Intestinal Microbiota and Bacterial Translocation: Impact of Diet, Obesity and Insulin Resistance

by

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ABSTRACT

Introduction:

The metabolic syndrome (MetS) is associated with factors that increase the risk of Type 2 Diabetes and cardiovascular disease. Recently, intestinal microbiota has attracted attention as an important environmental factor that plays a role in regulating weight gain and development of type 2 diabetes (T2D). Type 2 diabetes is characterized by insulin resistance, impaired glucose metabolism, low-grade inflammation and has also been linked to the quality of microbiota. However, studies in humans are yet to report a consistent relationship between intestinal bacterial composition and diabetes. We do know that altered gut microbial composition can result in the leakage of bacterial products [such as lipopolysaccharide (LPS)] and viable bacteria (known as the bacterial translocation). Additionally, studies have shown that low birth weight (LBW) and postnatal nutrition are risk factors for adult metabolic diseases. However, the interaction between LBW, diet and intestinal lipid absorption/secretion leading to adult metabolic disease remains unclear.

Hypothesis and objectives:

We hypothesized that over-nutrition would alter gut microbiota composition in a spontaneous animal model of insulin resistance (the JCR:LA-cp rat). Similarly, addition of an energy rich diet (high fat-HFD) would further exacerbate microbial dysbiosis and subsequently, facilitate increased bacterial translocation. Using a complementary approach in swine, we hypothesized that LBW pigs fed a western diet (high-fat, high-carbohydrate [HFHC] diet) would develop metabolic complications and induce dysbiosis in gut microbiota. We further proposed that a diet rich in a novel bioactive monounsaturated fatty acid [(vaccenic acid or VA) 1.7 % *w/w* of diet or 10% of the fat] would result in an improved gut dysbiosis and lipid metabolism profile.

Material methods:

For our rodent studies, 6-week-old obese homozygous and lean heterozygous JCR:LA-cp rats were randomly assigned to control or HFD diet for 6 weeks. Samples of lymph and portal vein blood were cultured and processed for sanger sequencing. For gut microbiota analysis from cecal samples, total DNA was extracted and further processed by next generation sequencing. For the first of two swine studies, pigs were fed a control or HFHC diet for 6 weeks, (post-weaning) until 13 weeks of age. In the second swine study pigs were fed either control, HFHC or HFHC enriched in VA for 6 weeks. For both swine studies, we combined a 2-step modified oral glucose tolerance and fat challenge test. Lymph was collected followed by exsanguination under anesthesia and tissue collection.

Results:

Experiments in rodents showed an effect of HFD on gut microbiota however, our results did not find any impact of insulin resistance per se. We found evidence of bacterial translocation but neither obesity nor HFD was found to exacerbate the condition. Studies in swine found that LBW pigs fed on HFHC diets displayed fasting and postprandial hypertriglyceridemia as well as a higher insulin excursion. In the second swine experiment, addition of VA showed mild insulin sensitizing effects. In terms of gut microbiota, HFHC diet showed significant shift in gut microbiota in LBW pig with increase ratio of *Firmicutes*: *Bacteroidetes*. Interestingly, the addition of VA in HFHC diet normalized gut microbiota dysbiosis in LBW pigs.

Conclusion:

Findings from this thesis provide evidence that increased energy intake and the obese genotype per se does not necessarily affect the gut microbiota composition (at least in JCR:LA-cp rats). Whereas the addition of HFD showed substantial changes in intestinal microbiota.

Experiments in pigs demonstrated that a HFHC diet also results in a dysbiotic gut microbiota, yet can be avoided with the addition of dietary VA. Our observations are consistent with both rodent and human studies and give reason for LBW swine to be utilized as a model for human translational research to better understand the relationship of microbiota and chronic disease.

PREFACE

Enclosed in this thesis is the original work of Vijay Pal Singh with contributions as detailed below: Chapter 3 assistance appreciated from students and staff from the Dr. Proctor labs. Sandra Kelly (lab manager proctor lab), Dr. Rabban Mangat (Research Associate) and Willing labs. Janelle Fouhse (Postdoctoral fellow), Tinting Ju (PhD student), Deanna Pepin (M.Sc. student), and Andrew forgie (M.Sc. student).

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"Science is a beautiful gift to humanity; we should not distort it."

—A. P. J. Abdul Kalam

Dedication

To the rats and pigs, with love and appreciation.

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LIST OF ABBREVIATIONS

AA	Arachidonic Acid
ABCG5/8	ATP-Binding Cassette Transporter G5/G8
AC	Abdominal Circumference
ACAT2	Acyl-Coa:Cholesterol Acyltranferase Enzyme 2
ACUC	Animal Care and Use Committee
ADF	Average Daily Feed Intake
ADG	Average Daily Weight Gain
ALA	α-Linoleic Acid
ANOSIM	analysis of similarity
Apo	Apolipoprotein
AUC	Area Under the Curve
BT	Bacterial translocation
CCAC	Canadian Council on Animal Care
CDC	Centers for Disease Control and Prevention
CE	Cholesterol Esters
CETP	Cholesteryl Ester Transfer Protein
CLA	Conjugated Linoleic Acid
CM	Chylomicron
CM-r	Chylomicron Remnant
CR	Crown-Rump
CVD	Cardiovascular Disease

DAG	Diacylglycerol
DGAT	Diacylglycerol Acyltransferase
DHA	Docosahexaenoic Acid
DOHaD	Developmental Origins Of Adult Health And Disease
DPP-IV	Dipeptidyl Peptidase-4
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-linked Immunosorbent Assay
EPA	Eicosapentaenoic Acid
ER	Endoplasmic Reticulum
FA	Fatty Acid
FAA	Fastidious anaerobe agar
FAAs	Free fatty acids
FABP	Fatty Acid-Binding Protein
FAME	Fatty Acid Methyl Ester
FAT/CD36	Fatty Acid Translocase/Cluster Determinant 36
FATP	Fatty Acid Transport Protein
FC	Free cholesterol
FCR	Feed Conversion Ratio
FIAF	Fasting-induced adipose factor
FGR	Fractional Growth Rate
FMO	Flavin monooxygenase
FPLC	Fast protein liquid chromatography
GCG	Proglucagon Gene

GF	Germ-free
GFP	Green fluorescent protein
GLP	Glucagon-Like Peptide
GOS	Galactooligosaccharide
GPR	G-protein coupled receptor
GLUT	Glucose Transporter
HDL	High Density Lipoprotein
HFD	High Fat Diet
HFHC	High-Fat, High-Carbohydrate
HIV	Human immunodeficiency virus
HL	Hepatic Lipase
HMGCR	HMG-CoA Reductase
HSL	Hormone-sensitive lipase
IBD	Inflammatory bowel disease
IDF	International Diabetes Federation
IDL	Intermediate-Density Lipoprotein
IGF	Insulin-like Growth Factor
IR	Insulin Resistance
IRS	Insulin Receptor Substrate
IS	Insulin Sensitivity
IUGR	Intrauterine Growth Restriction
LA	Linoleic Acid
LB	Luria Bertani

LBW	Low Birth Weight
LCAT	Lecithin-Cholesterol Acyl Transferase
LCFA	Long Chain Fatty Acids
LDL	Low Density Lipoprotein
LDLR	Low-Density Lipoprotein Receptor
LPL	Lipoprotein Lipase
LPS	lipopolysaccharide
MAG	Monoacylglycerol
MetS	Metabolic Syndrome
MGAT	Monoacylglycerol Acyltransferase
MLN	Mesenteric lymph nodes
MOGTT	Modified Oral Glucose Tolerance and Fat Challenge Test
mRNA	Messenger RNA
MTP	Microsomal Triglyceride Transfer Protein
MUFA	Monounsaturated Fatty Acid
NAFLD	Non-Alcoholic Fatty Liver Disease
NASH	Non-Alcoholic Steatohepatitis
NBW	Normal Birth Weight
NEFA	Non-Esterified Fatty Acid
NPC1L1	Niemann-Pick C1-Like 1
OUT	Operational taxonomic units
PAR	Predictive Adaptive Response
PC	Phosphatidylcholine

PCoA	Principal Coordinate Analysis
PDME	Phosphatidyldimethylethanolamine
PE	Phosphatidyethanolamine
PI	Phosphatidylinositol
PL	Phospholipid
PPAR	Peroxisome Proliferators Activated Receptor
PS	Phosphatidylserine
PUFA	Polyunsaturated Fatty Acid
PYY	Peptide YY
qPCR	Quantitative (Real-Time) Polymerase Chain Reaction
RA	Rheumatoid arthritis
RCT	Reverse Cholesterol Transport
SC	Snout-Crown
SCFA	Short-chain fatty acids
SD	Standard Deviation
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEM	Standard Error of the Mean
SM	Sphingomyelin
SR-B1	Scavenger Receptor Class B Member 1
SREBP	Sterol Regulatory Element-binding Protein
T2D	Type 2 Diabetes
TG	Triglyceride
TJ	Tight junction

TLR4	Toll-like receptor 4
TMA	Trimethylamine
TMAO	Trimethylamine-N-oxide
TSOD	Tsumura Suzuki obese diabetes
VA	Vaccenic Acid
VLDL	Very Low-Density Lipoprotein
WHO	World Health Organization
AA	Arachidonic Acid

Chapter 1: Review of literature:

1.1 Obesity and type 2 diabetes

In 2016 approximately 1.9 billion adult people were overweight (World Health, 2016). There has been significant investment to appreciate the pathogenesis of obesity and its potential cure, however, the exact mechanisms that propagate its metabolic complications (including insulin resistance and type two diabetes) have not been fully explained (Kahn, Hull, & Utzschneider, 2006).

1.2 Insulin resistance and metabolic syndrome

The increasing rates of obesity throughout the world have also shown a corresponding increase in the metabolic syndrome (MetS), (Idf, 2006). According to the International Diabetes Federation (IDF), MetS consists of fasting glucose greater than 100mg/dL, triglyceride concentrations over 150mg/dL blood pressure greater than 130/85mmHg, low HDL-cholesterol (less than 40mg/dL or 50mg/dLfrom men and women respectively) and BMI over 30kg/m^2 (Idf, 2006). Two main contributing causes for the development of MetS is excessive adiposity and over-utilization of glucose that leads to impaired insulin metabolism.

1.3 Impaired glucose and insulin metabolism during obesity

Increased adipocyte abundance results in increased release of free fatty acids (FFAs) (Boden & Shulman, 2002). Increased FFAs act as a source of energy and are thought to compete with glucose, resulting in less clearance of blood glucose (Ebbert & Jensen, 2013). It is also known that the insulin response can regulate FA uptake through stimulation of lipoprotein lipase (LPL) and concomitant reduction in hormone-sensitive lipase (HSL) activity (Sears & Perry, 2015). Insulin resistance is the driving factor that leads to many disease states, such as type two diabetes (T2D) and MetS (Saltiel & Kahn, 2001). Insulin resistance occurs specifically in the liver, adipose and

muscle tissues (Saltiel & Kahn, 2001). These specific tissues become resistant requiring higher insulin production to maintain plasma glucose (Saltiel & Kahn, 2001). Over time the pancreas develops a reduced capability to secrete insulin after prolonged periods of overproduction, which can result in an exogenous insulin-dependent condition and trajectory to T2D (Khan & Pessin, 2002).

1.3.1 Lipid metabolism physiology in humans

The transport of insoluble lipids in the aqueous blood is facilitated by macromolecules known as lipoproteins (Couture et al., 2014; Giammanco, Cefalù, Noto, & Averna, 2015). Lipoprotein particles consists of a bi-layer. The inner hydrophobic core is comprised of triglyceride (TG) and cholesteryl esters (CE), while the outer monolayer surface contains amphipathic phospholipids (PL), small amounts of free cholesterol (FC) and proteins (Ginsberg & Tuck, 2001). Lipoproteins can be differentiated based on density, size, and composition (as described in **Table 1.1**). The liver and intestine are the two main organs responsible for lipoprotein production and homeostasis.

Table 1.1. Physical properties of major classes of lipoproteins in human circulation.

	Chylomicron (CM)	Chylomicron Remnant (CM-r)	VLDL	IDL	HDL	LDL
Density (g/ml)	<0.930	0.930- 1.006	0.930- 1.006	1.006- 1.019	1.063- 1.210	1.019- 1.063
Size (nm)	75-1200	30-80	30-80	25-35	5- 12	18- 25
Major Lipids	Triglyceride (80-95%)	Triglycerides (70%) Cholesterol (13%)	Triglyceride (55-80%)	Triglyceride (20-50%) Cholesterol (20-50%)	Cholesterol (15-25%) Phospholipids (20-30%)	Cholesterol (40-50%)
Major Apo-proteins	B-48, C-I, C-II, C-III, E, A-I, A-II, A-IV	B-48, E	B-100, E, C-I, C-II, C-III	B-100, E, C-I, C-II, C-III	A-I, A-II, C-I, C-II, C-III, E	B-100

Adapted from Feingold and Grunfeld,2015 and Kritchevsky et al, 1986

1.4 Association between diabetic dyslipidemia and cardiovascular disease

Heart disease and stroke cause two-thirds of diabetic-related mortalities (Chehade, Gladysz, & Mooradian, 2013; Deshpande, Harris-Hayes, & Schootman, 2008). There are many factors associated with diabetes that increase the risk of a cardiovascular disease (CVD) related event. High concentrations of low density Lipoprotein (LDL) cholesterol, low concentration of high density lipoprotein (HDL) cholesterol, as well as elevated fasting plasma triglyceride (TG) are all independent risk factors of CVD (Battisti, Palmisano, & Keane, 2003). Oxidation of LDL is also thought to play an important part in the development of atherogenesis. During diabetic hyperglycemia, LDL oxidation and glycation is exacerbated, which promotes the development of atherosclerosis (Amir Ravandi, Arnis Kuksis, & Nisar A. Shaikh, 2000).

1.4.1 Hepatic lipid metabolism

The synthesis of lipids occurs in the liver through de novo lipogenesis, where carbohydrates are converted into FAs and subsequently packaged into TG (S. H. Choi & Ginsberg, 2011). The hepatocyte then assembles TG into VLDL particles for secretion to peripheral tissues (Nguyen et al., 2008). Each VLDL particle requires a single structural protein called apolipoprotein B100 (apoB100), which in turn can be used to quantify plasma concentration of VLDL (Sniderman, Couture, & de Graaf, 2010). Additionally, VLDL particles associate with other apolipoproteins such as apolipoprotein E, C-I, C-II and C-III.

ApoCII is an important regulator, as it allows LPL to recognize the molecule and hydrolyze TG (Nguyen et al., 2008). Thus, as VLDL particles become smaller and more dense, they are typically considered cholesterol-rich IDL particles. Subsequently, as IDL continues to distribute TG to tissues it becomes a smaller more dense LDL particle (Nguyen et al., 2008). By definition, both IDL and LDL are considered remnant lipoproteins of the larger VLDL that are inturn recycled

back to the liver via the LDL receptor (LDLR) (S. H. Choi & Ginsberg, 2011). In addition to the secretion of apolipoprotein B-containing particles, the liver also secretes apolipoprotein A-I (apoA-I) particles. Hepatic derived apoAI particles rapidly accept cholesterol from the plasma, peripheral tissues, and/or the arterial wall (Daniels, Killinger, Michal, Wright, & Jiang, 2009). The primary function of HDL is to transport cholesterol to organs, other lipoproteins or be cleared by the liver (Daniels et al., 2009). Cholesteryl ester transfer protein (CETP) exchanges cholesterol from HDL and LDL particles for TG in VLDL and Chylomicron (CM) particles (Daniels et al., 2009). Lecithin-cholesterol acyltransferase (LCAT) enzymes esterify free cholesterol and catalyze production of mature HDL (Daniels et al., 2009). HDL particles are thought to facilitate the 'removal' of cholesterol from peripheral tissues through the reverse cholesterol transport (RCT) pathway (Rye, Bursill, Lambert, Tabet, & Barter, 2009).

1.4.2 Lipid metabolism in intestine

Chylomicrons (lipoproteins synthesized in the intestine) facilitate the transport of ingested fats and lipid-soluble vitamins into the circulation (Dash, Xiao, Morgantini, & Lewis, 2015). During the fasted state, plasma TG is typically associated with hepatic derived lipoproteins. In contrast, during the fed state the secretion of newly synthesized CM result in a substantial and prolonged excursion of TG during the post-prandial state (Dash et al., 2015; Xiao & Lewis, 2012). Chylomicrons and liver-derived VLDL particles contain similar apolipoproteins, such as apoCI, CII, CIII and E. However, apolipoprotein B48 (apoB48) is essential for assembly of CMs. Each CM particle contains only one apoB48 protein and therefore it can be used to differentiate and quantify CM from hepatic-derived apoB100 particles (Dash et al., 2015; M.J. van Greevenbroek & W.A. de Bruin, 1998; M. L. Phillips et al., 1997). After hydrolysis of TG, the remaining (smaller, more dense) particle is considered a CM-remnant (CM-r) (Haidari et al., 2002). Like the liver, the

intestine is also able to secrete apoAI-associated particles (Mangat et al., 2018). The addition of cholesterol and phospholipids to apoA1 form a native HDL, which is thought to occur in the collecting duct and mesenteric lymphatics via the ATP-binding cassette, subfamily A, member 1 (ABCA1) (Green & Riley, 1981). It has been proposed that up to 30% of the whole plasma pool of HDL is derived from intestinal HDL biogenesis (Brunham et al., 2006). Interestingly, during conditions of insulin resistance and diabetes, over production of apoB particles by the intestine is thought to impact the secretion of intestinal-derived apoAI particles and is the focus of ongoing research in our laboratory (Mangat et al., 2018).

1.4.3 Absorption and reassembly of triglycerides

In the proximal intestinal lumen, dietary TG interact with bile acids and lipases to liberate FAs and the formation of monoacylglycerols (MAGs). The absorption of FAs and monoacylglycerols (MAGs) at the brush broader membrane is thought to occur with high-efficiency (more than 95%) (Pan & Hussain, 2012). Discrete transport proteins facilitate transportation of FAs and monoacylglycerols (MAGs) into the enterocyte (see **Figure** 1.1 for a summary). Transport proteins such as fatty acid-binding protein (FABP), fatty acid-transport protein 4 (FATP4) and fatty acid translocase/cluster determinant 36 (FAT/CD36) are thought to be primarily responsible FFA absorption (Nassir, Wilson, Han, Gross, & Abumrad, 2007; Stahl et al., 1999; Stremmel, 1988). Once absorbed, fatty acid-binding protein 2 (FABP2) facilitates the intracellular transport of long chain fatty acids (LCFAs) to the endoplasmic reticulum (ER) (Niot, Poirier, Tran, & Besnard, 2009). From the intracellular pool, the enzyme monoacylglycerol acyltransferase (MGAT) and diacylglycerol acyltransferase (DGAT) enzymes re-esterify and re-assemble MAGs and FAs into diacylglycerols (DAGs), respectively (Buhman et al., 2002; Grigor & Bell, 1982; Lukens et al., 2014; Mansbach & Siddiqi, 2010; Yen & Farese, 2003). Glucose can

be an alternate source of triglyceride produced via the glycerophosphate pathway (Pan & Hussain, 2012; Yang & Kuksis, 1991). Fatty acid-binding protein 1 (FABP1) assists the transportation of the molecules from the ER to the Golgi complex, where a pre-CM transfer vesicle is formed after addition of one apolipoprotein B48 (ApoB48) (Niot et al., 2009). It is also important to note that secretion of exogenous lipids via the CM pathway is usually constitutive, but not necessarily temporarily linear. For example, fats can also be shunted to be stored inside the enterocyte as neutral lipid droplets (Robertson et al., 2003) and can result in the delayed secretion of lipids from sequential ingested meals (Lambert & Parks, 2012).

