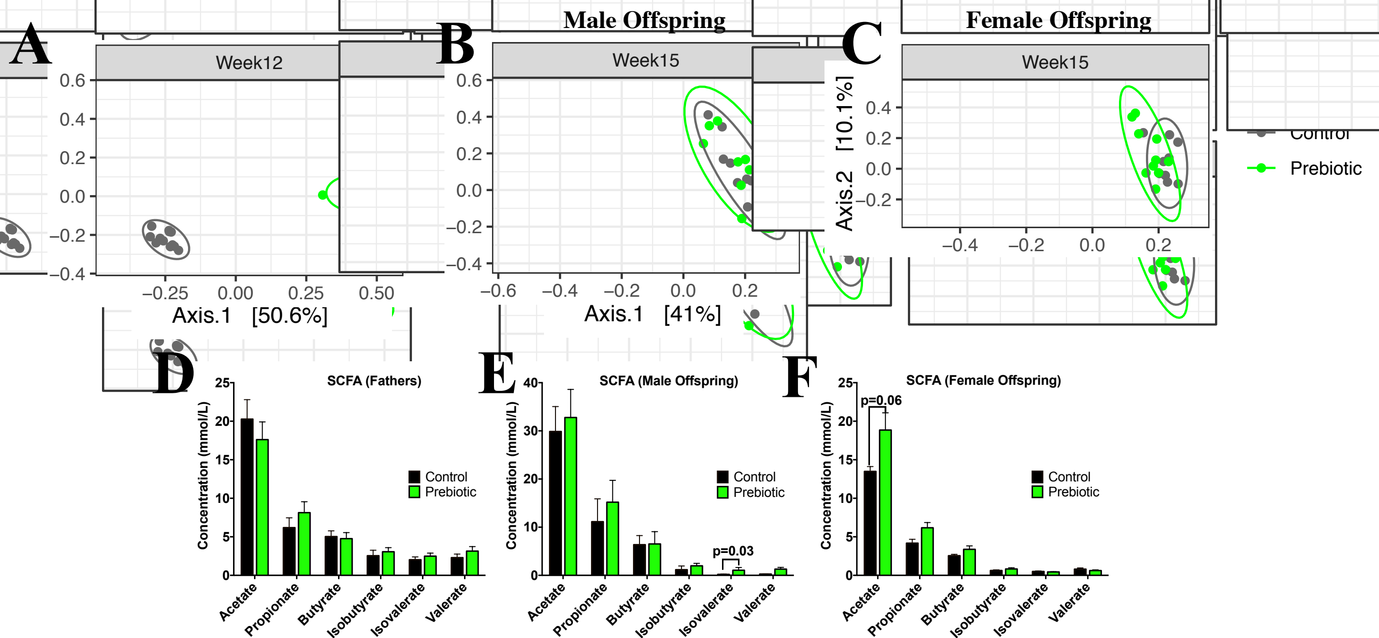

Figure 6.3 Fecal microbiota comparisons of fathers fed prebiotic or control diet and the intergenerational similarities in male and female offspring. Beta diversity of A) paternal at 12 weeks of age, B) male offspring and C) female offspring at 15 weeks of age, calculated with principal coordinates analysis (PCoA) using a Bray-Curtis distance matrix. Cecal short chain fatty acids in: D) fathers, E) adult male offspring and F) adult female offspring at euthanasia. Values are means ± SEM, n= 8-13 (p<0.05).

Table 6.5 Fecal microbiota summary in prebiotic fathers and offspring

Age	Phylum	Order	Family	Genus	Species	LDA Score
Fathers						
	[Firmicutes]	[Lactobacillales]	*[Lactobacillaceae]*	*Pediococcus*		4.7
	[Firmicutes]	Erysipelotrichales	*Erysipelotrichaceae*	*Faecalicoccus*	*pleomorphus*	4.4-4.6
9	**[Firmicutes]**	Erysipelotrichales	*Erysipelotrichaceae*	*[Facecalitalea]*	*cylindroides*	4.2-4.4
	[Proteobacteria]	[Betaproteobacteriales]	*[Burkholderiaceae]*	*Parasutterella*	*secunda*	3.9
	Actinobacteria	Bifidobacteriales	*Bifidobacteraceae*	*Bifidobacterium*	*animalis*	5.4-5.5
	[Firmicutes]	[Lactobacillales]	*Enterococcaceae*	*Enterococcus*	-	4.3
	[Firmicutes]	Erysipelotrichales	*Erysipelotrichaceae*	*Faecalitalea*	*cylindroides*	4.8-5.1
12	**[Firmicutes]**	Erysipelotrichales	*Erysipelotrichaceae*	*Faecalicoccus*	*pleomorphus*	4.8
	[Firmicutes]	[Lactobacillales]	*[Lactobacillaceae]*	*Pediococcus*	-	4.7
	Actinobacteria	Bifidobacteriales	*Bifidobacteriaceae*	*Bifidobacterium*	*animalis*	5.4-5.5
	Actinobacteria	[Coriobacteriales]	*Coriobacteriaceae*	*Enorma*	-	4.4-5.5
Male Offspring						
3	**No Differences**					
9	**[Firmicutes]**	[Clostridiales]	*Christensenellaceae*	-	-	4.5
	[Firmicutes]	[Lactobacillales]	*Streptococcaceae*	-	-	4.4
Female Offspring						
	[Firmicutes]	[Clostridiales]	*[Lachnospiraceae]*	*Butyrivibrio*	-	4.1
	[Firmicutes]	[Clostridiales]	*[Ruminococcaceae]*	*Negativibacillus*	-	3.5
3	**[Firmicutes]**	[Clostridiales]	*[Ruminococcaceae]*	*Candidatus Soleaferrea*	-	3.1
	[Firmicutes]	[Clostridiales]	*[Ruminococcaceae]*	*Anaerotruncus*	*colihominis*	3.0-3.1
	[Bacteroidetes]	[Bacteroidales]	*[Bacteroidaceae]*	*[Bacteroides]*	*salanitronis*	4.2
	Tenericutes	Anaeroplasmatales	*Anaeroplasmataceae*	*Anaeroplasma*	-	3.2-3.3
	[Firmicutes]	[Clostridiales]	*[Ruminococcaceae]*	*Ruminoclostridium_1*	-	3.9
	[Firmicutes]	[Clostridiales]	*[Lachnospiraceae]*	*Eisenbergiella*	-	3.8
9	**[Firmicutes]**	[Erysipelotrichales]	*[Erysipelotrichaceae]*	*Holdemania*	*filiformis*	3.8
	Bacteroidetes	Bacteroidales	*[Bacteroidaceae]*	*[Bacteroides]*	*barnesiae*	3.9-4.7
	Proteobacteria	-	-	-	-	4.0

