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Potential for Prebiotic Fiber to Attenuate Obesity Risk and Insulin Resistance in Rat Offspring Exposed to a Maternal Obesogenic Diet with Low-Dose Aspartame Consumption

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Potential for Prebiotic Fiber to Attenuate Obesity Risk and Insulin Resistance in Rat Offspring Exposed to a Maternal Obesogenic Diet with Low-Dose Aspartame Consumption

by

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A THESIS

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Abstract

Maternal diet during pregnancy has a lasting impact on offspring health. Our objective was to examine if offspring postnatal oligofructose prebiotic fibre (Pre) intake could mitigate metabolic risks of a maternal obesogenic diet plus aspartame (APM) consumption.

Following 11 weeks of obesity induction, female Sprague-Dawley rats (n=29) were randomized during pregnancy and lactation to a high fat/sucrose (HFS) diet with water control (CTR) or HFS with APM (7mg/kg/day) in drinking water. Offspring of each maternal group were weaned onto the following four groups: CTR-CTR, APM-CTR, CTR-Pre, and APM-Pre from 3-12 weeks of age. Energy intake, body weight, fecal microbiota, and metabolic outcomes including body composition, glucose tolerance, and liver triglycerides were assessed in dams and offspring.

Metabolic disturbances were not observed in APM dams yet maternal APM exposure with HFS diet increased hepatic triglyceride levels ($p=0.046$) and influenced gut microbiota beta diversity ($p=0.029$) in 3-week old weanlings, and increased body weight in young male offspring at 4-5 weeks of age ($p<0.05$). By week 12, females in the APM-Pre group had lower body weight ($p=0.046$), greater (%) lean mass ($p=0.044$), reduced (%) body fat ($p=0.047$) compared to CTR-CTR; The female APM-Pre group also had greater (%) lean mass ($p=0.006$) and reduced (%) body fat ($p=0.009$) than the APM-CTR group. Postnatal Pre intake improved glucose tolerance and AUC in males ($p<0.001$) with no effect in females and worsened insulin sensitivity in APM-Pre females ($p<0.05$) relative to CTR-CTR females with no effect in males. In adult offspring, Pre intake reduced alpha diversity ($p<0.001$), affected beta diversity ($p<0.001$), and increased the relative abundance of *Bifidobacterium, Blautia,* and *Streptococcus* while decreasing 5 other bacteria including *Romboutsia*.

Maternal APM intake with an HFS diet may disrupt weight homeostasis, increase hepatic triglycerides and gut microbiota in young offspring with no effect of APM seen in later life. Postnatal Pre intake by offspring exposed to a maternal obesogenic diet is linked to lower body weight and improved body composition predominantly seen in female rats with improved glucose control seen in adult male rats. Noticeable shifts in gut microbiota secondary to Pre consumption may mediate these changes.

Preface

This thesis is original, unpublished, independent work by the author, G. Venegas Silva. The thesis is presented in traditional thesis format.

The rat study reported in this thesis was covered by Ethics Certificate number AC22-0113 issued by the University of Calgary's Animal Care Committee for the project "Mitigation of obesity risk with prebiotics in offspring of maternal rats consuming aspartame" originally issued on October 06, 2022.

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Dedication

This thesis is dedicated to the 390 million children and adolescents living with overweight or obesity in the world with the aim that the information contained herein may add to the ever-growing body of knowledge on this subject. May you be treated with dignity, respect, and receive the same level of compassionate healthcare given to other chronic diseases.

And dad.

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List of Symbols, Abbreviations and Nomenclature

APM	Aspartame
Pre	Prebiotic fiber
T2DM	Type 2 Diabetes Mellitus
NCD	Noncommunicable Disease
GI	Gastrointestinal
LPS	Lipopolysaccharide
NAFLD	Nonalcoholic fatty liver disease
SD	Sprague Dawley
FDA	Food and Drug Administration
ADA	American Diabetes Association
IDF	International Diabetes Federation
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
cDNA	Complementary DNA
mRNA	Messenger RNA
HFS	High fat/sucrose diet
FAS	Fatty acid synthase
ACC	Acetyl CoA carboxylase
IL-6	Interleukin 6
IRS-1	Insulin receptor substrate 1
IRS-2	Insulin receptor substrate 2
TNF-α	Tumor necrosis factor alpha
FPG	Fasting plasma glucose
IGT	Impaired glucose tolerance
OGTT	Oral glucose tolerance test
IPGTT	Intraperitoneal glucose tolerance test
ITT	Insulin tolerance test
AUC	Area under the curve
BGL	Blood glucose level
TG	Triglyceride

DEXA	Dual energy x-ray absorptiometry scan
FDR	False Discovery Rate
ANOVA	Analysis of variance
ASV	Amplicon sequence variant
PCoA	Principal coordinate analysis
PERMANOVA	Permutational multivariate analysis of variance
SEM	Standard error of the mean
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon-like peptide 1
PYY	Peptide tyrosine tyrosine
PPAR-a	Peroxisome proliferator-activated receptor alpha
PPAR-y	Peroxisome proliferator-activated receptor gamma
NAc	Nucleus accumbens
FGF21	Fibroblast growth factor 21

CHAPTER ONE: INTRODUCTION

1.1 Background

Worldwide, the prevalence of obesity has increased dramatically since 1975. Once largely seen in high-income countries, today many low- and middle-income countries are also experiencing rising rates of obesity. According to the World Health Organization (WHO), in 2022, 2.5 billion adults aged 18 years and older were living with overweight, including over 890 million adults living with obesity which represents 43% and 16% of the worldwide population respectively (1). Obesity is not just a problem affecting adults, with global estimates in 2022 of 37 million children under the age of 5 and over 390 million children and adolescents aged 5-19 years of age living with overweight or obesity (1). Worldwide the prevalence of overweight and obesity among children and adolescents aged 5-19 rose dramatically from 8% in 1990 to 20% in 2022. The increases were similar among boys and girls at 21% and 19% living with overweight respectively in 2022 (1).

Overweight and obesity particularly when associated with increased abdominal fat distribution and increased intrahepatic and intramuscular triglyceride content are major risk factors for prediabetes and type 2 diabetes (T2DM) because this causes both decreased insulin sensitivity in the tissues and compromised insulin secretion in the β-cells of the pancreas (2). Thus, alongside increasing worldwide rates of obesity, there has also been a significant rise in cases of T2DM among adults and youth (3–5).

While obesity and T2DM are complex and multifactorial diseases, early life exposures play an important role in their development into adulthood (6), particularly maternal diet during gestation and lactation is a phenomenon known as the developmental origins of health and disease (DOHaD) (7). This 'programming' is the adaptation process whereby nutrition and environmental factors alter developmental pathways during growth and induce changes in postnatal metabolism and disease risk (8). There is existing evidence to suggest that a maternal obesogenic (high fat/sucrose) diet coupled with low-dose aspartame intake can alter gut microbiota in offspring (9). This in turn increases adiposity and impairs glucose tolerance in the early life of offspring even if they did not directly consume aspartame (9).

Prebiotics which are fibers preferentially utilized by beneficial gut bacteria have been shown to reduce body fat, improve appetite regulation, and increase health-associated *Bifidobacterium* in humans and rats (10,11). More recently, the International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics defined prebiotics as "a substrate that is selectively utilized by host microorganisms conferring a health benefit" (12). By improving the

gut microbiota profile, administering prebiotics postnatally may reduce offspring obesity risk associated with perinatal (i.e. intrauterine and during the suckling period) exposure to a maternal high fat/sucrose diet with low-dose aspartame consumption.

1.2 Purpose of the Research

The overall purpose of this thesis is to investigate the mechanisms (microbial and metabolic) by which postnatal consumption of prebiotic fiber potentially reduces the risk of obesity and insulin resistance in offspring of female rats consuming a high fat/sucrose diet plus aspartame during pregnancy and lactation.

1.3 Overview of Thesis Chapters

This thesis contains six chapters. The first chapter provides a brief introduction to this thesis. Chapter two is a literature review that covers the relationship between diet, the gut microbiota, and developmental programming and explores the current evidence for their direct and intergenerational influence on offspring health and metabolism via the maternal line. Chapter three outlines the methods and procedures used to conduct this research. Chapter four describes the results of this research study including the direct impacts of a high fat/high sucrose diet with low-dose aspartame consumption on maternal outcomes and the influence of this diet with postnatal prebiotic fiber provision on offspring outcomes. Chapter five discusses and interprets the findings and considers the strengths and limitations of this work. Finally, chapter six provides an overall conclusion of the findings of this study and proposes future directions for this research.

CHAPTER TWO: LITERATURE REVIEW

2.1 Metabolic Diseases: Emphasis on Obesity and Type 2 Diabetes

Obesity and type 2 diabetes (T2DM) have reached epidemic proportions globally and are a significant public health concern. The prevalence of these metabolic diseases continues to rise globally with obesity more than doubling from 1990 to 2022 (1). In Canada, the prevalence of obesity was the same for women and men at 30% each, an increase from just over one in five (21%) in 2003 (13). Recent evidence among Canadian adolescents shows a similar trend with one third now living with overweight or obesity (14). A body mass index (BMI) of 25 to 29.9 kg/m^2 is classified as overweight and a BMI ≥ 30 kg/m^2 is classified as obese (15). Obesity is a disease characterized by excess adiposity and contributes to extensive morbidity and mortality due to various weight-related complications (16). Obesity has adverse effects on overall health and is a risk factor for a number of non-communicable diseases (NCDs) like T2DM, cardiovascular diseases (hypertension, myocardial infarction, stroke), fatty liver disease, osteoarthritis, and cancer (liver, kidney, colon, breast) (17). Annually, NCDs account for 71% of all fatalities globally and represent a leading cause of premature disability (17).

Obesity, particularly when associated with increased abdominal and intra-abdominal fat distribution and increased intrahepatic and intramuscular triglyceride content, is a major risk factor for prediabetes and T2DM because it causes both insulin resistance and β-cell dysfunction (2). Prediabetes is a condition that causes a person's blood glucose level to be elevated but not high enough to warrant a diagnosis of T2DM and is defined as fasting plasma glucose (FPG) of 6.1-6.9 mmol/L (110 to 125 mg/dL) and impaired glucose tolerance (IGT) defined as 2 h of plasma glucose of 7.8-11.0 mmol/L (140-200 mg/dL) after ingestion of 75 g or oral glucose load or a combination of the two based on a 2 h oral glucose tolerance test (OGTT) according to the WHO (18). The American Diabetes Association (ADA) has an additional hemoglobin A1c (HbA1c) based criteria of a level of 5.7% to 6.4% for the definition of prediabetes (19). Diabetes is a chronic metabolic disease characterized by elevated levels of blood glucose which over time leads to damage of the heart, blood vessels, eyes, kidneys, and nerves (4). Diabetes is diagnosed at an A1c of greater than or equal to 6.5%, an FPG of greater than or equal than 6.9 mmol/L (126 mg/dL), and a 2 hour blood glucose greater than or equal than 11.0 mmol/L (200 mg/dL) (20). Insulin resistance in combination with defective insulin secretion leads to a persistent increase in blood glucose levels which in turn gives rise to T2DM (21). The risk of T2DM increases linearly with an increase in BMI (2). Currently, 540 million people are living with diabetes and recent projections from the International Diabetes Federation (IDF) estimated that 1 in 8 adults, approximately

783 million people, will be living with diabetes by 2045 (3). Of these figures, it is estimated that 223 million women worldwide currently live with diabetes and this number is projected to increase to 343 million by 2045 (22).

In 2021, approximately 21.1 million or 16.7% of live births had some form of hyperglycemia in pregnancy where an estimated 80.3% were due to gestational diabetes (GDM) (23),(24). GDM develops in women who are unable to mount a compensatory β-cell response, leading to hyperglycaemia. Here the mother is unable to make and use all the insulin she needs for pregnancy. Without enough insulin, glucose uptake by the cells is compromised and cannot be broken down for energy leading to a buildup of glucose in the blood (25). The Canadian Diabetes Association suggests a diagnosis of GDM is made if one plasma glucose value is abnormal following a 75 g glucose load (i.e. fasting ≥5.3 mmol/L, 1 hour ≥10.6 mmol/L, 2 hours ≥9.0 mmol/L) while the ADA recommends the following targets for women testing blood glucose levels during pregnancy: before a meal at 95 mg/dL or less, one hour after a meal at 140 mg/dL or less, and two hours after a meal at 120 mg/dL or less (25). Increasing maternal age, along with increasing rates of obesity worldwide, has led to rising rates of GDM (26). GDM increases the risk of developing metabolic syndrome, pre-eclampsia, and T2DM by seven times post-pregnancy, and cardiovascular and coronary artery disease by four times (27),(28). GDM is of particular concern because it impacts a significant proportion of pregnant women and it is suspected that its prevalence will continue to increase due to the increase in associated risk factors worldwide including obesity and physical inactivity (29). In addition to the large burden metabolic diseases such as obesity and T2DM place on the individual, they also place a significant burden on the healthcare system and the economy with obesity and T2DM accounting for 2.8% and 1.8% of the entire global gross domestic product (GDP) respectively (30),(31).

The development of obesity is multifactorial, and a complete understanding of this disease remains elusive. One factor that has been more recently implicated in the etiology of obesity and T2DM is the gut microbiota.

2.2 Gut Microbiota and Metabolic Disease

The simplistic view of the gastrointestinal (GI) tract as largely a digestive organ has shifted over the past two decades to now recognize its role in metabolism, immunity, and behaviour in large part due to the microbial inhabitants of the gut (32). The collection of bacteria, viruses, fungi, archaea, and eukaryotes colonizing the GI tract is called the 'gut microbiota'. The gut microbiota have co-evolved with the host over thousands of years to form a complex and mutually beneficial symbiotic relationship

(33). The microbes with their collective genomes are called the 'gut microbiome' and it encodes over 3 million genes which produce thousands of metabolites. By comparison the human genome only consists of approximately 23,000 genes (34). Gut microbiota profiles are unique to the individual and are composed of different bacterial species taxonomically defined by phyla, order, family, and genus (35). Despite variations in proportion, diversity, species and gene function, humans seem to all share a core microbiota (36).

In humans, the two most prevalent bacterial phyla are the *Bacteroidetes* (gram-negative, anaerobic, non-spore-forming) and *Firmicutes* (gram-positive, anaerobic, spore-forming) (37). Despite their high abundance, the relative quantities of microbes within these phyla vary enormously across individuals and can be affected by numerous factors such as age, diet, birth mode, breastfeeding versus formula feeding, antibiotic use, geographic area, disease states, exercise frequency, and lifestyle (35,38). Several animal and human studies have shown sex differences between male and female gut microbiota with changes mediated by sex hormones (39). In individuals with obesity there is typically an increase in *Firmicutes* and a decrease in *Bacteroidetes* although not all studies show this consistently (40). Several studies demonstrate the influence diet has on shaping the gut microbiota. In one study switching mice from a low-fat, plant polysaccharide-rich diet to a high fat, high sugar diet elicited changes to the gut microbiota within a matter of hours where an increase in members of the *Erysipelotrichi* and *Bacilli* classes of *Firmicutes* was observed (41). Another rodent study demonstrated significant alterations in the *Firmicutes/Bacteroidetes* ratio in Syrian hamsters along with changes in beta diversity as a result of a high fructose diet over 7 days (42). Similar findings were reported in children living in rural African areas, who consumed a traditional diet rich in fiber and showed higher proportions of *Bacteroidetes* and lower of *Firmicutes*, compared to children from western countries whose diet included large amounts of protein, fat, sugar, and starch (43). *Firmicutes* are more effective in extracting energy from food than *Bacteroidetes* thus promoting a more efficient absorption of calories and resultant weight gain (44). Additionally, *Actinobacteria* (*Bifidobacterium*), *Proteobacteria* (*Gammaproteobacteria* within *Enterobacteriaceae*), or *Verrucomicrobia* (*Akkermansia*) are less abundant phyla but also contribute to health and have been implicated in diseases ranging from obesity to inflammatory bowel disease (38,45,46). In fact, a select few opportunistic pathogens are considered a part of the normal intestinal microbiota and only become disease-causing in dysbiotic or imbalanced microbial states (38).

Given that the gut microbiota is malleable and susceptible to beneficial change via exogenous factors such as diet, it stands to reason that it can also be disrupted or exhibit dysbiosis. Dysbiosis refers to a state of imbalance wherein there are alterations to the composition and function of the microbiota

which result in detrimental effects to host health (47). Perturbations in gut microbiota and subsequent fluctuation in associated metabolic pathways (gut dysbiosis) have been linked to chronic disease states of interest including obesity and diabetes (48). Several mechanisms have been proposed by which gut dysbiosis contributes to metabolic disease. For example, individuals with an increased predisposition to obesity may harbor gut microbial communities possessing greater levels of catabolic genes that could increase the efficiency of energy extracted from the diet thereby providing additional calories to the host leading to weight gain (49). Gut dysbiosis can also alter the expression of tight junction proteins in the small intestine thereby increasing intestinal permeability and allowing leakage of endotoxins such as the bacteria-derived lipopolysaccharide (LPS) into the host circulation (11,50). Increased blood bacterial LPS concentration is linked to low-grade inflammation and contributes to insulin resistance and glucose intolerance in the host (50). Additionally, disruptions to the gut microbiota can alter the production of short-chain fatty acids (SCFAs) that act as signaling molecules for satiety, lipid metabolism, and insulin sensitivity (51). Gut dysbiosis can also alter bile acid metabolism which has implications for liver dysfunction and risk of obesity, insulin resistance, and non-alcoholic fatty liver disease (NAFLD) (51).

The gut microbiome is responsible for hundreds of metabolic pathways unique to microbiota and is essential for human health as it has been implicated to act both directly and indirectly (mediating the effects of diet) (34),(38). The gut microbiota is a central regulator of host metabolism and carries out many functions including harvesting energy in the form of SCFAs from nondigestible carbohydrates, regulating host lipid metabolism, maintaining the integrity of the mucosal barrier in the GI tract, acting as a barrier against pathogens by playing a role in immune development and maintenance, and synthesizing certain micronutrients (52,53). Understanding the lifelong development of the human microbiome in the context of health outcomes in conjunction with improving our knowledge of microbiome-host molecular interactions is of paramount importance. The early-life microbiome is gaining appreciation as a major influencer of human development and long-term health. Multiple factors are known to influence the initial colonization, development, and function of the neonatal gut microbiome (54).

2.3 Gut Microbiota in Infancy

The neonate gut microbiota is less diverse and stable than that of an adult, as a result, its development is dependent on many factors (55). The initial colonization of the GI tract during infancy is especially vital in programming the immune, nervous and metabolic systems and can alter risk for diseases later in life (38). While the initial colonization of the infant gut was thought to occur at birth,

there is some evidence (although controversial) that suggests this may occur in the *in utero* environment stemming from the discovery of microbes in infant meconium (first feces), amniotic fluid, placenta, and umbilical cord blood (56),(57). Nonetheless, with birth as the first major exposure to microbes, several environmental and host factors including perinatal, physiological, pharmacological, dietary, and environmental factors are known to shape the infant gut microbiota (58).

Immediately after birth, the gut microbiota of newborn infants is rapidly colonized by microorganisms from their mothers and their surrounding environment (59,60). During birth, infants are exposed to maternal vaginal, fecal, and skin bacteria which colonize the infant body sites that provide a suitable habitat and nutrients. Each bacterial species has specific habitat requirements and cannot permanently colonize inhospitable body sites (60). The infant gut is colonized at birth by maternal gut microbes notably *Bacteroides* and *Bifidobacterium* (61). While recent research findings suggest maternal vaginal microbes do not colonize the infant gut (instead they may provide 'transient priming') (62), there is a wealth of existing research suggesting that delivery mode has a strong effect on infant gut microbiota composition in the first weeks of life. Children born vaginally are colonized by the genera *Lactobacillus* and *Prevotella* whereas in cesarian delivery it is mostly the maternal skin microbes that colonize the infant's intestine as demonstrated by the dominance of *Streptococcus, Corynebacterium*, and *Propionibacterium* (59),(63). In cesarean section delivery the maternal transmission of the beneficial *Bacteroides* strains is disrupted whereas the high-level colonization of opportunistic pathogens (including *Clostridia, Enterococcus, Enterobacter,* and *Klebsiella* species) associated with the hospital environment is enhanced when compared to vaginally delivered babies (60),(64). Diseases in childhood and later in life are potentially implicated by perturbations to the early colonizers of the infant gut microbiota (65).

Establishment of the gut microbiota is also largely influenced by type of feeding whether it be breastmilk or infant formula, with additional influence from environmental factors including antibiotic use (66). In breast-fed infants *Bifidobacterium* and *Lactobacillus* dominate the gut microbiota. Milk contains numerous microbiota-friendly compounds including indigestible glycans referred to as human milk oligosaccharides (HMO) which are easily broken down by *Bifidobacterium*, secretory IgA which is an antibody that plays a role in the immune function of mucous membranes in the intestine, and antimicrobial factors (67). Human breast milk is a unique, concentrated source of key nutrients and bacterial species that contribute to the development of the infant gut microbiome. In formula-fed infants, *Enterococcus, Enterobacteria, Bacteroides, Clostridia*, and other anaerobic *Streptococcus* dominate the gut (68). A recent large-scale study on 903 children concluded that in their sample,

breastfeeding status was the most significant factor associated with microbiome structure in early life (69).

2.4 Developmental Programming

While the gut microbiota play a role in the development of metabolic diseases like obesity and T2DM, evidence suggests that they are also closely linked to genetic and environmental factors (70). Around 250 genes are now associated with obesity with the fat mass and obesity-associated (FTO) gene on chromosome 16 being the most important as it carries the highest risk of the obese phenotype (70–72). Notwithstanding, genetic mutations alone cannot completely explain the inheritance of obesity as it is now well-established that environmental influences are also an important factor in disease etiology. Epigenetics refers to a heritable change in the state of expression and function of a gene that does not involve alterations to the sequence of DNA. Numerous human and animal studies confirm a strong relationship between epigenetics and metabolic disease (21). Epigenetic modifications can arise as a result of various external behavioral and environmental influences such as from chronic heavy metal exposure or pollution, traumatic stress, and nutrition (73). Today, epigenetics is recognized as a major mechanism for the transmission of phenotype in developmental programming.

There is large body of evidence demonstrating the role of maternal nutrition in developmental programming effects on offspring. A landmark study conducted in 1989 found a link between low infant birth weight and weight at age one year with ischemic heart disease mortality at age 64 years in England (74). A subsequent study found that men with lower birth weight had greater body mass index (BMI), waist-to-hip ratio, blood pressure, and fasting plasma glucose concentrations in adulthood (75). These findings gave rise to the Developmental Origins of Health and Disease (DOHaD) theory, which recognizes the influence of the *in utero* environment on programming metabolic outcomes in children (76).

From DOHaD theory, Barker proposed the fetal origins of adult disease (FOAD) hypothesis which holds that events during early development have a profound effect on one's risk for the development of future disease in adulthood (77,78). Organs during fetal development are highly adaptable to environmental conditions, a phenomenon that is meant to improve offspring survival after birth. For example, undernutrition during gestation signals to the developing fetus that the outside environment is nutritionally inadequate and can lead to microsomia and increased energy retention (76). A possible explanation for this is that maternal undernutrition during pregnancy leads to the preferential allocation of energy to one organ (i.e. brain) at the expense of another organ (i.e. liver), which may increase survival but can lead to health issues and a diminished ability to respond to adverse environmental

impacts in adulthood (76). This phenomenon is also referred to as "brain sparing" (79). Undernutrition *in utero* may lead the fetus to develop energy conservation methods which could lead to insulin resistance (80). Conversely, a number of rodent studies show that maternal overnutrition and obesity during pregnancy and/or lactation have been linked with the development of increased adiposity and metabolic dysregulation in offspring compared to control dams in which the offspring are challenged with a high fat diet after weaning (81). High maternal energy intake in pregnancy may result in altered hypothalamic development driving appetite, energy intake, food preferences, and body composition after birth via alterations in leptin and insulin signaling (82). Additionally, maternal overnutrition results in greater levels of fetal free fatty acids (FFAs), placental levels of pro-inflammatory cytokines, and epigenetic modifications of genes involved in DNA methylation, histone modifications, and microRNAs variations that can collectively change organ structures, cell numbers, and metabolism in offspring contributing to metabolic disorders later in life (83).