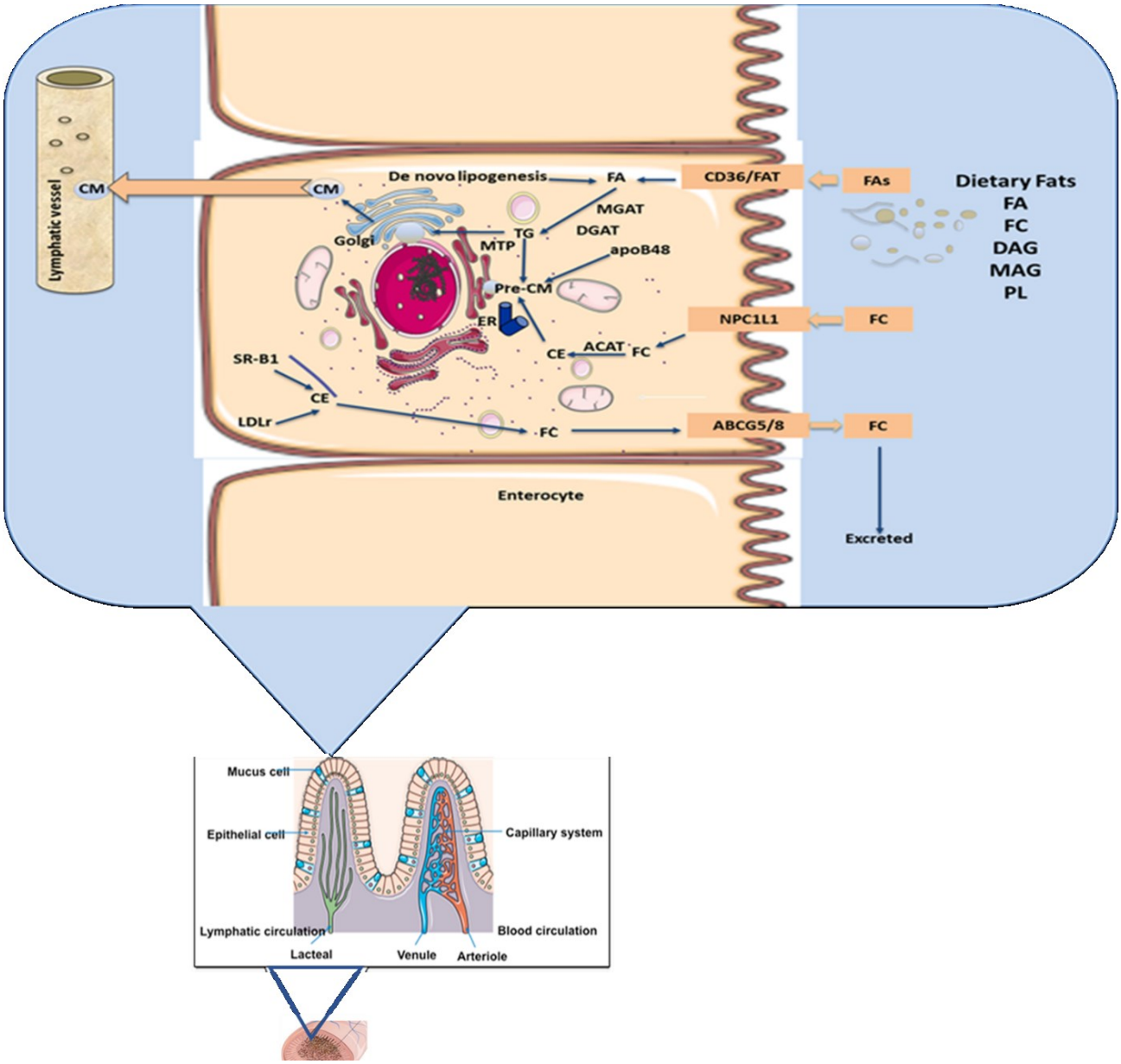


Figure 1.1 Dietary lipid processing in the intestinal enterocyte through absorption, assembly, and secretion of chylomicrons (CM).

CMs are synthesized in the enterocyte first in a pre-chylomicron formation step via MTP and addition of one apoB48, and then through lipidation of the lipoprotein core in the Golgi apparatus. CMs are then excreted into the lymphatic system. Adapted from Williams, 2008. This Figure was created in part using illustrations adapted from "Servier Medical Art" with permission. ABCG5/8, ATP-binding cassette transporter G5/G8; ACAT, acyl-coA:cholesterol acyltranferase; apo, apolipoprotein; CM, chylomicron; DAG, DGAT, diacylglycerol acyltransferase diacylglycerol; ER, endoplasmic reticulum; FA, fatty acids; FAT/CD36, fatty acid translocase/cluster determinant 36; FC, free cholesterol; LDLr, low-density lipoprotein receptor; MAG, monoacylglycerol; MGAT, monoacylglycerol acyltransferase; MTP, microsomal triglyceride transfer protein; NPC1L1, niemann-pick C1-like 1; PL, phospholipid; SR-B1, scavenger receptor class B member 1; TG, triglyceride

1.4.3.1 Chylomicron assembly

The synthesis of the pre-CM particle and later expansion of the core with TG are the main two stages of assembly of CMs. Formation of a pre-CM particle with TG, apolipoprotein AIV (ApoA IV), cholesterol, and PL is facilitated by triglyceride transfer protein (MTP) at the endoplasmic reticulum (Morel, 2003; Pan & Hussain, 2012). As described previously, the pre-CM requires a single apoB48 protein. The pre-CM reaches the golgi complex with the aid of FABP1. The pre-CM associates with apolipoprotein A1 (ApoA1) and is converted into a nascent CM to be transported out of the cell and into the lymphatic system (Mansbach & Siddiqi, 2010).

1.4.3.2 Chylomicron secretion

After production of nascent CMs, they move from the enterocyte into the lymphatic system by exocytosis (Dash et al., 2015). Through the thoracic duct, chylomicrons are delivered to the plasma. After reaching the circulation, CMs associate with additional apolipoproteins (apoC-II and apoE) to become more mature (Cohn, Marcoux, & Davignon, 1999). Once fully mature, the native CM particle rapidly populates density fractions with other hepatic-derived lipoproteins (making it difficult to differentiate between other lipoprotein fractions and assess biochemically). From a research standpoint, in order to overcome this problem one experimental approach is to collect native lymphatic CMs before they enter the circulation. Our research group has developed methods to sample lymph-derived native CMs in animal models. In smaller animal models such as rodents, lymph can be sampled acutely or collected chronically via the lymph fistulae procedure (Bollman, Cain, & Grindlay, 1948). For this thesis, I sought to take advantage of this lymph cannulation technique and assess the impact of a lipid-rich diet to gut microbiota during conditions of insulin resistance. More specifically, our interest was to understand whether a lipid rich diet might

facilitate gut bacteria infiltration (translocation) and/or gut bacterial products crossing the gut barrier to the circulation.

1.4.3.3 Chylomicron kinetics

In the circulation, hydrolysis of CMs occurs with the interaction of lipoprotein lipase (LPL) and hepatic lipase (HL). Lipoprotein lipase is present on the surface of endothelial cells and is catalyzed by the activity of apoCII (Willnow, 1997). During the hydrolysis step, CM donate both apoAI and apoCII to HDL. During this time CM become smaller and more dense and form cholesterol-rich CM-remnants (Dash et al., 2015). Chylomicron remnants interact with the LDL-Receptor (also known as the apoB100/E receptor) on hepatocytes and are cleared from circulation via receptor-mediated endocytosis (Hussain et al., 1991; Willnow, 1997).

1.4.3.4 Chylomicrons and their remnants in atherosclerosis and cardiovascular disease development

Cardiovascular disease and stroke are responsible for causing two-thirds of diabetic-related death (Chehade et al., 2013; Deshpande et al., 2008). There are many factors that increase the risk of CVD-related event in diabetes such as increase concentrations of low density lipoprotein (LDL) cholesterol, decrease high density lipoprotein (HDL) cholesterol, and elevated fasting plasma triglyceride (TG) (Battisti et al., 2003). After reaching the circulation, chylomicrons (CMs) quickly undergo hydrolysis to produce cholesterol-dense lipoprotein remnants which are taken up by the liver (Redgrave, 1983; van Beek et al., 1998). During the postprandial phase, there is an increased concentration of exogenous CMs, endogenously produced VLDL and their respective remnants. Under normal conditions, the liver quickly removes CM-remnants from the circulation (Cooper, 1997). However, during IR and T2D there is often a delay in the clearance of these remnant particles (Ginsberg & Tuck, 2001), which is known as postprandial dyslipidemia. Importantly,

during IR and diabetes, these independent risk factors increase the risk of CVD events (Mangat et al., 2011; Mero, Syvänne, & Taskinen, 1998; C. Phillips et al., 2000; Proctor, Vine, & Mamo, 2004). Both CMs and VLDL share the same metabolic pathway, for example, endothelium-bound lipoprotein lipase (LPL), hydrolyze both CMs and VLDL into glycerol and fatty acids. In the postprandial phase, the availability of LPL is limited, resulting in accumulation of triglyceride-rich lipoproteins (TRLs) (Klop, Proctor, Mamo, Botham, & Castro Cabezas, 2012).

The prolonged residence time of elevated TRL in the circulation increases the exchange rate of esterified cholesterol from HDL and LDL to TRL (Sposito et al., 2004). As a consequence, TG-enriched LDL particles can form. The hepatic lipase hydrolyses TG from TG-enriched LDL particles and are converted into smaller and more dense particles (Sposito et al., 2004; Weintraub et al., 1996). These smaller, more dense LDL and remnant lipoprotein particles can enter the subendothelial space and become entrapped inside the arterial wall. Later these LDL and remnant lipoprotein particles were taken up by macrophages and contribute to the development of atherogenic foam cells and promote atherosclerosis (Carmena, Duriez, & Fruchart, 2004; Twickler, Dallinga-Thie, Chapman, & Cohn, 2005).

1.5 Lipid metabolism and gut microbiota

1.5.1 Gut microbiota

Microbes are present in nearly every environment on earth, including deep below the earth's surface to miles overhead and are therefore adept at surviving diverse environments. Each environment harbors a unique population of microbes specifically adapted to their environment and together form an ecosystem. A community of microbes colonizing an environment is referred to as a microbiota. Microbes are present in different areas of the human body including the skin, mouth, nasal cavity and vaginal tract. All these microbial communities have been studied extensively, especially in the Human Microbiome Project. Through the Human Microbiome Project, a total 4788 specimen from 242 adults were collected from five body sites and sequenced by next generation sequencing methods, revealing that abundance and diversity of microbiota are widely varied both within and among individuals (Huttenhower & Human Microbiome Project, 2012). Inside the human intestine, microorganisms can have commensal and mutualistic relationships with their host and improve energy extraction from the diet and play a major role to maintain the intestinal immune system (J, 2010; Tremaroli & Bäckhed, 2012; Turnbaugh et al., 2006).

1.5.2 Spatial distribution of the human gut microbiota

Microbes inhabit the entire length of the gastrointestinal (GI) tract from the esophagus to the rectum and their density increases along the length of the intestine and reaches approximately 10^{11}-10^{12} cells per gram in the distal colon (Bäckhed, Ley, Sonnenburg, Peterson, & Gordon, 2005). The gut microbiota is mainly composed of bacteria from two major phyla: *Bacteroidetes* and *Firmicutes* but there are some less abundant phyla also present such as *Actinobacteria*, *Proteobacteria*, *Euryachaeota*, and *Verrucomicrobia* (Bäckhed et al., 2005; Eckburg et al., 2005).

Inside the GI tract gut bacteria number and diversity increase with an increase in pH from the stomach to distal colon. Among humans, there is variability between the total number of bacterial species. A study on 124 individuals estimated that there were over 1000 species in the cohort and each individual carried at least 160 species. This study also found a core of bacterial species that were common amongst all (18 species) or a majority (75 species) of individuals (Clemente, Ursell, Parfrey, & Knight, 2012).

1.5.3 Gut microbiota development during infancy

The microbiota is more similar between children and their parents as opposed to unrelated individuals (Yatsunenko et al., 2012). This indicates that common environmental exposures are important for shaping the gut microbiota community. Mode of delivery and whether the baby is breast or formula fed are important factors that determine the early colonization of the gut microbiota. This information is summarized in **Figure 1.2** which is adapted from articles published by Kumar M and Babaei P, 2016 (Kumar, Babaei, Ji, & Nielsen, 2016).

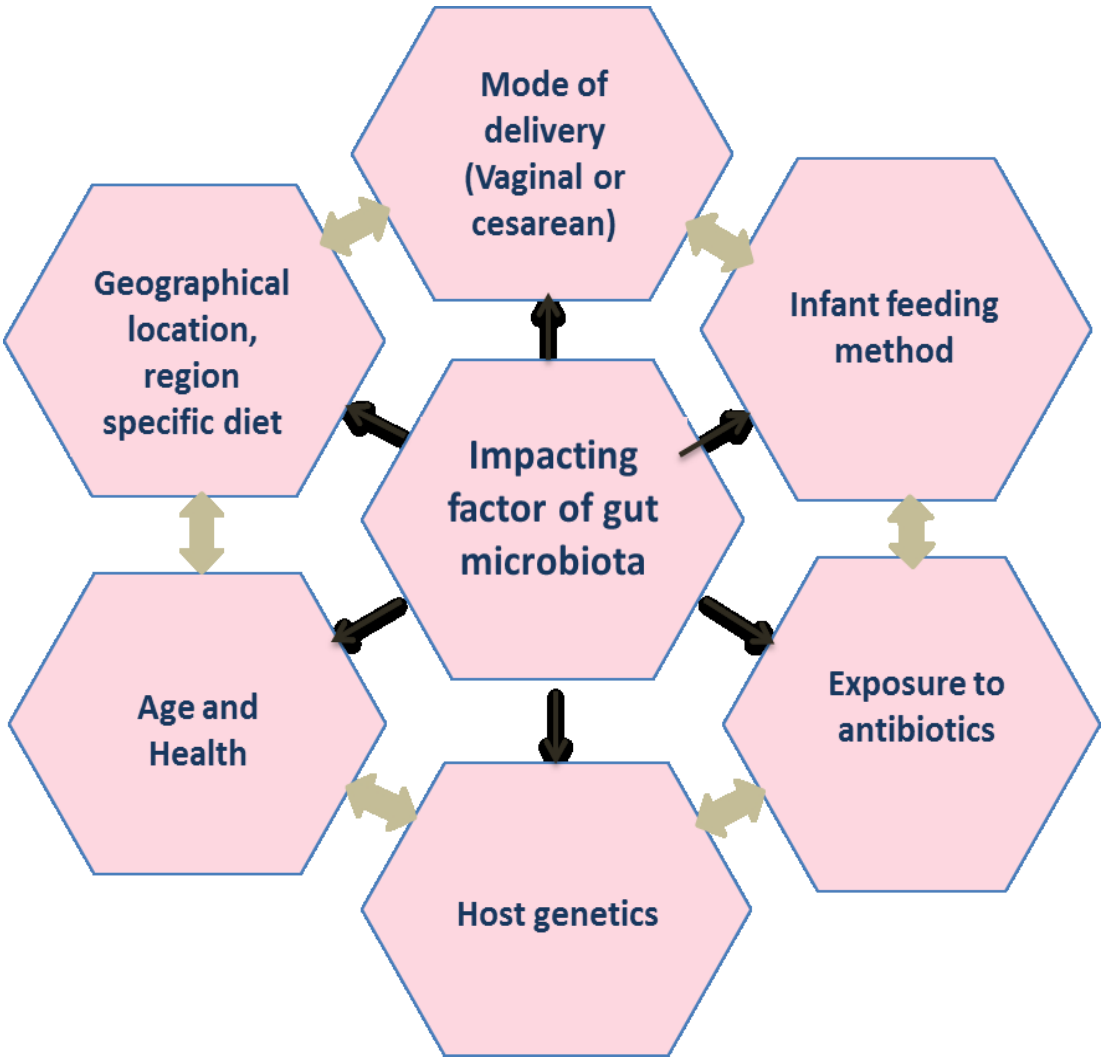


Figure 1.2 Factors regulating the composition of human gut microbiota
(Figure adapted from articles published by Kumar M and Babaei P, 2016 (Kumar et al., 2016)

1.5.4 First colonization of gut microbiota from the outside environment

Neonates are exposed to new microbes from the outside world during birth and the mode of delivery determines the colonization of the first batch of microbiota. The comparison of the microbiota of four vaginally born and six C-section born babies showed that the fecal microbiota of vaginally born differed from those of C-section born babies (Clemente et al., 2012). Vaginally born infants build their first intestinal microbiota from the vagina and include *Bacteroides*, *Bifidobacterium*, *Escherichia*/*Shigella*, and *Parabacteroides* (Bäckhed et al., 2015). Whereas neonates born by C-section acquire their first gut colonizers from their mothers' skin, and include *Enterobacter hormaechei*, *Haemophilus parainfluenzae*, *Enterococcus faecalis*, and *Streptococcus australis* (Clemente et al., 2012). Anotheer study showed that the vaginally delivered babies gain genera similar to vaginal microbiota such as *Lactobacillus* and *Prevotella*, however, skin surface bacteria for example *Staphylococcus*, *Corynebacterium*, and *Propionibacterium spp* were more predominant in the baby born with C-section babies' (Dominguez-Bello et al., 2010). These first colonizing bacteria can have a remarkable impact on the intestinal development and newborn health (Bäckhed et al., 2015).