Data derived from LefSe analysis. [] is indicative of taxonomy that is not significant.

6.5 Discussion

Although clear beneficial metabolic effects have been observed for both mother and offspring with maternal prebiotic intake during gestation and lactation[41,174,193–195], our findings suggest that paternal prebiotic intake before conception, improves metabolic and gut microbial status in fathers but has only limited impact on offspring health. The effects seemed to be largely confined to increased serum PYY, a trend towards decreased hepatic triglyceride concentrations and increased cecal acetate in female offspring and only increased serum GLP-1 in male offspring. Minimal gut microbiota alterations were seen in male and female prebiotic offspring compared to control offspring.

We did not observe any differences in bodyweight or adiposity in fathers consuming oligofructose, which is not entirely consistent with previous work in rodents although it is important to note that the majority of studies showing reduced body fat involves the addition of a prebiotic to an obesogenic high fat/high sucrose diets rather than a control diet such as we did[175]. It is possible therefore, that the metabolic impact of paternal oligofructose consumption could be more evident in an obese paternal model where fathers are consuming a high fat/high sucrose diet although this remains to be examined.

Despite the lack of effect of paternal oligofructose intake on body weight or body composition, we did see a notable decrease in hepatic triglyceride concentrations in fathers and female offspring. The neutral storage form of fatty acids are triglycerides which takes place mostly in hepatocytes[397]. The liver is the primary organ that modulates lipid homeostasis using complex biochemical, signaling and cellular mechanisms[397]. In a healthy subject, the liver processes vast amounts of fatty acids and only stores a small amount as triglycerides[398]. Excess triglyceride accumulation is typical of diseased states that affect the liver, including type 2 diabetes, dyslipidemia as well as increased incidence of insulin resistance. Nevertheless, we did not observe any differences in measures of insulin resistance, as determined by HOMA-IR, OGTT or ITT, which may not be surprising given that the rats were all fed a control diet and this was not an obese, insulin resistant model which might make differences more apparent between groups.

Our paternal microbial results reflect that of multiple animal and human studies over the past few decades, showing that prebiotic intake promotes the proliferation of *Bifidobacterium* [174,174,217,399,400]. Specifically, we saw an increased abundance of *B. animalis*, a species

previously linked to reductions in or slowing down the accumulation of adiposity[401] and low-grade, chronic and systemic inflammation[402]. Prebiotic consumption is also typically accompanied by the proliferation of *Lactobacillus*[403], which was similarly consistent with our findings in fathers. This is a biological advantage, since *Lactobacillus* microbes, like *L. plantarum*[404] and *L. reuteri*[405] are natural producers of B vitamins, including B1-3, B6, B8, B9 and B12, all of which play a vital role in immune regulation and metabolic health.

Prebiotics, and particularly chicory root-derived oligofructose and inulin have been shown to increase the L cell numbers in the distal gut which are responsible for the production of the satiety hormones GLP-1 and PYY[183,406,407]. This fits with the increased serum PYY and reduced energy intake we observed in fathers consuming oligofructose in our study. Interestingly, male offspring of prebiotic fathers had increased GLP-1 while female offspring had increased PYY, although this did not translate into reduced energy intake in either sex.

Given that prebiotics are known to exert a substantial effect on gut microbiota[405], it is not surprising that the fathers who directly consumed the oligofructose would have substantial shifts in their gut microbiota compared to controls. What is more intriguing is that we also observed some although limited microbiota compositional shifts in their offspring. First, female offspring showed significantly increased alpha diversity compared to controls at weaning and 9 weeks of age. Female prebiotic offspring at weaning showed a significant increase in *Anaeroplasma*. *Anaeroplasma* belongs to the Tenericutes phylum, which has been associated with beneficial effects on gastrointestinal (GI) health, modulating intestinal integrity[408]. In instances of GI inflammation in previous work, Tenericutes were substantially reduced[407]. Moreover, a reduced abundance of *Anaeroplasma* spp. has been associated with fecal hardness and gut microbial dysbiosis[409].