In addition to the powerful lifelong influence exerted by maternal diet on offspring, the gut microbiota is an important environmental factor that warrants further research given its ability to modulate obesity and T2DM risk. Vertical transmission of gut microbiota from a mother's birth canal and feces to the sterile gut of offspring at birth is one way metabolic health can be programmed in early life (59,63). It is therefore equally important to understand the lifelong implications a dysbiotic gut may have on offspring in early life and into adulthood. Thus, exploring the potential impact of nutrition on the maternal gut microbiota is of paramount importance. One dietary factor that has been shown to alter the gut microbiota and is gaining interest from a public health standpoint is artificial sweeteners.

2.5 Artificial sweeteners: Aspartame

Perceptions and trends in artificial sweetener consumption vary between countries (84) yet in response to increased worldwide rates of obesity, consumption of artificial sweeteners has risen in the USA and is on the rise worldwide in particular among women and children (85–87). Secondary to increased rates of obesity and diabetes and calls to reduce sugar intake, the global market for artificial sweeteners grew 5.1% annually between 2008 and 2015 (88). Artificial sweeteners have become an increasingly popular alternative to sugar due to their sweet taste and absent/negligible calories. They are now used worldwide in beverages, food products, drugs, and even mouthwashes (89–91). In Canada, nine artificial sweeteners are permitted to be sold as tabletop sweeteners including advantame, acesulfame-potassium, aspartame, erythritol, monk fruit extract, neotame, saccharin, steviol glycosides,

and sucralose (92). Of these, aspartame is one of the most widely used and popular artificial sweeteners approved for use in more than 100 countries (90,93).

Aspartame was accidentally discovered in 1965 by chemist James Schlatter and by 1981 was approved for use in certain foods in 1981, soft drinks in 1983, and authorized as a general-purpose sweetener for foods and beverages in 1996 (94). Aspartame is not without controversy and continues to be studied for its effects on the body. There is mixed evidence associating its use with obesity (95,96), and T2DM in humans (97,98). An acceptable daily intake (ADI) of aspartame was established at 40 mg/kg per day by Health Canada whereas the Food and Drug Administration (FDA) establishes an ADI of 50 mg/kg per day (90). The Canadian ADI is recognized internationally and was recently reaffirmed by the Joint Expert Committee on Food Additives of the Food and Agriculture Organization/World Health Organization (91).

Aspartame metabolism produces approximately 4 kcal/g of energy, which is negligible in the diet as aspartame is estimated to be up to 200 times sweeter than table sugar [137]. In contrast with table sugar which is made up of two simple sugars glucose and fructose, aspartame is composed of two amino acids linked together, aspartic acid and phenylalanine. Digestion of aspartame occurs in the small intestine by enterases and peptidases and releases phenylalanine (50%), aspartic acid (40%), and a small amount of methanol (10%) (99). Only the hydrolyzed components are absorbed into the circulation and metabolized following their normal metabolic pathways (100). Methanol is metabolized in the liver, while phenylalanine and aspartic acid enter the free amino acid pool (93). Thus, these components are taken up by peripheral tissues, utilized for protein synthesis, metabolism, and excreted hence aspartame does not accumulate in the body as it is rapidly digested (101) and it does not reach the colon (102,103). The resultant compounds of methanol, phenylalanine, and aspartic acid are not unusual as they are also found in much larger quantities in foods that are consumed frequently such as meat, milk, fruits, and vegetables (93). Caution, however, must still be exercised as evidence suggests that aspartame's metabolites can be harmful at high doses therefore prolonged aspartame exposure may be a risk factor (104,105) as they are believed to be more toxic than the original substance itself (106,107).

Interestingly, human studies found that aspartame was not present in breastmilk likely due to its rapid metabolism in the duodenum however future studies should examine if aspartame consumption could alter the presence of its metabolites in breastmilk [168]. Studies in humans reported, however, that the metabolic products of aspartame cross the placenta (108–110). During its transient existence, the placenta performs essential actions that are later taken on by the lungs, liver, gut, kidneys, and

endocrine glands (111). It acts as a gateway between the fetal and maternal circulation and is subject to metabolic, endocrine, and inflammatory changes as a result of maternal nutrition (112). The human placenta mediates net transfer of amino acids to the fetus, with amino acid concentrations being higher in fetal plasma compared to maternal plasma, indicating an active transfer process across the placenta (113). A number of human studies have found that the amino acid contribution from aspartame is minimal compared to other food sources (114,106,115,116). One study found that an abuse dose of 200 mg/kg of aspartame was not found to result in methanol poisoning or mental retardation caused by increased levels of phenylalanine in the offspring (117). Evidence suggests the body utilizes the amino acid metabolites and methanol consistently irrespective of the food source (93).

Aspartame consumption during gestation and lactation has been studied in rodents. In rats, increased body weight, fasting glucose, and impaired learning performance were seen in males exposed to high doses of aspartame (50 mg/kg) beginning in utero and continuing with chronic direct postnatal exposure (118). In another study, male rats expressed significant memory and learning impairment compared to control when exposed to excessively high doses of aspartame (200 mg/kg) which the authors posited was due to higher phenylalanine concentrations in utero. The same study found that high dose chronic consumption of aspartame prenatally could contribute to development of a less effective satiety mechanism leading to compensatory over-eating and positive energy balance in later life (119). These studies are controversial because their high dosage does not represent a reasonable consumption of aspartame in humans within ADI levels. A study on the impact of low-dose artificial sweetener consumption coupled with an obesogenic diet on rat dams and their offspring found that consumption of aspartame during lactation and gestation increased obesity in their offspring at weaning (9). A detrimental effect on glucose and insulin tolerance was noted in young male offspring. Additionally, the gut microbiota in rat dams and their offspring in early life were also altered with changes in the expression of genes in the mesolimbic reward system seen in offspring (9). The mesolimbic pathway is a dopaminergic pathway in the brain and regulates motivation and desire for rewarding stimuli, facilitates reinforcement, reward-related motor function learning, and may also play a role in the perception of pleasure (120). The dysregulation of the mesolimbic pathway and its output neurons in the nucleus accumbens (NAc) plays a significant role in the development and maintenance of an addiction (121). Furthermore, while central resistance to leptin commonly seen in obesity is known to occur in the hypothalamus, it can also occur in the mesolimbic pathway due to changes in the NAc mu-opioid receptor as a result of maternal diet and artificial sweetener consumption and impact food preference (9). Recent research in mice suggests that exposure to artificial sweeteners *in utero*

stimulates postnatal weight gain, insulin resistance, increased adipocyte accumulation, and adipocyte differentiation via a temporally regulated set of gene-expression events that create a large reservoir of adipocytes capable of supporting the development of obesity in response to over-feeding in later life (122). Notwithstanding these findings, a recent systematic review and meta-analysis of rodent models exploring the metabolic and behavioural effects of exposure to artificial sweeteners found that the balance of evidence suggests that a maternal diet with artificial sweeteners during pregnancy and lactation did not increase body weight in offspring (123).

While aspartame has been linked to increasing T2DM risk (124), a systematic review of observational studies and clinical trials argues that the true relationship between aspartame and metabolic disease is unclear (98). Meta-analyses based on randomized controlled trials (RCTs) showed no significant effect of aspartame alone on caloric consumption or body weight compared to sugar or water in individuals with either obesity or T2DM (125). However, when chronic long-term artificial sweetener consumption is examined in prospective studies, associations with increased body weight and increased of T2DM are evident (126). Although the evidence is not unanimous, it has been suggested that the use of artificial sweeteners such as aspartame can lead to body weight gain, increased prevalence of obesity, an altered metabolic profile, and an increased sensation of hunger in humans (127) and rodent models (9). These results challenge the long-held belief that artificial sweeteners like aspartame are “benign” and pass through the body with no metabolic effect (102). Assessing the influence of aspartame on the growth in the number of people living with obesity remains challenging because it is difficult to elucidate whether the occurrence of obesity is specifically associated with the consumption of artificial sweeteners including aspartame or with the overconsumption of calories due to compensation and increased sensation of hunger (127).

In 2014, a landmark study by Suez, Korem, and Zeevi (2014) demonstrated that consumption of artificial sweeteners including aspartame alters the gut microbiota in lean rats thereby offering a potential mechanism for the development of metabolic syndrome, glucose intolerance, and obesity seen in the rats. In this study, lean rodents that were fed artificial sweeteners (aspartame, sucralose, or saccharin) in their drinking water within acceptable daily intake levels presented with glucose intolerance compared to control groups receiving either water alone or water containing glucose. Saccharin presented with the most profound effect on glucose tolerance and considerable gut dysbiosis, therefore it was the primary sweetener examined in a subsequent fecal microbiota transplant experiment (128). The glucose intolerant phenotype could be transferred to germ free mice confirming the role of altered microbiota in the effects of this artificial sweetener. In rats, aspartame consumption

increased the abundance of *Clostridum leptum* and *Enterobacteriaceae* and metabolomics analysis revealed an increase in the short-chain fatty acid propionate, a gluconeogenic substrate (10). In a 2020 rodent study, fecal microbiota was impacted by artificial sweetener consumption including aspartame in dams and early life in their offspring where they showed a higher relative abundance of *Clostridium leptum* where this feature was absent in the offspring of dams who did not consume artificial sweeteners (9). Collectively, evidence suggests that artificial sweeteners, potentially via different mechanisms (129) can perturb the gut microbiota. Whether other dietary compounds can compensate for this is not known but will be investigated through prebiotic fiber.

2.6 Prebiotic Fiber

Several dietary compounds including prebiotics have been investigated to mitigate the programmed risk of obesity conferred to offspring via maternal obesity. Prebiotics have been shown in multiple rodent studies to attenuate the deleterious effects of obesogenic diets on both maternal and offspring adiposity and metabolism (130). In animal and human studies, increased consumption of prebiotics has shown potential to improve metabolic health including decreased body weight, improved insulin sensitivity, reduced inflammation, and an increase in beneficial microbiota (131).

The International Scientific Association for Probiotics and Prebiotics defines prebiotics as "a substrate that is selectively utilized by host micro-organisms conferring a health benefit" and it is its selective utilization that distinguishes prebiotics from other substances or compounds that may affect the gut microbiota more broadly (12). The guiding principle of this criterion is that a limited number of microbial groups are affected and that the microbial groups and metabolites affected are linked to a beneficial effect on health (12). The majority of prebiotics can be categorized as inulin-type fructans (ITF), galactooligosaccharides (GOS) (132), and lactulose, and these are accepted by ISAPP as prebiotics (12). ITF is a general term referring to all beta (β) (21à) linear fructans and includes native inulin, short-chain fructooligosaccharides (scFOS), and oligofructose. ITF and GOS promote the growth of *Lactobacillus* and *Bifidobacterium*, among other less abundant, but beneficial, microbial species (*Faecalibacterium prausnitzii*) (12). Native inulin and oligofructose are found in artichokes, asparagus, bananas, chicory root, garlic, onions, leeks, and wheat (133). They possess 30-50% of the sweetness of table sugar (134).

Due to the nondigestible nature of prebiotics, they are metabolized by gut bacteria in the distal small intestine and proximal large intestine via fermentation. Fermentation of prebiotic fiber in the gut is an anaerobic process that produces energy and metabolites (135). The principal end products of said

fermentation are short-chain fatty acids (SCFAs) primarily acetate, propionate, and butyrate (12). SCFAs play important roles in intestinal health and metabolism including glucose homeostasis, lipid metabolism, the immune system, and appetite (136). Acetate makes up at least half of the total SCFAs produced and is a central metabolite in human metabolism involved in many biochemical pathways including fatty acid and glucose metabolism (135). Animal and human data demonstrated that acetate beneficially affects host energy and substrate metabolism via secretion of gut hormones like glucagon-like peptide-1 (GLP-1) and peptide YY (PYY) which influence appetite through increased satiety and parasympathetic activity. A reduction in whole-body lipolysis, systemic pro-inflammatory cytokine levels, and an increase in energy expenditure and fat oxidation are also associated with acetate (137). Propionate has been linked to the regulation of appetite, maintenance of blood glucose levels, and reducing inflammation (138–140). The regulation of immune cells that assist in controlling intestinal inflammation may be beneficial in maintaining the gut barrier (139) and be protective in guarding against diseases like irritable bowel syndrome (141). Butyrate has been linked to beneficial effects on intestinal homeostasis and energy metabolism by supporting the gut barrier and supporting colonocytes respectively. Butyrate has also shown protective actions in the context of inflammatory diseases like inflammatory bowel disease and colon cancer (142). A large body of evidence suggests butyrate has an effect on alleviating high-fat diet-induced obesity and insulin resistance by downregulating the expression and activity of PPAR-γ and promoting a change from lipogenesis to lipid oxidation (143). Butyrate also enhances the secretion of GLP-1 and PPY which help reduce appetite (144). Butyrate is the most studied of the SCFAs and although many studies support alleviation of obesity, there are a few studies that show the opposite effect (145).

The chemical structure of prebiotics notably chain length and branching plays a role in their fermentation in the intestines thus impacting their effects on the intestinal microbiota and/or health outcomes (146). Prebiotic fibers with shorter lengths like oligofructose are fermented more rapidly in the distal small intestine or proximal colon, whereas longer chain inulin is fermented more slowly in this area (147,148). Further research is warranted to determine the influence of prebiotic chain length on human health parameters like gastrointestinal physiology, glucose homeostasis, cardiovascular, and immune health although both short- and long-chain length prebiotic fibers have been associated with a bifidogenic effect (149). *Bifidobacterium* is an important gut bacterium known to confer health benefits on the host when administered in adequate amounts (150).

Consumption of prebiotics in clinical trials in children and adults with obesity or overweight demonstrated improvements in metabolic health and microbial species including improved appetite

control, decreased adiposity, decreased body weight, increased abundance of *Bifidobacterium* and decreased abundance of *Bacteroides vulgatus* (bacteria associated with adiposity) (151). Kellow, Coughlan, and Reid (2014) conducted a systematic review and noted various metabolic benefits of prebiotics in the absence of weight loss as the latter is not universally achieved across all clinical trials (152). Le Bourgot, Apper, Blat, and Respondek (2018) found that animal studies have also confirmed the benefits of prebiotics on host metabolism including increased numbers of enteroendocrine L-cells (specialized cells found within the GI tract that produce and release hormones in response to a number of stimuli and are involved in appetite and metabolism), recovery of gut barrier function, reduced hepatic accumulation of triglycerides and cholesterol, decreased *Firmicutes/Bacteroidetes* ratio, lower body fat, improved weight maintenance, and weight loss (153).

Studies in rodents suggest that dietary supplementation with prebiotic fiber modifies the gut microbiota, reduces body fat, and improves glucose tolerance (154,155). One study recently showed that antibiotic use during pregnancy increased adiposity and compromised post-partum weight loss in dams, whereas co-administering prebiotics with antibiotics in rat dams attenuated obesity risk in offspring associated with altered gut microbiota (156). While the benefits of prebiotic supplementation are encouraging, evidence regarding the ability of prebiotic fiber to 'rescue' metabolic health in compromised offspring, such as those of obese rat dams fed an obesogenic diet with low-dose aspartame during pregnancy and lactation, is lacking. We have previously shown that postnatal intake of prebiotic fibre by offspring can attenuate some of the detrimental metabolic effects of a maternal low protein diet (157). Whether prebiotics can likewise rescue the metabolic health of offspring of dams fed an obesogenic diet with aspartame, is not known but the goal of the proposed work.

2.7 Research Justification

Obesity and diabetes are serious health problems that are becoming increasingly common worldwide. The corresponding rise in the use of artificial sweeteners like aspartame has also compounded the problem with mounting evidence showing it may increase insulin resistance and thereby increase body weight. Maternal adiposity, metabolism, and diet have been linked to the transmission of obesity and insulin resistance to offspring. Given that the gut microbiota is particularly vulnerable to perturbation during pregnancy and/or in early postnatal life, identification of protective interventions that could mitigate microbial and metabolic risks of a maternal obesogenic diet coupled with aspartame consumption is of great interest.

Prebiotics, which are non-digestible carbohydrates preferentially utilized by beneficial gut microbiota, have been shown to have multiple metabolic benefits and could help to rescue metabolic and microbial health if consumed directly by offspring in early life. Identifying postnatal strategies, such as prebiotics, to mitigate the risks associated with *in utero* and perinatal exposure to obesogenic diets and aspartame is critically needed. Therefore, our overarching objective is to examine whether postnatal prebiotic fiber consumption can reduce the risk of obesity and insulin resistance in offspring of female rats consuming a high fat/sucrose diet plus aspartame during pregnancy and lactation.

2.8 Research Objectives and Hypotheses

Primary Objective: To examine metabolic outcomes (adiposity, insulin resistance) in dams and offspring exposed to a maternal obesogenic diet throughout gestation and lactation with aspartame and postnatal prebiotic fiber intake

Secondary Objective: To assess gut microbiota composition in dams and offspring fecal matter using 16S rRNA sequencing.

Tertiary Objective: To assess liver triglycerides and the expression of genes regulating hepatic fat synthesis in offspring.

Hypothesis: We hypothesize that postnatal consumption of prebiotic fiber by rat offspring mitigates the detrimental effects of a perinatal maternal obesogenic diet with low-dose aspartame consumption in rat dams.

CHAPTER THREE: METHODS

3.1 Ethical Approval

All animal experiments were approved by the University of Calgary Animal Care Committee (AC23-0061) and conducted in accordance with the principles outlined in the Canadian Council on Animal Care *Guide to the Care and Use of Experimental Animals*.

3.2 Animals, Diets and Experimental Design

Eighty-four female Sprague Dawley (SD) rats, aged 6 weeks, were purchased from Charles River Laboratories (Montreal, QC, Quebec). SD rats are frequently used in health research as they are an outbred strain of rat possessing a high degree of genetic variability similar to a genetically diverse human population (158). Thus, SD rats provide an effective model to study polygenic diseases like obesity (159) Rats were housed in a temperature and humidity-controlled room with a 12-hour light/dark cycle in the Life and Environmental Sciences Animal Resource Centre (LESARC). The rats underwent an obesity induction phase for 11 weeks where they consumed a custom obesogenic high-fat, high sucrose (HFS) diet (Dyets #102412, Dyets Inc., Bethlehem, PA) *ad libitum*. The diet is composed of 39% of calories from fat (lard and soybean oil) and 44% of calories from sucrose and resembles a Western diet given its high saturated fat and sugar content. The HFS diet has been used in previous studies to induce obesity with metabolic disturbances and deleterious effects on the liver including hepatic steatosis and mild inflammation observed in rodents (9,130,160). Body weight in the females was measured weekly. After the 11-week obesity induction phase, the top 50% of weight gainers were considered obesity-prone and remained in the study. The obesity-resistant female rats were euthanized.

The obese females were paired randomly with one 11–14-week-old male Sprague Dawley rat (n=15) and mated in wire bottom cages during the dark cycle for consecutive nights until pregnancy was confirmed through the identification of a copulation plug in the morning. During mating, the rats had access to a standard pellet rat chow diet (5001 – Laboratory Rodent Diet, Canadian Lab Diets, Inc., Leduc County, AB) and water *ad libitum*.

Plug-positive females were randomly allocated to one of two groups throughout pregnancy (3 weeks) and lactation (3 weeks): (1) HFS + water (control group, CTR); (2) HFS + aspartame (experimental group, APM) (5-7mg/kg; Fluka, Charlotte, North Carolina, USA). Aspartame (APM) was administered in drinking water (changed weekly) throughout pregnancy and lactation and the dose was adjusted based on body weight and fluid intake. The dose of APM administered at 7mg/kg/day falls well below the Canadian ADI of 40mg/kg/day and the duration of its consumption in the study resembles the normal

intake of APM via diet sodas throughout gestation and lactation in humans. Baseline body weight was measured to ensure there were no significant differences between the two groups prior to the start of the intervention. The HFS diet was provided in a powder form and purchased from Dyets Inc. (Bethlehem, PA, USA). Detailed composition of the diets used in this study is provided in Table 1.

Table 1. Experimental diet compositions

	HFS (Dyets # 102412)	HFS-Pre (Dyets # 102412)
Casein	200	180
Sucrose	499.48	499.532
Soybean Oil	100	90
Lard	100	90
Cellulose	50	45
AIN-93M Mineral Mix	35	31.5
AIN-93-VX Vitamin Mix	10	9
DI-Methionine	3	2.7
Choline bitartrate	2.5	2.25
t-Butyllhydroquinone	0.02	0.018
OFS	0	100
Total (g)	1000	1000
Protein (kcal%)	15.8	15.3
Carbohydrate (kcal%)	45	46.9
Fat (kcal%)	39.2	37.8
Energy Density (kcal/g)	4.60	4.29

Due to effects of obesity on fertility (161), not all females became pregnant which resulted in n=15 dams in each of the CTR and APM groups. The body weight of dams was measured weekly throughout gestation and lactation and food intake was measured for 5 consecutive days every 2 weeks throughout the study.

Following delivery, litter sizes and offspring sex ratios were recorded. Litters were culled to n=10 within 24 hours after birth, with an equal number of males to females (5 male and 5 female pups) where possible to minimize metabolic impacts associated with differences in nutrition and maternal care between varying litter sizes. Pup body weights were measured at birth as postnatal day (PND) 0 and again at weaning at PND 21. At weaning (3 weeks of age), one dam, one male, and one female pup from each litter were tested according to the procedures described below. An additional two males and two females from each dam, doubly housed by sex and within treatment, were carried forward and assigned to: (1) CTR-CTR (water control offspring consuming HFS diet), (2) CTR-Pre (water control offspring consuming HFS diet + prebiotic fiber as oligofructose (OFS) (10% wt/wt)), (3) APM-CTR (aspartame offspring consuming HFS diet), (4) APM-Pre (aspartame offspring consuming HFS diet + prebiotic fiber

oligofructose (OFS) (10% wt/wt)). Prebiotic diets were mixed in-house by combining 900 g of HFS diet with 100 g of OFS. The offspring consumed these diets for nine weeks. The remaining unused pups at weaning were used in another concurrent study. Offspring body weight was measured weekly and food intake was measured for 5 consecutive days every 3 weeks in the offspring.

While prebiotic fiber consumption is associated with improvements in metabolic health, body composition, and inflammation (12), prebiotic oligofructose was specifically chosen as the postnatal diet as it is known to enhance the growth of bifidobacteria (12). A "bifidogenic effect" is important given the role of bifidobacteria in early gut microbiota development and the promotion of a lean phenotype with potential to mitigate obesity risk (156).

In total, 1 female (receiving dietary intervention) was mated with 1 male breeder to yield a single litter, adjusted to equal size to control for litter effects, and then the dam, 1 male and 1 female offspring from each dam was tested at weaning (3 weeks of age). The remaining offspring of each litter, 2 males and 2 females, were allocated to their respective groups and were tested at the end of the study (12 weeks of age). Testing followed the procedure described below. Please see Appendix A for the experimental flow chart and Appendix B for the condensed version.

3.3 Oral Glucose Tolerance Test (OGTT)

Two days before euthanasia, in the morning following a 12 hour overnight fast, fasted blood glucose was measured from a tail nick sample using a Contour Next One Blood Glucose Meter (Ascensia Diabetes Care, Mississauga, Canada) in the dams and adult offspring (12 weeks of age). Additional blood glucose measurements were made at 15, 30, 60, 90, and 120 minutes following administration of a 2g/kg glucose load given by oral gavage. Total glucose area under the curve (AUC) was calculated based on the mathematical models described by Tai (162).