1.5.5 Establishment of microbiota after birth

After birth, the first three years of the development are crucial for the establishment of intestinal microbiota and intestinal immune system. There are some main influential factors in these processes, which include feeding pattern (breastfeeding or formula-feeding), solid food introduction, and antibiotic treatment (Rodríguez et al., 2015; M. Tanaka & Nakayama, 2017). Feeding methods for the infant greatly affect the colonization of gut microbiota in early life. Human milk contains carbohydrate, fat, protein and immunoglobulins and endocannabinoids. Lactose is one of the main oligosaccharides in human milk (HMO). Human oligosaccharides act

as a prebiotic and stimulate the development of *Bifidobacterium* rich microbiota (Matsuki et al., 2016). One study observed that microbiota of breastfeeding infants are colonized by aerobic bacteria while formula-fed infants are colonized by anaerobic bacteria such as *Bacteroides* and *Clostridium* (Marcobal et al., 2011).

Antibiotics also have a profound effect on the development of gut microbiota in infants. Antibiotic use in early life can increase the *Proteobacteria* and decrease the *Actinobacteria* population (Guinane & Cotter, 2013; S. Tanaka et al., 2009). According to some epidemiological surveys, antibiotic exposure in early life has a great impact on the development of neonatal intestinal microbiotas and increases the risk of diseases such as asthma, atopic disease, eczema, and type 1 diabetes (Langdon, Crook, & Dantas, 2016). Besides antibiotics and breast feeding, the introduction of the solid food into the infant diet can also impact causing a shift from a *Bifidobacterium* dominant community to a *Bacteroidetes*- and *Firmicutes*-dominant community (Fallani et al., 2011; Palmer, Bik, DiGiulio, Relman, & Brown, 2007; Vallès et al., 2014). At approximately three years of age the child develops complex gut microbiota composition and diversity and becomes similar to that of an adult.

1.5.6 Other factors to regulated gut microbiota development

1.5.6.1 Gender

It has been reported in animals that gender also impacts the diversity and richness of gut microbiota (Markle et al., 2013; Yurkovetskiy et al., 2013). Human data exploring the effect of gender on gut microbiota has been inconclusive although some studies have showed that women have lower *Bacteroides* phylum than men. Interestingly, this prevalence has been confirmed in studies using rodents (Yurkovetskiy et al., 2013).

1.5.6.2 Environmental factors and lifestyle

The environmental factors and geographical location also have an influence on intestinal gut microbiota colonization. Studies have shown that infants from the northern area of Europe have higher *Bifidobacterium* spp. and fewer *Clostridium* spp., while infants from southern Europe have higher *Eubacteria*, *Lactobacillus*, and *Bacteroides* (Fallani et al., 2011). There are also significantly higher *Bifidobacterium* in Finnish and Swedish compared to German and Estonian infants (Grzeskowiak et al., 2012; Sepp et al., 1997). Another study showed that Malawi infants have higher *Bifidobacterium* spp, *Prevotella*, and *Clostridium histolyticum* than Finnish infants (Grzeskowiak et al., 2012). Furthermore, *Bacteroides* have been shown to be higher in infants from the rural village of Burkina Faso while in Italian children *Enterobacteriaceae* has been reported to be predominant (De Filippo et al., 2010).

1.5.7 Age and intestinal microbiota development:

During adulthood, the gut microbiota composition is more complex and stable. *Bacteroidetes* and *Firmicutes* phyla are more dominant in adults (Turnbaugh et al., 2006) and three different enterotypes have been described, including *Prevotella*, *Ruminococcus*, and *Bacteroides* enterotypes (Arumugam et al., 2011; Benson et al., 2010). In contrast, we know that the elderly have a significant difference in gut microbiota compared to earlier lifecycle stages (Arumugam et al., 2011). Characterization of gut microbiota in 141 elderly Irish subjects found variation in *Firmicutes* from 8 to 80% and 14 to 92% in *Bacteroidetes* (Claesson et al., 2011).

1.5.8 Diet and intestinal microbiota

The intestinal microbiota can increase the availability of energy and vitamins for intestinal absorption (Laureys & De Vuyst, 2014; G. D. Wu et al., 2011). The gut microbiota can be rapidly and reproducibly shaped by diet. For example, in mouse studies, consumption of a high fat diet (HFD) results in changes to gut microbiota (Walker et al., 2014). In western countries,

epidemiological studies show that inadequate exposure to food and microbial metabolites increased the risk of immune-mediated diseases (Lukens et al., 2014; Thorburn, Macia, & Mackay, 2014). Dietary fiber is a key substrate for intestinal microbes and results in the production of short-chain fatty acids (SCFA), such as acetate, propionate, and butyrate. These SCFA play an important role in maintaining intestinal homeostasis and immunity (Thorburn et al., 2014). SCFA are an important source of energy for the host, but also act as signaling molecules via G-protein coupled receptor (GPR) activation and regulate lipid and glucose metabolism **(summarized in Figure 1.3).** SCFA activate G-protein coupled receptors 43 (GPR43) and G-protein coupled receptors 41 (GPR41) and increase secretion of peptide YY (PYY) and glucagon-like peptide-1 (GLP-1) (Tolhurst et al., 2012). The increased secretion of GLP1 regulates energy homeostasis in tissues, facilitates insulin secretion by pancreatic-B cells, and alters gastric motility (MacDonald et al., 2002). GPR41 and GPR43 activation also suppress insulin signaling in adipose tissues and reduce fat accumulation (Ang & Ding, 2016). Thus, activation of GPR by SCFA play a major role to regulate fat accumulation and glucose homeostasis.

While studying gut microbiota during obesity and IR, *Bacteroidetes* and *Firmicutes* are the two most abundant phyla which received the most attention (R. Ley, P. Turnbaugh, S. Klein, & J. Gordon, 2006). Analysis of gut microbiota between lean and obese twin pair showed that the obese individual had decreased *Bacteroidetes* and impaired expression of genes involved in lipid metabolism (Turnbaugh & Gordon). There are many studies that have reported that intestinal microbiota play key role in adiposity and affect host metabolism by controlling energy extraction and lipid metabolism (K. Harris, Kassis, Major, & Chou, 2012; R. Ley et al., 2006).

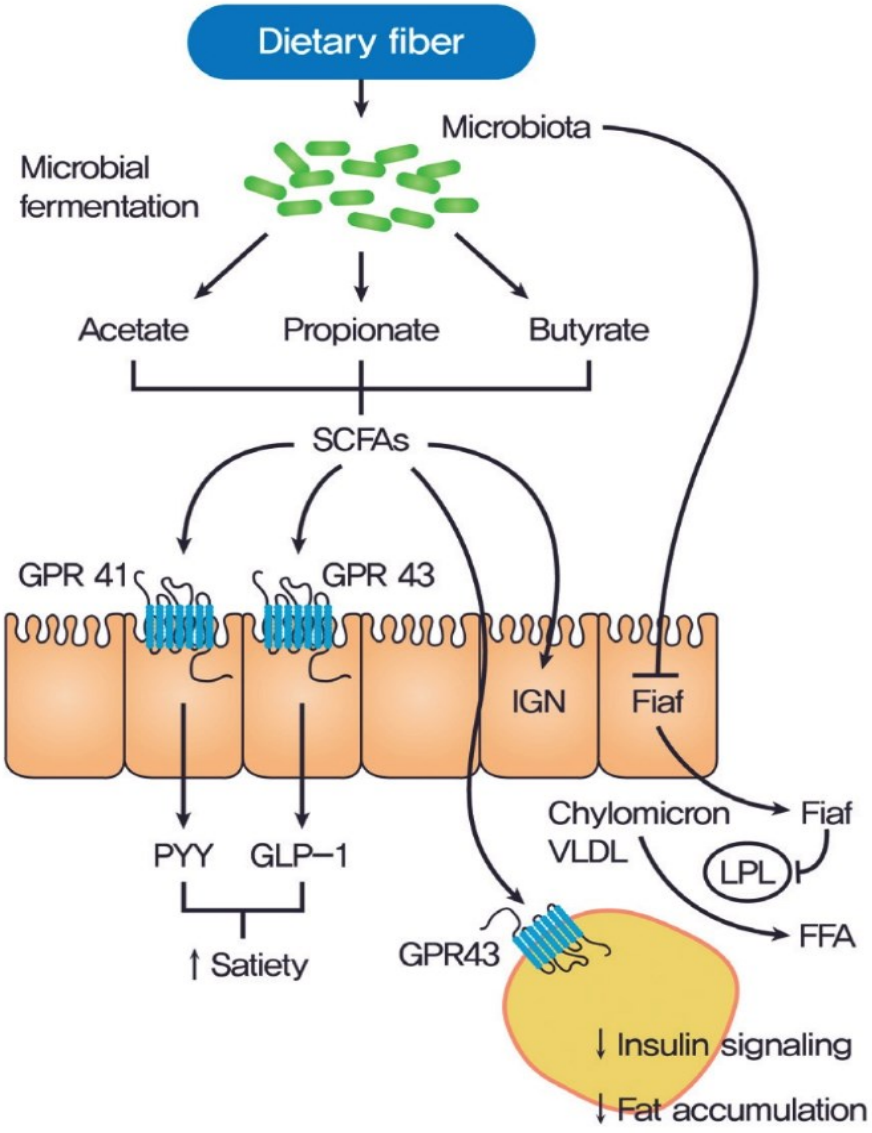


Figure 1.3 Gut microbiota regulation of host metabolism.
Bacterial glycoside hydrolases cleave complex carbohydrates into short-chain fatty acids (SCFAs) such as acetate, propionate, and butyrate. SCFA-dependent activation of G-protein coupled receptor 41 (Gpr41) induces the expression of peptide YY, an intestinal hormone that inhibits gut motility, increases intestinal transit rate, and reduces the harvest of energy from the diet. Engagement of G protein-coupled receptor 43 (Gpr43; and Gpr41) by SCFAs has been shown to trigger the incretin hormone glucagon-like peptide 1 (GLP-1) to increase insulin sensitivity. Gut microbiota efficiently suppresses fasting-induced adipose factor (Fiaf) expression in the ileum, which inhibits lipoprotein lipase (LPL) activity and fat storage in white adipose tissue. SCFAs mediated activation of GPR43 results in suppression of insulin signaling in the adipose tissue and subsequent prevention of fat accumulation. SCFAs also activate intestinal gluconeogenesis (IGN) via a gut-brain neural circuit, which can improve glucose metabolism and reduce food intake. VLDL, very low-density lipoprotein; FFA, free fatty acid. (adapted from articles published by Kyu yeon et al (2015)

1.6 High Fat Diets (HFD) and microbiota

Consumption of high dietary fats is a recognized factor that can induce an alteration in gut bacteria. Higher fat diets decreases *Bacteroidetes* phyla and increase *Firmicutes* phyla in the gut and this change is closely related to the development of obesity (Turnbaugh, Bäckhed, Fulton, & Gordon, 2008). Furthermore, it has been demonstrated that germ-free lean mice have increased total body fat after colonization with an obese mouse gut microbiota (R. Ley et al., 2006). Lipid-rich diets can alter the composition of gut microbial community by changing competition for fermentable substrates or indirectly by altering bile acid composition and secretion. Transportation, emulsification, absorption, and oxidation of dietary fats can be regulated by intestinal microbiota (Kübeck et al., 2016; Martinez-Guryn et al., 2018; Semova et al., 2012). Typically, HFD studies do not usually consider fat quality, however, fatty acids distinctly affect host physiology and as a result the microbial community. n-6 PUFA changes the gut microbial composition and can exacerbate colitis in mice. Whereas ingestion of a n-3 polyunsaturated fat PUFA rich diet increased adhesion of *Bifidobacteria* to the intestine and increases *Bifidobacterium* spp. (Ghosh, DeCoffe, et al., 2013; Ghosh, Molcan, DeCoffe, Dai, & Gibson, 2013). Diets high in saturated fat (such as palm oil), increase total SCFA concentration compared to monounsaturated (such as olive oil), or polyunsaturated fats (such as safflower or flax/fish oil) (de Wit et al., 2012; Patterson et al., 2014). The understanding of a high fat dietary intervention on gut microbiota in human studies is limited. A clinical trial by Brinkworth et. al showed that high fat low carbohydrate diet decreases the fecal SCFA concentration and *Bifidobacterium* spp compared to a high-carbohydrate, high-fiber, low-fat diet in overweight/obese individuals (Brinkworth, Noakes, Buckley, Keogh, & Clifton, 2009). While many studies have shown that altered types of fat can affect intestinal gut microbiota differently, the full characterization of fat induced changes in gut

microbiota is not fully understood. An overview of the effect of HFD on gut microbiota is shown in **Figure 1.4**.

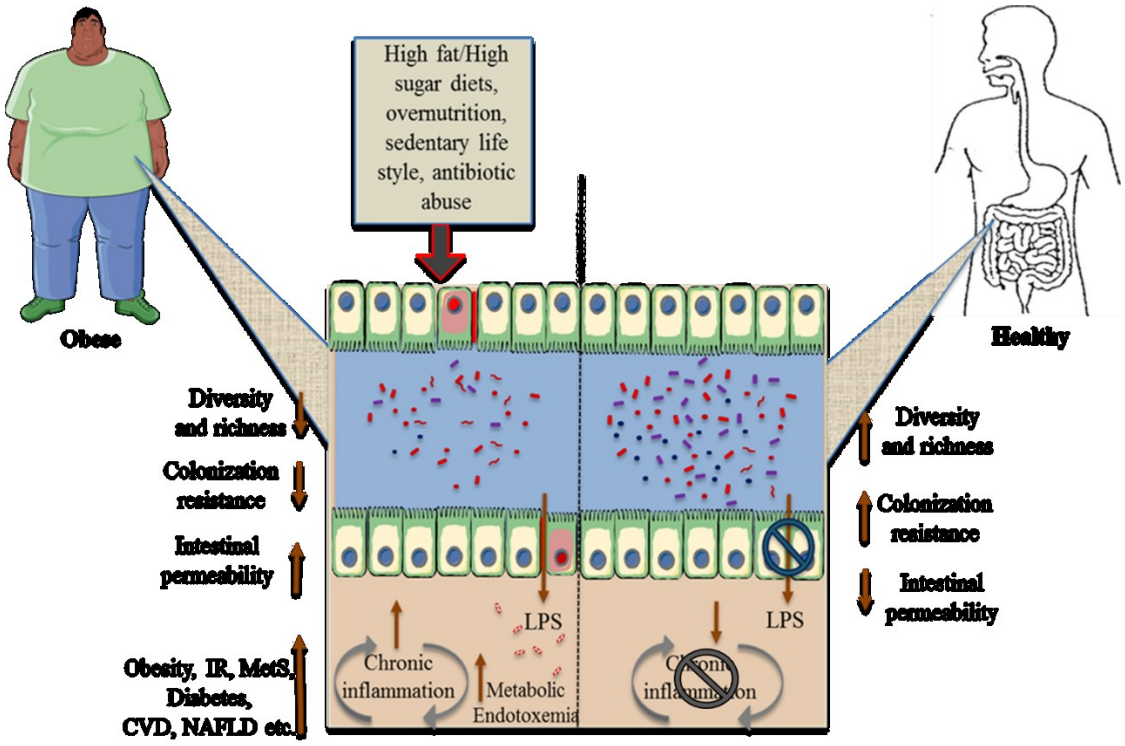


Figure 1.4 Effect of high fat diet (HFD) and on gut microbiota and intestinal environment.
A HFD induces gut microbial alteration (dysbiosis), As a result gut permeability increase, and the expression of tight junction protein reduced, such as zonal occludes (ZO)-1 and occludin, in the intestinal epithelial cells. It facilitates the passage of bacterial product in portal blood circulation and contribute to the pathogenesis of obesity, insulin resistance, and type 2 diabetes mellitus.This figure was created in part using illustrations adapted from "Servier Medical Art" with permission.

1.6.1 The effect of gut microbiota on human health

Interactions between the host and microbiota are largely mutualistic. The host facilitate the growth of gut microbiota by providing an anaerobic, homothermal and nutritious intestinal environment. While the gut microbiota helps to improve metabolism, energy harvest, produce vitamins and the essential compounds from ingested food (Donohoe et al., 2011). The gut microbiota can be both beneficial and deleterious to their host. For example, bacteria convert choline into TMAO, which may increase the chance of CVD and non-alcoholic fatty liver disease (NAFLD) (Tremaroli & Bäckhed, 2012). The gut microbiota also help shape the host immune system and maintain mucus layer thickness resulting in reduced direct contact and entry of pathogenic microorganisms (Cornick, Tawiah, & Chadee, 2015; Desai et al., 2016; Hansson, 2012; Littman & Pamer, 2011). The intestinal microbiota are also impact several diseases such as mental health (autism and anxiety neurosis), inflammatory bowel disease (IBD) as well as autoimmune disease such as rheumatoid arthritis (RA) (Chervonsky, 2013; Grenham, Clarke, Cryan, & Dinan, 2011; Vázquez-Castellanos et al., 2015; Wang et al., 2014).

1.6.2 Gut microbiota during obesity and insulin resistance

The role of the intestinal microbiota in obesity and IR has been shown in many animal model studies including pigs, rodents and zebra fish. A series of experiments in germ-free (GF) mice showed that an increase in adiposity after transplantation of gut microbiota compared to conventionally raised mice. After eight-weeks of HFD intake, conventional mice showed increased monosaccharide uptake from the intestine and increased fatty acid oxidation in muscles compared to GF mice (Bäckhed et al., 2004; Bäckhed, Manchester, Semenkovich, & Gordon, 2007). Moreover, the presence of gut microbiota reduced expression of fasting-induced adipose factor (FIAF) in the intestine, resulting in an increased activity of lipoprotein lipase in adipose tissue

(Mandard et al., 2006). In humans, obese individuals can have lower gene richness of intestinal microbiota compared to lean individuals and this has been correlated to a higher risk of obesity, IR and inflammation (Le Chatelier et al., 2013).

1.6.3 Lipid metabolism and gut microbiota

During obesity, intestinal microbiota affects lipid metabolism. The gut microbiota help to increase lipid digestion and modify bile salts that facilitate fatty acid emulsification and absorption (Kosa & Ragauskas, 2011). In rodent studies, intestinal microbiota affects expression of genes including fatty acid activation (Fatp4), phospholipid transfer protein – (Pltp), TG re-esterification (Dgat1 and 2), fatty acid and phospholipid binding (Fabp2,) and CM formation (Apob precursor, MTP) (Yu, Li, Huang, Yao, & Shen, 2018). Presence of selective gut microbiota also result in the downregulation of a majority of genes involved in lipid metabolism, while many of the adaptive immunity genes can be upregulated. *Shulzhenko et al* showed a similar trend of microbial transcriptional regulation of host genes involved in intestinal steroid, lipid, cholesterol metabolic processes as well as lipid transport in immune-deficient mice (Shulzhenko et al., 2011). Metabolomic studies by *Velagapudi et. al* showed that lipidomes of adipose tissue, serum and liver tissue (TG, FFA, and cholesterol) were different in germ-free versus conventionally raised mice. In conventionally raised mice, TG in adipose and liver were higher but in serum, TG, FFA and cholesterol were lower than GF mice (Velagapudi et al., 2010), which indicate higher lipid clearance and storage. Interestingly, after 4 hours of fasting, circulating concentrations of CM-TG in serum was reduced in conventionally raised mice, suggesting increased clearance. The conventionally raised mice produced greater VLDL TG secretion, the net concentration of plasma VLDL and HDL cholesterol did not differ compared to GF mice (consistent with increased clearance of lipid from the circulation). Despite these data, others have reported alteration of lipid

profiles may result as a consequence of changes to intestinal microbiota (Allayee, 2015). Consequently, the exact mechanism whereby gut microbiota can regulate intestinal lipid metabolism is not complete and forms part of the rationale for this this thesis.