Female offspring at 9 weeks of age showed enriched microbial composition of Bacteroidetes. Sonnenburg and colleagues[410] showed significantly increased abundance of species belonging to the Bacteroidetes phylum, specifically *Bacteroides* spp., which proliferated in response to prebiotic fructans. Specifically, *B. caccae*, *B. fragilis*, *B. ovatus*, *B. unofrmis*, and *B. vulgatus* all increased[410]. Importantly, bacterial species feed on non-digestible dietary fibers, like oligofructose, producing metabolites like SCFA[411]. SCFA confer beneficial effects on the intestinal mucosa[411]. Members of the Bacteroidetes phylum primarily produce acetate and propionate[412]. Female offspring in our study, although they did not directly consume the

oligofructose, showed increased abundance of the Bacteroidetes phylum as well as a concurrent increase in cecal acetate levels. Acetate plays a role in cholesterol metabolism and lipogenesis as well as satiety regulation, and has recently been shown to play a role in the browning of white adipose tissue[413,414]. One study in a rabbit model showed that acetate decreased hepatic triglyceride concentration by inhibiting fatty acid synthesis and promoting fatty acid oxidation[415]. They also found beneficial effects of acetate on skeletal muscle and adipose tissue triglyceride levels and fat content[415]. This may provide an explanation of the reduction in triglyceride content in liver in female offspring who also exhibited increased acetate levels. Moreover, a large correlation cohort involving 893 subjects, showed that fecal alpha diversity was negatively correlated with triglycerides[416], which may further explain the pattern ($p=0.07$) of decreased hepatic triglycerides we saw in female offspring.

Multiple studies have assessed the impact of maternal prebiotic intake on offspring health[174,195]. This study expands the parental impact of prebiotic diets to also include father's intake of oligofructose before conception. The effects of the direct consumption of prebiotics by fathers is consistent with the known bifidogenic and satiety-promoting effects of oligofructose. In offspring there were relatively few metabolic changes but female offspring were impacted to a greater extent than males. Future work is warranted to assess the sex-dependent intergenerational transmission of microbial and metabolic impacts of parenteral diets.

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CHAPTER SEVEN: CONCLUSION

7.1 Introduction

Obesity worldwide has nearly tripled since 1975, where 39% of adults 18 years or older are overweight and 13% are obese[417]. Childhood obesity rates have similarly risen markedly in the last 30 years and the numbers are still rising. Given that genetic changes in human populations occur too slowly to be responsible for the obesity epidemic, it is likely the complex interplay between environmental, sociocultural, physiological and epigenetic factors that has contributed to rising obesity rates. Epigenetic changes induced by modern regimes of excess energy intake, excess fat intake, lack of exercise and an increase in stress is one recognized contributor to obesity[418]. Many other factors have been identified that contribute to the increased incidence of obesity including formula-feeding instead of breastfeeding as well as paternal experiences before conception. Additionally, animal[312,419] and human[420–423] studies have implicated the gut microbiome in the pathogenesis of obesity. Given that the gut microbiota is highly malleable to dietary influence, nutritional interventions have been proposed as one strategy to target the gut microbiota and potentially reduce obesity.

Breastmilk is the optimal first and primary nutrient source for the infant. As such, it is evident that deviations in the infant's first nutrient source from this gold standard might contribute to obesity or the development of metabolic syndrome in adulthood[121]. Breastmilk is abundant in nutrients, vitamins and bioactive compounds that affect infant growth and development. Among these bioactive molecules are human milk oligosaccharides (HMOs). Nearly a century ago, scientists postulated that oligosaccharides in human milk might serve as a growth factor that enrich healthy *bifidus* gastrointestinal bacteria in breast fed infants[135]. Sela and colleagues[140] sequenced the genome of *Bifidobacterium longum* subsp. *infantis* (*B. infantis*), a bacterium known to flourish when HMOs are the only carbohydrate source. They found that *B. infantis* contains entire gene clusters that control the expression of specific glycosidases, sugar transporters and glycan-binding proteins for HMO utilization, suggesting a critical functional benefit of the HMO component of breastmilk [140]. Recently, research has shown that HMOs are more than just a substrate that promotes the growth of desired bacteria, as HMOs also serve as antiadhesive antimicrobials, immune modulators, and nutrients for brain development. Given that it is difficult to produce human breast milk on a large scale, it has

become increasingly important to find a functional equivalent that reproduces the beneficial effects of human breastmilk.

Moreover, paternal nutrition has been put forth as yet another modulator of offspring obesity risk. In one of the early studies examining paternal nutrition, a paternal high fat diet for 10 weeks prior to mating, impaired pancreatic β cell function and insulin secretion, and increased bodyweight in their offspring[264]. These impairments were associated with increased risk for obesity in adulthood[264]. In addition to the detrimental effects of paternal high fat diet, low protein diets during the periconceptional period also elicit metabolic complications in offspring, including increased adiposity. In a mouse model, consumption of a low protein diet by fathers for 8 weeks prior to mating, upregulated the expression of genes involved in offspring hepatic lipid and cholesterol biosynthesis[112]. The offspring also displayed increased BMI, adiposity, glucose intolerance and altered gut bacterial profiles[112]. Researchers attributed these detrimental effects to alterations in sperm-DNA methylation and non-coding RNA[112].

Numerous dietary interventions that manipulate macronutrient composition and/or supplement the diet with potential anti-obesogenic compounds have been proposed. For example, high protein diets have been used for weight loss strategies in attempts to improve lipid metabolism and increase satiety [254,255]. Additionally, prebiotics have been used in supplemental form to help manage obesity in part by increasing the secretion of gastrointestinal hormones that suppress hunger and increase satiety[183,424]. Importantly, maternal programming studies have shown that supplementation with a prebiotic during gestation or lactation improves bodyweight, body composition, colon length[193], and improves immunity[194] in offspring. Moreover, previous work has demonstrated the benefits of folic acid supplementation in reducing the occurrence of neural tube defects, which resulted in national and international health policies requiring pre-natal supplementation of folic acid[425]. Suboptimal intake of other dietary methyl donors such as choline and B12 as well as methylation processes have now been associated with metabolic impairments as well[425]. To our knowledge, no previous research had examined whether paternal dietary interventions that involve high protein intake, prebiotic or methyl donor supplementation influence health outcomes in offspring.