Following ingestion of an oral glucose load, the incretin effect is conveyed by secretion of two incretin hormones glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) from enteroendocrine cells in the gut which elicit secretion of insulin from the beta cells of the pancreas (163). The incretin effect is a phenomenon where oral glucose elicits a higher insulin response than an administration of an identical amount of glucose via an intraperitoneal glucose tolerance test (IPGTT) as administration of glucose utilizing the latter method would not travel through the gastrointestinal tract and would not involve the secretion of incretin hormones (164). The OGTT determines how effectively insulin is secreted from the beta cells of the pancreas and how quickly glucose is cleared from the blood (20).

3.4 Insulin Tolerance Test (ITT)

Four days before euthanasia, following a 6 hour fast, fasted blood glucose was measured in the dams and adult offspring (12 weeks of age) from a tail nick sample using the Contour Next One Blood Glucose Meter. In the 3-week-old offspring, the ITTs were conducted 1 day before euthanasia following a 3 hour fast. Using an intraperitoneal injection, a 0.75 U/kg dose of insulin was administered to the rats, and blood glucose concentrations measured via tail nick at 15, 30, 60, 90 and 120 minutes following the insulin injection. Total glucose area under the curve (AUC) was calculated.

By measuring blood glucose levels before and after the administration of insulin, the ITT is designed to assess the peripheral sensitivity of insulin receptors (165). The ITT is a standard test used to assess insulin sensitivity in humans and animal models (166).

3.5 Body Composition and Tissue Harvest

To assess body composition, a Dual energy X-ray Absorptiometry (DXA) scan (GE Lunar Prodigy, AymesMedical) was performed 1 day before euthanasia (on the day of euthanasia for the weanlings). The rats were placed under light anesthetic (isoflurane) to ensure they remained stationary during the scan. Weight (g), lean mass (%), body fat (%), and relative bone mass (g/kg) were quantified using GE Lunar Prodigy (Mississauga, ON) software for small animals.

All adult rats in the study including dams were euthanized following an overnight fast via over-anesthetization with isoflurane and subsequent aorta cut. Organs and tissues were excised and weighed (heart, liver, kidney, jejunum, ileum, colon, and cecum). Samples from the liver and intestinal segments were immediately flash frozen using liquid nitrogen and stored at -80˚C until further analysis. Weanling rats were not fasted overnight and these were sacrificed immediately following their DXA scan.

3.6 Hepatic Triglyceride Analysis

To assess triglyceride content, 25mg portions of liver tissue were analyzed according to the manufacturer's instructions for the Stanbio™ Triglyceride (GPO)(Liquid) Reagent Set (Fisher Scientific™, Ottawa, ON).

3.7 Hepatic mRNA Expression

Total RNA was extracted from 100 mg of frozen liver tissue using TRIzol reagent (Invitrogen, Carlsbad, CA) and absorbance was measured at 260 and 280 nm (NanoDrop; Thermo Fisher Scientific). A total of 1 µg of RNA was used for reverse transcription. The resulting cDNA was amplified using primers listed in Table 2. The PCR reaction consisted of heating for 1 min 30 s then 40 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 20 s. Actin primers were included as a suitable housekeeping gene. mRNA levels were calculated using the $2^{-\Delta CT}$ method where:

$$\Delta C_T = C_T(gene\ of\ interest) - C_T(reference\ gene\ )$$

The liver genes that were assessed are listed in Table 2. Fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC) are master enzymes in fat synthesis whose genes are expressed in the liver with the former catalyzing the last step in the fatty acid biosynthetic pathway. Upregulated levels of hepatic FAS and ACC may be linked to the development of obesity (167). Insulin receptor substrate 1 (IRS-1) and insulin receptor substrate 2 (IRS-2) are signaling proteins involved in the signal transduction pathway of insulin. IRS-1 and IRS-2 play a key role in the eventual translocation of the GLUT-4 transporter protein from the cytoplasm to the plasma membrane. The presence of IRS-1 and IRS-2 is crucial for proper insulin function (168). Interleukin 6 (IL-6) and tumor necrosis factor alpha (TNFa) are proinflammatory cytokines responsible for a wide range of signaling events within cells involved in the immune response. Upregulated expression levels of IL-6 and TNFa are linked to the development of obesity and related metabolic disorders like T2DM (169). Actin was chosen as the housekeeping gene because it is highly stable, expressed in all cells and conditions, essential, belongs to cellular maintenance pathways, and is well conserved (170,171). All of these properties make actin an excellent internal control gene.

Table 2. Quantitative PCR primers for mRNA in liver tissue

Genes	**Forward primer**	**Reverse Primer**
FAS	5'-GCCGTGGTGCTGGAGATTG-3'	5'-TGCCGAGGTTGGTGAGGAAG-3'
ACC	5'-CCTTCTTCTACTGGCGACTGAG-3'	5'-TAAGCCTTCACTGTGCCTTCCA-3'
IRS-1	5'-CTCTGCTTCTGCTTCTGTTAC-3'	5'-TGGTTATGGTTGGGACTTAGG-3'
IRS-2	5'-AAGATAGCGGGTACATGCGAAT-3'	5'-GCAGCTTAGGGTCTGGGTTCT-3'
IL-6	5'-CTCCGCAAGAGACTTCCAG-3'	5'-GGTCTGTTGTGGGTGGTATC-3'
TNFa	5'-GTCGTAGCAAACCACCAAG-3'	5'-AGAGAACCTGGGAGTAGATAAG-3'
Actin	5'-AGATCAAGATCATTGCTCCTCC-3'	5'-ACATCTGCTGGAAGGTGGAC-3'

Note: Actin was used as the housekeeping gene.

DNA quantity and quality were assessed using a NanoDrop spectrophotometer which measures nucleic acid concentration and quality. The ratio of DNA's absorbance maxima at 260 nm to the absorbance at 280 nm is used as a measure of purity (172). DNA purity was assessed using the ratio of absorbance at 260 nm and 280 nm where a ratio of ~1.8 is generally accepted as "pure" for DNA with lower values (<1.6) indicating the presence of proteins, phenol, or other contaminants that absorb strongly at or near 280 nm (173).

3.8 Bacterial DNA Extraction and 16S rRNA Gene Sequencing

Maternal fecal matter was collected at week 2 of pregnancy and week 2 of lactation. Offspring fecal matter was collected at 3 (weaning), 6, 9, and 12 weeks of age. Once collected, the fecal matter was stored at -80˚C until further analysis. Bacterial DNA was extracted using the FastDNA spin kit for feces (MP Biomedicals, Lachine, QC, Canada) from ~75 mg of stool with bead beating. The samples were then diluted to a concentration of 4ng/μL and stored at -30˚C. DNA samples were then sent to the Centre for Health Genomics and Informatics at the University of Calgary (Calgary, AB, Canada) for sequencing. Microbial composition was quantified using the MiSeq Illumina 16S rRNA gene sequencing platform with amplification of the V3 and V4 hypervariable regions of the bacterial 16S rRNA gene (Illumina, San Diego, CA, USA). Microbiota was assessed for alpha and beta diversity, taxonomic composition, and differential abundance as detailed below.

3.9 Statistical and Bioinformatics Analysis

All biological data are presented as mean ± standard error of the mean (SEM). Normality of the data was assessed using Shapiro Wilk's normality test and assessment of quantile-quantile plots and histograms. Boxplots were utilized in the identification of outliers. Confirmed data entry errors were corrected when present(174). If data were skewed, a non-parametric test was used. The Mann-Whitney U Test was employed when comparing two groups of non-parametric continuous measures. This approach was used to compare the means of hepatic triglyceride data of dams which was not normally distributed. Notwithstanding the non-parametric analysis, the corresponding data was presented as mean ± SEM and illustrated in a bar graph consistent with prior work in the field. Outcomes with multiple time points (e.g., body weight, OGTT) demonstrating normal distribution were analyzed using repeated-measures ANOVA, where time was the *within-subject* factor and diet was the *between-subject* factor. If a significant interaction effect was identified, a one-way analysis of variance (ANOVA) with Tukey's post hoc where appropriate was used to determine differences between

groups. Outcomes with a single time point (e.g., fat mass, mRNA levels) demonstrating normal distribution were analyzed with a one-way ANOVA with Tukey's post hoc test where appropriate. In adult offspring, both males and females were assessed, therefore a multivariate general linear model was first used to determine whether there was a significant effect of sex on each outcome measure. If a significant sex effect was found, male and female offspring were assessed separately for that outcome as per the description above. Statistics were performed with IBM SPSS Statistics version 28.0 (SPSS Inc., Chicago, IL, USA) and significance set at $p \leq 0.05$.

Analysis of the gut microbiota data was performed as follows. Sequencing data from the MiSeq platform was de-multiplexed and converted to Fastq format using Illumina bcl2fastq software. All of the data analysis and taxonomy assignments were completed using R version 4.3.2. Primer removal and initial quality trimming using Cutadapt v4.1 was conducted with a minimum quality score of 20 to ensure high-quality reads were kept. Next, paired-end Fastq data was processed using the DADA2 pipeline (dada2 v 1.30.0) wherein forward and reverse reads were cleaned, denoised, and merged to generate an amplicon sequence variant (ASV) feature table. Chimeras were removed from the reads, and taxonomy was assigned to the representative sequences Silva database 138.1 was used to classify the sequences and assign their taxonomy.

For diversity analysis, analyses were carried out using the R package phyloseq (version 1.46.0). Alpha diversity was estimated using the Shannon, Chao1, and Observed species indices, normality was tested using Shapiro Wilk, as data were not normally distributed significance determined using Kruskal-Wallis and Wilcox test for p23 and p84. As alpha diversity data from dams was normally distributed, we used a T-test to compare means. Beta diversity was estimated using PCoA (principal coordinates analysis) on a matrix of Bray-Curtis distances. Before ordination, ASV counts were normalized using a variance stabilizing transformation 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 microbes were analyzed with DESeq2 (version 1.42.0), using a Wald significance test with alpha = 0.05 and correcting for multiple comparisons using the False Discovery Rate (FDR). All statistical outcomes were considered significant at $p \leq 0.05$, unless otherwise stated.

CHAPTER FOUR: RESULTS

4.1 Maternal Outcomes

The overall objective of this study is to examine the potential for prebiotic fiber to attenuate obesity risk in rat offspring following maternal consumption of an obesogenic diet with low-dose aspartame. We first examined the anatomical, glycemic, hepatic triglyceride, and gut microbial phenotypes of the maternal rats directly exposed to the diet.

4.1.1 Aspartame did not affect maternal body composition

In this study, body composition parameters such as weight (g), lean mass (%), body fat (%), and relative bone mass (g/kg) in dams at weaning (i.e. end of lactation) were not influenced by aspartame (Table 3). Similarly, aspartame did not affect maternal weight at any point in time throughout gestation and lactation (Figure 1).

Table 3. Anthropometrics of dams at weaning

	Diet	
	CTR	APM
N	13	15
Weight (g)	346.1 ± 3.6	344.9 ± 3.5
Lean Mass (%)	65.2 ± 1.2	66.0 ± 1.2
Body Fat (%)	32.6 ± 1.1	31.9 ± 1.2
Relative Bone Mass (g/kg)	13.5 ± 0.8	14.8 ± 0.7

Values are means ± SEM. No significant differences.

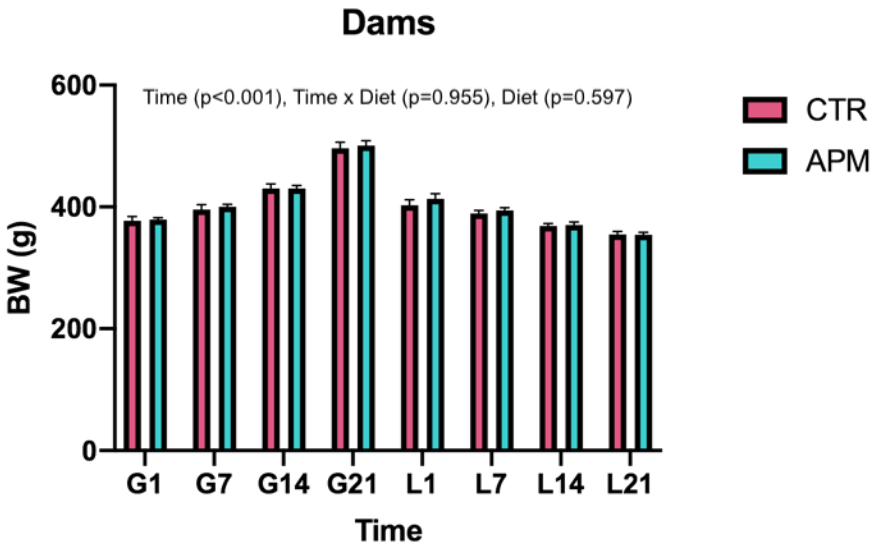

Figure 1. Maternal body weight from initiation of the dietary intervention at the beginning of gestation (3 weeks) throughout lactation (3 weeks). Values are mean ± SEM, n=13 CTR, n=15 APM.

4.1.2 Aspartame did not influence maternal relative organ weights

Organ weights were expressed relative to total body weight. There were no differences in relative liver or cecum weight (Figure 2) between aspartame and control dams.

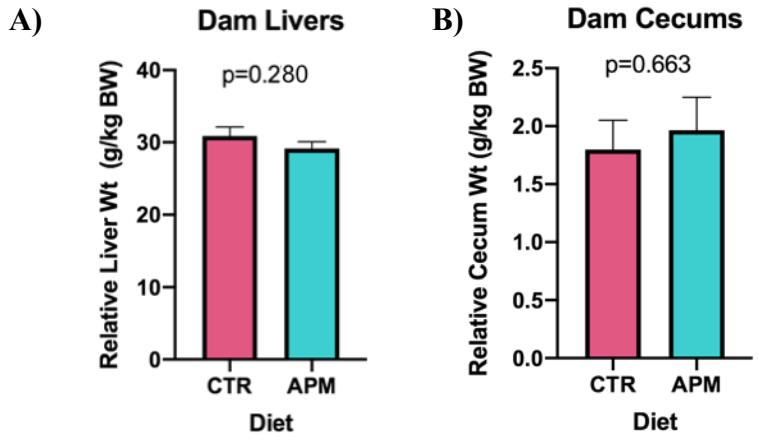

Figure 2. Maternal liver (A) and cecum (B) weight relative to total body weight. Values are mean ± SEM, n=13 CTR, n=15 APM.

4.1.3 Aspartame did not affect maternal insulin sensitivity and glucose tolerance

As expected during the OGTT, there was a significant main effect of time ($p<0.001$) on blood glucose with concentrations increasing at 15 minutes and falling thereafter (Figure 3A). Blood glucose levels (BGL) during the OGTT were not affected by diet ($p=0.106$) or a time×diet interaction ($p=0.106$). Moreover, there was no difference in glucose area under the curve (AUC) during the OGTT (Figure 3B).

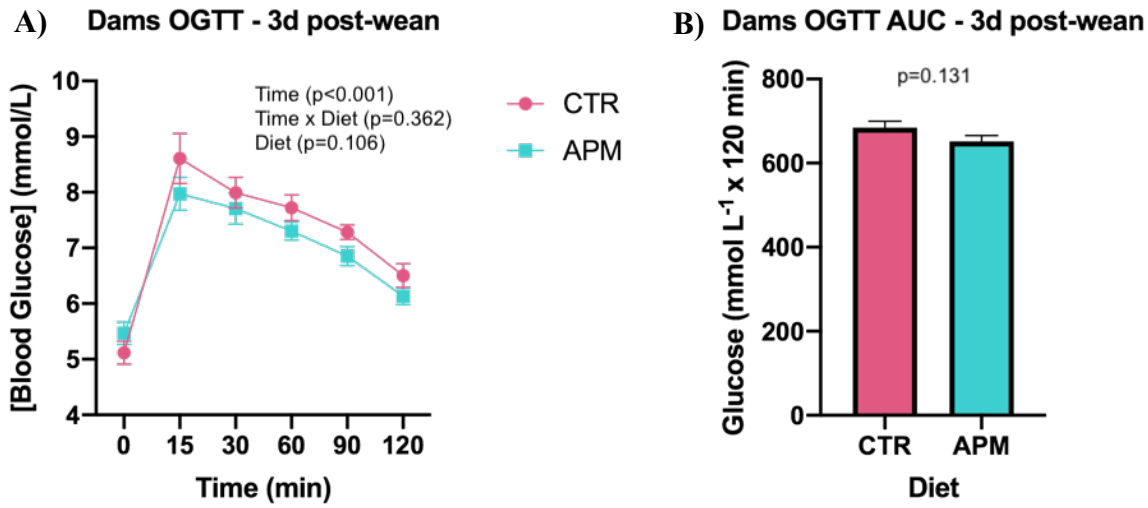

Figure 3. Maternal blood glucose concentrations (A) and glucose AUC (B) during an oral glucose tolerance test (OGTT). Values are mean ± SEM, n=13 for CTR, n=15 for APM.

BGLs during the ITT were significantly affected by time ($p<0.001$) with the lowest values observed at 60 minutes that then increased thereafter to 120 min in both groups (Figure 4A). BGLs during the ITT were not affected by diet ($p=0.273$) or a time×diet interaction ($p=0.963$). There was no difference in glucose AUC during the ITT (Figure 4B).

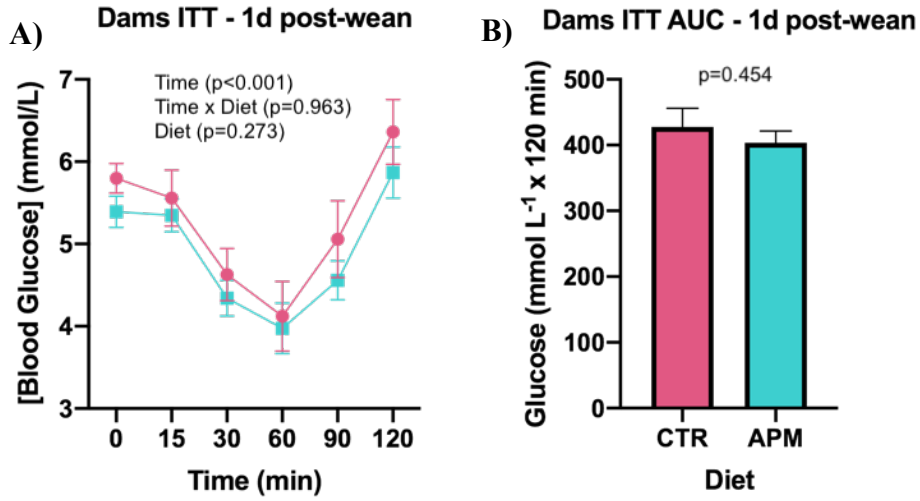

Figure 4. Maternal blood glucose concentrations (A) and glucose AUC (B) during an insulin tolerance test (ITT). Values are mean ± SEM, n=13 for CTR, n=15 for APM.

4.1.4 Aspartame did not affect maternal daily fluid intake

Fluid intake in dams was measured daily throughout gestation and lactation. Fluid intake was not affected by diet (p=0.663).

Table 4. Mean daily fluid intake in dams

	Diet	
	CTR	APM
N	13	15
Mean daily fluid intake (mL/day)	45.8 ± 1.7	44.9 ± 1.2

Values are mean ± SEM. No significant differences.

4.1.5 Aspartame did not affect energy intake

Food intake (grams of intake converted to kcal) was measured every other week through gestation and lactation for a five-day period (Figure 5). There was a significant main effect of time ($p<0.001$) with a significant increase occurring towards the end of lactation at L14-L18 across both groups. Food intake was not affected by diet (p=0.245) or a time×diet interaction (p=0.459).

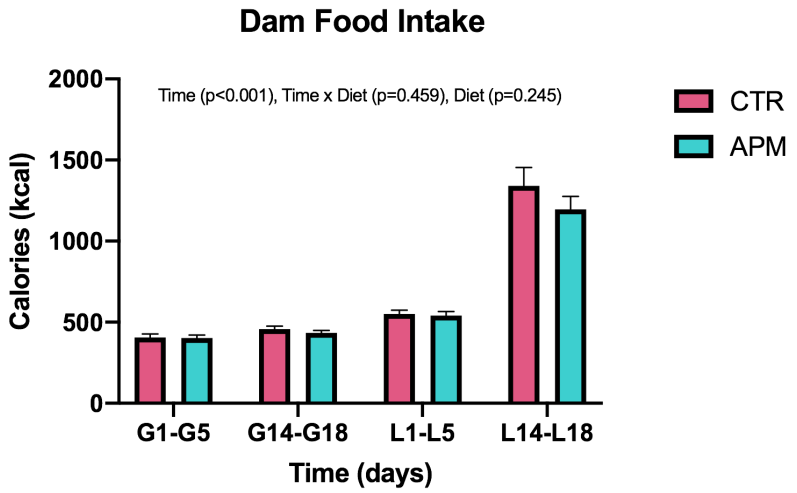

Figure 5. Energy intake (kcal) in dams measured from the initiation of the dietary intervention at the onset of gestation (3 weeks) throughout lactation (3 weeks). Values are mean ± SEM, n=13 CTR, n=15 APM.

4.1.6 Diet had no effect on maternal hepatic triglyceride levels

Hepatic triglyceride concentration (mg triglyceride/g liver) did not differ between CTR (32.2±3.6) and APM (31.7±3.4) dams measured at euthanasia (p=0.928, Figure 6).

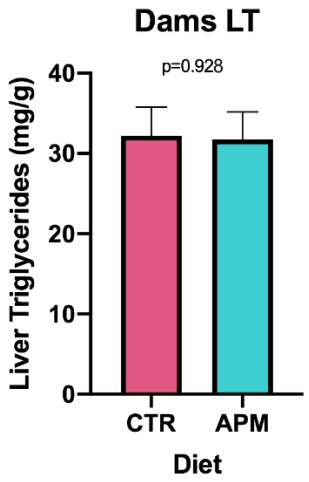

Figure 6. Maternal hepatic triglyceride levels. Values are mean ± SEM, n=13 CTR, n=15 APM.

4.1.7 Maternal reproductive stage but not diet significantly affected maternal gut microbial composition

Although there were no differences in Shannon and Chao1 indices of alpha diversity between CTR and APM dams, both Shannon (p=0.002; Figure 7A) and Chao1 (p<0.001, Figure 7B) were lower during lactation versus gestation.

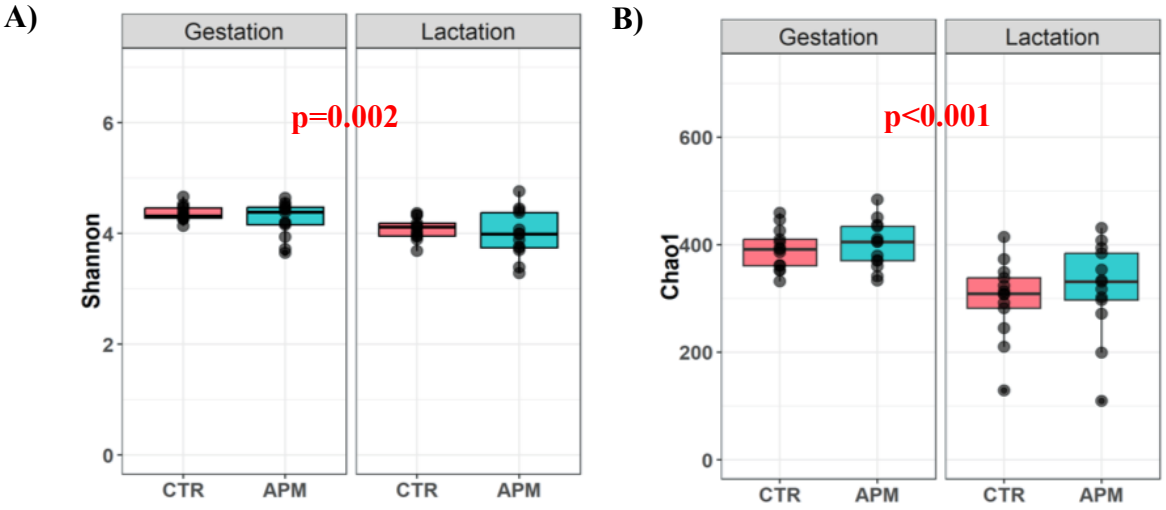

Figure 7. Alpha diversity indices in control and aspartame dams measured during gestation (G14) and lactation (L14) respectively according to Shannon (A), and Chao1 (B) indices. P-values represent gestation vs lactation, n=13 for CTR, n=15 for APM.