1.6.4 Metabolic inflammation and intestinal microbiota

Over the last twenty years, studies have shown that chronic, low-grade inflammation contributes to metabolic diseases such as obesity, diabetes, non-alcoholic steatohepatitis (NASH), and CVD.

1.6.4.1 Metabolic endotoxemia

During obesity, there is an increase in adipose tissue, elevated TG and inflammation of metabolic tissues. There is a strong link between weight gain, lipid metabolism and endotoxemia in T2D (Creely et al., 2007). Bacterial lipopolysaccharides (LPS), also known as endotoxin (a cell wall component from gram-negative bacteria), has been shown to be one of the major factors causing inflammation during obesity. A conserved lipid A domain activates inflammation through Toll-like receptor 4 (TLR4) expressed on the cell membrane of immune cells, epithelial cells and adipocytes (Devaraj et al., 2008; Frantz et al., 1999; Ioannidis, Ye, McNally, Willette, & Flaño, 2013). In healthy individuals, LPS concentration is usually low in circulation, but studies have shown that serum LPS level are elevated in obesity and diet induced obese animals (Cani et al., 2007; Pussinen, Havulinna, Lehto, Sundvall, & Salomaa, 2011). In particular, HFD consumption can increase endotoxin absorption in the intestinal epithelium and alter the concentration of endotoxin in circulation (Cani et al., 2010). Interestingly, Erridge et. al showed that consumption of high fat meal can increase plasma endotoxin concentrations within 30 minutes (Erridge, Attina, Spickett, & Webb, 2007). Obesity and IR can be induced with chronic systemic LPS infusion without feeding HFD. This low-level but chronic increase in LPS concentration in blood plasma

is known as “metabolic endotoxemia”(Neves, Coelho, Couto, Leite-Moreira, & Roncon-Albuquerque, 2013). Metabolic endotoxemia is associated with increased pro-inflammatory cytokines such as IL-6, TNF-α, and adipokines. Adipokines in plasma can in turn disrupt intestinal permeability (Cani et al., 2008). There are three different mechanisms by which endotoxin reaches the blood: (1) co-absorption of endotoxin by the intestinal mucosa during lipid absorption, (2) passive diffusion via paracellular passage through the impaired intestinal barrier, and (3) translocation to peripheral tissue via intestinal mucosal barrier.

1.6.5 Bacterial translocation (BT)

Bacterial translocation is defined as ‘the migration of live bacteria across the intestinal epithelium to the periphery such as mesenteric lymph nodes (MLN) and distant extra-intestinal sites’ (Berg & Garlington, 1979). Select aerobic and anaerobic gut bacteria have show more frequent translocation including: *Clostridium*, *Enterococcus* and *Enterobacteriaceae* (Laffineur et al., 1992). In the host, translocated bacteria can induce inflammation as well as sepsis (Roy & Gatt, 2012). Bacterial translocation also occurs in healthy individuals, without any negative consequences. During healthy conditions, bacterial translocation is hindered by phagocytes (Balzan, de Almeida Quadros, de Cleva, Zilberstein, & Cecconello, 2007). The translocation of bacteria is dependent on the physiological condition and bacterial species. The intestinal bacteria can translocate through paracellular (between cells) and/or transcellular (through cells) routes (Kalischuk, Inglis, & Buret, 2009). Normally, paracellular translocation is controlled by tight junction (TJ) complex, but disruption in intestinal permeability or hyper-permeability facilitate paracellular bacterial translocation**.** For transcellular bacterial translocation, both endocytosis and intracellular trafficking facilitate bacterial entry (Balzan et al., 2007; MacFie, 2004).

1.6.5.1 Factors responsible for an increase in the bacterial translocation

There are three main factors that promote bacterial translocation (a) an alternation in normal gut microbiota, (b) gut mucosal barrier physical disruption, (c) impaired host defense. (O'Boyle et al., 1998).

1.6.5.2 Alternation of normal gut microbiota or bacterial overgrowth

One of the major factors that is thought to facilitate bacterial translocation is alternation in intestinal microbial population that results in bacterial overgrowth (O'Boyle et al., 1998). There are normally low numbers of bacteria that inhabit the stomach, duodenum, jejunum, and proximal-ileum relative to the colon. For some situations, bacterial overgrowth can exacerbate lower gastric acidity, decrease peristalsis movement and damage the mucosal membrane. Bacterial overgrowth allows pathogenic bacteria to adhere to the epithelial wall of intestine facilitating penetration through the gut and entry into the circulation and distant organs (Stechmiller, Treloar, & Allen, 1997). Both obligate anaerobes and facultative anaerobes translocate at the same rate from the gut, but facultative anaerobes are better able to persist due to the presence of oxygen (O'Boyle et al., 1998). Antibiotic therapy can also induce bacterial overgrowth or generate a disturbed bacterial community. Antibiotic use can increase the growth of opportunistic pathogens such as Enterobacteriaceae, Enterococci, and Clostridium and facilitate bacterial translocation (Deitch, Maejima, & Berg, 1985). Bacterial overgrowth and altered small bowel motility has also been reported during obesity. Obese patients displayed a 41% higher prevalence of bacterial over growth (Mushref & Srinivasan, 2013; Xing & Chen, 2004). A randomized controlled study of 44 gastric bypass patients, showed a strong association between bacterial overgrowth and obesity, where administration of a probiotic significantly improved bacterial overgrowth, weight loss and inflammation (Woodard et al., 2009).

1.6.5.3 Gut mucosal barrier physical disruption

The other major factor influencing bacterial translocation is physical disruption of gut mucosal barrier, which leads to increased intestinal permeability. Bischoff et al showed that the intestinal barrier can be disrupted by many factors such as trauma, critical illness, severe malnutrition, jaundice, burns, shock, immunosuppression and intra-abdominal surgery (Bischoff et al., 2014; Cheng et al., 2013)**.** Though it is difficult to measure bacterial translocation, increased gut permeability can be measured by D-lactate in patients who are undergoing intraperitoneal operations**.** D-lactate is produced by gut bacteria but cannot be metabolized by mammals therefore it remains in plasma. After abdominal surgery, 19% of patients had higher endotoxin and D-lactate in plasma (Pessione, 2012)**.** The disruption to intestinal permeability can facilitate the passive movement of bacteria via the pathway of large molecules (O'Boyle et al., 1998).

1.6.5.4 Impaired host defense

Impaired host defense is another important factor that enhances bacterial translocation. According to Centers for Disease Control and Prevention (CDC) immunodeficiency is a state in which are individual's immune system is weakened or absent. Failure in defense mechanisms facilitates gut bacteria to moves to the extra intestinal sites (O'Boyle et al., 1998). A decrease in macrophages or T cells and lower production of immunoglobulin has been correlated to the bacterial translocation (Gautreaux, Deitch, & Berg, 1994).

1.6.6 Impaired hepatic lipid metabolism during insulin resistance

Impaired lipid metabolism is a major contributor to metabolic complications such as obesity and insulin resistance. As mentioned previously, conditions of IR and obesity result in the release of FAs (Ebbert & Jensen, 2013). Accumulation of lipid in the liver is described as a non-alcoholic fatty liver disease (NAFLD). NAFLD is a marker of hepatic IR and closely correlated

with T2D and MetS (Paschos & Paletas, 2009). Obesity and IR further exacerbate dyslipidemia by upregulation of hepatic VLDL production and secretion (Paschos & Paletas, 2009).

1.6.7 Impaired intestinal lipid metabolism during insulin resistance

Insulin resistant, obese and/or T2D patients display high fasting concentrations of plasma TG-rich CMs (Fielding et al., 1996; Xiao & Lewis, 2012). The main cause for this observation is thought to be altered CM production and/or secretion as well as impaired particle clearance. However, the exact mechanisms have not been extensively studied. Recent reports have suggested this phenomenon is multifactorial. *Adeli et. al* have demonstrated that TNFα induces inflammation in the intestine and promote IR (Adeli & Lewis, 2008). Additionally, CM assembly and secretion genes, such as CD36, MTP, MGAT, DGAT and apoAIV have increased expression in enterocytes during a state of IR (Hsieh, Hayashi, Webb, & Adeli, 2008; Vine, Glimm, & Proctor, 2008). Under normal conditions, insulin suppresses production of apoB48-containing CM particles in the intestine. However, with excessive adiposity, this suppression is diminished, due to high level of FFAs in circulation (Federico, Naples, Taylor, & Adeli, 2006; Lewis, Uffelman, Szeto, Weller, & Steiner, 1995; Pavlic, Xiao, Szeto, Patterson, & Lewis, 2010). Delayed CM clearance can increase the amount of lipid in circulation and this can ultimately be stored as lipid droplets in peripheral tissues including the arterial wall (Karpe, 1999).

1.7 Effect of diet during insulin resistance

To study obesity and early aberrations of insulin resistance, animal models often require dietary induction. Higher dietary fat intake (high-fat diet) is used on a regular basis to induce conditions of over-nutrition, obesity and early insulin resistance. One of the challenges using this approach is the variability in the quality and quantity of lipid and macronutrient composition (Buettner, Schoelmerrich, & Bollheimer, 2007). A ‘western-type diet’ is also a common approach

that consists of poor quality carbohydrates (excessive quantity and/or higher amounts of fructose), in combination with higher proportions of saturated and trans fatty acids (Forbes et al., 2013). The following section will provide some background on a number of factors that justify the use of certain dietary induced models.

1.7.1 Effect of dietary carbohydrate on insulin resistance and dyslipidemia

Historically, the literature has demonstrated that a reduction in dietary fat intake (caloric restriction) may prevent obesity and associated insulin resistance. Conversely, other studies have shown that diets with higher proportions of carbohydrate (for example fructose) may also induce fasting hypertriglyceridemia and increase the development of insulin resistance. We know that glucose intake stimulates insulin production and can provide subsequent biochemical inhibitory feedback, whereas fructose does not (Schaefer et al., 2009). Short-term feeding of fructose reduces plasma glucose levels and show apparent benefits in terms of insulin sensitivity. However, chronic intake of fructose dysregulates the glycolytic pathway. In the liver, fructose enters the glycolytic pathway as a glyceraldehyde, which down stream of the phosphofructokinase step (Basciano, Federico, & Adeli, 2005). The impact of this results in increased TG synthesis, de novo lipogenesis and dyslipidemia (Mayes, 1993). Studies using the hamster model have reproducibility that a diet high in fructose increases lipogenesis, dyslipidemia and insulin resistance (Haidari et al., 2002). The hamster model has also revealed a number of mechanisms that explain overproduction of intestinal TG and apoB48 post-prandially (Schaefer et al., 2009). For the above stated reasons, a diet with higher proportions of fructose in combination with higher dietary fat appear to be very effective at inducing insulin resitance (at least in animal models) (Rutledge & Adeli, 2007).

1.7.2 Effect of dietary fat on insulin resistance and dyslipidemia

High-fat diet (HFD) is known to modulate the intestinal absorption of various nutrients (Farvid et al., 2014; Rivellese et al., 2003). Dietary fat is typically absorbed with a high-efficiency, while cholesterol has a much wider physiological range (absorbed only 30-80%) (Bosner, Lange, Stenson, & Ostlund, 1999). After consumption of a fatty meal, CMs are secreted and accumulate in circulation, however the composition and source of meal fatty acids can also affect postprandial lipid metabolism (Ooi, Ng, Watts, & Barrett, 2013). Fatty acids (FAs) are generally classified as either saturated (SFA), monounsaturated (MUFA) or polyunsaturated (PUFA) (Vannice & Rasmussen, 2014).

1.7.3 Effects of unsaturated fatty acid on lipid metabolism

Nomenclature for unsaturated FAs is premised on the location of the double bond on the FA backbone (,e.g. n-3) (Vannice & Rasmussen, 2014). Eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and α-linoleic acid (ALA) are examples of n-3 series, of FAs. The n-3 series of FAs are often considered essential as they are not produced endogenously in sufficient quantities by humans to maintain health and must be consumed through diet. The main source of EPA and DHA are sardines, salmon, and herring, while flaxseed oil canola and leafy green plants also have higher quantity of EPA (Vannice & Rasmussen, 2014). Other PUFA series such as n-6 and n-9s are also very bioactive and considered essential. Vegetable oil is a rich source of n-6 series including linoleic acid (LA) and arachidonic acid (AA). Rich sources of the n-9 series include avocado and nut oils (Vannice & Rasmussen, 2014). Importantly, the intake of diets rich in n-3 PUFA (EPA, DHA and ALA) have been show to decrease plasma TG through inhibition of hepatic VLDL and TG secretion (Nestel et al., 1984; Sanders & Hochland, 1983; Schectman, Kaul, & Kissebah, 1989; Wijendran & Hayes, 2004). The supplementation of dietary n-3 PUFA

improves fasting and postprandial lipid profiles by downregulating PPAR and c-Jun pathways in the intestine (Hassanali, Ametaj, Field, Proctor, & Vine, 2010).

1.7.4 Effects of saturated fatty acid and industrial trans-fat on lipid metabolism

Saturated and *trans* fats are often obtained from farm animal-derived lipids such as lard and butter but can also be found in some vegetable, cocoa and coconut oils collected through natural or hydrogenation processes (Vannice & Rasmussen, 2014). According to Canada's food guide high intakes of saturated fat should be minimized (Government of Canada, Health Canada). Saturated FAs such lauric, myristic and palmitic acids can increase cholesterol levels in circulation by inhibiting LDL clearance (Grundy & Denke, 1990). Industrially produced *trans* fats (TFAs) or partially hydrogenated vegetable oil (PHVOs), have been shown to substantially increase both fasting TG (by a CETP-mediated pathway), and increase LDL as well as lower HDL (Sun et al., 2007; van Tol, Zock, van Gent, Scheek, & Katan, 1995).

1.7.5 Effects of ruminant-derived *trans* fats on lipid metabolism

Trans Fatty acids found in primary produce (meat and dairy from ruminant animals) may have very different bioactivity compared to that described for industrial produced *trans* fatty acids (from partially hydrogenated vegetable oil processing. Vaccenic acid (18:1 *trans 11*) , is a monounsaturated FA with a double bound in the *trans* position that is highly abundant in foods derived from ruminant animals (e.g. dairy products). The vaccenic acid (VA) may have health benefits associated with activating peroxisome proliferator-activated receptor (PPAR) pathways (Jacome-Sosa et al., 2016). Supplementation of VA in pre-clinical rodent models was shown to improve lipid profiles and beneficially impact pathways in both the liver and intestine (Gebauer et al., 2011; Kim et al., 2012; Y. Wang et al., 2009). It is hypothesized that VA activates peroxisome proliferator-activated receptor alpha (PPARα), which is present in high abundance in liver,

intestine, muscle and heart tissues. PPARα works as a regulator of lipid oxidation (Yu, Correll, & Vanden Heuvel, 2002) and increased expression of CD36 as well as increase β-oxidation, resulting in increased FA uptake. For activation of this process peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) work in coordination and activate genes involved in the citric acid cycle and regulate transcription of genes involved in oxidative phosphorylation. Studies have shown that ingestion of diet in a high fat may reduce expression of PGC-1α, as a result, incomplete β-oxidation and leads to increase in IR (Nagatomo et al., 2012). However, the addition of VA in the diet can potentially counter the effect of high-fat diet and improve insulin sensitivity.

1.7.6 Experimental models of impaired intestinal lipid metabolism during IR

Animal models are a critical pre-clinical tool that enable a better physiological and mechanistic understanding of human diseases, including obesity and insulin resistance (**summarized in Figure 1.5).** Even though cell-based applications are constantly improving and being refined, it is also vital to carefully assess the metabolic state and physiology (Kleinert et al., 2018).

Small animal models are often the first choice due to the ability to be manipulated genetically, short reproductive cycle, sexual maturity within 4–8 weeks of birth and can be surgically and/or monitored under controlled conditions. Small animal models are used regularly to test the bioactivity of agents or nutrients. All these characteristics make rodents an economical choice for researchers. In **Table 1.2**, I combined key aspects and considerations for performing and analyzing rodent studies under the umbrella of obesity and diabetes research.

A wide range of animal models with their distinct advantage and disadvantage is used in obesity and type 2 diabetes mellitus (T2DM) research. The non-mammalian models, such as (*Zebrafish*, *Caenorhabditis elegans* and *Drosophila*) have the advantage of easy availability of

diverse gene editing, short life and low maintenance cost. However, due to their distinct anatomy and physiology, their translational value is limited. By contrast, the large animal model such as pigs, dogs and especially non-human primates have physiology that closely resembles humans. In contrast these models have high maintenance costs and long-life cycle (Kleinert et al., 2018).