The overarching goal of this dissertation was to assess the impact of dietary manipulations during the pre-conceptional period or post-natal period on metabolic and microbial health status. To accomplish this goal, four major studies were performed. The first

study examined the impact of 2’FL and 3’SL HMO supplementation alone or in combination post-natally to determine intestinal barrier function and microbial composition in the gut of young male and female rats. The second study assessed the link between paternal high protein diet nine weeks before conception and the intergenerational impact on body composition, insulin sensitivity, epigenetic status and gut microbial composition. The third study examined whether a paternal methyl donor supplementation in a rodent model improved fertility, physiological outcomes, gut microbiota and epigenetic status altered by a high fat/ high sucrose diet. Finally, the fourth study examined whether prebiotic supplementation in fathers before conception affects measures of fat accumulation and gut microbial composition intergenerationally.

7.2 General Discussion

The major findings from the research presented in this thesis include:

1. 2’FL and/ or 3’SL HMO supplementation in females decreased intestinal permeability, mRNA expression of genes involved in maintaining gut barrier function, and gut microbial composition. Males supplemented with HMOs displayed reductions in weight gain at the end of an eight-week intervention, improved pro-inflammatory cytokine profiles and showed an increased abundance in beneficial gut microbes.
2. A paternal diet high in protein derived from casein before conception elicits protective effects on male and female offspring that persist into adulthood. These protective effects of paternal HP diet include reduced paternal adiposity, consequently influencing offspring bodyweight and adiposity and improving insulin sensitivity and gut microbial/ epigenetic signatures intergenerationally.
3. Supplementing a paternal HF/S diet with a methyl-donor cocktail of betaine, choline, folic acid and vitamin B12 reduced energy intake, fasting insulin levels, and insulin resistance alongside alterations in gut microbial signatures, epigenetic markers modulating metabolism, and reproductive outcomes in fathers. Paternal HF/S+M also appears to reduce fat mass and alter microRNA and gut microbial signatures in adult male and female offspring compared to HF/S intake alone.
4. Paternal prebiotic intake before conception, improves metabolic and gut microbial status in fathers. However, paternal prebiotic supplementation has only limited effects on offspring that are more evident in female compared to male offspring.

7.3 Sex specific impact of HMO supplementation on intestinal health

We were interested in assessing whether supplementing the regular weanling diet of rats with two HMOs highly prevalent in human milk would influence gastrointestinal health in a similar way to that described in breastfed infants. Based on evidence that HMOs are prebiotics that confer advantageous biological outcomes in the gastrointestinal tract and throughout the body, we examined gut microbial composition, intestinal permeability and inflammatory gene expression. There is a critical move to include both male and female rats in research studies, which proved to be important in our study. To our surprise we found key sex differences between groups, where females showed improvements in intestinal permeability as well as mRNA expression of important genes involved in maintaining gut barrier function, and improved gut microbial composition. Males exhibited reduced weight gain as well as marked improvements in their proinflammatory cytokine profile. This sexual dimorphic response could be due to sex-dependent expression and modulation of tight junction proteins (TJP) like occludin, and ZO-1. In an effort to investigate how estrogen receptors α/β affect intestinal permeability, previous work examined primary human gut tissue ZO-1 mRNA expression and inflammatory activation *in vitro*[234]. They found that males and females differed significantly in mRNA expression and that estrogen treatment downregulated ZO-1 mRNA and protein expression[234]. Their results uncovered important sex differences, wherein, sex hormones have the ability to modulate gastrointestinal TJPs[234]. In our study, female control rats showed significantly lower ZO-1 mRNA expression, which was upregulated following supplementation with 3'SL HMO, alone or in combination with 2'FL. Male control rats on the other hand showed four times higher mRNA expression in ZO-1 compared to females. Therefore, it is interesting that in females, with normally lower ZO-1 mRNA levels compared to males, supplementation with 2'FL reduced intestinal inflammation. It is known that ZO-1 selectively modulates the permeability of the gastrointestinal epithelium, thereby blocking the translocation of bacteria from the lumen into circulation[426]. We found HMO supplementation to differentially influence ZO-1 mRNA levels, thereby reducing intestinal permeability in females. This finding is corroborated by previous work in breastfed infants, who derive an abundance of HMOs from breastmilk, that showed a 2.8-fold reduction in intestinal permeability compared to infants who were exclusively formula fed[197]. Altogether, we found that females were more sensitive to the actions of HMOs in attenuating measures of intestinal permeability. We are not aware of any

research in human infants that has shown a difference in males and females in the utilization of HMOs. Furthermore, the biological rationale for such a sexual dimorphism, should it exist, in response to HMO supplementation is not known but certainly warrants further investigation.

7.4 Paternal nutrition during preconception affects body composition and insulinotropic factors

Based on evidence that certain dietary patterns affect body composition and insulin sensitivity, we sought to investigate whether these metabolic outcomes would extend beyond the generation consuming them directly and extend to first generation offspring. Weight loss programs based on high protein diets, like the Atkins or the Zone diet, have yielded positive effects on body composition and weight loss[289,290]. Moreover, previous high protein diet rodent models have displayed improved hepatic metabolism with reductions in lipogenesis and increases in gluconeogenesis and glycogenesis in the liver[287,288]. Results derived from our second manuscript, where fathers consumed a high protein diet, showed protective effects intergenerationally. Male and female offspring exhibited reductions in bodyweight and adiposity which likely accounts in part for the improved insulin sensitivity observed. In contrast to the detrimental effects of a paternal low protein diet which includes greater adiposity, glucose intolerance, and cardiovascular dysfunction[155], our study is the first indication that a paternal high protein diet can positively impact metabolic outcomes in adult offspring. Our findings appear to be in line with human population studies showing that high protein consumption derived from dairy sources protects against obesity[427,428], which may also extend to offspring via the paternal line.