Similarly, beta diversity was not affected by maternal diet given that Bray Curtis Dissimilarity plots did not reveal significant compositional dissimilarity between CTR and APM groups (Figure 8). However, similar to alpha diversity, there was a significant (p=0.001) difference in community structure between gestation and lactation (Figure 8).

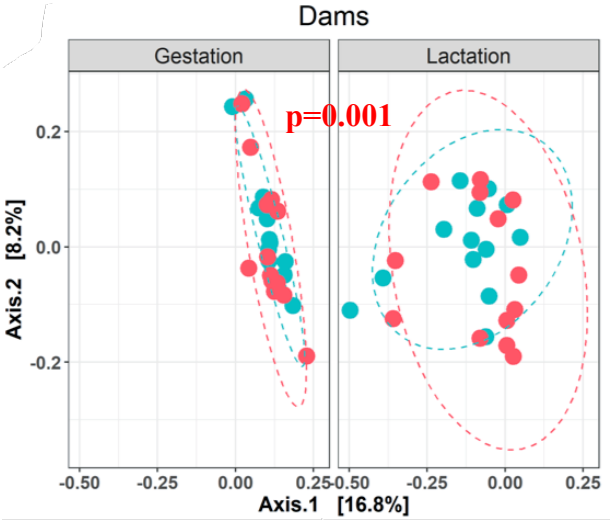

Figure 8. Principal Coordinate Analysis (PCoA) plot visualizing Bray-Curtis dissimilarity between control and aspartame dams measured during gestation (G14) and lactation (L14) respectively. Diversity was compared between groups using PERMANOVA with significance set at $p<0.05$, n=13 for CTR, n=15 for APM.

Next, taxonomical bar plots based on the 16S rRNA sequencing analysis are shown at the genus level between dams consuming CTR versus APM (Figure 9).

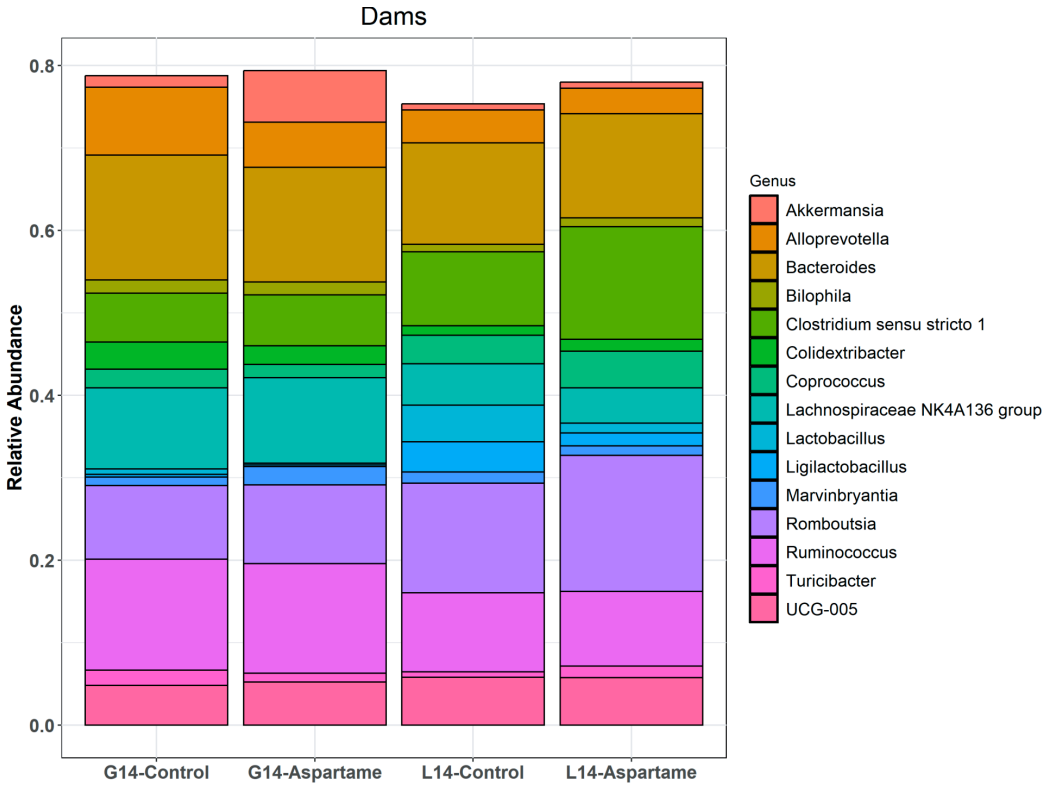

Figure 9. Taxonomical bar plot displaying gut microbial community composition at the genus level in dams consuming the control diet or aspartame at the time points of gestation and lactation. n=13 for CTR, n=15 for APM.

To investigate if specific microbiota in the dams were altered by aspartame, differential abundance analysis using DESeq2 was conducted. Similar to alpha and beta diversity, numerous bacteria were differentially abundant between the periods of gestation and lactation but there was no effect of aspartame (Figure 10).

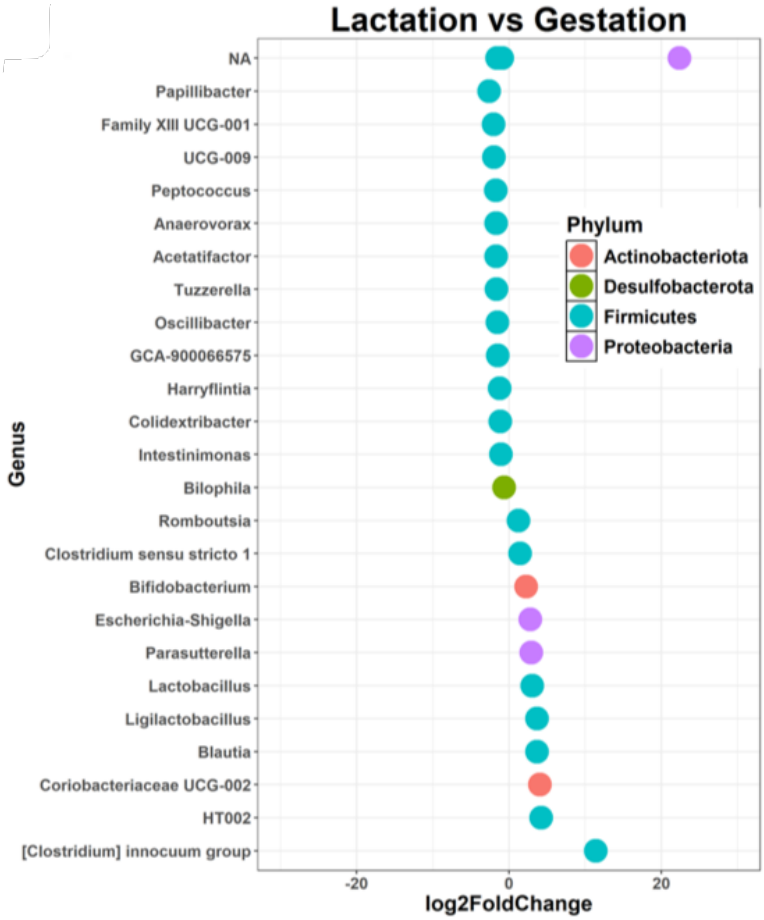

Figure 10. Differential abundance plot comparing dams at gestation versus lactation. n=13 for CTR, n=15 for APM. Taxa to the right of 0 log2FoldChange are higher in abundance in lactation versus gestation while those with a negative log2FoldChange are lower during lactation versus gestation.

4.2 Weanling Outcomes

We next sought to examine the effects of maternal aspartame consumption on their male and female offspring at weaning and into young adulthood. Thus, offspring birth weight, body composition, glycemic characteristics, hepatic triglyceride content, hepatic mRNA expression, and gut microbial composition were assessed at weaning (week 3) in 1 male and 1 female from each litter.

4.2.1 Maternal diet did not influence birth weight

Birth weight differed significantly by sex ($p<0.001$) therefore males and females were assessed separately. There was, however, no influence of maternal diet on offspring birth weight (Table 5).

Table 5. Birth weight of male and female offspring

	Diet	
	CTR	APM
N (males)	112	105
Male weight at birth (g)	6.4 ± 0.1	6.6 ± 0.1
N (females)	87	99
Female weight at birth (g)	6.1 ± 0.1	6.1 ± 0.1

Values are means ± SEM, n=199 for CTR and n=204 for APM.

4.2.2 Maternal diet had no influence on weanling body composition

No sex differences were detected for body composition at weaning, therefore males and females were analyzed together. No differences in any components of body composition were observed between CTR and APM offspring at 3 weeks of age (Table 6).

Table 6. Anthropometrics of offspring at weaning (3 weeks old)

	Diet	
	CTR	APM
N	28	30
Weight at weaning (g)	59.0 ± 2.1	59.2 ± 2.0
Lean Mass (%)	27.9 ± 1.8	29.9 ± 0.7
Body Fat (%)	66.2 ± 0.0	64.0 ± 0.0
Relative Bone Mass (g/kg)	39.1 ± 1.0	38.0 ± 0.4

Values are means ± SEM. No significant differences.

4.2.3 Maternal diet did not influence weanling relative organ weights

Organ weights were expressed relative to total body weight. No sex differences were detected. There were no differences in liver or cecum weight (Figure 11) between aspartame and control weanlings.

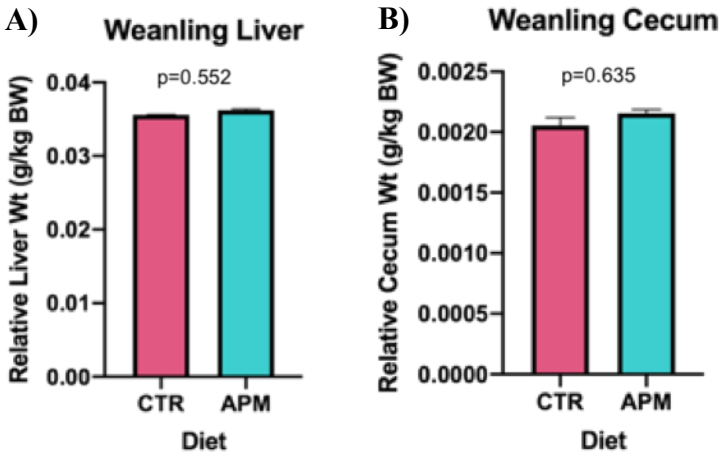

Figure 11. Weanling liver (A) and cecum (B) weights relative to total body weight. Values are mean ± SEM, n=28 for CTR, n=30 for APM.

4.2.4 Maternal diet did not influence weanling insulin sensitivity

No sex differences were detected therefore males and females were analyzed together. Weanling BGLs during the ITT were significantly affected by time ($p<0.001$) with the lowest value observed at 60 minutes with an increase thereafter to 120 minutes (Figure 12A). Weanling BGLs during the ITT were not significantly affected by maternal diet but a trend was observed ($p=0.069$) with APM having greater BGLs than CTR. No time×diet interaction ($p=0.614$) was observed. There were no significant differences in weanling glucose AUC during the ITT although a trend was observed ($p=0.092$) with APM having a greater AUC than CTR (Figure 12B).

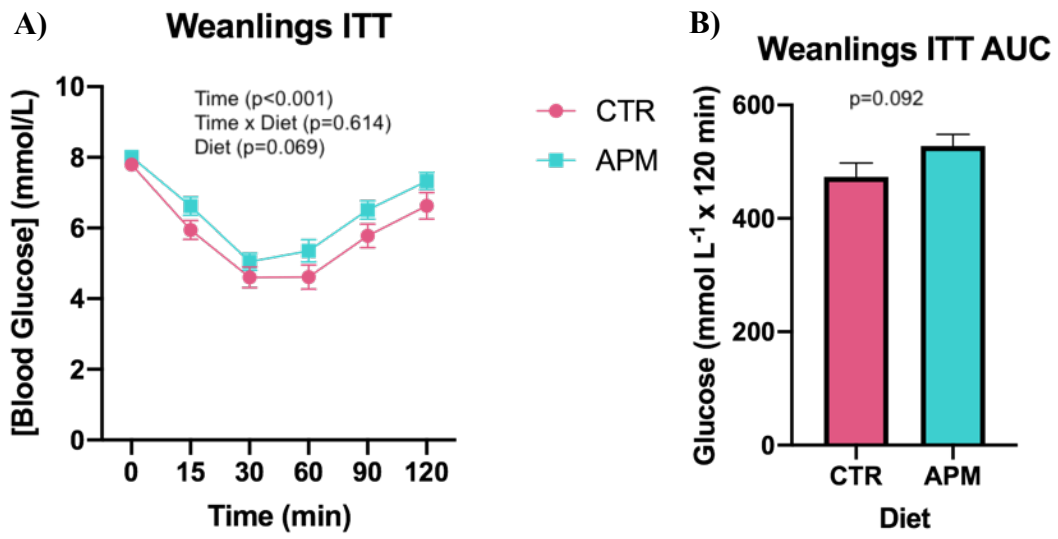

Figure 12. Weanling blood glucose concentrations (A) and glucose AUC (B) during an insulin tolerance test (ITT). Values are mean ± SEM, n=28 for CTR and n=30 for APM.

4.2.5 Maternal aspartame diet increased weanling hepatic triglyceride levels

No sex differences were detected but hepatic triglyceride concentration (mg triglyceride/g liver) was significantly affected by maternal diet in the weanlings (p=0.046, Figure 13) with APM (17.1±0.5) having a greater concentration than CTR (15.4±0.6).

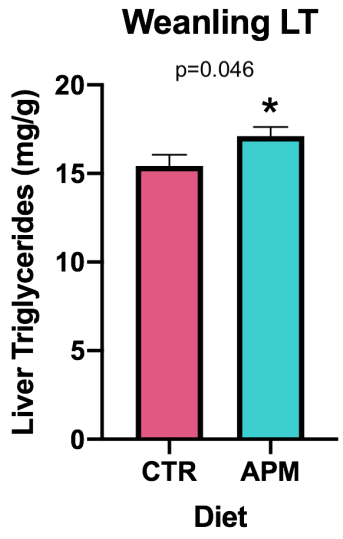

Figure 13. Weanling hepatic triglyceride levels. Values are mean ± SEM, n=28 for CTR and n=30 for APM.

4.2.6 Maternal aspartame diet did not upregulate weanling hepatic mRNA expression

Given the differences in hepatic triglyceride concentrations in the 3-week-old weanlings, we measured the expression of hepatic mRNA genes related to metabolism, lipogenesis, and inflammation in their liver tissue (i.e., FAS, ACC, IL-6, IRS-1, IRS-2, and TNFα). There was a significant sex effect ($p<0.05$) for all of the genes except TNFα therefore all genes except TNFα were assessed separately in males and females. Despite a generally worse profile in APM offspring, in male weanlings (Table 7) there were no significant differences due to maternal diet across FAS, ACC, IL-6, IRS-1, and IRS-2 although a trend ($p<0.079$) was observed for FAS with higher mRNA levels seen in male APM versus CTR weanlings. The similarly worse profile in APM female weanlings (Table 7) did not reach significance for any of the genes. For the inflammatory gene, TNFα, no difference was detected (Table 7).

Table 7. Hepatic mRNA levels in male and female offspring at weaning (week 3).

	Males		**Females**	
Diet	CTR	APM	CTR	APM
N	12	13	12	13
FAS	2.20 ± 0.6	3.45 ± 0.6	3.42 ± 0.6	4.86 ± 0.6
ACC	5.72 ± 0.9	5.32 ± 0.9	7.92 ± 0.9	8.59 ± 0.9
IL-6	0.30 ± 0.4	0.41 ± 0.4	0.68 ± 0.4	1.53 ± 0.4
IRS-1	1.58 ± 0.6	1.52 ± 0.6	2.47 ± 0.6	3.82 ± 0.6
IRS-2	1.39 ± 0.6	1.32 ± 0.5	2.38 ± 0.6	3.34 ± 0.5
Diet	CTR		APM	
N	24		26	
TNFα	0.71 ± 0.3		1.14 ± 0.3	

Values are means ± SEM. No significant differences.

4.2.7 Maternal aspartame diet influenced weanling gut microbial beta but not alpha diversity

Alpha diversity in the weanlings did not differ between groups as measured by Shannon's Index (Figure 14A) and the Chao1 Index (Figure 14B).

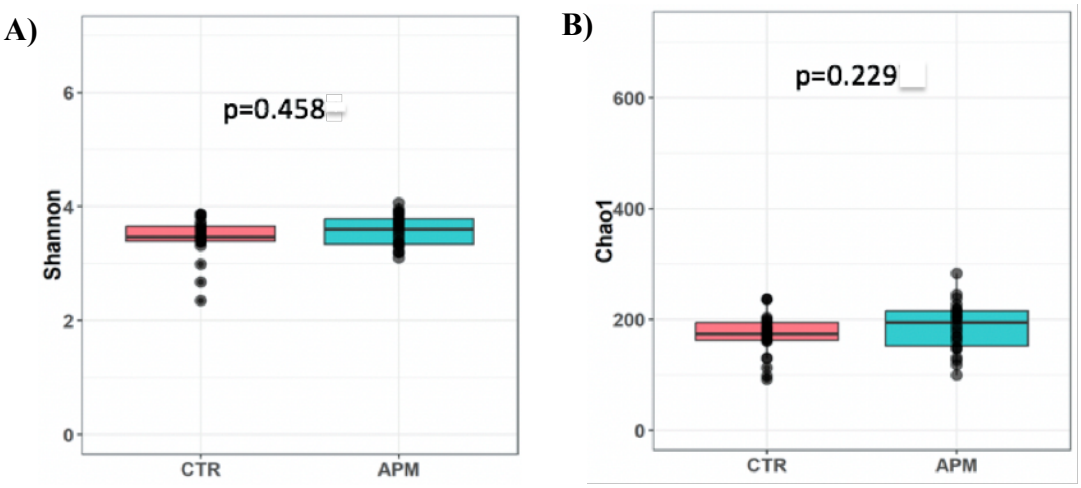

Figure 14. Alpha diversity indices in offspring measured at weaning (week 3) according to Shannon's (A), and Chao1 (B) indices. Significance was set at $p<0.05$, n=24 for CTR, n=26 for APM.

Conversely, beta diversity was significantly influenced by diet ($p<0.029$) where Bray Curtis Dissimilarity analysis revealed significant compositional dissimilarity between CTR and APM offspring. Clustering of the weanling CTR and APM groups indicates different community structure as a result of maternal diet (Figure 15).

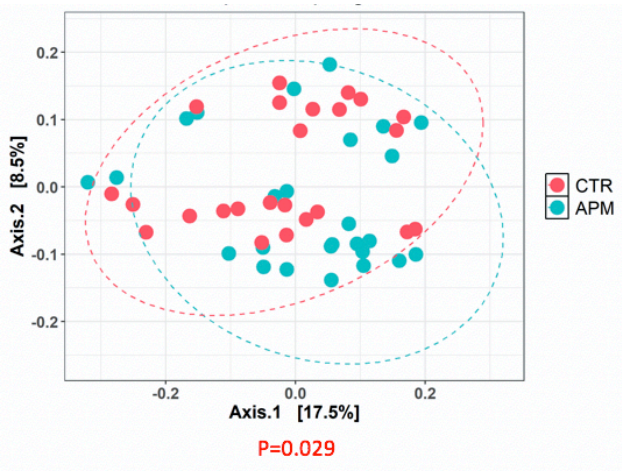

Figure 15. Principal Coordinate Analysis (PCoA) plot visualizing Bray-Curtis dissimilarity between APM and CTR weanlings. Diversity was compared between groups using PERMANOVA with significance set at $p<0.05$, n=24 for CTR, n=26 for APM.

Taxonomical bar plots are shown at the genus level in male and female weanlings exposed maternally to a CTR or APM diet (Figure 16).

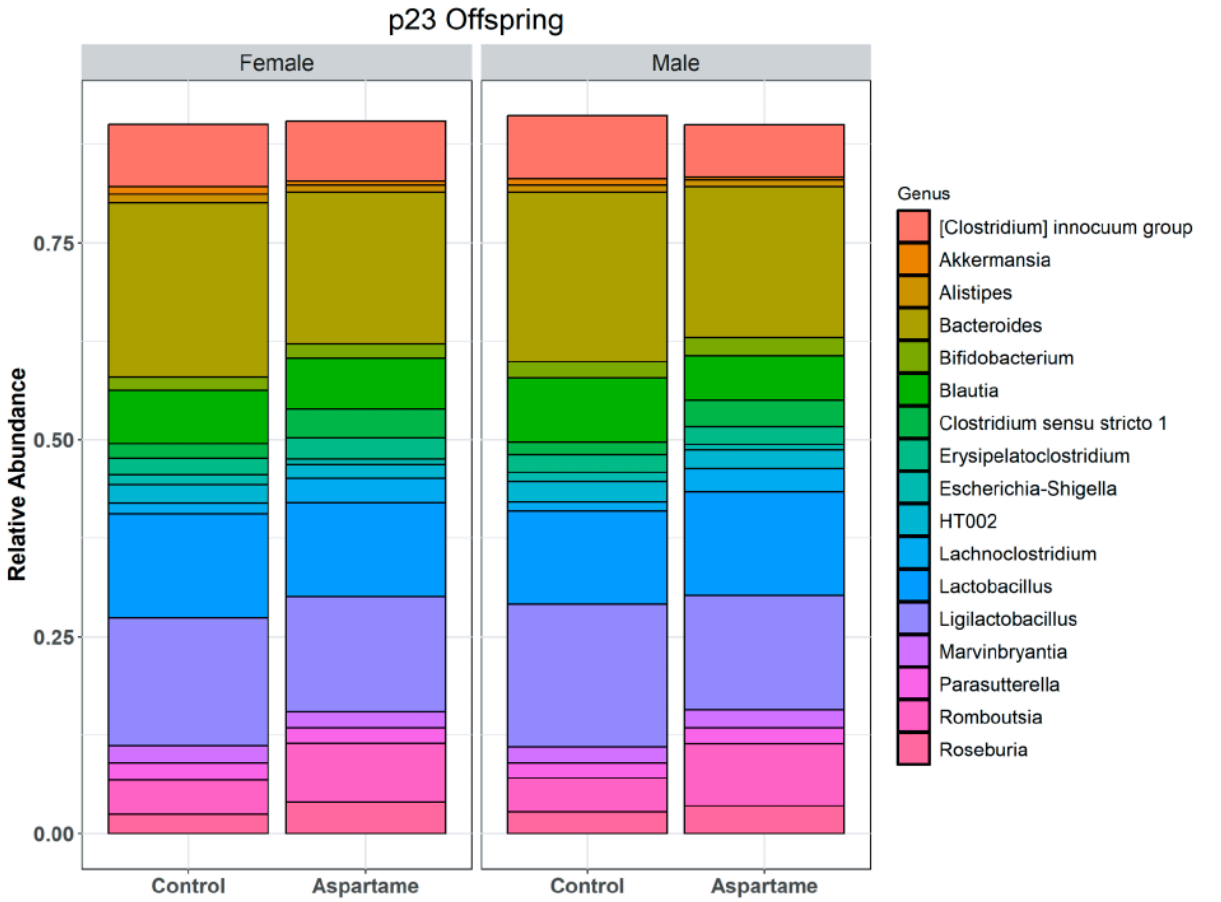

Figure 16. Taxonomical bar plot displaying gut microbial community composition at the genus level in male and female weanlings (week 3) exposed to maternal control or aspartame diet. Females and males were both n=12 for CTR and n=13 for APM respectively.