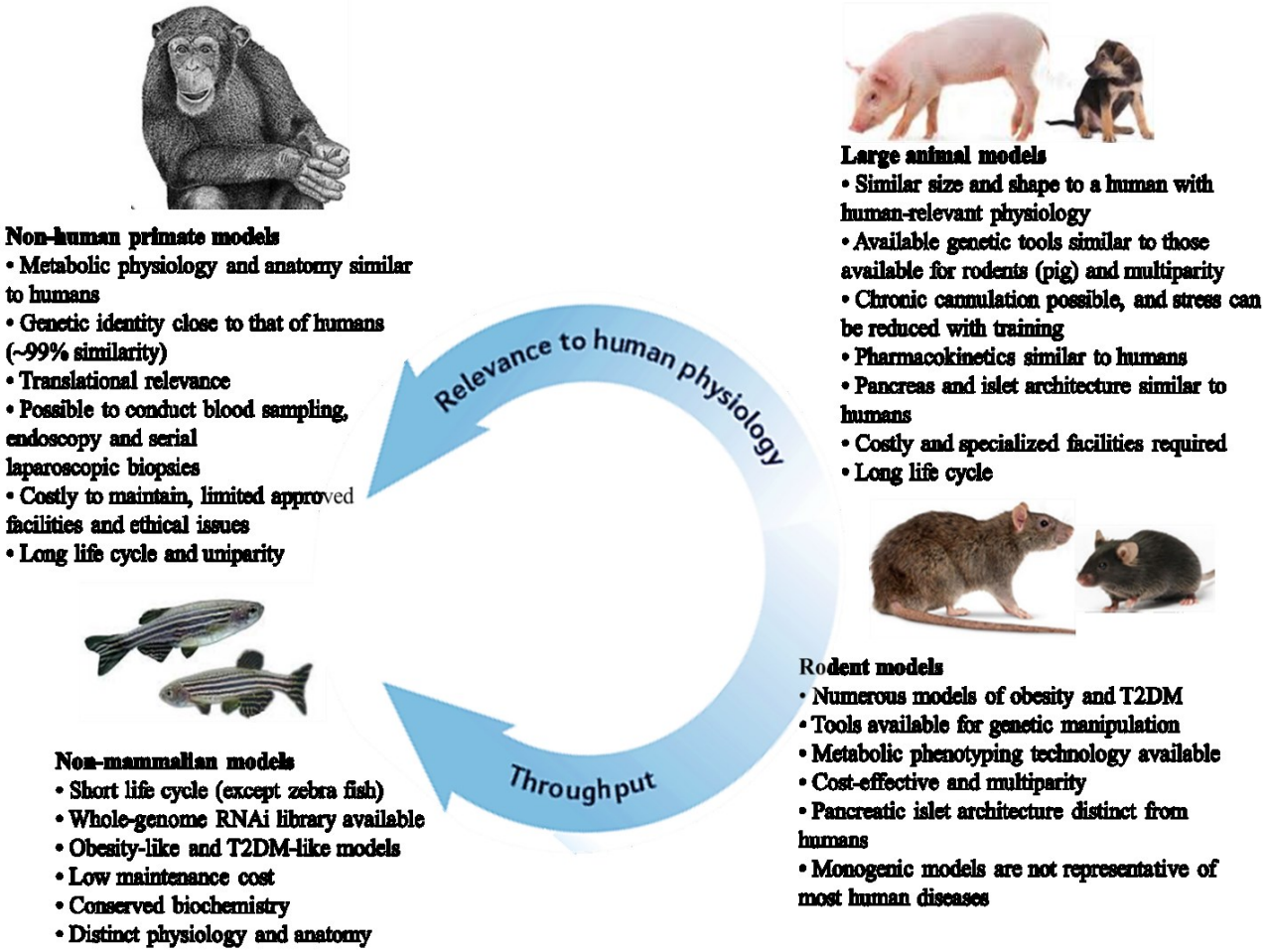


Figure 1.5 Advantages and disadvantages of different classes of animal models used in obesity and diabetes research (adapted from(Kleinert et al., 2018).

Table 1.2. List of selected rodent models potentially useful in obesity, insulin resistance and type 2 diabetes mellitus research

.

Strain or method	Diet	IR	Hyperglycemia	Obesity	T2DM	Dyslipidemia	Pathologic islet changes
Polygenic							
C57BL/6J mice	HFD	Y	N	Y	N	Y	N
SWR/J mice	HFD	N	N	N	N	Y	N
A/J mice	HFD	N	N	Y	N	Y	N
C3H/HeJ mice	HFD	Y	N	Y	Y	Y	N
DBA/2J mice	HFD	Y	N	Y	N	N	N
NZO mice	CHO	Y+	Y	Y+	Y	Y	Y
TALLYHO/Jng mice	SD, HFD	Y+	Y	Y	Y	Y+	Y
DIO-sensitive Sprague dawley rat	HFD	Y	N	Y	N	Y	N
DR Sprague dawley	HFD	N	N	N	N	N	N
UCD-T2DM rat	SD, HFD	Y+	Y	Y	Y+	Y	Y
Sand rat (Gerbil)	SD, HFD	Y	N	Y	Y	Y	Y
Goto-kakizaki rat	SD, HFD	Y	Y	N	Y	Y	Y
Monogenic							
C57BL/6J–ob/ob mice	SD, HFD	Y+	N	Y+	N	Y	N
C57BLKS/J–db/db mice	SD, HFD	Y+	Y	Y+	N	Y+	Y
fa/fa rat	SD, HFD	Y+	N	Y+	N	N	N
Zucker diabetic fatty rat	SD, HFD	Y+	Y+	Y+	N	Y+	Y+
Koletcky	SD, HFD	Y	N	Y+	N	Y	N
Experimental							
Streptozotocin mice or rat	HFD	Y	Y	Y	Y	Y	Y
VMH lesion mice and rat	SD, HFD	Y	N	Y	N	Y	N

Adapted from (Kleinert et al., 2018)Y, mild; Y+, severe; N, absent; CHO, carbohydrate enriched diet; DIO, diet-induced obesity; DR, diet resistant; HFD, high-fat diet; IR, insulin resistance; SD, standard diet; T2DM, type 2 diabetes mellitus; VMH, ventromedial hypothalamus.

1.7.6.1 Small animal rodent models

Rodents play major role in laboratory research, due to their similarities in biochemical pathways and high reproductive rate (Gautreaux et al., 1994). Almost 60% of diabetes research use small rodent models, however some research studies need genetic manipulation in order to mimic the human condition (Gautreaux et al., 1994). Although rodents are useful and cost-effective research models, the development of GI tract is not same as humans **(Figure 1.6)**. At birth, the human GI tract is more developed but mature more slowly than a small rodents (Sangild et al., 2006). Particularly for lipid research, rodent lack CETP, therefore are unable to transfer cholesterol from the HDL fraction to LDL other fractions. As a consequence, HDL is the main lipoprotein cholesterol fraction in rodents, which is not the case in humans. Most rodents are resistant to developing human-based dyslipidemias and usually require genetic modification such as apolipoprotein-E (apoE) or LDLR deletion (Russell & Proctor, 2006). There are many differences in the development of disease when comparing humans and rodents.

As a result of the limitations of commonly used rodent models, this thesis considered the application of a unique strain of rat that spontaneously develops obesity and insulin resistance; as well as contrasting data with a larger swine model.

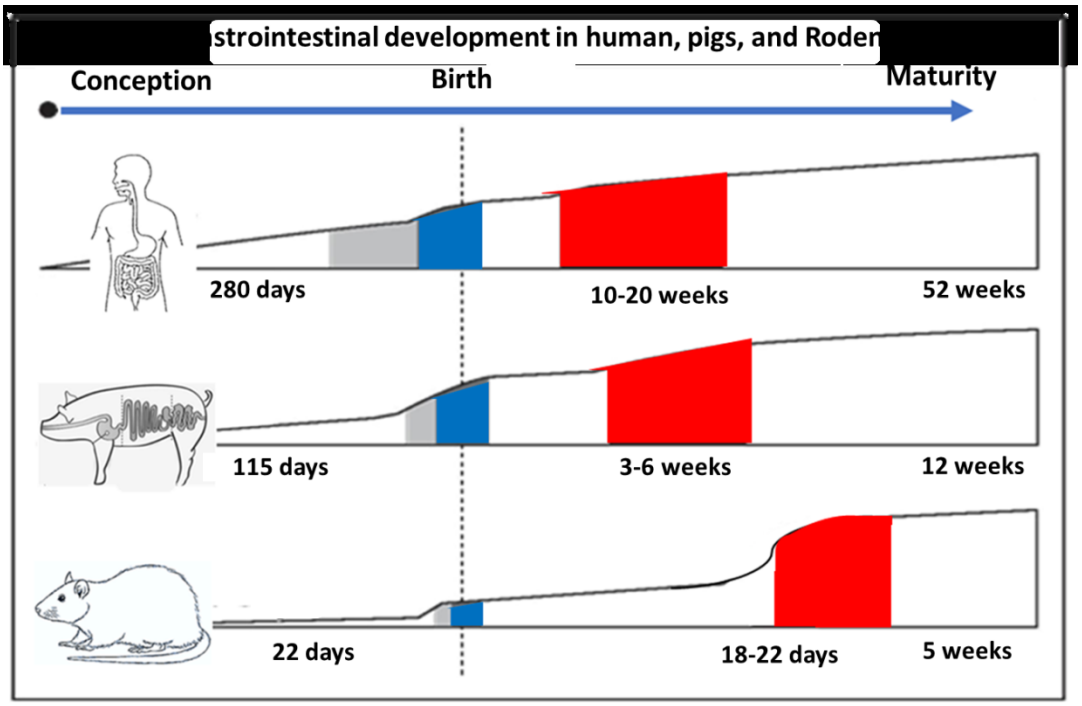


Figure 1.6 Comparison of gastrointestinal maturation in relation to birth and weaning between human, swine, and rodent.

Light gray areas refer to preterm birth of viable newborn, blue areas refer to a birth cluster of gastrointestinal tract maturational changes and red areas refer to weaning cluster of maturational changes. This **Figure** was adapted from (Sangild, 2006)

1.7.6.2 The JCR:LA-cp rat, a model of obesity and insulin resistance

As mentioned above, lipoprotein metabolism in rodents is primarily based on the HDL fraction and typically confer resistance to atherosclerosis and vascular diseases. However, while the technology to genetically engineer mice has been available for several decades, the subsequent technology for the larger rat has not been as forthcoming. Historically, there have been a handful of rat strains that were developed by cross breeding in the 1950s and 60s and displayed traits (polygenic) of abnormal carbohydrate/lipid metabolism (Russell & Proctor, 2006). The JCR:LA-cp strain was developed to incorporate an autosomal recessive corpulent trait (cp), first isolated by Koletsky in 1973 (Koletsky, 1973). This corpulent trait was later described to include a stop codon in the extracellular domain of the leptin receptor (ObR). JCR:LA-cp rats that are homozygous (cp/cp) for the trait spontaneously develop MetS-associated symptoms including, hyperphagia, obesity, IR and dyslipidemia (Brindley & Russell, 2002). The JCR:LA-cp rat also shows spontaneous development of ischemic lesions of the heart. Therefore, the JCR:LA-cp rat is a useful small rodent model to study the mechanisms related to the development of IR and MetS. The heterozygous (+/cp) or homozygous normal (+/+) JCR:LA-cp rats are lean and metabolically similar to their parents. JCR:LA-cp rats develop obesity at approximately three weeks of age and do not require additional high fat feeding. Once obese, JCR:LA-cp rats rapidly develop hyperinsulinemia (Emilsson, Liu, Cawthorne, Morton, & Davenport, 1997). Metabolic consequences of the JCR:LA-cp rat include overproduction of hepatic VLDL, hypertriglyceridemia and postprandial lipemia (Russell & Proctor, 2006). The cp/cp rat also displays overproduction of intestinal-derived nascent CM in lymph (Hassanali et al., 2010; Y. Wang et al., 2009), which offers a unique opportunity to study aberrant intestinal lipid absorption

under conditions of insulin resistance. The JCR:LA-cp strain has been used in a wide variety of approaches to study the consequences of MetS (Diane et al., 2016).

1.7.6.3 The Low Birth Weight (LBW) Swine models

In contrast to small animal models, large animal models can provide a more robust and reliable translation relative to human physiology and disease. For example, the pig has been developed as useful model to study human metabolism as it offers similar fetal and gastrointestinal tract development to that found in humans. Swine mimic many other general physiological similarities to that of humans and have been a good model to establish clinical/surgical manipulations and sampling techniques (Kararli, 1995).

High caloric diets used to induce MetS in different strains of pigs, have included mostly 15–25% (by weight) fat (mainly lard supplemented with hydrogenated soya bean and coconut oil). In most swine models of swine, features of MetS appear after 3–6 months on diet, but some can show symptoms of MetS as early 2–5 weeks of high-fat/high-energy diet (Christoffersen, Golozoubova, Pacini, Svendsen, & Raun, 2013). At 12 weeks after high-fat/high-sucrose dietary intervention, there are usually apparent manifestations of MetS (Xi et al., 2004) and prolonged intervention of high-fat/ high-fructose diet and are typically as long as 6 months (McKenney et al., 2014).

Miniature pigs, (such as the ossabaw and yucatan strains) are still considered a large animal and frequently used in diabetes and gastrointestinal research (Michael E. Spurlock & Nicholas K. Gabler, 2008). The Ossabaw pig readily develops obesity, IR and dyslipidemia after feeding on high fat, high cholesterol and high fructose diet. While the yucatan minipig is selectively bred, it needs multiple generations to select this phenotype (Bellinger, Merricks, & Nichols, 2006; R. W. Phillips, Westmoreland, Panepinto, & Case, 1982). Not only miniature pigs but other strain of

swine such as yorkshire breed can also show similar symptoms of diabetes, but it also requires some pharmaceutical induction (Bellinger et al., 2006). Many of these unique swine strains have limited availability, are costly and/or require specialized import/export permits. **(For summary see Table 1.3)** (X. Zhang & Lerman, 2016). On the otherhand, standard or production pigs such as (Landrace-Large White x Duroc) are more readily available and also provide a model for obesity (Fouhse, Zijlstra, & Willing, 2016).

Table 1.2. List of selected pig strain and their manifested metabolic syndrome (MetS) component induced by dietary intervention.

Breed	Gender	Age	Diet duration	Reported MetS components
Yucatan	M and F	3 m	6 m	Obesity, hypertension, increased cholesterol, increased TG, and insulin resistance
Ossabaw	M	6 w	9 w	Obesity, hypertension, increased cholesterol, and impaired glucose intolerance
Ossabaw	M	12 m	3–6 m	Obesity, hyperglycemia, hyperinsulinemia, increased cholesterol, increased LDL/HDL ratio, and increased TG
Ossabaw	F	3 m	14 w	Obesity, insulin resistance, increased cholesterol, and increased LDL/HDL
Domestic pigs	F	3 m	16 w	Insulin resistance and hypertension
Ossabaw	F	3 m	16 w	Obesity, insulin resistance, and inflammation
Yorkshire			11 w	Obesity and increased cholesterol impaired glucose tolerance
Ossabaw	M and F	6–8 m	6 m	Ossabaw, obesity, hypertension, hyperglycemia, increased cholesterol, increased TG, increased LD/cholesterol ration
Göttingen	M and F	9 w	4 m	Obesity, insulin resistance, impaired glucose tolerance, increased cholesterol, increased TG, visceral adiposity appeared after 2 weeks
Chinese Guizhou	M	3–4 m	6 m	Obesity, hyperglycemia, hyperinsulinemia, increased cholesterol, and increased TG
Ossabaw	M		6 m	Obesity and impaired glucose tolerance
Domestic pigs	F	3 m	16 w	Obesity, hypertension, increased cholesterol, insulin resistance, and visceral adiposity
Yorkshire x landrace		11 w	10 w	Hyperlipidemia and insulin resistance
Göttingen	F	9–10 m	5 w	Obesity, increased TG, hyperinsulinemia, and impaired glucose tolerance
Ossabaw		5–10 m	24 w	Obesity, insulin resistance, hyperglycemia, and hypertension
Lee-Sung	M and F	5 m	6 m	Hypercholesterolemia, hypertriglyceridemia, obesity, hypertension, and hyperglycemia
Ossabaw	M	6 m	6 m	Obesity, hypertension, increased LDL/HDL ratio, insulin resistance, increased TG, and coronary artery atherosclerosis

Adapted from (X. Zhang & Lerman, 2016)M, male; F, female; m, months; w, weeks; TG, triglyceride; LDL, low density lipoprotein; HDL, high density lipoprotein.

1.7.6.4 Definitions and prevalence of low birth weight and susceptibility to insulin resistance

According to WHO approximately 16% (22 million in 2013) of birth fall into low birth weight (LBW) category. However, 95.6% of these LBW are from developing countries (WHO & UNICEF, 2004). Low birth weight infants are considered as less than 2.5kg at birth or less than the 10th percentile. The prevalence of LBW specifically in Canada is approximately 6% (Stats Canada, 2013). The developmental origins of adult health and disease (DOHaD) theory hypothesizes that during fetal development a poor in utero environment results in modification in genes and pathways or organs that help the infant to survive in the short-term. However, in later life, this may cause chronic disease in the infant (Barker, 1997; Wadhwa, Buss, Entringer, & Swanson, 2009). Observational studies have been shown that during pregnancy the timing of dietary intervention affects the phenotype at birth and throughout life. The birth weight and T2D and MetS development was inversely proportional under normal range (Barker et al., 1993; Batterham et al., 2002; Lakshmy, 2013; Vuguin, 2007).

1.7.6.5 Low birth weight swine as a model of insulin resistance and dyslipidemia

Among all mammals, swine has high rate of intrauterine growth restriction (IUGR) due to the lack of placental capacity (Vuguin, 2007). The LBW offspring showed three times more mortality and reduced early postnatal growth than NBW offspring (Morise, Louveau, & Le Huërou-Luron, 2008). Studies have reported associations between one-year old LBW piglets and glucose intolerance (K. R. Poore & a L. Fowden, 2002). In swine and human studies, LBW is typically associated with an increased deposition of adipose tissue (Martorell, Stein, & Schroeder, 2001; Sarr, Louveau, Le Huërou-Luron, & Gondret, 2012). LBW swine also display reduced intestinal surface area and impaired intestinal absorption (Morise et al., 2008).

1.7.6.6 Animal model used in this thesis

In order to validate discoveries learned from small animal models, reproducible data from larger models are required. Part of the objectives of this thesis was to develop a comparable larger animal model to study early aberrations of lipid metabolism in insulin resistance. We hypothesized that by using an approach to feed a high fat, high carbohydrate diet to low birth weight production piglets (maintained on South Campus at the University of Alberta) we would be able to induce impaired lipid metabolism and early insulin resistance.

The general approach for this thesis was to use the spontaneous model of MetS (JCR:LA-cp) and see the effect of obese phenotype and HFD on gut microbiota and later to contrast this effect in LBW swine.

Chapter 2: Thesis rationale and gap in literature:

The prevalence of obesity and its related metabolic complications are increasing globally. These complications lead to T2D, MetS and CVD. The major underlying factor for the development of these non-communicable diseases (NCDs) is insulin resistance (IR). Lipid metabolism is negatively affected during obesity and IR and increases the risk of atherosclerosis and CVD (Mangat et al., 2011; Proctor et al., 2004). Although the liver and intestine have been the main regulating organs in these disease states, evidence suggest that the intestinal microbiota also act as a key environmental factor. Alteration in microbiota are known to affect weight gain and energy metabolism and offer new opportunities as a therapeutic target. The association between gut microbiota and MetS has been shown in both human and animal studies (Festi et al. 2014). Studies using rodents have reported that transplantation of gut microbiota from conventionally raised mice to the GF mice can increase adiposity. The comparison between GF and conventionally raised mice indicate increased monosaccharide uptake from the intestine and increased fatty acid oxidation in muscles and liver (Bäckhed et al., 2004; Bäckhed et al., 2007). Moreover, obese individuals are thought to have low gene richness of intestinal microbiota compared to lean individuals (Le Chatelier et al., 2013).