Results from the third study presented in this thesis also showed notable differences in body composition and insulin function. High fat/ high sucrose diets have been linked with poor metabolic outcomes across multiple species and with a variety of study designs. In 2016, researchers induced obesity in fathers using a high fat diet and examined whether the deleterious effects of diet induced obesity extended transgenerationally[8]. They found that paternal exposure to a high fat diet before conception resulted in metabolic syndrome-like characteristics across multiple generations that was in part due to epigenetic modifications of genes modulating lipogenesis and insulin resistance[8]. Our goal was to attenuate some of these known deleterious metabolic effects by supplementing fathers' diet with choline, betaine, folic acid and vitamin

B12. We uniquely showed that a paternal high fat diet supplemented with methyl-donors mitigates the accumulation of fat mass in adult male and female offspring. It is possible that this protection could be attributed to the observed reduction in the fasting level of the anabolic hormone insulin in fathers which contributed to the lower HOMA-IR we observed. Nevertheless, we did not see any differences in glycemia during the OGTT and ITT and therefore, better indicators of insulin sensitivity such as the euglycemic-hyperinsulinemic clamp[429] or conditions that challenge the homeostatic system like high fat feeding might be needed to confirm the extent of protection on insulin sensitivity.

7.5 Paternal nutrition impacts offspring's gut microbiota

Paternal nutrition during pre-conception yields notable gut microbial profiles in offspring, although the biggest microbial impact will always be observed in fathers, resultant of direct gut microbial manipulation by diet. Moreover, all three paternal studies showed convergence of the gut microbiota across dietary groups by 15 and 16 weeks of age. One possible reason is that all of the offspring consumed control diet throughout the duration of their life which drove the eventual uniformity, or it is possible that the most notable differences were at lower taxonomic groups which cannot be reliably detected using high throughout 16S rRNA sequencing technology.

Still, paternal high protein and high prebiotic diets independently increased female offspring alpha diversity at nine weeks of age. Increased alpha diversity has been credited with positive health-related outcomes although it should be noted that debate has emerged in recent years as to whether diversity is a good singular indicator of health[430]. Perhaps more meaningful is the shift in particular taxa that have been shown to have positive metabolic effects. In our paternal high protein and high prebiotic fiber diet studies, we observed a proliferation in *Bifidobacterium*. *Bifidobacterium* are commensal bacteria that have health-promoting properties[431] including their anti-obesogenic properties[301]. Chapter 4 showed a persistent increase in *Bifidobacterium* in fathers as well as female offspring.

Obesity and/or metabolic syndrome have been characterized by lower abundance of Bacteroidetes and an increase in Firmicutes[432]. Chapters 4 and 6 showed paternal protein or prebiotic consumption result in an increased abundance in Bacteroidetes phylum and a marked reduction in the Firmicutes phylum in both fathers and young adult offspring. An obesity-related

microbiota is typically linked with increased energy harvest from the diet, notably derived from carbohydrates, and a subsequent increase in short chain fatty acid production[312]. SCFA can be readily absorbed via intestinal mucosal surfaces contributing an energy source and maintaining the colonic epithelium[312], most notably butyrate[433]. Paternal methyl-donor supplementation as seen in chapter 5 resulted in significantly increased levels of cecal butyrate in fathers and male offspring, which has the potential to contribute to gastrointestinal health. Future work is needed to substantiate whether this similarly reduces gut permeability and contributes to other metabolic benefits. Nevertheless, it remains unclear how a paternal gut microbial signature could be transmitted from fathers to offspring since the direct vertical transmission that occurs in the case of maternal microbiota to the infant during birth simply does not occur with the paternal line. While no one has addressed this, it is possible that by simply improving the metabolic health in the offspring there will be an associated improvement in the gut microbial signatures in offspring. Certainly, fecal microbiota transplant studies that transfer stool from offspring to germ-free mice would help to causally relate offspring gut microbiota to metabolic outcomes. Nevertheless, even though this would help establish whether gut microbial changes drive metabolic changes in the offspring, it would remain to be discovered precisely how paternal microbiota patterns influence offspring microbiota patterns.

7.6 Strengths and Limitations

7.6.1 Animal Models

The genetic background of animal models is an important research variable in health research[434]. There are a myriad of different strains including inbred, outbred, mixed breeds, hybrid, recombinant hybrid, etc.[435]. Inbred strains provide genetic uniformity, however, outbred strains that exhibit genetic variability are commonly used as animal models of polygenic human diseases like obesity and cardiovascular disease[436–438]. Sprague Dawley (SD) rats are outbred rats and are not genetically uniform. Since the aim of our research was to mimic human populations, the genetic variability in SD rats provided a better model of the genetic heterogeneity observed in human cohorts. Additionally, in the context of developmental programming research, rats have short gestational periods and rapid growth into adulthood. This allows for the investigation of metabolic aberrations across multiple generations within a

reasonable period of time. Moreover, the larger size of rats compared to other animal models like mice allows for added physiological manipulations and measurements. For example, we chose to assess glucose tolerance, insulin tolerance and intestinal permeability just before euthanasia of our animals to assess a wide range of metabolic outcomes. This required a volume of blood that would not have been possible to obtain in smaller animals like mice. Similarly, we required a substantial amount of fecal/ cecal matter, blood derived from the portal vein, and gastrointestinal subsections that would have been harder to obtain in a smaller animal.

Nevertheless, using rats in general is not without limitations. Rats for example exhibit differences in hypothalamic development. While many neuropeptides and hormonal signals are similarly observed in rodents and in primates, the neurocircuitry controlling feeding varies between the two species[439]. In humans, the hypothalamus develops *in utero* whereas in rodents the development continues postnatally[440]. The human brain is also much larger and higher functioning compared to rats[441], likely explaining the notable disparity between organ growth and development.