Visually in the plots among the aspartame groups, there appears to be increases in the following genera belonging to the *Firmicutes* phylum in both sexes: *Roseburia*, *Romboutsia, Lachnoclostridium,* and *Clostridium sensu stricto 1.* A decrease in the *Lactobacillus* genus belonging to the phylum *Bacillota* was seen only in females. This is consistent with the DESeq analysis, although this statistical test of differentially abundant bacteria only identified a difference at the highest taxonomical level (phylum) with *Firmicutes* seen in greater abundance in APM compared with CTR weanlings (Figure 17).

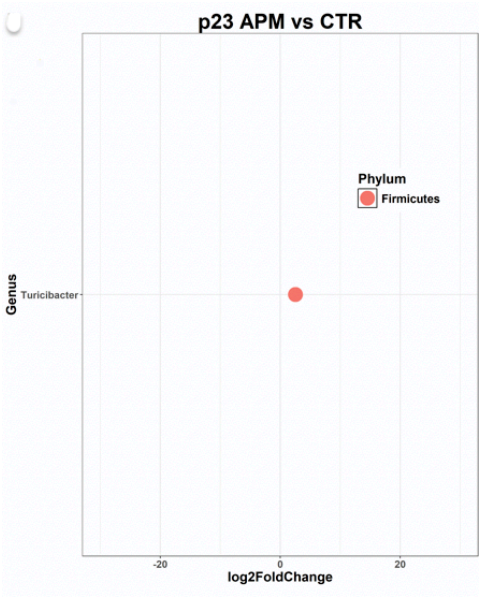

Figure 17. Differential abundance plot comparing APM versus CTR weanlings. n=24 for CTR, n=26 for APM.

4.3 Adult Offspring Outcomes

To examine the effects of the maternal diets on their offspring in young adulthood, we assessed offspring body composition, metabolic characteristics, hepatic triglyceride content, and gut microbial composition at 12 weeks of age.

4.3.1 Maternal aspartame diet increased early life body weight in males with no effect in females

There was a significant effect of sex for body weight ($p<0.001$) therefore males and females were assessed separately. In males, differences in body weight were significantly influenced by time ($p<0.001$) and a time×diet interaction ($p<0.05$). A trend ($p=0.065$) for differences in body weight due to diet was also observed. At p28 (week 4) the APM-CTR group had higher body weight compared to the CTR-Pre and APM-Pre group ($p<0.05$) and this continued at p35 (week 5). By p42 (week 6) the APM-CTR group continued to have higher body weight than the APM-Pre group ($p<0.05$) but not the others. At p56 (week 8) the APM-CTR group once again displayed significantly ($p<0.05$) higher body weight than the APM-Pre group. By p84 (week 12), the CTR-CTR group had higher ($p<0.05$) body weight than the CTR-Pre group. In all cases, postnatal prebiotic exposure (Pre) reduced body weight regardless of maternal diet. A summary of these findings is depicted in Figure 18.

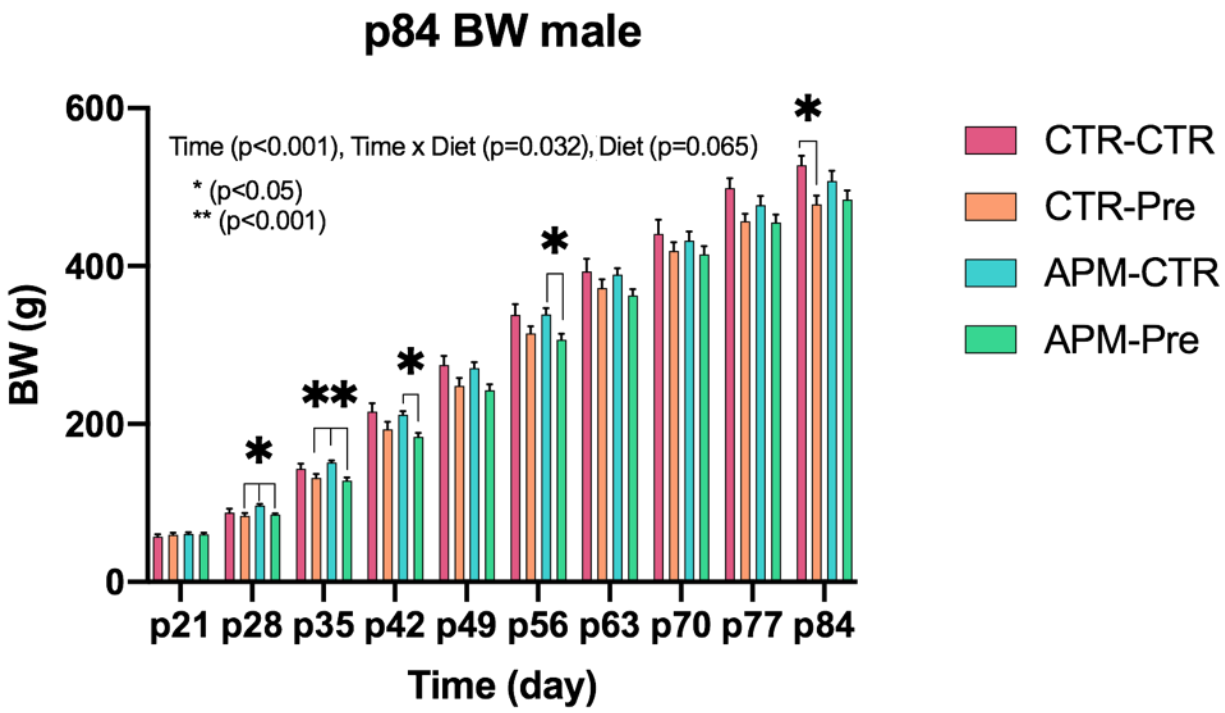

Figure 18. Male offspring body weight from p21-p84 (week 3-week 12). Values are mean ± SEM, n=13 for CTR-CTR, n=14 for CTR-Pre, n=15 for APM-CTR, n=15 for APM-Pre. *indicates a significant difference at p<0.05. **indicates a significant difference at p<0.001.

In females, differences in body weight were significantly influenced by time (p<0.001) and a strong trend for a time×diet interaction (p=0.052) but not diet (p=0.184). While body weight in those postnatally exposed to prebiotic trended lower, the only statistically significant (p<0.05) difference was found at p84 (week 12) with APM-Pre having lower body weight than CTR-CTR (Figure 19).

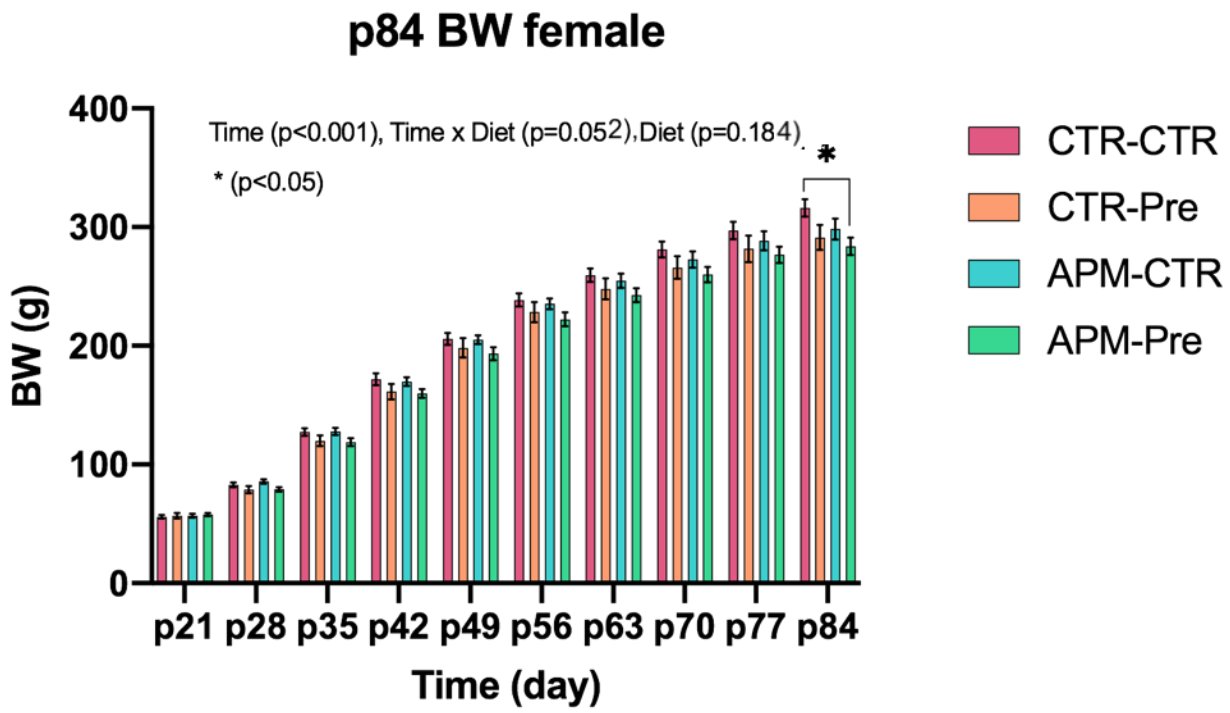

Figure 19. Female offspring body weight from p21-p84 (week 3-week 12). Values are mean ± SEM, n=14 for CTR-CTR, n=12 for CTR-Pre, n=15 for APM-CTR, n=15 for APM-Pre. *indicates a significant difference at $p<0.05$.

4.3.2 Offspring postnatal prebiotic diet improved body composition in females with no effect in males

Due to the presence of sex differences ($p<0.05$), body composition was assessed separately in male and female adult offspring at the time of DEXA scan during week 12.

There were no statistically significant differences in body composition in male offspring. With respect to body weight the CTR-Pre ($p=0.088$) group trended lower than the CTR-CTR group at week 12 (Table 8). No statistically significant differences were seen between groups with respect to lean mass (%). For body fat (%), the APM-Pre ($p=0.099$) group trended lower than the APM-CTR group (Table 8). No differences were seen in relative bone mass. Overall, postnatal prebiotic intake did not improve body composition in males with trends only seen in lower body weight and reduced body fat (%) among the CTR-Pre and APM-Pre group respectively (Table 8).

Table 8. Anthropometrics of adult male offspring (12 weeks old)

Diet	CTR-CTR	CTR-Pre	APM-CTR	APM-Pre
N	13	14	15	15
Weight (g)	532.4 ± 12.5[a]	487.2 ± 11.9[b]	512.3 ± 13.1	490.9 ± 13.8
Lean Mass (%)	62.5 ± 2.1	67.2 ± 1.1	62.3 ± 1.6	67.6 ± 1.6
Body Fat (%)	35.5 ± 2.0	30.8 ± 1.1	35.8 ± 1.5[a]	30.8 ± 1.5[b]
Relative Bone Mass (g/kg)	30.4 ± 0.6	29.5 ± 0.4	30.4 ± 0.4	29.1 ± 0.4

Values are mean ± SEM. Significance is set at ($p<0.05$) with Tukey post hoc. Trends shown.

In adult female offspring, body weight in the APM-Pre group was significantly ($p=0.046$) lower than the CTR-CTR group (Table 9). Lean mass (%) was significantly greater in the APM-Pre group relative to the CTR-CTR ($p=0.044$) and APM-CTR ($p=0.006$) groups (Table 9). Also, body fat (%) was significantly lower in APM-Pre offspring when compared with the CTR-CTR ($p=0.047$) and APM-CTR ($p=0.009$) (Table 9). There was no difference in relative bone mass. Overall, postnatal prebiotic intake improved female body composition.

Table 9. Anthropometrics of adult female offspring (12 weeks old)

Diet	CTR-CTR	CTR-Pre	APM-CTR	APM-Pre
N	14	12	15	15
Weight (g)	315.7 ± 8.0[a]	293.3 ± 10.9[ab]	300.0 ± 8.8[ab]	282.8 ± 7.3[b]
Lean Mass (%)	58.9 ± 2.0[a]	62.6 ± 1.8[ab]	57.4 ± 2.1[a]	66.0 ± 1.4[b]
Body Fat (%)	37.9 ± 1.9[a]	34.4 ± 1.7[ab]	39.2 ± 2.0[a]	31.1 ± 1.4[b]
Relative Bone Mass (g/kg)	35.3 ± 0.6	35.1 ± 0.4	36.2 ± 0.4	35.6 ± 0.4

Values are mean ± SEM. Significance is set at ($p<0.05$) with Tukey post hoc.

4.3.3 Postnatal prebiotic diet increased cecum but not liver weight in offspring

There were no significant effects of sex or diet on relative liver weight (Figure 20A). Cecum weight was affected by sex ($p=0.013$) therefore males and females were assessed separately (Figure 20B). Diet significantly affected ($p<0.001$) relative cecum weight in males and females with postnatal prebiotic consumption increasing cecum weight in males and females.

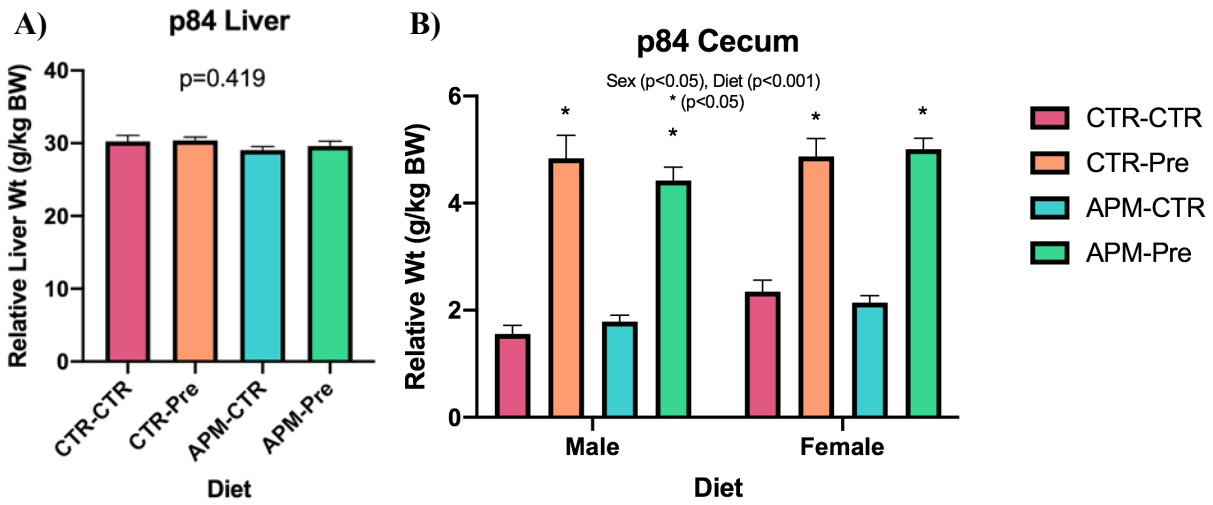

Figure 20. Adult offspring (week 12) liver (A) and cecum (B) weight relative to total body weight. Values are mean ± SEM, n=27 for CTR-CTR, n=26 for CTR-Pre, n=30 for APM-CTR, and n=30 for APM-Pre. *indicates a significant difference ($p<0.05$).

4.3.4 Postnatal prebiotic diet improved glucose tolerance in males with no effect in females

Offspring glucose control was measured utilizing OGTT at p84 (week 12). BGLs were significantly affected by sex ($p<0.05$) therefore males and females were assessed separately.

Male offspring BGLs were significantly affected by time ($p<0.001$) as expected during the OGTT (Figure 21). There was no interaction of time×diet ($p=0.141$), yet a significant effect of diet ($p<0.001$) was observed with BGLs lower ($p<0.001$) in offspring consuming prebiotic versus control diet (Figure 21A). For glucose AUC, significant differences due to diet ($p<0.001$) were also seen (Figure 21B). Postnatal prebiotic consumption decreased glucose AUC compared to control ($p=0.01$). Overall, in male adult offspring, postnatal prebiotic fiber consumption improved glycemic control regardless of maternal consumption of CTR or APM.

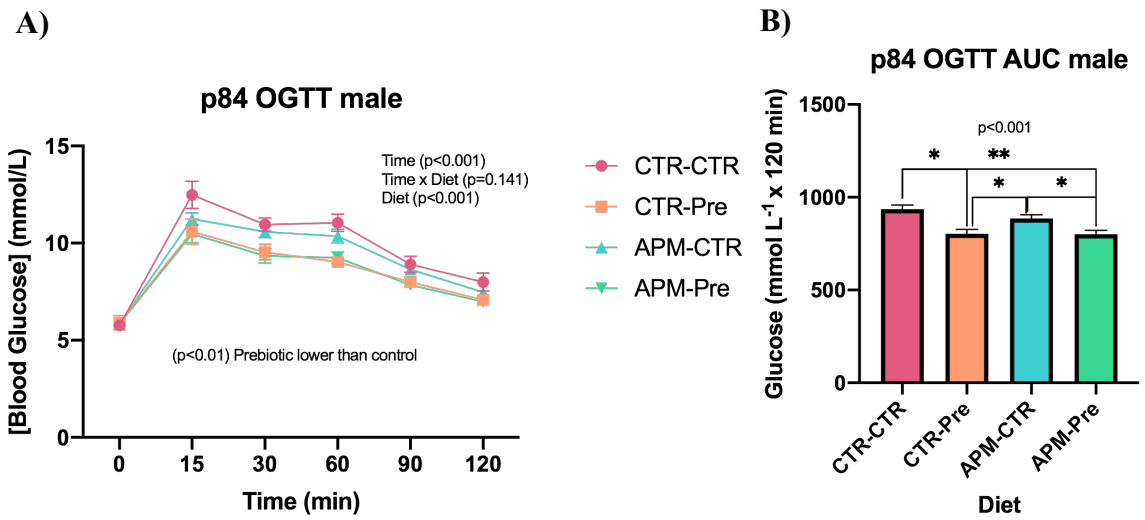

Figure 21. Adult male offspring at p84 (week 12) blood glucose concentrations during an oral glucose tolerance test (OGTT) (A) and glucose area under the curve (AUC) (B). Values are mean ± SEM, n=13 for CTR-CTR, n=14 for CTR-Pre, n=15 for APM-CTR, and n=15 for APM-Pre.

*indicates a significant difference at p<0.05. **indicates a significant difference at p<0.001.

With respect to adult female offspring, as expected there was a significant main effect of time (p<0.001) during the OGTT (Figure 22A). BGLs during the OGTT were not affected by a time×diet interaction (p=0.703) or diet (p=0.172). Moreover, no difference in glucose AUC (p=0.179) during the OGTT was detected (Figure 22B).

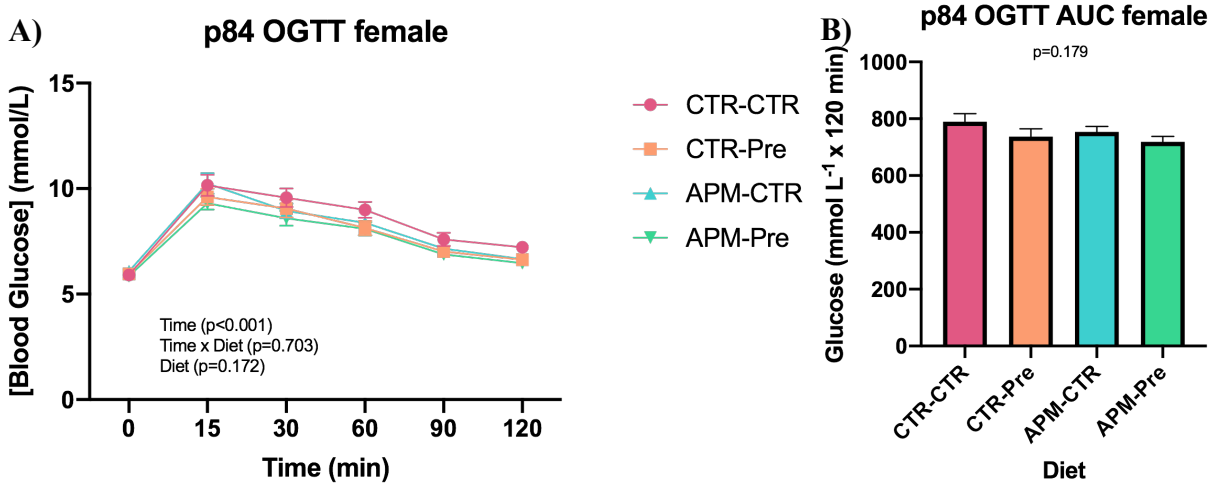

Figure 22. Adult female offspring at p84 (week 12) blood glucose concentrations (A) and glucose AUC (B) during an oral glucose tolerance test (OGTT). Values are mean ± SEM, n=14 for CTR-CTR, n=12 for CTR-Pre, n=15 for APM-CTR, and n=15 for APM-Pre.

4.3.5 Postnatal prebiotic diet worsened insulin sensitivity in females with no effect in males

Offspring insulin sensitivity was measured utilizing ITT at p84 (week 12). BGLs were significantly affected by sex ($p<0.05$) therefore males and females were assessed separately.

Male offspring BGLs were significantly affected by time ($p<0.001$) but not diet ($p=0.908$) or time×diet ($p=0.384$) during the ITT (Figure 23A). Similarly, no difference ($p=0.726$) was found for glucose AUC during the ITT in male offspring (Figure 23B).

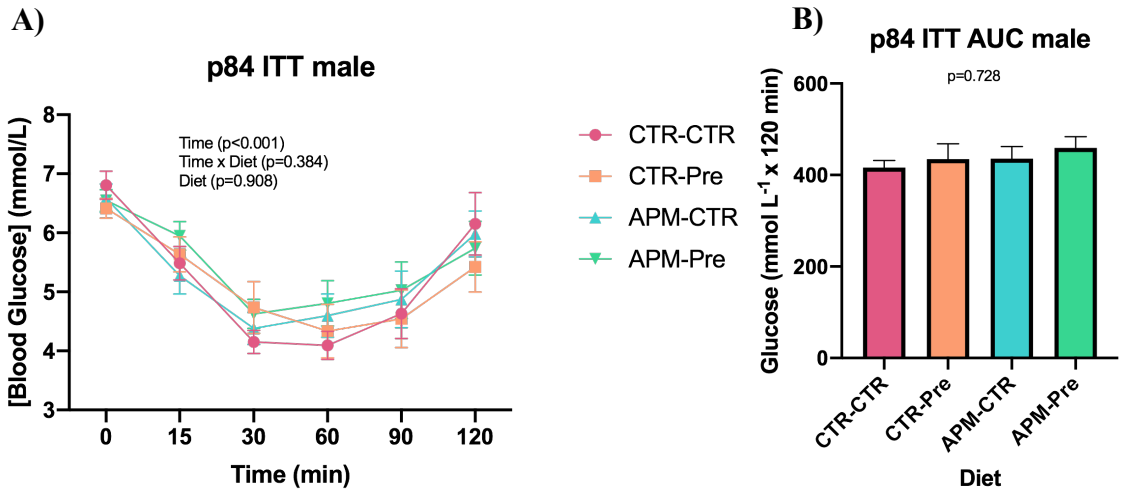

Figure 23. Adult male offspring at p84 (week 12) blood glucose concentrations (A) and glucose AUC (B) during an insulin tolerance test (ITT). Values are mean ± SEM, n=13 for CTR-CTR, n=14 for CTR-Pre, n=15 for APM-CTR, and n=15 for APM-Pre.