Obese individuals have significantly different *Firmicutes*: *Bacteroidetes* ratio than lean individuals. However, while there are numerous reports documenting the association between the ratio of *Bacteroidetes*/*Firmicutes* and obesity (Duncan et al., 2008; Jumpertz et al., 2011), the clinical relevance is still not clear. The gut microbiota can affect the host metabolism by controlling energy extraction and lipid metabolism (Harris et al., 2012; Ley et al., 2006). In rodents, gut microbiota affects the expression of genes such as fatty acid activation (Fatp4), phospholipid

transfer protein (Pltp), TG reesterification (Dgat1 and 2), fatty acid and phospholipid binding (Fabp2,) and chylomicron formation (Apob precursor, MTP) (Shulzhenko et al., 2011).

There is increasing evidence to suggest that diet is also a regulatory factor that can rapidly and reproducibly shape the gut microbiota. Some studies have reported that gut microbiota can regulate the transportation, emulsification, absorption and peroxidation of dietary fat. However, in most of the high-fat diet studies, saturated fatty acids is the main source of fat. Considering the quality of dietary fat and its impact on host physiology and gut microbiota during obesity is still not clear. A n-6 PUFA rich diet can alter the gut microbial composition and exacerbated colitis in mice (Ghosh, 2013), whereas a n-3 PUFA rich diet increases adhesion of *Bifidobacteria* to the intestine in mice. It has been demonstrated that gut microbiota of obese individuals harvest more energy from a high fat diet compare to a low fat diet (Turnbaugh et al., 2006).

Rodents have been considered as a good animal model to study MetS (Ai et al., 2005; Chen & Wang, 2005; Speakman, Hambly, Mitchell, & Krol, 2007). Rats can develop obesity and have increased basal and postprandial blood sugar when fed on diet with 70% energy coming from fat. (Budohoski et al., 1993; Harris & Kor, 1992; Samuels, Gilmore, & Reinecke, 1948). To study obesity and early aberrations of insulin resistance, animal models often require dietary induction. Higher dietary fat intake (high-fat diet) is used on a regular basis to induce conditions of over-nutrition, obesity and early insulin resistance (Buettner, Schoelmerrich, & Bollheimer, 2007). In most diet induced rodent studies, there is association between gut microbiota and IR. However, there are some strains of rodents for example JCR:LA-cp rats, which can develop obesity at approximately three weeks of age and do not require high fat feeding (Emilsson et al., 1997). The JCR:LA-cp rat also displays overproduction of intestinal-derived nascent CM in lymph (Hassanali

et al., 2010; Wang et al., 2009), which offers a unique opportunity to study aberrant intestinal lipid absorption under conditions of insulin resistance.

In contrast to small animal models, large animal models can provide a more robust and reliable translation relative to human physiology and disease. For example, the pig has been developed as useful model to study human metabolism as it offers similar fetal and gastrointestinal tract development to that found in humans. Studies have been showing that LBW offspring are predisposed to become obese, insulin resistant and develop T2D in adulthood (Armitage, Khan, Taylor, Nathanielsz, & Poston, 2004; Barker, 2001; Gluckman & Hanson, 2004; McMillen & Robinson, 2005; Varvarigou, 2010). Additionally, intake of HFD in LBW offspring can exacerbate the phenotype (Dulloo, 2008; Morrison, Duffield, Muhlhausler, Gentili, & McMillen, 2010) . Due to the larger litter sizes, swine have highest rate of LBW offspring among mammals (Karolina Ferenc et al., 2014).

However, few rodent studies have shown that quality of dietary fat also known to affect gut microbiota composition (Patterson et al., 2014). Due to the divergent nutritional requirements of various bacteria colonizing the gut, it is also well established that diet shapes the composition of the intestinal microbiota in mice and humans (Faith et al., 2011; Hildebrandt et al., 2009; Li et al., 2017b). Specifically, consumption of a western type diet has been shown to alter the composition of the gut microbiota (Carmody et al., 2015; Faith et al., 2011; Hildebrandt et al., 2009; Murphy et al., 2010), which in turn may promote the development of obesity and associated chronic diseases (Turnbaugh, Backhed, Fulton, & Gordon, 2008).

Hence, the focus of this thesis was to use the spontaneous model of MetS (JCR:LA-cp) and see the effect of obese phenotype and HFD on gut microbiota and later to contrast this effect in LBW swine.

2.1 Study rationale, objectives and hypotheses

2.1.1 Chapter 3. Investigation the impact of phenotype or dietary intervention on the gut microbiota in a spontaneous model of insulin-resistance (the JCR:LA-cp rat)

2.1.1.1 Rationale

Insulin resistance is often associated with increased risk for developing type 2 diabetes (T2D). Preclinical (animal model) studies have demonstrated that the intestinal microbiota has a major role in the pathogenesis of obesity and insulin resistance (IR). Observational studies have shown that obese individuals have significantly different gut microbiota compared to lean individuals, including a commonly described increase in the *Firmicutes* phylum and reduction in *Bacteroidetes* (Armougom, Henry, Vialettes, Raccah, & Raoult, 2009; Ley, Turnbaugh, Klein, & Gordon, 2006; Santacruz et al., 2010). However, it should be noted that there are numerous reports documenting the association between the ratio of *Bacteroidetes*/*Firmicutes* ratio between and obesity (Duncan et al., 2008; Jumpertz et al., 2011), the clinical relevance remains under debate (Sze & Schloss, 2016). Diet has also been shown to be a major factor that can affect the composition of the gut microbiota. High-energy or high fat diets (HFD) have been shown to increase LPS as well as bacterial nucleic acid in blood (Panasevich, Peppler, Oerther, Wright, & Rector, 2017). While increased circulating bacterial products have been described in metabolic diseases, bacterial translocation (BT) has been minimally characterized.

2.1.1.2 Hypothesis

I hypothesized that hyperphagia (overnutrition) in the JCR:LA-cp rat would alter gut microbiota composition. Similarly, addition of an HFD would further exacerbate microbial dysbiosis. Subsequent to this, we hypothesized that hyperphagia and/or HFD would facilitate increased bacterial translocation and appearance of live bacteria in lymph.

2.1.1.3 Objective

To test these hypotheses, the microbiota of both obese and lean JCR:LA-cp rats was first characterized under chow and high fat diet conditions. Mesenteric lymph was sampled to direct assess bacterial translocation. We sought to verify the source of bacterial translocation using GFP tagged *E. coli* during lymph collection.

2.1.1.4 General approach

We selected Male JCR:LA-cp rats, obese homozygous (cp/cp) and lean heterozygous (cp/+), born and raised in identical condition with free access to food and water in our established breeding colony at the University of Alberta. At 6 weeks of age, obese homozygous and lean heterozygous JCR:LA-cp rats were randomly assigned to control or HFD diet for 6 weeks. Samples of lymph and portal vein blood were cultured and processed for sanger sequencing. For gut microbiota analysis from cecal samples, total DNA was extracted and further processed by next generation sequencing

2.1.1.5 Personal contribution for this chapter

I took a lead role in this project with the assistant of students and staff from the Proctor and Willing labs. Chapter 3 is an original scientific contribution and one of the main studies contributing to this thesis.

2.1.2 Chapter 4. Investigating the effect of high-fat, high-carbohydrate diet and dietary fatty acid composition on gut microbiota in LBW swine

2.1.2.1 Rationale

LBW offspring are also prone to get MetS such as obesity, IR and T2D in adulthood (Armitage et al., 2004; Barker, 2001; Gluckman & Hanson, 2004; McMillen & Robinson, 2005). Among mammals, swine have a high rate of intrauterine growth restriction (IUGR) due to the lack

of placental capacity and large litter size (Vuguin, 2007). The post-natal diet, such as a high fat western diet, greatly contributes to metabolic complications and early insulin resistance in LBW offspring (Dulloo, 2008; Morrison et al., 2010; Poore & Fowden, 2002). A change in diet clearly affect the composition of gut microbiota and these changes may contribute to their host's metabolic phenotype. However, the interaction between LBW, diet and intestinal lipid absorption/secretion leading to adult metabolic disease remains unclear.

2.1.2.2 Hypothesis

Given findings from chapter 3, I hypothesized that LBW pigs fed a western (high fat, high fructose and cholesterol) [HFHC] diet would develop metabolic complications, contribute to impaired post-prandial dyslipidemia and induce dysbiosis in gut microbiota relative to their NBW littermates. I further hypothesized that supplementation of HFHC diet rich in VA fat would result in an reduced gut dysbiosis and improved lipid metabolism profile.

2.1.2.3 Objective

The overall objective was to utilize the novel low birth weight swine model with HFHC diet to determine the lipid lowering benefits of VA to reduced gut dysbiosis and improved lipid metabolism profile

2.1.2.4 General approach

In this study NBW and LBW piglets were selected from the University of Alberta SRTC at birth and weaned. For the first of two swine studies, pigs were fed a control or HFHC diet for 6 weeks, (post-weaning) until 13 weeks of age. In the second study swine were fed either control, HFHC or HFHC enriched in VA for 6 weeks. For both studies a combined 2-step modified oral glucose tolerance and fat challenge test (MOGTT) was performed. Lymph was collected followed by exsanguination under anesthesia and tissue collection.

2.1.2.5 Personal contribution for this Chapter

In this study, I have been equally involved with the assistance of one MSc and other laboratory staff from the Proctor and Willing labs. Chapter 4 is an original scientific contribution and a main contribution to this thesis.

2.2 Ethical considerations, and procedure developments

A major part of this thesis has been dedicated to observe the effect of diet in two different species such as swine and rats. My laboratory invested significant time during the course of my program to make sure that both animals in both study were cared for with a high quality of life. In addition, staff ensuring each animal is healthy and eating well took daily records. The documentation of both the model and the SOPs developed during this thesis has contributed significantly to research and knowing that the ethical handling and quality of life has been held at the highest regard.

Chapter 3: Investigation the impact of phenotype or dietary intervention on the gut microbiota in a spontaneous model of insulin-resistance (the JCR:LA-cp rat):

3.1 Personal contribution

I took a lead role in this project with the assistance of students and staff from the Proctor and Willing research laboratories. I assisted in the surgical procedures, developed new protocols, standardized certain procedures and performed all culture-based and molecular based microbiology work. I presented data from this study at the ALES Graduate Research Symposium, University of Alberta, March 2017.

3.2 Introduction

Metabolic Syndrome (MetS) is a cluster of conditions that includes high fasting triacylglycerol, low levels of high-density lipoprotein cholesterol, obesity, elevated plasma insulin and impaired glucose tolerance (Kaur, 2014). The prevalence of MetS globally ranges from <10% to 84%, varying by region (urban or rural environment), culture, and population composition (Desroches & Lamarche, 2007). Recently the intestinal microbiota has drawn growing attention as an important environmental factor regulating weight gain and energy metabolism. An association between gut microbiota and MetS has been reported in both preclinical and clinical studies, some with inconsistent findings (Festi et al., 2014).

Gut microbiota, obesity, diabetes, and other metabolic diseases

The first study to report that gut microbiota may play a role in the development of obesity proposed that germ free mice were protected from adiposity despite recieving a high-fat diet (HFD) (Backhed, Manchester, Semenkovich, & Gordon, 2007). Consistant with this, the reconstitution of

germ-free mice with gut microbiota from conventional mice resulted in significant weight gain (Bäckhed et al., 2004). Observational studies have also shown that obese individuals have significantly different gut microbiota composition compared to lean individuals, including a commonly described increase in the *Firmicutes* phylum and reduction in *Bacteroidetes* (Armougom et al., 2009; Ley et al., 2006; Santacruz et al., 2010). However, it should be noted that although there are numerous reports documenting the association between the ratio of *Bacteroidetes*/*Firmicutes* ratio between and obesity (Duncan et al., 2008; Jumpertz et al., 2011), the clinical relevance remains under debate (Sze & Schloss, 2016). Despite this, a major translational discovery has been that the gut microbiota is not only associated with obesity but can actually transfer an obese phenotype via gut microbiota transplantation (Turnbaugh et al., 2008; Turnbaugh et al., 2006; Vijay-Kumar et al., 2010).

3.2.1 Dysbiosis and Diabetes

Type 2 diabetes (T2D) is characterized by insulin resistance, impaired glucose metabolism as well as low-grade inflammation and has also been linked to the quality of microbiota. However, studies in humans are yet to report a consistent relationship between intestinal bacterial composition and diabetes. The slight increases in phylum *Bacteroidetes* in diabetic subjects compared to non-diabetic subjects (Larsen et al., 2010). A case control study showed reduced *Bacteroides vulgatus* and *Bifidobacterium* in the T2D group relative to healthy controls (Kalischuk, Inglis, & Buret, 2009; X. Wu et al., 2010). Although it is still not clear what microbes are important in T2D, we do know that microbial transplantation from lean donors to overweight recipients has been shown to improve insulin sensitivity (Vrieze et al., 2012).

3.3.2 Dysbiosis and Metabolic endotoxemia

Altered gut microbial composition, known as dysbiosis (Ley et al., 2005; Turnbaugh et al., 2006), can increase gut permeability, facilitate leakage of bacterial products [such as lipopolysaccharide (LPS)], activate the innate immune system and cause chronic low-grade inflammation during metabolic disease (Cani et al., 2007; Turnbaugh et al., 2006). An increase in LPS concentration in blood is known as "metabolic endotoxemia" (Boutagy, McMillan, Frisard, & Hulver, 2016). Studies have shown that increased endotoxin concentration can in turn increase intestinal permeability (Cani et al., 2008; de La Serre et al., 2010). Diet also plays an important role in the disruption of gut permeability and leakage of LPS. High-energy diet or high fat diets (HFD) have been shown to increase LPS as well as bacterial nucleic acid in blood (de La Serre et al., 2010; Panasevich et al., 2018; Panasevich et al., 2017). In a healthy human cohort, it has been shown that as much as 10 to 40 g of fat (either alone or in a mixed meal) can increase endotoxemia within 30 min post-prandially (Erridge, Attina, Spickett, & Webb, 2007; Michalski, Vors, Lecomte, & Laugerette, 2016), suggesting that these effects can be very acute.

3.3.3 Gut microbiota and bacterial translocation (BT)

While increased circulating bacterial products have been described in metabolic diseases, bacterial translocation (BT) has been minimally characterized. Bacterial translocation is defined as the passage of viable bacteria from the GIT to extra intestinal sites (Berg, 1995). There are many factors, that influence the prevalence of bacterial translocation. These include, (a) an alternation in normal gut microbiota or bacterial overgrowth (b) gut mucosal barrier physical disruption, (c) impaired host defense (O'Boyle et al., 1998). *Campylobacter jejuni* promote translocation of non-invasive bacteria in IBD patients via lipid rafts (Kalischuk et al., 2009), however but the exact mechanism of translocation is unknown.

3.3.4 Animal Model (JCR:LA-cp) rat

The JCR:LA-corpulent (JCR:LA-cp) strain of rats has been developed to have a recessive corpulent (cp) trait, originally identified by Koletsky (Koletsky, 1973). Homozygous (cp/cp) JCR:LA-cp rats spontaneously develop symptoms of MetS and pre-diabetes (Russell & Proctor, 2006; Vine, Takechi, Russell, & Proctor, 2007). Vine et. al showed that JCR:LA-cp rats have significant postprandial lipemia, caused by the overproduction and secretion of lymphatic chylomicron particles from the intestine (Diane et al., 2016). The JCR:LA-cp rat is therefore a useful model to study association between gut microbiota, impaired intestinal lipid metabolism and insulin resistance.

3.3.5 Hypothesis and objectives

While the intestinal microbiota has been studied in a diverse number of rodent models, the association between IR and gut microbiota is still unclear. I hypothesized that hyperphagia (overnutrition) in the JCR:LA-cp rat would alter gut microbiota composition. Similarly, addition of an energy rich diet (high fat) would further exacerbate microbial dysbiosis. Subsequent to this, I hypothesized that hyperphagia and/or HFD would facilitate increased bacterial translocation and appearance of live bacteria in lymph. To test these hypotheses, the microbiota of both obese and lean JCR:LA-cp rats was first characterized under chow and high fat diet conditions. Mesenteric lymph was sampled to direct assess bacterial translocation. We sought to verify the source of bacterial translocation by enteral infusion of GFP tagged *E. coli* during lymph collection.

3.4 Materials and methods

3.4.1 Animals

Male JCR:LA-cp rats, homozygous obese (cp/cp) and heterozygous lean (?/+), were provided via our established breeding colony at the University of Alberta (Vine et al., 2007). All

experimental procedures were approved by the Animal Ethics Committee at University of Alberta, and procedures followed the Canadian Council on Animal Care guidelines.

3.4.2 Study design

The experimental design was divided into two pilot studies. For study 1 (**please see Figure 3.1 for study design**), at 6 weeks of age, obese homozygous (cp/cp) (n=6) and lean heterozygous (cp/+) (n=6) rats were selected for chow diet and remaining obese homozygous (cp/cp) (n=6) and lean heterozygous (cp/+) (n=6) were selected for HFD (**Table 3.1)** for 6 weeks.

In Study 2 (please see **Figure** 3.2 for study design), involving prelabelled bacteria experiment, we randomly selected (n=12) obese homozygous (cp/cp) and (n=12) lean heterozygous (cp/+) rats. At 6 weeks of age (n=6) animals from each group were placed on either a HFD or a chow diet for 6 weeks. Food intake and body weight (BW) were monitored weekly throughout the study. At the end of the treatment period (12 weeks of age), rats were fasted overnight.

3.4.3 Lymph cannulation

To assess the extent of translocation into lymph, rats were subjected to lymph fistulae procedure. Intestinal mesenteric lymph cannulation surgery was performed as previously described (Hassanali et al., 2008; Vine, Croft, Beilin, & Mamo, 2002). We used aseptic techniques and sterile conditions throughtout the whole procedure. Prior to surgery equipment was sterilized (such as forceps, scissors, lymph collection tubes, cannula and surgical suture) and/or autoclaved. To ensure sterile surgical procedures, on the day of surgery, rats were anesthetized with isofluorane, abdomen hairs were shaved and skin was cleaned with sterilant. Prior to the placement of cannula, a surface swab sample was collected for post-anlayses. In addition, lymph cannula were pre-flushed with sterile saline propr to placement. During lymph collection all tubes were

kept on ice and samples cultured on the same day. All samples that showed contamination in swab or flushed saline samples were excluded from subsequent analysis. A second cannula was inserted in the upper duodenum, lipid (20% intralipid, 4.0% glucose in saline) was infused and lymph was collected for a total of 4 hours during the course of lipid infusion. (Hassanali et al., 2008; Vine et al., 2002). After lymph surgery, rats were euthanized under isofluorane anesthesia. Cecal samples were collected for microbiota analysis **(Figure 3.1)**.