These noticeable differences in developmental stages between rodents and humans, while ideal for shortening the period required to see effects from gestation into adulthood, reduce the translatability of the research. Similarly, rodents typically have multiple fetuses per pregnancy, which does not resemble typical human reproduction. In this respect, using guinea pigs, who more closely mirror that of human fetal growth and development, could provide better developmental programming insights compared to rats[442].

7.6.2 Diet Composition and Treatment

In 1976, the American Institute of Nutrition developed a purified, fixed formulation diet for rodents known as AIN-76A diet[443]. The formulation was revised in 1993 to AIN-93 diet[444]. AIN-93G diet is to be used during growth and during pregnancy while AIN-93M is to be used as a maintenance diet[444]. The revised formulation provided a better balance of essential nutrients aimed at improving the performance of the rodents consuming it. However, unpublished work in our lab has suggested there may be a potential obesogenic property of AIN-93 diet due to the abundance of refined/ processed ingredients like sucrose, dyetrose, soybean oil as well as the low presence of fibers. Using AIN-93 diet may have potentially created a type of metabolic challenge in studies 1, 2 and 4 of this thesis that yielded higher body weight and

increased fat mass and potential differences in glycemic control and gut microbiota profiles compared to if a standard chow would have been used as the control diet.

In our first study, we examined individual and combined HMO supplementation. We selected the most abundant fucosylated HMO, 2'fucosyllactose (2'FL)[215] as well as the most predominant sialyllactose that remains stable throughout lactation, 3'sialyllactose (3'SL)[216]. While this was the first study to look at supplementation with these synthesized HMOs alone or in combination, there were some key limitations. First, fucosylated milk oligosaccharides, while prevalent in human milk, are virtually absent in rodent milk[445], therefore supplementation of a rodent diet with these HMOs could result in outcomes that differ from what would be observed in a human infant given an HMO-fortified diet. Furthermore, HMOs have co-evolved with the genetic function of specific intestinal bacteria[222] and are also co-regulated with one another[446]. The HMOs we used are not as efficient as other HMOs in promoting the growth of symbiotic gut microbes and eliciting a strong immune response[447]. Findings from a randomized controlled trial of healthy term infants given the HMOs 2'FL and LNnT, showed a shift in the gut microbiota towards that of breast-fed infants[448]. We did not supplement our rodents with LNnT HMO which could explain some of the differences we noted with the two HMOs we selected for examination. Finally, we only supplemented with a small fraction of HMOs found in breast milk which are unlikely to provide the exact benefits conferred from the evolutionary forces perfecting the process of exclusive breast feeding, ensuring the greatest health benefit for the infant[40].

Models of animal obesity can be characterized based on their etiology, however no animal model may optimally replicate human obesity[449]. Rodent models of obesity often utilize some of the same components of human obesogenic diets, namely high fat/ high sucrose, as seen in chapters 2 and 3 of this thesis. Animals introduced to these diets typically develop obesity and may show reductions in insulin and leptin sensitivity[450] as well increased hepatic/ plasma glucose and free fatty acids[449]. HF/S diets most resemble that of Western Diets, causing increased energy intake and hyperphagia[449], making them a valuable model for investigation of obesity and potential therapies to attenuate some of the deleterious effects associated with this condition.

In our third study, we examined a cocktail of nutrients that affect one-carbon metabolism. As noted previously, HF/S diets induce deleterious metabolic outcomes and this was the first

study to examine how paternal supplementation with four methyl donors may attenuate some of these adverse outcomes. Still, animal, human, and in vitro studies suggest that the epigenetic effects of methyl donors are highly complex[451]. We did not assess each methyl donor individually or at different doses, rather we only assessed them in combination using one dose, therefore it remains unclear how these methyl donors impact specific physiological outcomes independently.

In study 4, we utilized a 10% wt/wt prebiotic oligofructose concentration, similar to that of previous work in our lab and others showing significant differences in body weight, adiposity, satiety hormones, hepatic steatosis and gut permeability in rats[34,175,452,453]. While animal studies have shown promising results, this dose would not be possible in a human cohort due to the gastrointestinal side effects (i.e. osmotic diarrhea, bloating and flatulence)[454]. In humans, a daily dose of 2.5-10 g is required to elicit a bifidogenic effect but higher doses are typically needed to achieve therapeutic health benefits[454]. While doses up to 21 g/day have been shown to result in some minor gastrointestinal side effects, none are considered serious and building up the dose over time appears to help with adaptation[177]. In our model we gave our rats 10% wt/wt prebiotics without gradually building up the prebiotic dose, therefore, we cannot deduce whether this high prebiotic concentration caused adverse effects in the rats. Based on the extensive use of this dose in rodent studies and the general health of our rats, we suspect that the rats did not have any issues with tolerance which is in large part due to the differences that exist anatomically in the rat GI tract compared to humans, where rat cecums are substantially larger than humans relatively speaking[455]. This allows for increased capacity for the fermentation of non-digestible carbohydrates like oligofructose[455]. Anecdotally, we did see substantially more bloated cecums in prebiotic-rats compared to controls at euthanasia, which could simply be excess gas production from increased fermentative activity.

7.6.3 Intervention Duration

As described in Chapter 3, we started our HMO supplementation at three weeks of age which may have affected HMO metabolism within the gut during postnatal development. Based on oligosaccharide profiles seen in the stool of human infants, HMO metabolism undergoes three stages: 1) dominance of acidic and neutral oligosaccharides; 2) increased presence of metabolites, like SCFA and 3) reductions in both HMOs and corresponding metabolites. Translating these metabolic pathways to Sprague Dawley rats, stage 3 starts at 21 days of age.