Female offspring BGLs were significantly affected by time ($p<0.001$) and time×diet ($p<0.001$) but not diet ($p=0.142$) during the ITT (Figure 24A). Insulin sensitivity was significantly worse ($p<0.05$) in the APM-Pre offspring group compared to CTR-CTR at 30 and 60 minutes respectively. Glucose AUC differed ($p=0.046$) between groups with a posthoc trend ($p=0.055$) for APM-Pre to be higher than CTR-CTR (Figure 24B).

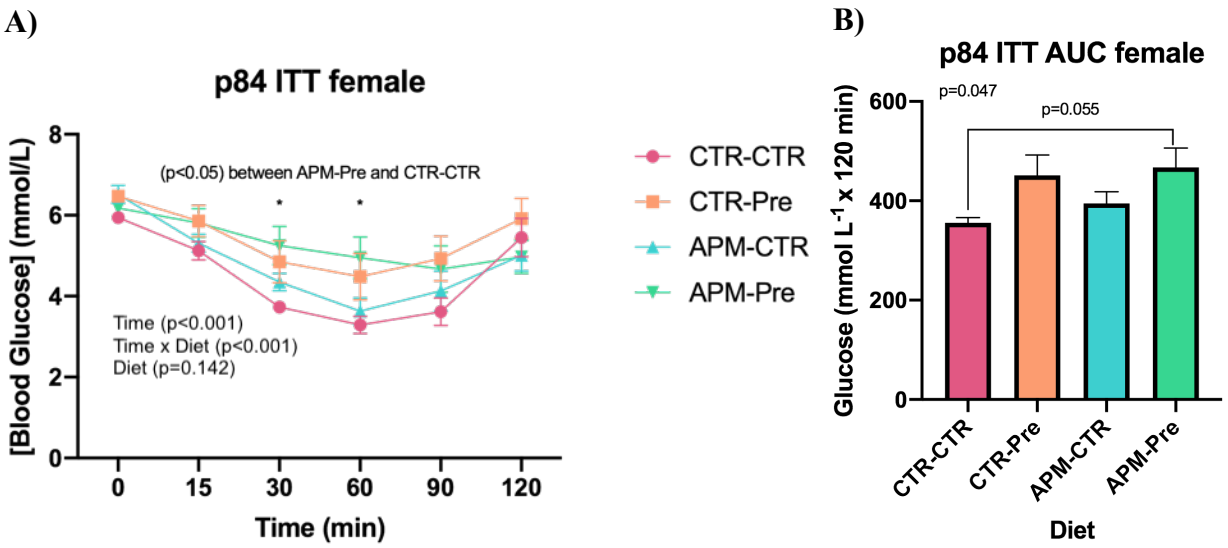

Figure 24. Adult female offspring at p84 (week 12) blood glucose concentrations (A) and glucose AUC (B) during an insulin tolerance test (ITT). Values are mean ± SEM, n=14 for CTR-CTR, n=12 for CTR-Pre, n=15 for APM-CTR, and n=15 for APM-Pre. *indicates a significant difference at p<0.05.

4.3.6 Postnatal prebiotic intake affected energy intake in females but not males

Food intake (grams converted to kcal) in adult offspring was measured at three time points: p28-p32 (week 4), p56-p60 (week 8), and p77-p81 (week 11). Energy intake was significantly affected by sex (p<0.05) therefore males and females were assessed separately.

In male adult offspring, there was a significant main effect of time (p<0.001) but not diet (p=0.370) or time×diet interaction (p=0.083) (Figure 25).

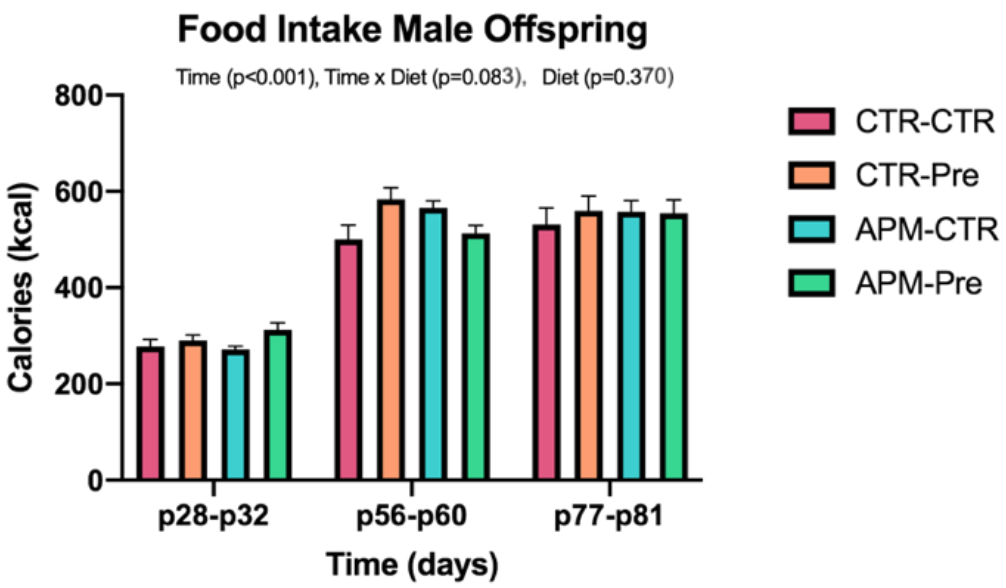

Figure 25. Energy intake (kcal) in adult male offspring measured at three time points: p28-p32 (week 4), p56-p60 (week 8), and p77-p81 (week 11). Values are mean ± SEM, n=13 for CTR-CTR, n=14 for CTR-Pre, n=15 for APM-CTR, and n=15 for APM-Pre.

Interestingly, when energy intake was measured in female adult offspring, there was a significant main effect of time ($p<0.001$), a significant effect of a time×diet interaction ($p<0.001$), and a significant effect of diet ($p<0.001$). Postnatal prebiotic consumption increased energy intake ($p<0.05$) in females exposed prenatally to APM at p56-p60 and at p77-p81. Specifically, at p56-p60 APM-Pre had greater energy intake than CTR-CTR ($p=0.004$), CTR-Pre ($p=0.047$), and APM-CTR ($p=0.003$). At p77-p81, APM-Pre consumed more energy compared to the CTR-Pre ($p<0.001$) and APM-CTR ($p=0.001$) offspring with a trend observed relative to the CTR-CTR group ($p=0.082$). (Figure 26).

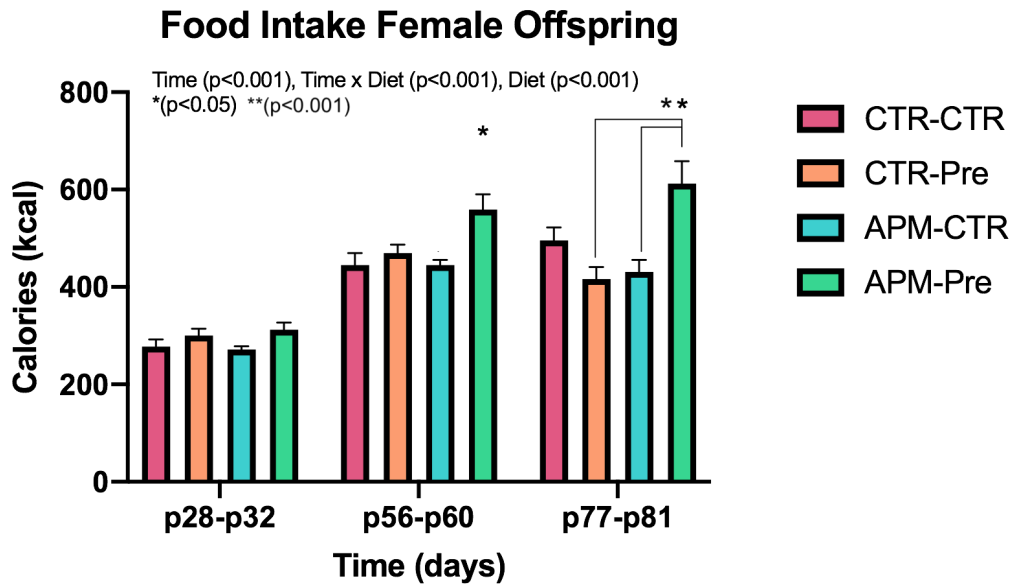

Figure 26. Energy intake (kcal) in adult female offspring measured at three time points: p28-p32 (week 4), p56-p60 (week 8), and p77-p81 (week 11). Values are mean ± SEM, n=14 for CTR-CTR, n=12 for CTR-Pre, n=15 for APM-CTR, and n=15 for APM-Pre. *indicates a significant difference at p<0.05.

4.3.7 Offspring prebiotic diet did not influence hepatic triglyceride levels

There was a significant sex effect (p<0.001) for hepatic triglyceride concentration (mg triglyceride/g liver), therefore males and females were analyzed separately. In adult male offspring, hepatic triglyceride concentration did not differ by diet although males had a significantly greater concentration when compared to females across all treatment groups (Figure 27). No effect of diet was detected in adult female offspring either (Figure 27). Overall, there was no effect of diet (p=0.122) or an interaction of diet×sex (p=0.416).

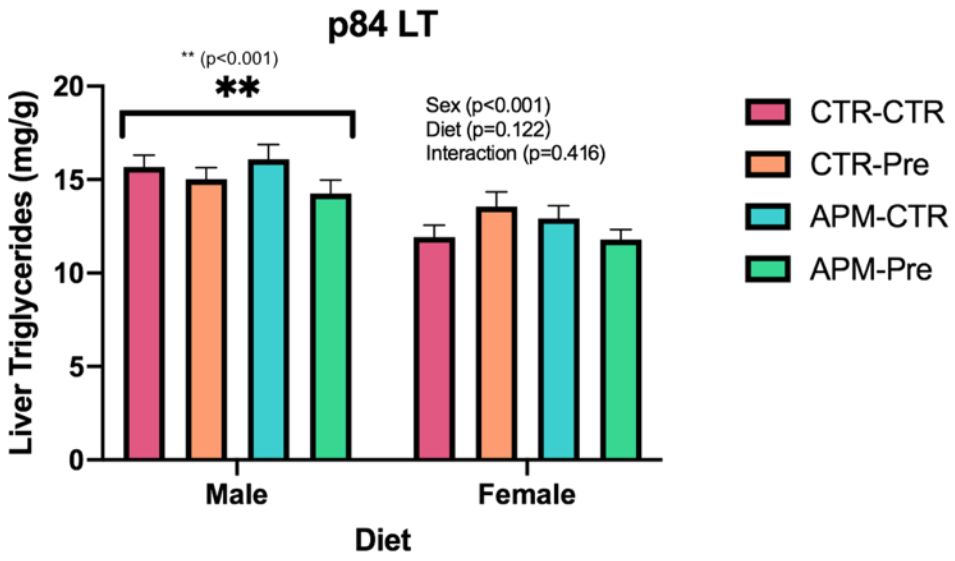

Figure 27. Adult offspring hepatic triglyceride levels. Values are mean ± SEM. For males, values are n=13 for CTR-CTR, n=14 for CTR-Pre, n=15 for APM-CTR, and n=15 for APM-Pre. For females, values are n=14 for CTR-CTR, n=12 for CTR-Pre, n=15 for APM-CTR, and n=15 for APM-Pre.** indicates a significant ($p<0.001$) difference between male and female rats.

4.3.8 Postnatal prebiotic diet significantly influenced adult offspring gut microbiota

Alpha diversity in the adult offspring differed ($p<0.001$) between groups as measured by Shannon (Figure 28A) and Chao1 indices (Figure 28B) because of postnatal prebiotic exposure. CTR-Pre had lower alpha diversity than CTR-CTR and similarly, APM-Pre had lower diversity than APM-CTR. Maternal diet had no effect on alpha diversity in adult offspring.

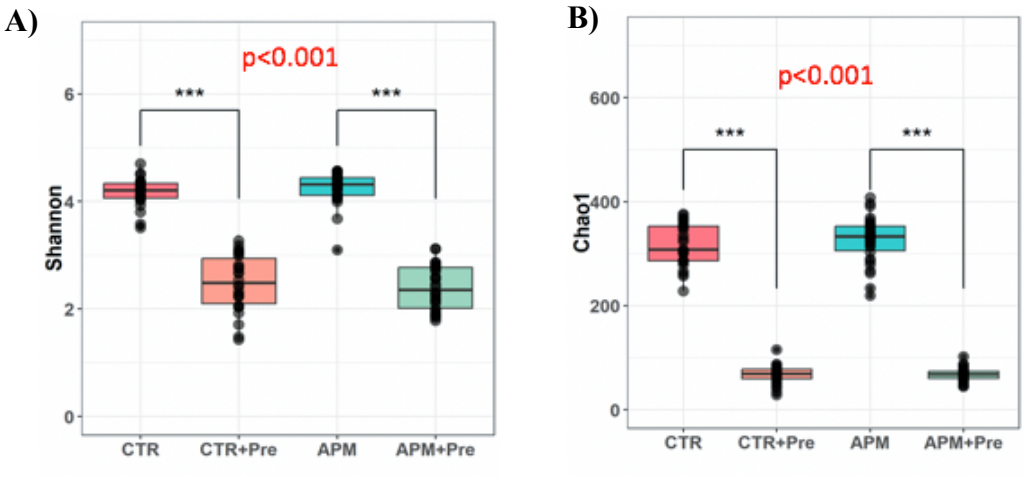

Figure 28. Alpha diversity indices in adult offspring measured at 12 weeks (p84) according to Shannon (A), and Chao1 (B) indices. Significance was set at p<0.05, n=27 for CTR-CTR, n=26 for CTR-Pre, n=30 for APM-CTR, and n=30 for APM-Pre. *** indicates a significant difference at p<0.001.

Diet was a significant factor (p<0.001) in adult offspring beta diversity measured at 12 weeks of age as calculated using Bray Curtis Dissimilarity. This analysis revealed significant compositional dissimilarity between groups exposed postnatally to prebiotic fiber versus those who were not. Figure 29 shows clustering of the prebiotic-consuming groups (CTR-Pre and APM-Pre) regardless of maternal diet. These findings suggest that groups demonstrated significantly different community structure at 12 weeks of age as a result of postnatal diet with no significant influence from maternal diet (Figure 29).

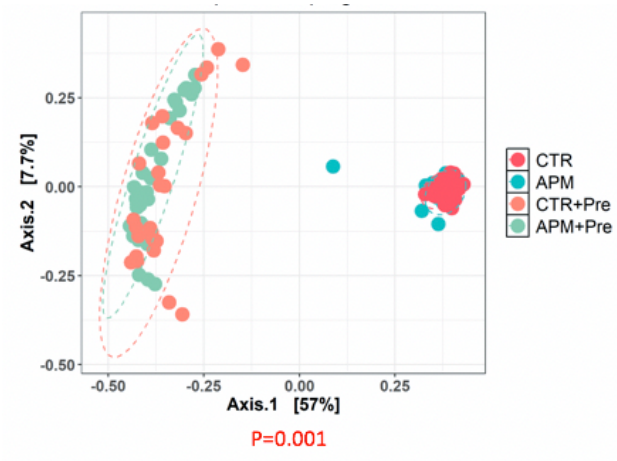

Figure 29. Principal Coordinate Analysis (PCoA) plot visualizing Bray-Curtis dissimilarity between diet groups in the adult offspring measured at week 12. Diversity was compared between groups using PERMANOVA with significance set at p<0.05, n=27 for CTR-CTR, n=26 for CTR-Pre, n=30 for APM-CTR, and n=30 for APM-Pre.

Taxonomical abundance plots are shown at the genus level during week 12 in male and female adult offspring. While there are no significant differences due to sex, the taxonomical data is graphically represented within sex across all four groups for visual simplicity: CTR-CTR, CTR-Pre, APM-CTR, APM-Pre (Figure 30).

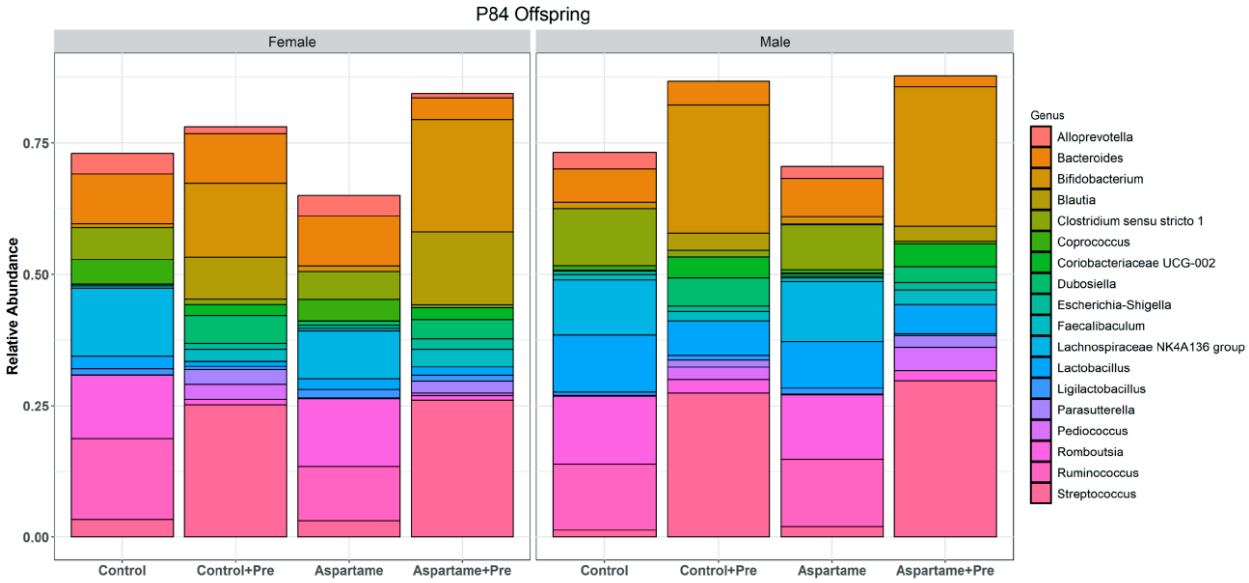

Figure 30. Taxonomical bar plot displaying gut microbial community composition at the genus level in adult offspring separated by sex (week 12, day 84) across all four groups. For males, values are n=13 for CTR-CTR, n=14 for CTR-Pre, n=15 for APM-CTR, and n=15 for APM-Pre. For females, values are n=14 for CTR-CTR, n=12 for CTR-Pre, n=15 for APM-CTR, and n=15 for APM-Pre.

To investigate whether any differences existed between the microbial communities, differential abundance analysis using DESeq2 was conducted at the genus level at 12 weeks of age in the adult offspring. There was no significant sex effect, therefore the analysis represents pooled male and female data. When the influence of maternal diet alone is compared by looking at APM versus CTR, APM offspring harbored a greater abundance of one unidentified genus in the *Proteobacteria* phylum and 4 genera in the *Firmicutes* phylum (Figure 31).

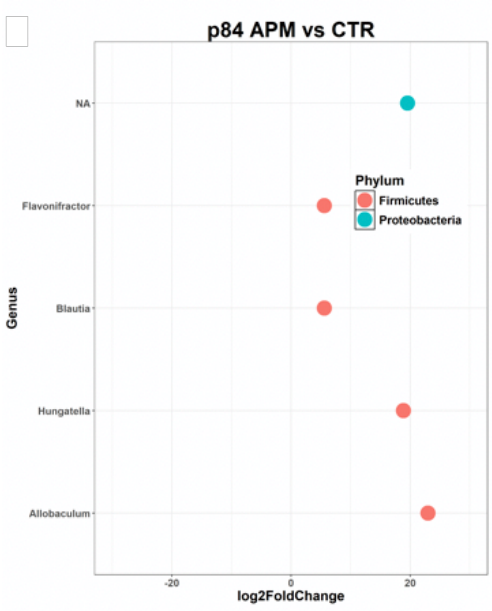

Figure 31. Differential abundance plot of adult offspring (male and female together) exposed prenatally to APM or CTR at week 12, n=53 for CTR and n=60 for APM.

Further differential abundance analysis showed that APM-Pre had lower abundance of six known genera in the *Firmicutes* phylum when compared to CTR-Pre suggesting a mild influence of maternal diet on offspring gut microbiota in adulthood (Figure 32A). For postnatal diet influences, however, numerous bacteria were differentially abundant between CTR-Pre and CTR-CTR (Figure 32B) and between APM-Pre and APM-CTR (Figure 32C) illustrating the strong influence of postnatal prebiotic fiber consumption on gut microbiota at 12 weeks. Prebiotic fiber consumption resulted in a greater abundance of multiple genera in the following phyla: *Actinobacteria, Proteobacteria,* and *Verrucomicrobiota*. Prebiotic effects dominated the changes in microbiota at 12 weeks of age. Regardless of maternal diet, CTR and APM offspring responded to prebiotics with major shifts in bacterial abundance.

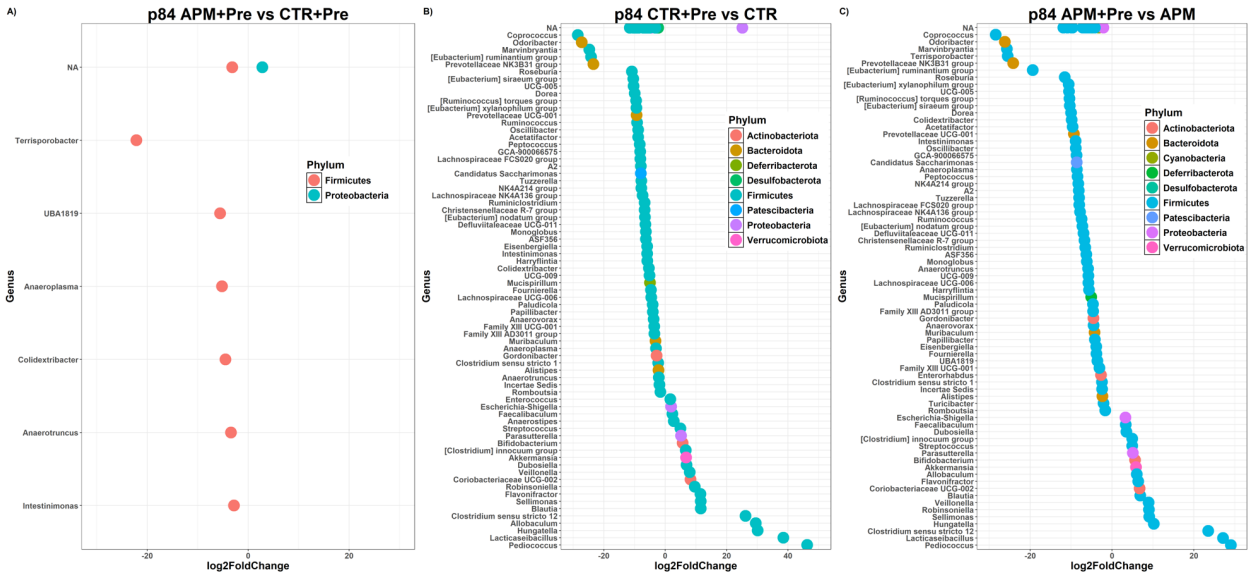

Figure 32. Differential abundance plot of various phyla at week 12 between APM-Pre (n=30) vs CTR-Pre (n=26) (A), CTR-Pre (n=26) vs CTR-CTR (n=27) (B), and APM-Pre (n=30) vs APM-CTR (n=30).