In study 2, following surgery, lymph and portal vein blood samples were sampled. All collected blood and lymph samples were plated on fastidious anaerobe agar (FAA) and incubated in an anaerobic chamber for 5-6 days at 37°C **(Figure 3.2)**. After incubation, 2-3 bacterial colonies were randomly selected and processed for sanger sequencing using the 16S rRNA gene amplicon. Briefly, colonies were picked from positive plates using a sterile pipette tip and mixed with 25 µl of water in a 600 µl microcentrifuge tube and placed in boiling water for 10 minutes. The lysate was amplified targeting the V1-V6 regions of the bacterial 16Sr RNA gene with the following conditions: 94°C for 10 min, followed by 40 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 60 sec, and 72°C for 7 min with universal primers (forward 8F 5'-AGAGTTTGATCMTGGCTCAG-3' and reverse 926R: 5'-CCGTCAATTCCTTTRAGTTT-3'). After amplification, a 1.5% agarose gel was used to identify the amplified product, the PCR product was cleaned up using the QIAquick PCR purification kit and was subjected to sanger sequencing. All the sequenced isolates were identified using the online database tool Ribosomal Database Project (RDP).

Table 3.1. Ingredients of control and high-fat diets used for feeding obese JCR:LA-cp rats and their lean littermates.

	Control diet (chow)	850 g of 85% basal mix+15% fat
		g/850g
Casein	281	269.9
l-Methionine	2.94	2.499
Dextrose, monohydrate	273.417	234.104
corn starch	255	224.4
cellulose	58.82	49.99
Mineral Mix,	59.82	50.84
Sodium Selenite (0.0445% in sucrose)	0.353	0.3
Magnese sulfate, Monohydrate	0.28	0.23
Vitamin Mix, A.O.A.C. (40055)	11.8	10.03
Inositol	7.35	6.247
Choline Chloride	1.62	1.377
Fat 15%		
Hydrogenated fat (Lard)	47.0	138.5 g
Flaxseed Oil	0.5	1.5 g
Cholesterol	0.0	10.0g
total	1000 g	1000 g

Study 1

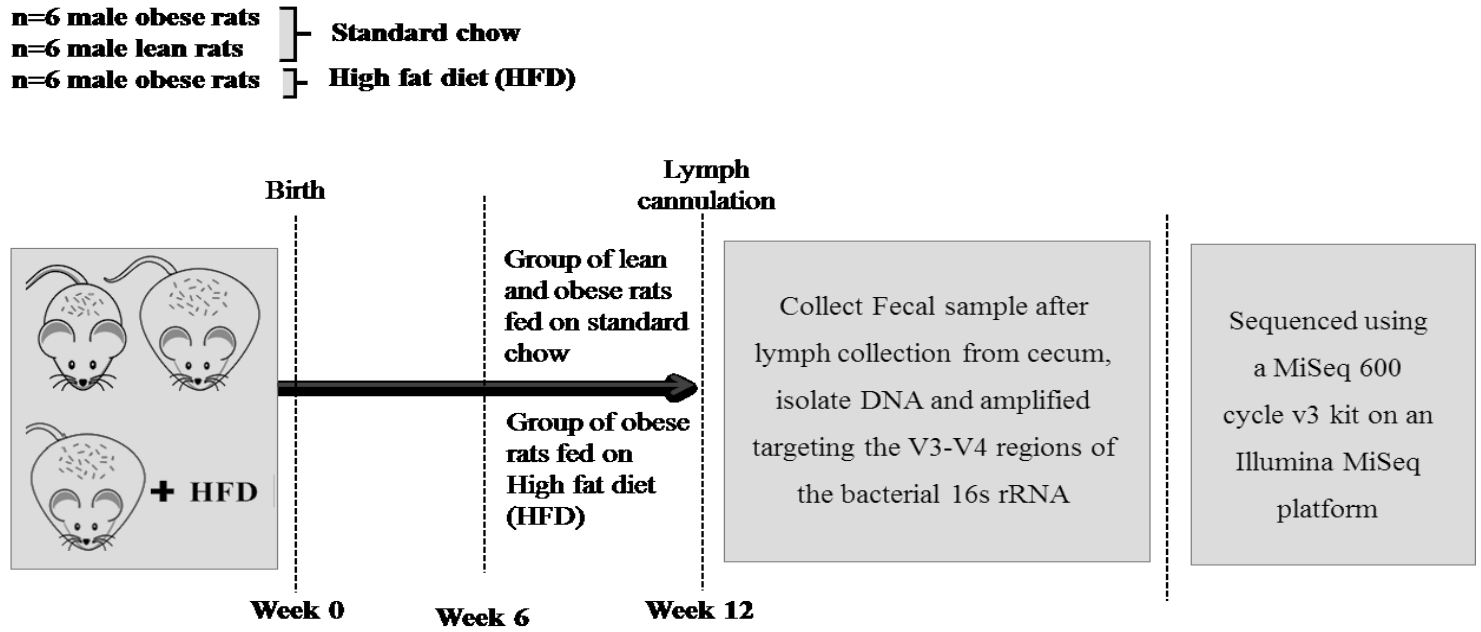


Figure 3.1 Study design to investigate the effect of JCR:LA-cp rats trait (overconsumption) on gut microbiota

Study 2

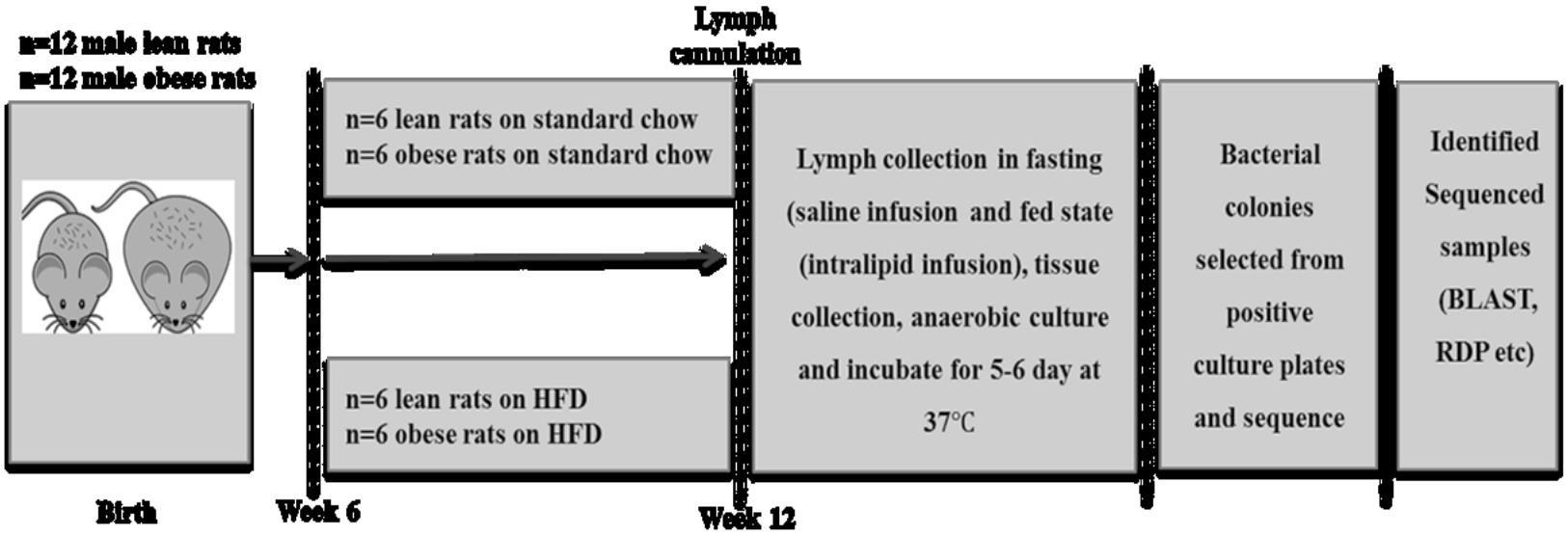


Figure 3.2 Study design to investigate the bacterial translocation in lymph under healthy and insulin resistance conditions.
high fat diet ; HFD, basic local alignment search tool; BLAST, ribosomal database project; RDP.

3.4.4 Green fluorescent protein (GFP) labeled *E. coli* study

To confirm bacteria cultured from lymph had directly translocated and originated from the gastrointestinal track, GFP labeled *E. coli* were introduced to the intralipid infusion during lymph collection. The *E. coli* used in this experiment was isolated from a JCR:LA-cp rat fecal sample by selective culture on MacConkey agar and subsequent Sanger sequencing of 16S rRNA gene as described above.

3.4.4.1 Green fluorescent protein *E. coli* labeling

A single colony of *E. coli* was inoculated into 5ml of Luria Bertani (LB) broth media and grown overnight at 37°C with shaking at 250 rpm. The overnight preculture was diluted with 100 ml of LB broth with 1 ml of saturated culture and subsequently incubated at 37°C with shaking at 250 rpm until an OD600 = 0.4 was achieved (2-3 hours). The bacterial suspension was placed on ice for 15 min, poured in prechilled 50 ml falcon tubes and centrifuged at 2700 × g for 10 min at 4°C. All further steps were performed in cold room and on ice. The supernatant was removed, and the bacterial pellet resuspended with 1.6 ml ice cold 100 mM $CaCl_2$-$MgCl_2$ by gentle swirling. The suspension was subsequently incubated on ice for 1 hour, centrifuged at 2000 rpm for 10 min at 4°C and resuspended with 1.6 ml ice cold 100mM $CaCl_2$-$MgCl_2$ by gentle swirling. Suspension was returned to ice for 20 minutes and combined with 0.5 ml ice cold 40% glycerol (glycerol: $CaCl_2$=1:1). Aliquots of 100 µl were placed in precooled 1.5 ml microcentrifuge tubes and directly frozen in liquid nitrogen and stored in -80°C. Competent cells were then transformed with a GFP plasmid FUA1028; with gene coding for eGFP and carrying an antibiotic resistance gene (ampicillin) (Froger & Hall, 2007) (**experimental sequence shown in Figure 3.3)**. During lymph collection GFP labeled *E. coli* (10^8 CFU/50 µl) was mixed with intralipid and infused through the duodenum. *E. coli* infusion was performed on obese and lean rats with and without HFD

(n=6/treatment). After completion of lymph collection, all samples were cultured on FAA and MacConkey agar with 100 μg/ml ampicillin. Portal vein blood was collected for culture and LPS quantification.

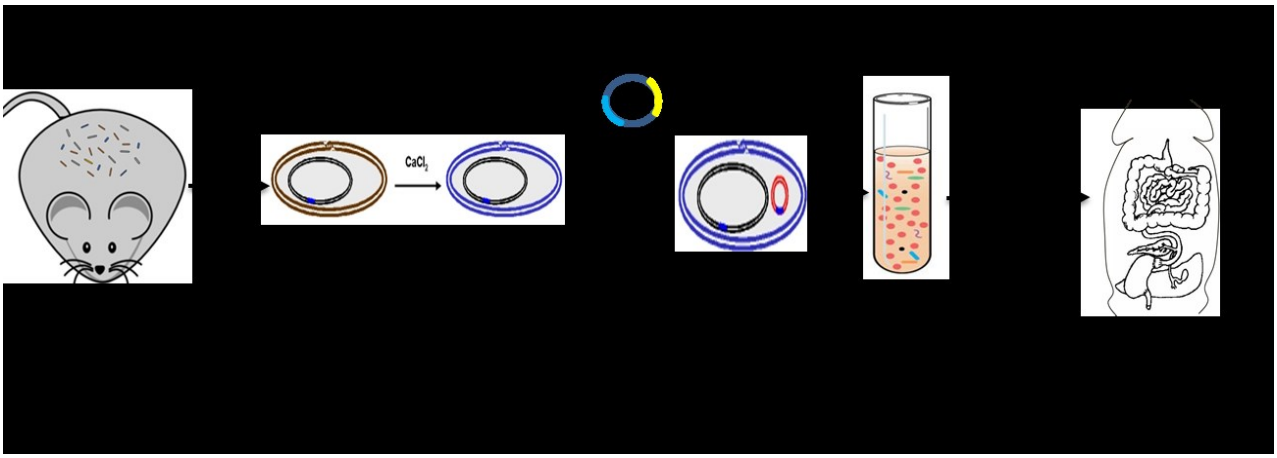


Figure 3.3 Isolation of GFP tagged *E. coli* and intestinal infusion

3.4.4.2 Circulating plasma endotoxin

To determine the effect of genotype and HFD on serum LPS, portal blood was collected at termination into pyrogen-free tubes (Thermo Fischer Scientific). Endotoxin concentration was measured using the PYROGENT-5000 kit following the protocol as per manufacturer's instructions (Lonza, Mississauga, Canada). At 37°C absorbance was measured at 340 nm per minute for 1 hour using a SpectraMax® M3 Microplate Reader (Molecular Devices, LLC. Sunnyvale, CA, USA). The required time for the absorbance to increase 0.03 absorbance units was considered as reaction time.

3.4.5 Cecal bacterial community characterization

Total DNA was extracted from cecal digesta using the QIAamp DNA Stool Mini Kit (Qiagen, Inc.) according to the manufacturer's instructions, with the addition of a bead-beating step to lyse gram positive bacteria (FastPrep instrument, MP Biomedicals, Solon OH). DNA concentration was measured using a Quant-iT™ PicoGreen® dsDNA Assay Kit (Thermo Fischer Scientific). Extracted DNA was amplified targeting the V3-V4 regions of the bacterial 16Sr RNA genes using KAPA HiFidelity Hot Start Polymerase (Kapa Biosystems, Inc. Wilmington, MA, USA) with the following conditions: 95°C for 3 min, followed by 25 cycles of 95°C for 30 seconds, 55°C for 30 second, and 72°C for 30 second, and 72°C for 5 min with universal primers (Forward 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3' and Reverse 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG -3') for amplification (Klindworth A; et al 2013). After amplification, AMPure XP beads were used to purify the 16S amplicon to remove primers and primer dimer species. Dual indices and Illumina sequencing adapters were attached using the Nextera XT Index Kit, followed by a second PCR cleanup and quantification. Samples were diluted to 4 nM and 5μL aliquots of each diluted DNA sample were pooled and size-selected

and denatured with NaOH, diluted to 4pM in Illumina HT1 buffer, spiked with 2-PhiX and heat denatured at 96°C for 2 min. The library was sequenced using a MiSeq 600 cycle v3 kit on an Illumina MiSeq platform (Illumina USA), according to the manufacturer's instructions.

Sequence Data Processing

I analyzed our sequenced by using QIIME pipeline (MacQIIMW 1.8.0 OS10.10) (Caporaso JG, et al. 2010). For quality filtering PANDAseq was used to assemble paired end reads into contigs with miscalled or uncalled bases discarded. UCHIME and UPARSE workflows were used to clear chimeras and singletons from resulting sequences and subsequently clustered into operational taxonomic units (OTUs) having >97% similarity with USEARCH (Edgar RC, 2010). QIIME default setting and Ribosomal Database Project classifier V2 were used to assigned taxonomy (Wang Q, et al, 2007). Alpha-diversity and beta-diversity estimations were conducted using the QIIME workflowcore_diversity_analysis.py (Seda A, et al 2015). Chao-1, simpson and shannon indices were used to estimate the alpha diversity (Colwell RK, et al .2012). All tests and comparisons with $p<0.05$ were considered statistically significant. Differences in microbial communities were investigated using Bray-Curtis dissimilarity. One-way analysis of similarity (ANOSIM) was applied to compare the differences of bacterial community structure between control and HFHC diets and birth weight. Diversity and abundance were compared using Kruskal-Wallis test.

Statistical analysis

Result are presented as means and standard deviation of the mean (stdev). Kruskal–Wallis rank sum test was used to analyse the non-normally distributed relative abundance of microbial taxa. GraphPad Prism v. 6.02 (La Jolla, CA) and SAS (University Edition) were used to analyse the results.

3.5 Results

3.5.1 High fat diet alters the profile of cecal gut microbiota

To determine whether the JCR:LA-cp phenotype was associated with altered gut microbiota composition, 16S rRNA amplicon sequencing results were compared with cecal samples of lean rats fed on chow diet. A total of 823,141 reads were obtained. Sequences were clustered into operational taxonomic units (OTU) with USEARCH resulting in a total of 560 OTUs. Principal Coordinate Analysis (PCoA) of cecal microbiota composition using Bray-Curtis dissimilarity **(Figure 3.4)** did not report any significantly difference between groups (ADONIS, $p = 0.125$). In contrast, the addition of a HFD shifted gut microbiota relative to rats fed chow diet (ADONIS, $p = 0.001$) **(Figure 3.5)**.

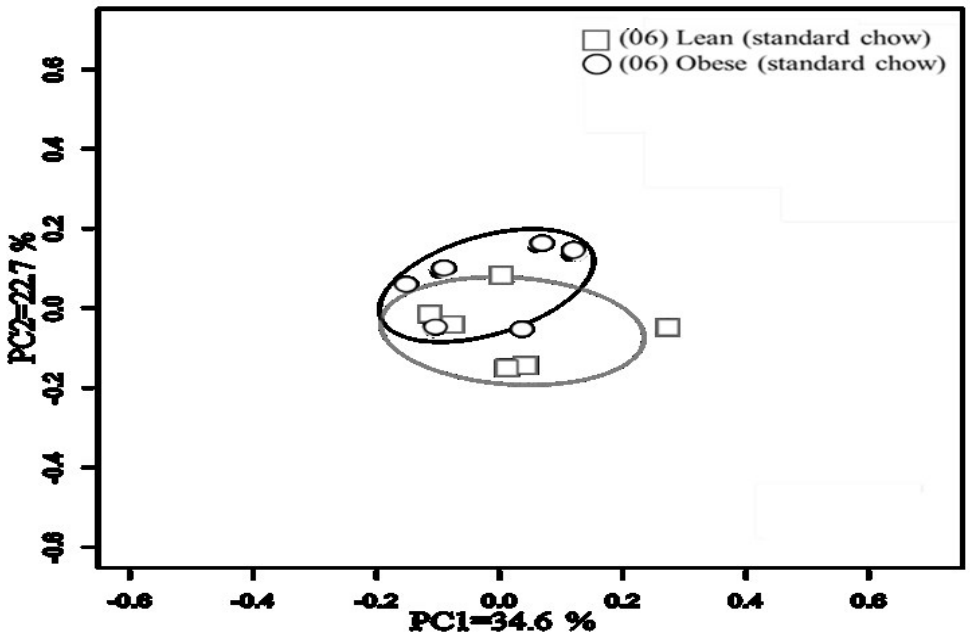


Figure 3.4 Principle coordinate analysis of cecal bacterial community composition using Bray-Curtis dissimilarity.

Cecal microbiota was not significantly different in lean versus obese rat fed on chow diet (ADONIS, p = 0.125).