We supplemented HMOs at approximately stage 2 of HMO metabolism and continued this intervention well beyond stage 3. Therefore, we cannot know whether the age at which we decided to introduce the HMO supplementation was optimal from the perspective of the maturation of the gut microbiota. Using metagenomic analysis of the gut microbiota in addition to quantification of the HMOs in the fecal matter of the rats at various ages would provide more information on how well matched the particular HMOs were to the gut microbiota present at that particular developmental stage.

Interventions in chapters 4, 5 and 6 started at 3 weeks of age, in attempts to elicit the greatest epigenetic changes induced by diet in fathers. However, we recognize manipulating father's diet during childhood, the adolescent period and adulthood might have all elicited varying epigenetic and metabolic results. Future work is warranted to investigate paternal dietary manipulation at different periods of development as well as transiently, pre-ceding conception by a short period. Additionally, paternal age has been positively associated with reduced fertility and increased incidence of disease in offspring[456]. In an effort to reduce the confounding factor of age and increased disease occurrence, we mated our rats relatively shortly after they reached sexual maturity at 12 weeks of age. The fathers in our experiments received their diets from three to twelve weeks of age accounting for a nine week dietary intervention. Previous parental programming research demonstrated more pronounced metabolic effects across two generations when mothers and fathers adhered to their respective diets for 16 weeks[457] suggesting that we could have extended our feeding period even further. Additionally, we followed the offspring for 4 months. We recognize that assessing the offspring for a longer duration may have yielded more pronounced metabolic differences. This might have been especially true if the offspring were subjected to a high fat/ high sucrose metabolic challenge that has been shown to unmask any latent metabolic effects or increased metabolic disease risk.

7.6.4 Gut Microbiota

To determine microbial community structure, we classified microbial DNA sequences taxonomically from fecal matter using the highly conserved 16S ribosomal RNA gene, in our case specifically the V3 and V4 region[458,459]. This method offers insights pertaining to intra- and inter-sample diversity and bacterial community composition[458]. However this method is known to produce short read lengths, sequencing errors[460,461], and differences based on which variable region is selected for amplification[462]. The bioinformatics pipeline can also

influence outcomes, although we tried to mitigate this by using DADA2. DADA2 is a software that infers the exact ASV from amplicon data, while denoising and removing sequencing errors[274]. This program offers the best sensitivity, however at the expense of ASV specificity[462]. Similarly, the utility of a single gene to determine diversity is limited considering the frequency of horizontal gene transfer [463].

Another widely used comparative approach is whole genome shotgun sequencing (WGS). WGS provides greater microbial resolution at the species and potentially at the strain level as well and includes the functional characterization of the gut microbiome[464]. While 16S rRNA amplicon sequencing and WGS have both provided invaluable information about DNA sequences, microorganism presence and diversity/ abundance in a myriad of different environments, there are still major hurdles that need to be addressed, primarily global standardization of methods used, storage of data and data sharing[464].

7.6.5 Real-Time PCR MicroRNA and DNMT analysis

MiRNAs have been identified to be involved in a plethora of biological mechanisms as well as the pathophysiology of human diseases[465,466]. Real-time PCR is a valuable, relatively quick tool that provides information pertaining to the expression of particular miRNAs. However, this method is not without limitations. MiRNAs can degrade very quickly via ribonuclease activity. Efforts were made to prevent possible degradation by excising tissue as quickly as possible, snap freezing tissue and maintaining samples on dry ice until the homogenization step in the microRNA extraction protocol. We chose to assess a panel of microRNAs involved in metabolic outcomes like insulin resistance and lipogenesis, albeit not all microRNAs are involved in metabolic dysfunction. In future, rather than selecting individual miRNAs for RT-PCR analysis, it could be useful to utilize a miRNA microarray that can measure the expression of thousands of miRNA at once. Given that over 5000 miRNAs have been identified, this technology would provide a more comprehensive picture of intergenerational or transgenerational miRNA expression following dietary intervention[467].

DNA methylation has yielded a great deal of interest as an epigenetic modifier since it experiences rapid changes during development which contribute to cellular differentiation and organogenesis[468]. However, it is important to consider DNA methylation is tissue- and species-specific[469]. We only investigated DNA methyltransferase expression in adipose tissue and we did not assess the unique *de novo* methylated sites of each DNMT isoform. Although

arduous, future work could examine cells derived from different metabolically relevant tissues to address differences in morphology and function, to determine if DNA methyltransferase gene expression has a direct impact on those cells. A genome-scale map of methylation kinetics could additionally infer actual rates of methylation and demethylation at specific CpG sites at the scale of the genome. So far, it has been established that these processes are context-specific[470] and cannot be broadly applied.

7.6.6 Glucose Measurements

To study glucose and insulin homeostasis in our rats, we used glucose and insulin tolerance tests. While these tests are a mainstay in pre-clinical research, they are not without limitations, primarily because of their low-reproducibly due to many extraneous factors that are difficult to control. Animal husbandry variations is among these confounding variables. For example, environmental stressors will increase adrenaline and noradrenaline, which have noticeable physiological effects on glucose homeostasis[471]. These stressors could include cage handling, repeated bleeding or even noise. The room in which the experimental animals were housed was adjacent to the rather noisy facility dishwasher, undoubtedly increasing animal stress. Recognizing this, efforts were made to minimize the stress on the animals. LaFollette *et al.*[472] demonstrated that rat tickling decreases stress hormones and anxiety measures, therefore we employed this method regularly in our rats to reduce stress hormones induced by environmental stressors.

Moreover, metabolic functions are controlled in a sex-specific manner[473]. In models of insulin resistance and glucose intolerance, males show a stronger phenotype compared to females; females exhibit better glucose tolerance[474] believed to be owing to their greater insulin sensitivity in liver, adipose tissue and muscle[474]. These differences are likely owing to differences in sex hormones. We did not control for variations in sex hormones like estrogen or testosterone, however, we did utilize statistical modelling to determine if there was a sex effect. In adult offspring, a significant sex effect was often seen, therefore we analyzed the sexes separately.