A deeper examination of eight differentially abundant bacteria is shown in Figure 33. While there were no significant differences due to sex, there were statistically significant differences ($p<0.001$) due to diet as a result of postnatal prebiotic fiber consumption. Following the 12-week dietary intervention, prebiotics increased *Bifidobacterium, Blautia*, and *Streptococcus* (Figure 33A, B, and H respectively), while decreasing five other bacteria including *Romboutsia* (Figure 33C-G).

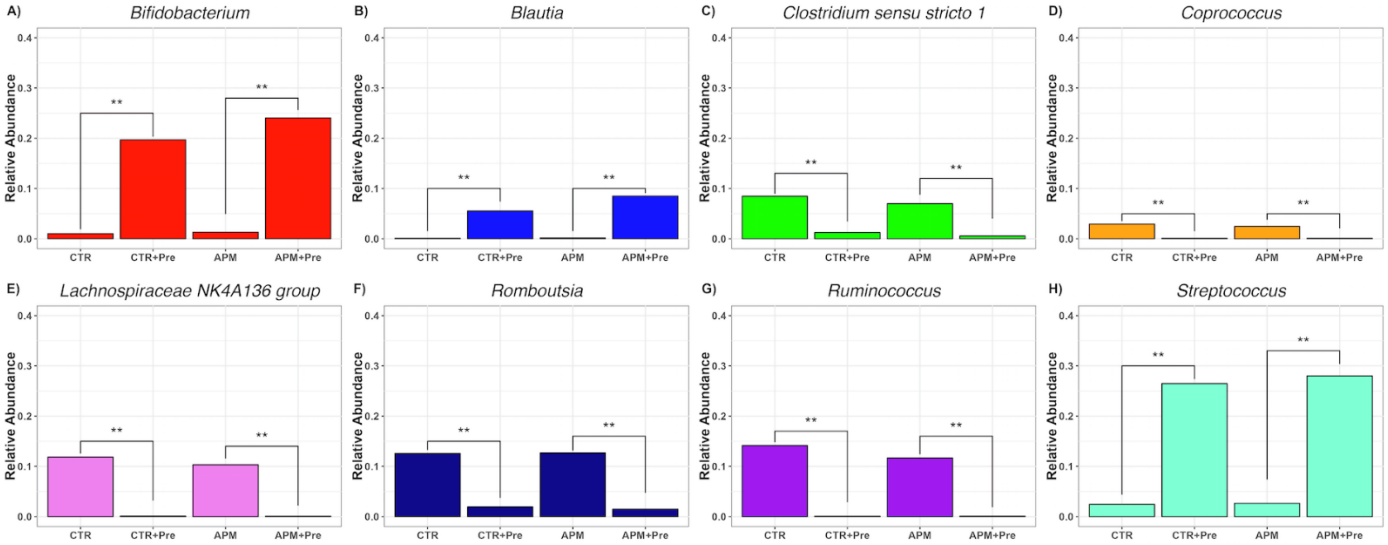

Figure 33. Genus graph of eight distinct bacteria (A-H) at week 12 in adult offspring across all four groups, n=27 CTR-CTR, n= 26 CTR-Pre, n=30 APM-CTR, and n=30 APM-Pre. ** indicates a significant ($p<0.001$) difference in microbial communities between groups.

CHAPTER FIVE: DISCUSSION

5.1 General Discussion

The results from our study assessing 6 weeks of maternal consumption of aspartame are consistent with existing human evidence from meta-analyses based on small randomized controlled trials (RCTs) with short follow-up (median 6 months), that there is no difference or a slight decrease in body weight with consumption of artificial sweeteners (126). With respect to aspartame consumption specifically, additional meta-analyses based on RCTs showed no effect on body weight compared to sugar or water in individuals living with either obesity or T2DM as well (125). We find that maternal aspartame consumption during pregnancy and lactation does not significantly disrupt insulin sensitivity and glucose tolerance in dams measured at weaning which is consistent with findings from Nettleton et al (9). Our work found that aspartame consumption did not differentially affect maternal energy intake. There is an existing assumption that less satiety associated with artificial sweetener consumption may drive food seeking behaviour contributing to increased energy intake (119) yet RCTs have shown that reduced caloric intake by replacing natural sugars with artificial sweeteners is not completely compensated and has led to decreased energy intake in some cases (175). Furthermore, meta-analyses of acute (<1 day), moderate (4-10 weeks), and long-term (4 weeks to 40 months) RCTs suggest that compensatory energy intake during artificial sweetener consumption does not seem to occur supporting our findings in dams (176,177). Artificial sweetener-induced gut microbiota dysbiosis via saccharin consumption has been linked to metabolic syndrome and the development of an inflammatory state in rodents by increased LPS synthesis and a loss of gut mucosal integrity through the reduction of tight junction proteins (128,178). Demonstrating the unique structure and properties of each of the artificial sweeteners, studies looking at aspartame, acesulfame-K, and sucralose did not find these to alter intestinal permeability (178). Compromised intestinal permeability allows LPS to translocate from the gut into the portal and systemic circulation and stimulate the activation of pro-inflammatory macrophages and cytokines which infiltrate the peripheral tissues and release TNFa, IL-1B, and IL-6 which may interfere with insulin signaling and insulin-stimulated glucose uptake. Some rodent studies found that saccharin, acesulfame-K, and sucralose may increase inflammation (179,180). The inflammatory molecules may inhibit adipogenesis by reducing adipocyte turnover and adipose tissue expansion (181). This may lead to lipid overflow and fat accumulation in non-adipose tissue. This accumulation of ectopic fat in organs such as the liver, skeletal muscle, and heart seems to disrupt metabolic processes and impair organ function ultimately contributing to insulin resistance and reduced

pancreatic B-cell function (182). Interestingly aspartame was not implicated in these findings and our study reflects this as there was no significant increase in maternal hepatic triglyceride accumulation nor were there significant alterations in maternal gut microbial composition with respect to alpha and beta diversity suggesting it was hydrolyzed into its corresponding metabolites of methanol, aspartic acid, and phenylalanine by the time it reached the colon. As a result, low-dose aspartame consumption did not modulate gut microbiota in dams and it did not appreciably affect the metabolic health of the dams when consumed for 6 weeks during pregnancy and lactation.

While many national authorities have deemed artificial sweeteners to be safe for human consumption under appropriate doses, their use remains controversial regarding human health (183). Specifically, in contrast to the largely neutral effect of artificial sweeteners seen in meta-analyses of RCTs, data from 30 cohort studies showed significant increases in weight and waist circumference, and higher incidence of obesity, hypertension, metabolic syndrome, type 2 diabetes and cardiovascular events (126). While it has been suggested that these findings are largely explained by reverse causation bias where an increase in artificial sweetener intake is to compensate for an unhealthy diet or lifestyle (184), animal studies have been able to show a casual relationship between artificial sweetener intake and worse glucose tolerance (128). Suez et al showed that the negative metabolic effects of artificial sweetener intake by mice was mediated by the gut microbiota and that fecal microbiota transplant transferred the glucose intolerant phenotype to germ free mice (128). However, glucose intolerance was worst with saccharin which reinforces that each sweetener has a distinct chemical structure and can be metabolized or absorbed differently by the body (183). As a result, not all artificial sweeteners elicit the same metabolic effect as some metabolites may affect the gut micriobiota composition directly and others are digested and absorbed more easily (183). For example, aspartame is broken down in the small intestine and its metabolites are taken up by peripheral tissues and do not enter the colon therefore they do not impact gut microbiota directly (102). This effect was present in our study which showed no difference in gut microbial alpha and beta diversity in dams who consumed aspartame compared to water. Rather, changes in alpha and beta diversity in dams were the result of gestation and lactation. This can be explained by sizeable shifts in the concentration of hormones estrogen and progesterone during pregnancy which alter the composition of gut microbiota relative to non-pregnant (185). Throughout the time of gestation the gut microbiota change considerably to optimize fetal development with observed increases in *Akkermansia*, *Bifidobacterium*, and *Firmicutes* as well as increases in proinflammatory *Proteobacteria* and *Actinobacteria* to protect both mother and fetus (186). Lactation also exerts an influence on gut microbial community structure with bacterial communities

corresponding to increased SCFA production known for its health benefits in the host (187). While our study did not examine the effects of saccharin, this sweetener is not metabolized by the body. Instead 85-95% is absorbed and bound to plasma proteins to be distributed via blood and then excreted in urine while the remaining 5-15% passes unchanged through the gut to be eliminated in feces (102). This fraction of saccharin that is not absorbed is therefore able to affect the gut microbiota composition directly (102). As a result, the different metabolic fates of each artificial sweetener may help explain some of the conflicting findings that have been reported when the metabolic effects of a single artificial sweetener are extrapolated to include all artificial sweeteners collectively (183).

Prior studies have shown the relationship between an exposure to stressors during the critical phases of development and health outcomes in later life including the development of NCDs such as obesity (188). Epigenetic modifications including histone modifications, DNA methylation, and posttranscriptional noncoding microRNA and their subsequent modulation of gene expression are the driving factors behind these outcomes (83). The obesogenic HFS maternal diet with low-dose aspartame consumed by our obese-prone dams throughout gestation and lactation would be viewed as a stressor capable of programming phenotypic changes in their offspring without changing their genetic code directly. Preconception health and quality of life should not be overlooked as it is a period of time that is important developmentally for next-generation health (189). Studies in rodents have shown that maternal obesity causes alterations in the DNA methylation patterns of oocytes in mouse female offspring, with an increase in the methylation of the *LEP* promoter region responsible for the production of leptin and a reduction in PPARa promoter methylation, leading to an imbalance in energy regulation (190). Another rodent study showed that a maternal high fat diet during the preconception period induced enhanced hedonic behaviour and obesity in the offspring via tRNA-derived small RNAs in paternal sperm which propagated the phenotype across three generations (191). The modifications of the epigenome via gametic modifications can lead to the transmission of these alternations through new epigenetic marks in their offspring influencing their susceptibility to the development of obesity (188). In our study, the preconception period was marked by an 11-week DIO phase capable of inducing some epigenetic changes in dams although these markers were not measured in our study. Gestation is a critical stage of development sensitive to intrauterine insults and environmental factors that can program the fetus for the development of metabolic disease in adulthood secondary to organ plasticity (192). From the beginning of gestation, there is a period of plastic development where the fetus can be affected by environmental changes which is subsequently able to alter its phenotype (193). One study showed that male offspring programmed by a high-fat diet (60%) prenatally during gestation showed

increased food consumption and increased adiposity without changes in body weight along with hyperinsulinemia, hyperglycemia, dyslipidemia, and hyperleptinemia at 14 weeks (194). DNA methylation and miRNA are epigenetic mechanisms more frequently implicated in gestation (188). While our study employed an HFS diet as opposed to a high-fat diet, young male offspring exposed prenatally to APM had increased body weight at week 4 and 5. Lactation also constitutes an important window of development highlighted by the importance of breastfeeding duration and its protective effects (195). This is largely mediated by the presence of hormones such as leptin, insulin, and fibroblast growth factor 21 (FGF21) which interact with the gut receptors of the pup leading to developmental changes (196). Leptin is absorbed by the neonate gut where it reaches they hypothalamus via systemic circulation where it is able to regulate the energetic homeostasis of the pup (196). In one rodent study, a high-carbohydrate formula intake during suckling by pups led to the development of hyperphagia and obesity during adulthood (197). Decreased methylation of the NPY gene in the hypothalamus during early life, decreased acetylation in histone, H3K9, of the POMC gene, and increased acetylation of the same residue for NPY gene may account for the hyperphagia and obesity seen in the model (198). While our study did not measure the marker genes referenced in these studies, the influence of diet on epigenetics in establishing the phenotype seen in offspring cannot be ignored.

While the dams consumed the aspartame directly, interest in artificial sweeteners also extends to the potential indirect or the effects of these sweeteners on their offspring. Early safety work found that aspartame consumption poses no risk to mother and fetus in terms of toxicity if consumed within FDA-established adequate daily intake (ADI) levels of 50 mg/kg of bodyweight (10,114,128). More recent studies have moved beyond toxicity endpoints to examine the metabolic consequences of nutritionally relevant doses. One Australian study showed evidence of transplacental fetal exposure to artificial sweeteners and raised concern of possible dose accumulation within the fetus when human amniotic fluid and cord blood were measured yet unlike acesulfame-K, saccharin, and sucralose, aspartame was not implicated (199). In humans, aspartame is completely hydrolyzed by digestive enzymes into phenylalanine, aspartate, and methanol and does not enter the blood nor large intestine intact and aspartame itself is therefore not present in the placenta, amniotic fluid, or breast milk (102). Despite its' complete digestion, earlier work showed that maternal aspartame and stevia consumption during pregnancy and lactation increases adiposity in male and female offspring at weaning (9). While maternal aspartame consumption did not worsen weanling body composition parameters in our study, weanling hepatic triglyceride accumulation was upregulated in both sexes. It has been reported that following the breakdown of aspartame, unabsorbed phenylalanine that reaches the large intestine can

be metabolized by gut microbiota to form phenylpyruvic acid and subsequently phenylacetic acid. After absorption into the portal system, phenylacetic acid is readily metabolized in the liver to produce phenylacetylglycine (PAG). Interestingly, the PAG metabolite has been associated with obesity, diabetes, and cardiovascular diseases, and particularly glucose intolerance in mice (200). Furthermore, it has recently been suggested that consumption of artificial sweeteners is related to non-alcoholic fatty liver disease (NAFLD) with gut microbiota dysbiosis identified as a potential novel mechanism (201). Our study also demonstrates that maternal aspartame consumption influences weanling gut microbiota beta diversity suggesting a possible mechanism for increased hepatic triglycerides and the trend for worsened glucose control seen in both sexes respectively during the ITT.

Metabolic programming by the gut microbiota may precede birth as illustrated by a recent study in mice showing that SCFAs from the maternal microbiome cross the placental barrier and bind to GPR41 and GPR43 receptors in the developing embryo capable of impacting downstream development (202). In this case, pups born to mothers harbouring microbiomes deficient in SCFA production owing to germ-free status, antibiotic treatment, or low-fiber diets had higher risks of metabolic syndrome upon exposure to high fat diet as adults when compared to pups born to mothers harbouring SCFA producing microbiomes (202). Alterations to the gut microbiota are linked to increased energy harvest from food and can result in increased fat accumulation in tissues like the liver ultimately leading to NAFLD (203). In our study, weanlings exposed to maternal aspartame had a greater relative abundance of *Firmicutes* with observed increases in both sexes at the genus level for *Roseburia*, *Romboutsia, Lachnoclostridium,* and *Clostridium sensu stricto* and a decrease in the genus *Lactobacillus* observed in females (Figure 16). C*lostridium sensu stricto* is considered a harmful bacteria which could also have adverse effects on the human intestinal tract (204) and has been connected with oxidative stress in mice secondary to gut dysbiosis (205). Oxidative stress is implicated in the pathogenesis of metabolic chronic liver diseases such as NAFLD (206). *Lactobacillus* has been found to play important roles in many aspects of human health (207). It has been reported that *Lactobacillus* ameliorates the progression of NAFLD through the modulation of the gut microbiome by the production of valeric acid which is accompanied by improved intestinal barrier integrity (208) and with subsequent reduction of inflammatory cytokines TNFa, IL-1B, and IL-6 in the liver (209).

Gut dysbiosis can also lead to the disruption of the gut epithelial barrier known as a leaky gut which leads to the passage of pro-inflammatory molecules and bacterial endotoxins such as LPS, as well as microbially-produced ethanol into the bloodstream. These ultimately reach the liver through the portal vein and increase hepatic inflammation and susceptibility to NAFLD (50). In our study, the

expression of hepatic mRNA genes related to metabolism, lipogenesis, and inflammation in the liver tissue (i.e., FAS, ACC, IL-6, IRS-1, IRS-2, and TNFα) revealed a significant sex effect for all of the genes except TNFα with all the others being higher in females. No significant differences due to maternal aspartame consumption were detected although several genes trended upwards. Interestingly, a rodent study showed that female mice are more susceptible to NAFLD than male mice primarily due to alterations in the adiponectin-AMPK-PAI-1 signaling cascade seen in female livers suggesting a stronger effect of sex on leaky gut and enhanced endotoxemia associated with a poor diet which provides a plausible explanation for our findings (210).

The subtle effects of maternal aspartame consumption on offspring were not just limited to weanlings but were also present early on in the lives of young male rats who exhibited increased body weight between weeks four and five. While the females exhibited a strong trend in the interaction between time×diet for body weight, there was no significant effect due to maternal diet. These findings are somewhat consistent with sex differences seen in the developmental literature, with significant changes seen in males and trends seen in female rats with lifetime exposure to aspartame starting *in utero* (211). The increased body weight in young male rats is explained by the notion that male fetuses are more vulnerable to intrauterine insults and adverse perinatal conditions, like diet and stress (212).

While the effect of aspartame cannot be ignored especially in early life, our study findings show its subtle influence dissipated into adulthood. Independent of maternal aspartame consumption, postnatal prebiotic fiber intake had a notable influence on body composition, cecum weights, glucose, and insulin tolerance of rats. Animal studies have demonstrated that intake of fermentable fibers like inulin-oligofructose can improve body composition by decreasing visceral and liver fat content via mechanisms that modulate gut microbiota, increase production of SCFAs (acetate, propionate, and butyrate) by gut microbiota, increase production of anorexigenic gut hormones (PYY and GLP-1), and reduce energy intake (149). Prebiotics markedly improve body composition and metabolic parameters like adiposity and glycemia respectively in obese rat models (213). Our study employs an obese rat model and its findings show that offspring prebiotic fiber consumption improves body composition in adult female rats as these displayed lower body weight, lower percent body fat, and higher percent lean mass while male rats trended lower in body weight and percent body fat. Surprisingly energy intake in adult female offspring increased as a result of postnatal prebiotic fiber consumption which is not consistent with previous work in rodents (154,155). A recent review of inulin-type fructan intake reported mixed effects on energy intake and reduced levels of anorexigenic (leptin) and orexigenic (ghrelin) hormones following consumption of oligofructose over 12 weeks (149). These findings would

not explain the improved body composition associated with increased energy intake observed in our rodent study as the reduction in leptin would reduce appetite which would be offset by the comparable reduction in the hunger hormone ghrelin. However previous studies in humans have shown that hunger is increased by fat free mass and muscle mass (214,215). Moreover, a cross-sectional analysis known as the HEARTY trial involving 304 adolescents living with obesity demonstrated that the magnitude of the body's lean muscle mass is the strongest predictor of energy intake out of a number of other factors including fat mass (216). These studies provide evidence for an existing correlation between lean mass and energy intake albeit in the context of human obesity independent of consideration of diet and appetite hormones, the latter factor not being accounted for in our study which would be a limitation.

Intriguingly in regards to metabolic parameters, postnatal prebiotic fiber intake worsened female insulin sensitivity in the APM-Pre group relative to controls and had no effect on glucose tolerance. This suggests that it was the interaction between postnatal prebiotic fiber, maternal aspartame, and female sex that led to this deleterious effect. Future studies should explore the mechanisms involved in this interaction more carefully. With respect to males, prebiotics did not affect insulin sensitivity but rather improved glucose control. It has been shown that improvements in glucose tolerance in rodents after prebiotic fiber supplementation are related to changes in the microbiota (217). Prebiotic fiber supplementation can increase the secretion of the anorexigenic and insulinotropic hormone, GLP-1 from L cells located in the distal gut (218) GLP-1 stimulates insulin secretion and inhibits glucagon secretion thereby limiting postprandial glucose excursions and improving blood glucose control (219). Cani *et al.* (220) demonstrated that improvements in glucose tolerance and hepatic insulin sensitivity seen with oligofructose consumption require a functional GLP-1 receptor. Although GLP-1 levels and its receptors were not measured in this study, the potential that their function was in some way compromised provides a possible explanation for the worsened findings seen in our female rats and could be looked at in a future study. Moreover, Delmee *et al.* (221) showed that advanced metabolic disturbances induced by a prolonged obesogenic diet reduce the ability of oligofructose to decrease energy intake, body weight, or glycemia in mice. Our rats consumed the obesogenic HFS diet continuously from weaning until they were sacrified in early adulthood. In addition, there is evidence suggesting that a "mismatch" between early environment (dietary or otherwise) and an organism's later environment can increase disease risk (222). This "mismatch" was built into our study with aspartame supplementation exclusively in the maternal diet but not in the offspring diet subject to prebiotic fiber. Interestingly this effect was only observed in females belonging to the APM-Pre group with respect to insulin sensitivity suggesting a key role of sex.

Prebiotic fibers like oligofructose are not susceptible to digestion by human enzymes but are metabolized by bacteria in distal segments of the gut including the cecum and proximal colon (223). As a result, postnatal prebiotic fiber exposure resulted in a noticeable increase in cecum weight in both sexes in our study. This change in cecum weight is attributed to the stimulation of bacterial populations and fermentative process in the presence of prebiotics (224). Moreover, since prebiotics exert a substantial effect on gut microbiota (154,155), it is not surprising that postnatal prebiotic fiber consumption resulted in substantial shifts in offspring gut microbiota compared to controls in terms of alpha diversity, beta diversity, and relative bacterial abundance. Prebiotic effects dominated the changes in offspring microbiota at 12 weeks of age with no effect of maternal diet (aspartame consumption). Lower alpha diversity similar to what was seen in offspring exposed to prebiotic fiber has been previously reported in similar studies and is associated with improved metabolic health (225). Prebiotic fiber consumption has been shown to significantly affect the beta diversity of gut microbiota (226,227). This effect was seen at 12 weeks with prebiotic groups clustering together regardless of maternal diet suggesting no influence of maternal aspartame consumption on gut microbiota in adulthood. These changes were characterized by increases in *Bifidobacterium, Blautia*, and *Streptococcus* and decreases in *Clostridium sensu stricto 1, Coprococcus, Lachnospiraceae NK4A136, Romboutsia,* and *Ruminococcus*.

Prebiotic fiber is known to reduce low-grade inflammation caused by dysbiotic gut microbiota thus improving the intestinal barrier function and promoting the abundance of beneficial gut bacteria (228). A leaky gut occurs when tight junction proteins (claudins, occludens) involved in gut barrier functions of the epithelial lining of the small intestine become compromised leading to inflammation (229). Multiple animal and human studies show that prebiotic intake confers beneficial changes in the host gut microbiota and promotes the proliferation of *Bifidobacterium* (130,230). Improved gut microbial community composition with prebiotics has been linked in cell lines and rodents to reduced fat accumulation, improved nutrient absorption, and anti-inflammatory effects brought about by decreasing intestinal permeability, upregulating the expression of tight junction proteins, and reducing inflammatory cytokines (IL-6 and TNFa) (150,231). More specifically, prebiotic fiber consumption is linked to increases in *Blautia* which is negatively correlated with inflammatory diseases, metabolic syndrome, obesity, and T2DM, however caution must be exercised in drawing general conclusions as these studies have only focussed on the genus level (232). In our rats, *Streptococcus* was also increased secondary to prebiotic fiber supplementation. While this bacteria is typically associated with respiratory tract infections, a retrospective cohort study found that high dietary fructose in 57 teenagers in the

United States living with overweight or obesity was associated with depletion of *Streptococcus* and *Eubacterium* in the gut microbiome which are involved in carbohydrate metabolism (233,234). Future longitudinal studies in humans and animal studies could be used to further examine the association between prebiotic fiber, an HFS diet, and *Streptococcus* and its effect on SCFAs and metabolic outcomes.

In another retrospective cohort study involving 1,914 Chinese adults *Romboutsia, Ruminococcus, Clostridium sensu stricto 1,* and *Dorea* were positively and significantly associated with body weight, serum lipids, and uric acid (235). Moreover, *Coprococcus* was seen in the gut microbiota of adults living with obesity-associated metabolic abnormalities (235). *Clostridium sensu stricto 1* is an opportunistic pathogen that can cause intestinal inflammation and decrease the content of SCFAs seen in a human and animal model (236). It is also responsive to obesogenic diets in rodents (237). These findings suggest a deleterious association of these genera with health in humans which supports our findings where these genera with the exception of *Dorea* were found in significantly lower abundance in our rats exposed to postnatal prebiotic fiber intake. A further microbial shift to consider in our rats fed prebiotic fiber was a lower relative abundance of *Lachnospiraceae NK4A136*. This is similar to Zhang et al (238) who fed mice a high-fat diet alone or supplemented with a traditional Chinese medicine herbal formula for 24 weeks. They found that the traditional medicine group had significantly reduced liver steatosis alongside a reduced relative abundance of *Lachnospiraceae NK4A136* (238). There was a strong and positive correlation between *Lachnospiraceae* NK4A136 and the expression of several inflammatory cytokines and chemokines in the liver suggesting a reduction in *Lachnospiraceae* NK4A136 was beneficial in these mice. A signature microbiome configuration attributed to optimal health has not yet been identified, however when the distinct shifts in gut microbiota of our adult offspring exposed to prebiotic fiber postnatally are viewed in conjunction with improvements in body weight, body composition in females, and glucose tolerance in males, a generally beneficial relationship between diet, microbiota, and health can be seen.

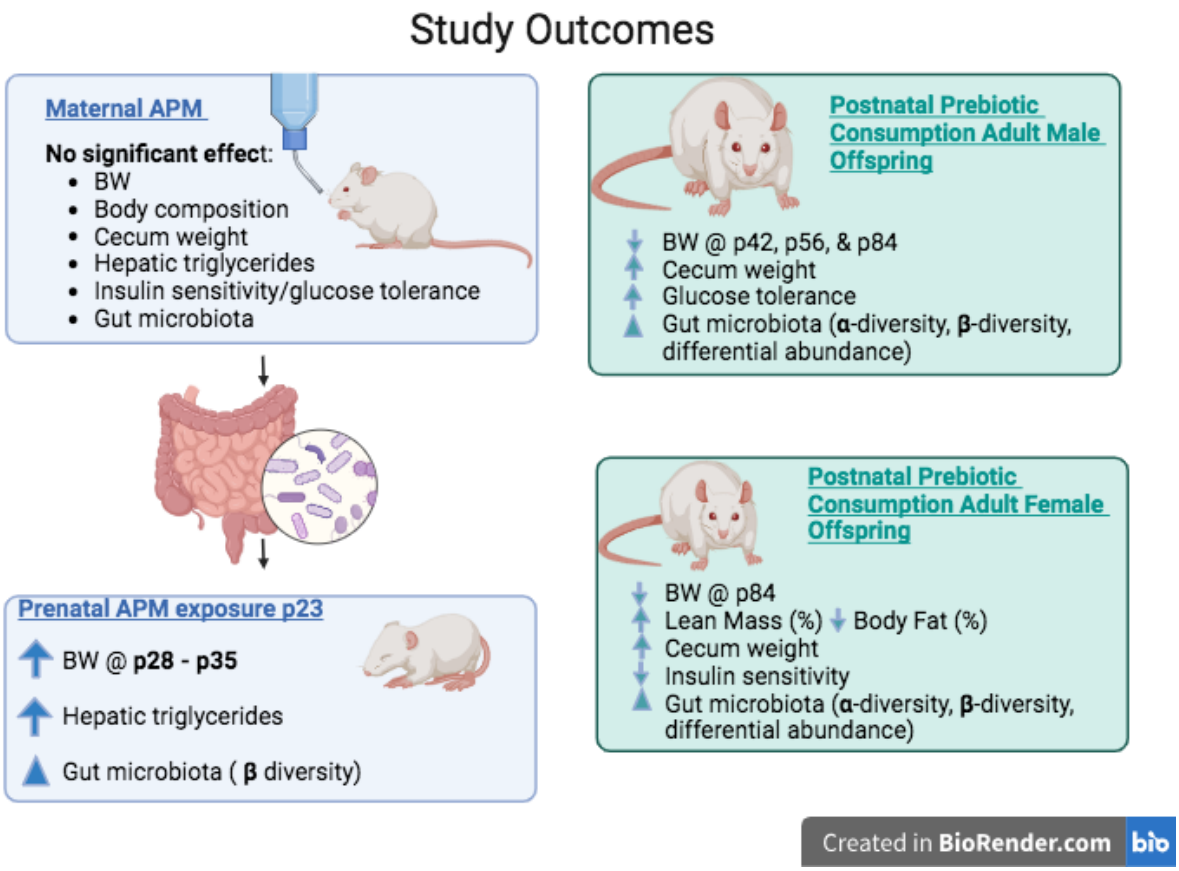

Figure 34. Summary of study outcomes.

5.2 Strengths and Limitations

5.2.1 Rodent Model

The Sprague Dawley (SD) rodent model used in this study is frequently used in health research as it provides several advantages over other rodent species and rat strains. SD rats are an outbred rat strain that exhibit genetic variability which better represents the genetically diverse human population (158). Outbred strains are effective animal models of polygenic human diseases like obesity and cardiovascular disease (159). SD rats are typically used in studies involving diet-induced obesity (DIO) as they acquire this disorder more easily when compared to other rat strains signifying that some genetic basis is essential in body weight gain (239). Like humans, SD dams demonstrate the potential to transmit an obese phenotype to their offspring (130). At birth, pups are exposed to maternal microbiota through the birth canal and fecal matter similar to the vertical transmission of microbiota experienced during human birth. SD rats possess similar metabolic responses to obesity as humans as obese rats have greater levels of circulating leptin and tend to be resistant to its effects on energy intake and body composition in central circuits regulating food intake (240). SD rats also metabolize aspartame similar to humans (241), although they do not taste the sweetness of aspartame like humans do (242). Aspartame is broken down into aspartic acid, phenylalanine, and methanol by enzymes in the gut lumen (esterases and peptidases) and rapidly absorbed into the portal circulation (241). Rats are well known for their docile temperament, social behaviours, and their ability to breed well in the laboratory environment (243). SD rats have a long lifespan and reach sexual maturity at an early age allowing for multiple generations of research (244). The larger size of the SD rat compared to other animal models allows for greater collection of blood, tissue, and fecal samples used in the analysis of glycemic control and insulin sensitivity, hepatic triglycerides and expression of hepatic fat synthesis genes, and gut microbiota respectively. For example, 16S rRNA sequencing for gut microbiota analysis requires a 150 mg fecal sample per rat which would be more challenging to obtain in a smaller animal model like mice and in the event the DNA yield is not good additional fecal samples would be required which may be difficult to obtain.

The utilization of the SD rodent model is not without its limitations. While ideal for shortening the overall study period, gestation period for SD rats is 21 days compared to 9 months for humans. Rats also display routine behaviours not observed in humans such as cannibalism of siblings and offspring and coprophagy which would impact gut microbiota (243). Nonetheless, the use of a rodent model has important strengths which allow the researcher a high degree of control over factors that would otherwise confound human studies such as food intake, diet composition, and the environment.

5.2.2 Experimental Diets

Female SD rats were fed an HFS diet (Dyets #102412, Dyets Inc., Bethlehem, PA) used in previous studies (9,130) ad libitum in a DIO model prior to breeding spanning 11-weeks . This diet was in powdered form and composed of 39% of calories from fat (lard and soybean oil) and 44% of calories from sucrose. The high sucrose and saturated fat content present in the HFS diet used in our study resembles a Western diet characterized by consumption of large amounts of processed red meat, high fat dairy products, sugary drinks, and refined carbohydrates (245). Metabolic disturbances observed in rats exposed to DIO via HFS consumption in prior studies (9,130) make the HFS diet an ideal choice for the investigation of metabolic disorders and potential therapies.

Aspartame was administered through drinking water in doses that resemble normal intake via diet sodas. Doses were chosen based on average consumption reported from individuals who consume sweeteners regularly to reflect potential outcomes resulting from a long-term nutritionally relevant consumption. In all cases, the amount of aspartame was kept within established safe limits (i.e. within human acceptable daily intake levels at 7mg/kg/day well below the ADI of 40mg/kg/day).

We provided our adult offspring with a 10% wt/wt prebiotic oligofructose dose, similar to that of previous work in our lab and other findings showing that this dose significantly alters adiposity, body weight, food intake, and gut permeability (246,11). While effective in rats, an equivalent dose in humans would be more difficult for the modern gut that has adapted to low fiber diets and would likely produce gastrointestinal side effects including diarrhea, bloating, and flatulence (247). Evidence suggests that a dose of approximately 8 grams/day in children 7-12 years of age (230) and 16-21 grams/day in adults (152) results in reductions in body fat but at a lower magnitude than the reductions seen in rats with a 10% dose. Rats' larger relative cecum size likely explains their ability to better tolerate a 10% wt/wt dose of prebiotic oligofructose than humans via their increased fermentative capacity (248). Aversion to this concentration of prebiotic is ruled out in our study as rats consuming prebiotic fiber consumed larger quantities of food and had greater overall energy intake in our study when their food was adjusted for calories. Based on the well-documented use of this dose of prebiotic in rodent studies, we did not anticipate tolerance issues in our rats yet we did observe bloated abdomens in vivo and visually during DEXA scan. Additionally, their cecums were larger and heavier at euthanasia compared to controls suggesting excess gas production from increased fermentative activity.

5.2.3 Intervention Duration

Previous work has demonstrated that 50% of SD rats develop diet-induced obesity when fed a high-energy HFS diet ad libitum with the remaining 50% resisting weight gain and gaining weight and adiposity at a rate similar to a chow-fed control diet (249). The phenotypic differences in the population are evident after a minimum of two weeks consuming a high-energy diet (249). Recent work in our lab employed a 10-week DIO model to achieve a desired mean weight of 450 g in female rats (9). In an attempt to replicate the same characteristics in our study and identify the top 50% weight gainers ("responders") we extended DIO by one week as the female rats were not at an ideal heavy weight by week 10. Weight did not increase appreciably by week 11 as the "responder" female rats had a mean weight of 374 g, still considerably lower than previous work in our lab (9). As a result, we decided to conclude DIO and begin breeding as females were 19 weeks old at that point compared to 18 weeks old in the Nettleton et al. study. Given the smaller baseline body weight values in female dams prior to breeding at week 11 in our study (374 g) compared to the baseline weight (450 g) in the previous study (9), it is hypothesized that even our "responder" female rats may have had a degree of obesity resistance and that this may have been transmitted to the offspring. This would be a limitation in our study despite our extended DIO protocol that intended to screen the top 50% of weight gainers or "responders" from the "non-responders". Some researchers have suggested that there is genetic drift in commercial vendors colonies (250) and it is possible they have become more obesity-resistant although we do not have concrete evidence for this. Another factor that has been suggested to affect the ability of rats to gain weight and fat mass is the housing temperature (251) where differences can affect the microbiota as well as weight gain (i.e. rodents gain more weight at thermoneutral 30°C than cooler temperatures).

Prior parental programming research demonstrated stronger intergenerational metabolic effects when parents adhered to their specific diets for 16 weeks (252). While this study focussed on maternal programming of obesity via an 11-week DIO phase with male breeders fed a normal rat chow diet, it is possible that extending DIO and/or including aspartame in the pre-conception period may have conferred a stronger obese phenotype to offspring although prior work typically does not involve DIO longer than 10 weeks (9,161,253). Additionally, we followed the offspring for 12 weeks. We recognize that assessing the offspring for a longer period of time may have yielded more pronounced metabolic differences and a stronger obese phenotype.

5.2.4 Outcome Measures

While cost-effective and straightforward, the manual weighing of a food cup before and after a feeding period employed in our study is not without its limitations. This approach is time and labour-intensive requiring daily readings during the prescribed food intake measurement period. While every effort to closely pack the food into the food cups was made, rodents can create crumbs, defecate, and urinate in their food cup which reduces the accuracy of the measurements (254). The toppling of food was observed yet care was taken to ensure the food and crumbs were restored to their food cups when possible.

Another limitation exists with the measurement of body composition utilizing the GE Lunar Prodigy DEXA scanner. The accuracy of the scanner is compromised with animals weighing less than 100 grams. Given that all of our weanlings weighed less than this threshold, assessing body composition in this age group was more challenging than in the adult rats. Accordingly, great attention to detail was exercised in the post-scan analysis which requires the identification of distinct body regions and lean body structures as the resolution in weanlings was not as sharp thereby opening up an opportunity for measurement bias. Use of a scanner with greater sensitivity such as a small animal DEXA scanner would overcome this limitation although animal transport limitations and other financial constraints did not make this a viable alternative.

Additionally, we employed both OGTT and ITT in our investigation of glucose tolerance and insulin sensitivity in SD rats respectively. These tests are a standard in pre-clinical research yet they are not without their limitations mainly because of their low-reproducibility due to many possible confounding variables related to animal husbandry and their environment. For example, environmental stressors will increase adrenaline and noradrenaline which directly affect glucose homeostasis (255). These stressors may include cage handling, repeated bleeding from the OGTT and ITT procedures, and even loud noise. At the time of our study, construction-related noise adjacent to the animal facility may have bled into the room along with unwanted vibrations during human daylight hours which is when rats are dormant. A noise-intensity-dependent increase in plasma adrenocorticotropic hormone (ACTH) and corticosterone in SD rats was observed with loud noise with levels of these stress hormones beginning to rise at approximately 85 dBA (ie. food blender) (256) although observed noise levels in the rat holding room did not reach this level.

Furthermore the first author of this study and the facility veterinarian are both biological males. Findings suggest that female rats display consistently exacerbated anxiety-like behaviours along with elevated body surface temperature during repeated exposure to male experimenters including

increased corticosterone and lower oxytocin levels (257). This may in part explain the unexpected results in OGTT and ITT where prebiotic fiber consumption had no effect on glycemic control and worsened insulin sensitivity in females as they would have been handled by a male at the time of these tests. Many measures were employed in this study to minimize disturbances to the rats' environment and reduce animal stress including limiting the number of people interacting with them in an effort to increase familiarity. Rats were also kept in a scent-free, temperature, and humidity-controlled room with a set light and dark cycle. Overall, great effort was taken to ensure the rats were well taken care of as they were under constant monitoring by the research team, animal facility staff, and the veterinarian. There were no overt signs of stress, distress, or aberrant behaviour throughout the duration of the study.

CHAPTER SIX: CONCLUSION

6.1 Future Directions

The prevalence of obesity continues to increase dramatically worldwide (1,258). Obesity can affect nearly every organ system and has been linked to the development of chronic metabolic conditions (259). Obesity is a complex health issue caused by a combination of individual factors including cultural, lifestyle, genetic, and environmental variables (260). In response to rising obesity rates and in an attempt to reduce the energy content of foods and beverages, artificial sweeteners like aspartame have become a popular replacement for sugar and are now found in thousands of products. Like sucrose, aspartame provides energy of 4 calories per gram however it is 200 times sweeter therefore it is only required in minuscule amounts to provide sweet taste (261). A 12-ounce can of diet soft drink contains around 200 mg of aspartame (262). Thus, while aspartame significantly reduces the energy content of foods and beverages, the body of evidence does not universally point to beneficial health outcomes. While randomized controlled trials, typically of shorter duration show weight benefits, longer-term prospective cohort studies show higher weight and increased risk of T2DM with artificial sweetener intake (126). Recently, a study by Suez et al., (263) provided causal evidence for artificial sweeteners' potential to induce glucose intolerance with saccharin and sucralose significantly impairing glycemic response in humans. Additionally, this study showed that there are responders and non-responders to artificial sweeteners in the development of glucose intolerance in humans and this phenotype can be transferred to germ free mice via fecal microbiota transplantation (263). Additional studies are necessary to further explore underlying mechanisms and possible existing sex differences in humans.

In our study the DIO non-responders were not included in the study. Future studies could include non-responder rats to DIO further identify key differences between the two groups and their respective offspring. Sex differences could also be studied. Additional tests not included in our study such as blood sample collection for the identification of blood triglycerides and satiety hormones, samples of the small intestine and colon to assess levels of intestinal barrier genes, and brain samples to assess the mesolimbic reward center and gut-brain axis, could all be obtained and compared between the groups. Causation could be explored via fecal microbiota transplant of responders and non-responders into germ-free mice. Refocussing the investigative lens on the non-responder rats provides an obese-resistant reference model whose underlying metabolic mechanisms can be studied to help combat obesity. Furthermore, while aspartame was the artificial sweetener of interest in this study, its

effects and the effects of other artificial sweeteners with a more potent glycemic effect (ie. saccharin) per Suez et al., (263) could also be explored in future studies in the context of non-responder rats.

Our study explored the impact of maternal aspartame consumption during gestation and lactation on offspring. Aspartame consumption did not significantly alter body composition and glycemic control in dams or offspring with the exception of increased liver triglycerides in weanlings. Future studies could explore the effect of lifelong aspartame consumption in dams by introducing it at the onset of a 10-week DIO challenge as this might more closely resemble artificial sweetener intake patterns of humans. Effects on gut microbiota and other health parameters could be compared in female rats and their offspring between lifelong aspartame consumption versus its consumption beginning in gestation only as studies to date have only investigated one or the other pattern of consumption within a single study. Microbial and metabolic dysfunction may accumulate over time.

Irrespective of diet, statistical analysis revealed several sex-specific metabolic differences which led to analyses being stratified by sex. Increased hepatic fat synthesis genes in female weanlings, greater blood glucose values in AUC following an OGTT in adult males, greater AUC in adult females following an ITT, and greater hepatic triglyceride content in adult males were seen. It is understood that there are sex-specific metabolic differences between males and females (165). These differences between males and females are likely attributed to distinct sex hormone levels between the two. While we did not control for variations in testosterone or estrogen, future studies could explore this difference by collecting primary sex organs and analyzing sex hormone levels in conjunction with other findings. Of particular interest is the significantly worsened blood glucose levels seen in the APM-Pre adult female rat group during ITT. Correlation between sex hormones, diet, and other parameters not tested in our study but suggested above could help explain this aberrant finding.

International standards focussing on the evidence-based treatment of obesity have been developed and the state of knowledge on this topic continues to expand (264). The goal of this research is to add to the existing body of knowledge by informing on the potential benefits of postnatal prebiotic oligofructose intake in attenuating obesity risk. Our study identifies the beneficial metabolic changes seen as a result of postnatal oligofructose consumption as improved body weight and body composition in rats with improved glucose tolerance observed in males. Our study posits that these changes secondary to prebiotic intervention are mediated by distinct changes in gut microbiota. Like oligofructose, galactooligosaccharides (GOS) another type of prebiotic fiber is also known to promote the growth of beneficial microbial species (12). GOS is an oligosaccharide and an important active substance in milk and is divided into α-GOS and β-GOS due to the different galactosidic bonds attached

(265). β-GOS is the more widely researched of the two and is produced commercially with few studies looking at α-GOS (265). α-GOS is widely found in legume seeds of plants such as soybean, lentils, and chickpeas (266). Future studies exploring the postnatal effect of α-GOS on gut microbiota could be explored to ultimately assess its potential for health relative to oligofructose under the context of treating obesity. Legumes are an essential component of plant-based diets which have shown potential in the treatment of obesity (267).

Our investigation into the attenuation of programmed metabolic risks through the introduction of prebiotic fiber into the diet in early life is novel and may lead to the generation of an innovative postnatal dietary strategy to improve long-term health. Given the increased consumption of artificial sweeteners and the current rise in diabetic obesity, identifying novel dietary strategies to mitigate the risks associated with maternal aspartame consumption are needed. While metabolic disturbances from aspartame consumption were not seen in our study outside of increased weight gain at weeks 4-5, significant improvements in several health parameters were observed secondary to postnatal prebiotic fiber intake. Taken together, the findings of this study may have potential to translate into a human trial and eventually inform dietary guidelines for prebiotic fiber intake in pregnancy and early childhood.

6.2 Concluding Statement

Indeed the gut microbiota represent a changing ecosystem that is shaped by many developmental and environmental factors with profound implications for biological and metabolic processes. In this study, the gut microbiota was influenced to a minor extent by exposure to maternal aspartame consumption but was markedly altered by postnatal prebiotic fiber consumption. Interestingly, metabolic and phenotypic changes were most clearly seen when microbial differences were detected (i.e. with prebiotic fiber intake). While the detrimental effects of offspring exposure to maternal aspartame were lesser than has been seen in previous studies, the findings of this study suggest that postnatal diet does convey a significant influence. Despite consuming a high fat/sucrose diet for 9 weeks postnatally, rats consuming prebiotic fiber were characterized by improved health parameters in body weight and body composition predominantly in females, improved glycemic control in males, and shifts in gut microbiota seen in adult offspring. Overall, this study provides justification for future work to examine other adverse developmental or maternal exposures and the potential for postnatal prebiotic fiber supplementation to improve metabolic outcomes.

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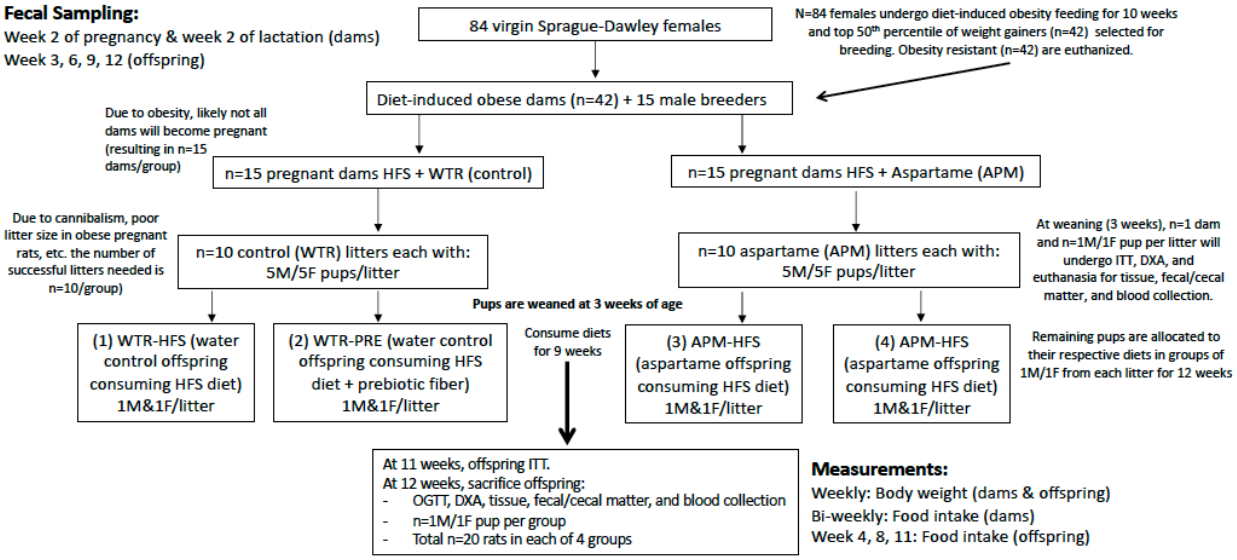
APPENDICES

Appendix A. Experimental flow chart



Total rats: 84 virgin females; 15 male breeders; 300 offspring

Note: We indicate an n=300 offspring which is the maximum we would generate if all n=30 pregnancies are successful. Given past experiments we know that not all obese pregnancies are successful and therefore the actual 'n' is likely to be lower (the required n=20). At birth, litters are culled to n=10 pups (5M/5F). The extra pups generated via breeding will be offered to others and not just euthanized. At weaning a total of 40 pups will be euthanized for testing (1M and 1F from 10 litters of control and 1M and 1F from 10 litters of aspartame). Dams are also tested and euthanized at weaning (likely n=20 but as high as n=30 dams total). At 12 weeks, 1M and 1 F from 10 litters in each of the 4 treatment groups are tested and euthanized (total of 80 rats). From each litter at weaning, there are n=4 pups not used and they will be offered to others prior to euthanasia.

Appendix B. Condensed experimental flow chart