7.7 Future Directions and Perspectives

Breastfeeding has been associated with reduced disease burden in offspring[475]. Still,

continued breastfeeding for the recommended length of time is not consistently practiced or may not be possible due to socio-economic, demographic or biological factors[475]. Therefore, infant formula options that contain nutrients and bioactive components that closely resemble human milk is needed. Such bioactives could include HMOs. Our first study, supplementing two key HMOs, demonstrated significant differences in food intake between the 3'SL supplementation and control group during the final week of the intervention, where 3'SL groups consumed significantly less energy in kcal/ day. This difference in energy intake could point to future investigation into the reward circuitry in the brain (Ventral Tegmental Area and the Nucleus Accumbens) and/ or leptin to determine whether HMO supplementation of 3'SL and 2'FL or others regulate hunger and satiety long term. No research to date has investigated the role of HMOs on the reward circuitry.

Moreover, sialyllactose HMOs have been recently credited with modulating neurological and cognitive function, more specifically, sialic acids (Sia) [476]. Sialyllactose HMOs exist in conjugated forms of Sia. Sia have been associated with embryonic neural development, neuronal plasticity, as well as long-term potentiation (LTP)[476]. Future work could supplement infant formula with 3'SL or other Sialyllactose HMOs, either alone or in combination, to determine whether they elicit changes in brain development as well as learning and memory.

Our understanding of the full impact of paternal influence on offspring health remains in its infancy. While we and others have attempted to shed light on potential mechanisms affecting offspring health derived from paternal environmental exposures, further investigation is required. For example, utilization of a Luminometric Methylation ASSAY (LUMA) would provide an assessment of global methylation using a small quantity of DNA [477]. LUMA offers high-throughput analysis, utilizing the Pyrosequencing™ platform to analyze 48 samples in less than 20 minutes without a reference genome[477]. Use of this platform would yield a much broader overview of the methylation status of father and offspring.

Beyond methylation status, it is important to investigate the mechanism by which the father alters the oviductal fluid surrounding the embryo and whether differences may be observed based on nutritional, metabolic and inflammatory factors. Will this alteration provide a microcosm that reflects the outside world? This is typically discussed in the context of maternal

factors, however, we postulate paternal seminal fluid will affect the oviductal fluid and consequently the developing embryo. In a recent study examining ram capacitation and in vitro fertilization success, they found varying volumes of oviductal fluid affect motility, sperm viability, acrosome reaction and IVF success[478]. Applying these conclusions beyond the scope of agricultural reproductive technologies, we can postulate this may also apply to rodents and humans. Future work should employ high-throughput sequencing technology on the seminal fluid, spermatocytes, oviductal epithelial cells and fluid to investigate the female response to seminal fluid and influence on the developing fetus. This investigation needs to go beyond just sperm motility and morphology and investigate epigenetic alterations and the direct impact on gene expression in these cells and tissue.

Moreover, since the fetal GI tract remains sterile until birth, it is unlikely the paternal microbiota can influence offspring's microbiota directly. Still, we did see notable differences in gut microbial signatures among offspring in our studies. One possible mode of transmission might be immune parameters transmitted via seminal fluid to the mother in preparation for gestation. Future work should determine if paternal dietary patterns affect innate immune cells, either via epigenetic programming or immunity transmission. Alternatively, a combination of the two are also possible, where epigenetic status, whether in histones, DNA or non-coding RNA, could potentially train immunity transcriptional factors and functional programs, thereby eliciting notable adaptive or maladaptive states in offspring. So far it remains unclear how fathers influence offspring health non-genetically. Immune memory and/ or epigenetic markers transmitted via seminal fluid, might be the best determinant for this heritability. It is possible certain immune parameters can create an ecosystem that is favourable of a certain gut microbial signature, as previously seen in mothers where maternal microbiota modulated early postnatal innate immunity[479].

Additionally, investigation into how co-habitation of pregnant mothers with fathers affects microbial profiles may be an avenue of interest. Co-habitation may induce a stronger paternal impact on the gastrointestinal system of neonates. We speculate this may occur via paternal transmission of microbiota via saliva or seminal fluid during gestation and a corresponding immune influence, creating an ecosystem that is favourable of a certain gut microbial signature in offspring post-natally.

Ultimately, the goal of animal research is translation to human clinical studies. Understandably, there are a myriad of ethical concerns related to paternal nutritional manipulations and reproductive and/ or offspring health, more specifically when comparing beneficial interventions to a deleterious HF/S or Western diet. Results derived from the studies in this dissertation warrant further investigation in humans. Similar to maternal programming research, the ultimate goal of paternal dietary work is to provide evidence that informs human clinical studies that result in guidelines to improve infant health.

7.8 Conclusion and Significance

In summary, the outcomes derived from this thesis highlight the metabolic, gut microbial and overall health status of animals that experience micro or macro-nutrient manipulations during pre-conception or during development. Supplementation of one or both of 2'fucosyllactose (2'FL) and 3'sialyllactose (3'SL) during early development showed complex, sex-dependent beneficial and/or deleterious outcomes on bodyweight, food intake, inflammation, gastrointestinal gene expression and/ or gut microbial composition. Intake of casein-derived protein, prebiotics or methyl-donor cocktail supplementation in fathers during pre-conception all elicited varying beneficial effects on body composition, satiety, insulin sensitivity, gut microbiota and/ or epigenetic changes in offspring. Further investigation could help inform researchers and health professionals on potentially pro- or retro-active dietary interventions and the mechanism by which they can influence physiology, metabolism and reproduction at varying levels of development.

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