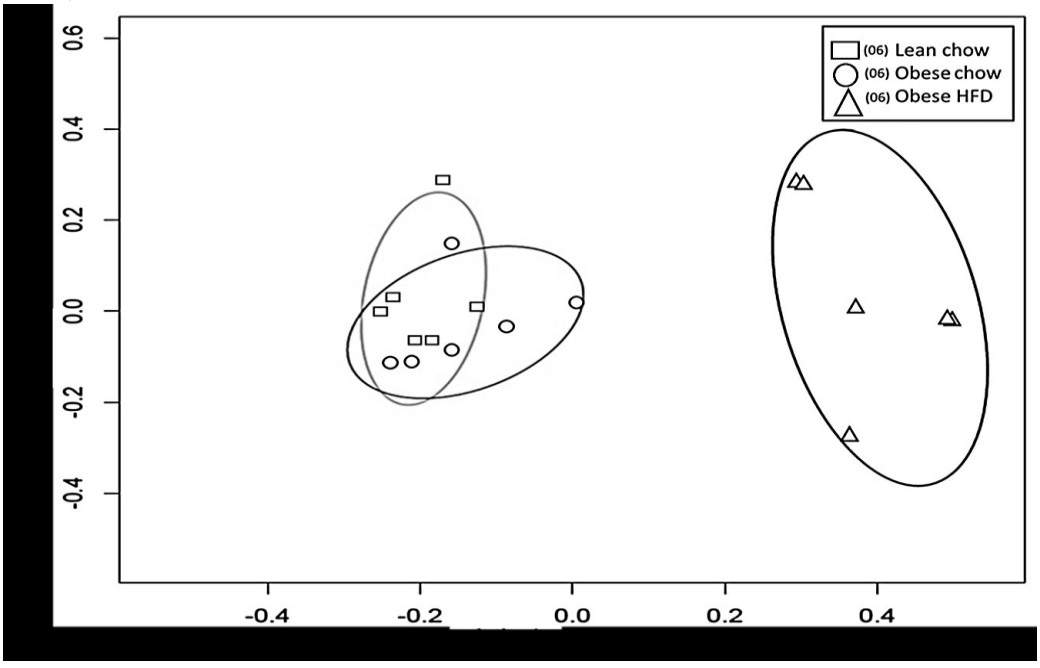


Figure 3.5 Principle coordinate analysis of cecal bacterial community composition using Bray-Curtis dissimilarity

(Obese HFD vs Lean Chow p = <0.001*ADONIS, p = 0.001* and Obese HFD vs Obese Chow p = 0.01*) HFD, High fat diet.Cecal microbiota was significant different in rats fed a HFD

3.5.2 Species richness and evenness of gut microbiota

Rarefaction curves for alpha diversity were also generated based on treatment and diet, to assess the depth of sequencing and comparison of microbial communities. Species richness was measured by chao-1 diversity index and species richness and evenness of taxa was measure by Shannon and Simpson diversity indeces **(Table 3.2)** (Faith, 2006). Chao1 analysis indicated reduced richness in obese rats fed a HFD diet as compared to rats fed chow **(Figure 3.6)**. I did not observe any significant difference between groups for Shannon and Simpson diversity indeces (**Figure** 3.7).

Table 3.2. alpha diversity by Shannon, Simpson and chao-1 diversity index.

	Lean Chow	Obese Chow	Obese HFD	p Value
Shannon	5.0 ± 0.32	4.7 ± 0.43	4.5 ± 0.28	> 0.10
Simpson	0.9 ± 0.00	0.9 ± 0.02	0.9 ± 0.03	> 0.10
Chao-1	246.7 ± 23.3[a]	238.6 ± 30.0[a]	154.2 ± 12.2[b]	<0.0001

Values are means ± SD. HFD, high-fat diet Chow. chow diet. a,b refers to statistical difference with a p-value <0.05 between different letters

3.5.3 Relative abundance of gut bacteria from phylum to genus in cecal digesta

The five most abundant phyla in cecal digesta of rats were *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Tenericutes*, and *Verrucomicrobia,* constituting 98.3% of all sequences. Bacterial communities in cecal digesta changed in response to HFD diet at phylum level with an increase in *Firmicutes*, ($p < 0.05$), *Verrucomicrobia* and *Proteobacteria* and a decrease *Bacteroidetes* ($p < 0.05$) as compared to chow diet fed rats. Relative abundance of bacteria at each taxonomic level was also assessed and is presented in **Table 3.3**. At the class level, *Bacilli*, *Clostridia* ($p<0.05$), *Verrucomicrobiae*, *Gammaproteobacteria* and *Mollicutes* ($p < 0.05$) were more abundant in HFD diet group compared to chow diet group, whereas *Bacteroidia* ($p < 0.05$) were less abundant. At the order level *Lactobacillales*, *Clostridiales* ($p <0.05$), *Enterobacteriales* and *Verrucomicrobiales* were significantly increased, whereas *Bacteroidales* were reduced ($p < 0.05$) in HFD diet fed rats compared to chow. At the family level *Streptococcaceae, Clostridiaceae* ($p < 0.05$) were more abundant while *Bacteroidaceae* ($p<0.05$), and *Porphyromonadaceae* ($p < 0.05$), were less abundant in HFD diet groups compared to the chow. Increased *Lactobacillus, Clostridium* ($p < 0.05$), and *Streptococcus* genera was consistent with the increase in *Firmicutes* with *Akkermansia* from phylum *Verrucomicrobia* in HFD fed rats. Genera including *Bacteroides* ($p < 0.05$) and *Parabacteroides* ($p < 0.05$) were increased in chow diet fed rats compare to HFD diet fed rats.

Table 3.3. Differences in relative proportion of bacteria from class to genus in cecal digesta of lean and obese rat fed on chow and high fat diet (HFD)

Phylum	Obese HFD	Obese Control	Lean Control	p Value		
				Phenotype	Diet	Treatment (HFD)
Bacteroidetes	10.27±7.10	46.24±8.36	56.73±8.45	0.01*	0.00*	0.00*
Firmicutes	68.06±17.75	38.16±7.02	31.45±10.81	0.04*	0.01*	0.02*
Proteobacteria	9.69±14.19	6.68±10.01	4.34±7.02	0.23	0.4	0.46
Tenericutes	0	0	0	0.22	0.24	0.37
Verrucomicrobia	11.02±6.11	8.32±3.28	6.65±4.44	0.19	0.34	0.4
Class						
Bacteroidetes						
Bacteroidia	10.1±7.1	46.24±8.3	56.7±8.5	0.01*	0.01*	0.04*
Firmicutes						
Bacilli	7.2±5.2	2.9±2.53	2.7±1.3	0.37	0.09	0.26
Clostridia	57.0±13.1	34±9.2	28.3±11.3	0.06	0.03*	0.09
Proteobacteria						
Gammaproteobacteria	8.9±13.6	4.99±5.8	3.1±5.8	0.27	0.17	0.17
Tenericutes						
Mollicutes	0.00	0.00	0.00	0.22	0.24	0.24
Verrucomicrobia						
Verrucomicrobiae	11.08±6.10	8.32±3.28	6.6±4.4	0.19	0.34	0.34
Order						
Bacteroidia						
Bacteroidales	10.17±7.10	46.24±8.36	56.73±8.45	0.00*	0.00*	0.00*
Bacilli						
Lactobacillales	5.34±5.31	2.19±1.81	1.97±1.34	0.54	0.14	0.33
Clostridia						
Clostridiales	56.96±13.13	34.92±6.88	28.25±11.29	0.06	0.01*	0.02*
Gammaproteobacteria						
Enterobacteriales	8.91±13.61	4.99±7.57	3.03±5.75	0.22	0.17	0.32
Pasteurellales	0.00±0.00	0.00±0.00	0.10±0.19	0.34	0.78	0.63
Mollicutes						
RF39	0	0	0	0.1	0.06	0.12
Verrucomicrobiae						
Verrucomicrobiales	11.02±6.11	8.32±3.28	6.64±4.44	0.19	0.34	0.4
Family						
Bacteroidales						
Bacteroidaceae	3.15±4.1	5.14±1.95	14.62±6.09	0.00*	0.02*	0.01*
Porphyromonadaceae	0.61±0.81	1.05±0.61	1.92±1.04	0.04*	0.03*	0.05*
Prevotellaceae	0.01±0.02	1.58±1.97	1.78±2.09	0.07	0.00*	0.01*
Lactobacillales						
Lactobacillaceae	3.39±4.56	1.95±1.78	1.91±1.31	0.76	0.92	0.92
Streptococcaceae	0.22±0.09	0.07±0.11	0.03±0.03	0.13	0.00*	0.02*

Clostridiales						
Clostridiaceae	4.90±3.56	0.81±0.65	0.28±0.31	0.01*	0.00*	0.00*
Pasteurellales						
Pasteurellaceae	0.00±0.01	0.00±0.01	0.11±0.19	0.34	0.78	0.63
Enterobacteriales						
Enterobacteriaceae	8.91±13.61	4.99±7.57	3.03±5.76	0.23	0.17	0.32
RF39						
RF39; f__	0.00	0.00	0.00	0.10	0.06	0.12
Verrucomicrobiales						
Verrucomicrobiacea	11.02±6.11	8.32±3.28	6.64±4.44	0.19	0.34	0.4
Genus						
Bacteroidaceae						
Bacteroides	3.15±4.11	5.13±2.57	14.6±6.09	0.00*	0.02*	0.01*
Porphyromonadaceae						
Parabacteroides	0.61±0.82	1.05±0.62	1.92±1.05	0.04*	0.03*	0.05*
Prevotellaceae						
Prevotella	0.01±0.02	1.32±1.97	2.00±2.09	0.07	0.00*	0.3
Lactobacillaceae						
Lactobacillus	3.39±4.56	1.00±1.72	1.80±1.27	0.76	0.92	0.92
Streptococcaceae						
Streptococcus	0.08±0.03	0.03±0.11	0.10±0.03	0.23	0.02*	0.07
Lactococcus	0.14±0.08	0.98±0.00	0.00±0.00	0.06	0.00*	0.00*
Clostridiaceae						
Clostridium	2.45±2.12	0.04±0.04	0.00±0.01	0.02*	0.00*	0.01*
Pasteurellaceae						
Haemophilus	0.05±0.10	0.00±0.00	0.11±0.19	0.34	0.78	0.63
Enterobacteriaceae						
*Enterobacteriaceae; g_*	8.90±13.61	4.99±7.57	3.03±5.76	0.23	0.17	0.32
RF39; f__						
RF39; f__; g__	0.00	0.00	0.01±0.01	0.10	0.06	0.12
Verrucomicrobiacea						
Akkermansia	7.45±6.32	8.32±3.28	6.64±4.44	0.19	0.34	0.4

3.5.4 Bacterial translocation

To investigate whether insulin resistance and/or HFD could facilitate bacterial translocation, lymph and portal blood samples were cultured on FAA. Samples from 3 of 18 rats were excluded due to contamination. Counts ranging from 1 - 8 individual colonies were found on 6 of the 15 lymph samples and 0 of 15 portal blood samples. These data indicate translocation of live bacteria to lymph (but not portal blood). Two rats per group had positive bacterial culture from lymph indicating no difference in frequency of translocation between groups. Sequencing of isolates from culture-positive samples identified that *Enterococcus* was present in each of the treatment groups with *Olsenella* present in chow fed lean rats **(Table 3.4)**, consistent with translocated bacteria being of intestinal origin .

3.5.4.1 Green fluorescent protein tagged *E. coli* experiment validates translocation from the gut

To verify the route of translocation, GFP tagged *E. coli* were generated and enterally infused with intralipid. Selective and non-selective culture was performed on lymph samples. The appearance of live *E. coli* in the lymph was relatively infrequent with only two lean and one obese rats identified as positive for GFP-labelled *E. coli*. GFP *E. coli* was not detected in lean and obese rats fed a HFD. Other bacteria isolated from lymph in this experiment included, *Lactobacillus, Clostridium, Enterococcus, Enterobacter, Clostridium*, and *Bacillus* **(Table 3.5)**. Consistent with the first lymph culture experiment, approximately 2/3 of samples were sterile, with 11/32 samples showing positive cultures with bacterial growth.

Table 3.4. Identified bacteria from lymph (Intralipid infusion) of lean and obese rats after Sanger sequencing

Rats	Included animals	culture positive samples in lymph samples	Identified gut bacterial genus lymph samples	culture positive samples in portal vein blood
Lean rats (chow)	5	2	*Enterococcus faecalis, Olsenella spp.*	0
Obese rats (chow)	6	2	*Enterococcus faecalis*	0
Obese rats (HFD)	4	2	*Enterococcus faecalis*	0

Table 3.5 Identified labelled GFP *E coli* and another gut bacterial genome collected from lymph sample during Intralipid infusion and portal vein blood of lean and obese rats on chow diet and HFD, after Sanger sequencing

Rats	Included animals	culture positive samples in lymph samples	Confirmed GFP *E coli* in lymph samples	Identified gut bacterial genus in lymph samples	Culture positive samples in portal vein blood	Confirmed GFP *E coli* in portal vein blood
Lean rats (chow)	11	4	2	*Lactobacillus johnsonii, Clostridium sartagoforme*	0	0
Lean rats (HFD)	6	2	0	*Clostridium sartagoforme Enterococcus gallinarum Enterobacter cloacae*	0	0
Obese rats (chow)	11	4	1	*Enterobacter cloacae, Clostridium sartagoforme, Bacillus licheniformis*	0	0
Obese rats (HFD)	4	1	0	No quality sequence obtained	0	0

3.5.5 High fat diet did not impact serum concentration of lipopolysaccharides

High fat diet consumption has been associated with modulation of endotoxin absorption in the intestinal epithelium and increased translocation of LPS in blood, therefore circulating LPS concentration in plasma was measured. Although mean values were numerically higher in serum samples from HFD fed animals as compared to control diet fed rats, there was no difference ($p = 0.715$) in serum LPS between treatment groups (**Figure 3.8).**

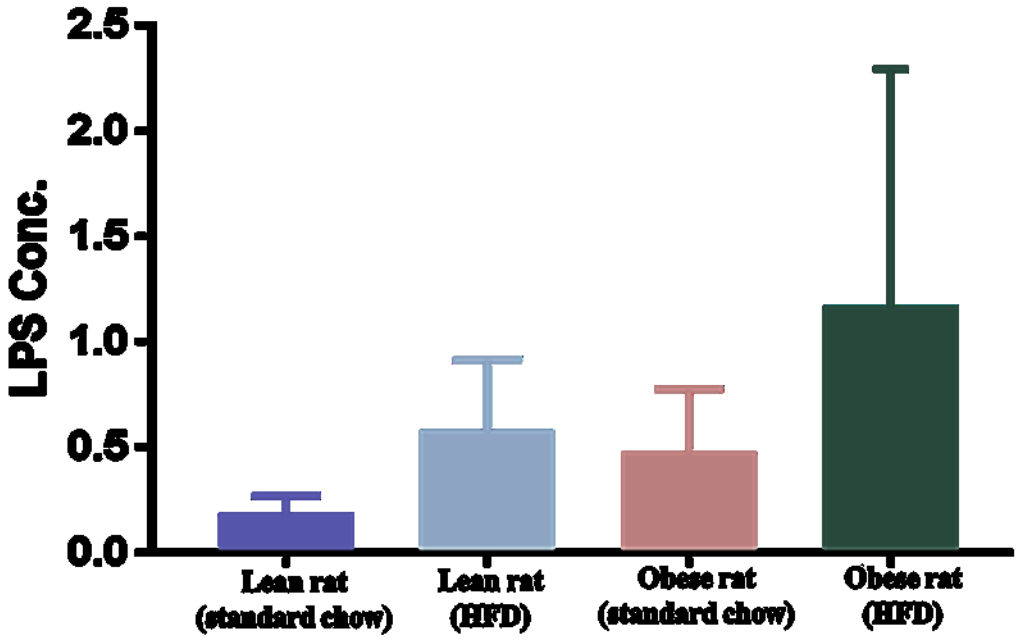


Figure 3.8 Concentration of serum lipopolysaccharide (LPS) in lean and obese rats with and without high fat diet.
LPS levels were measured by PYROGENT-5000 kit and followed the protocol as per manufacturer's instructions (Lonza, Mississauga, Canada). LPS were numerically higher in serum samples from HFD fed animals but there was no significance difference ($p=0.715$) in serum LPS

3.6 Discussion

In the current study, we used the JCR:LA-cp rat strain, a rodent model of insulin resistance and MetS (Koletsky, 1973) to characterize the effects of increased energy intake on the microbiome as well as bacterial translocation. Contrary to our hypothesis, the phenotype of the JCR:LA-cp strain did not show a significant alternation in intestinal microbiota when fed on a chow diet for 12 weeks. These results are not consistent with previous studies where male Tsumura Suzuki obese diabetes (TSOD) (mice spontaneously develop obesity and obesity-related metabolic syndrome) fed on chow diet for 24 weeks displayed an increased *Firmicutes*/*Bacteroidetes* ratio (Nishitsuji et al., 2017). In our study, we used young rats (12 weeks old) that were fed for shorter duration (6 weeks chow diet). The longer intervention period and older age of rats used by Nishitsuji et. al may explain the differential result on gut microbiota. Some human studies have reported shift in the ratio of *Firmicutes*/*Bacteroidetes* in obese relative to lean healthy individuals. This change in gut microbiota during obesity remains a matter of significant debate (Everard & Cani, 2013; Jumpertz et al., 2011).

3.6.1 High fat diet increases in *Firmicutes*/*Bacteroidetes* ratio

Overall, the data from the current study indicate that neither increased energy intake or the obese genotype per se influence the gut microbiota composition (atleast in JCR:LA-cp rats). In contrast feeding a HFD resulted in substantial changes in intestinal microbiota, including an increase in *Firmicutes*/*Bacteroidetes* ratio. At the genus level, a HFD increased both *Clostridium* as well as unclassified *Enterobacteriaceae*, while reducing *Prevotella* and *Bacteroides*. These observation are in accordance with recent studies utilizing HFD to induce obesity (HFD) (Hamilton, Boudry, Lemay, & Raybould, 2015; Lecomte et al., 2015).

3.6.2 Live gut bacteria appear in mesenteric lymph

During obesity, there is an increase in the concentration of circulating triglyceride and inflammation in metabolic tissues (Ventura et al., 2017). Lipopolysaccharide has been known as one of the major factors to cause inflammation during obesity (Hersoug, Moller, & Loft, 2018). HFD intake is also associated with gut dysbiosis and systemic low-grade inflammation, these features includes; impaired intestinal mucus production and decreased expression of tight junction proteins that inturn facilitate the translocation of bacterial products (Bleau, Karelis, St-Pierre, & Lamontagne, 2015). While the translocation of bacterial products have been extensively studied, the translocation of live bacteria has not been well characterized. This is the first study to explore the appearance of live bacteria directing into the lymphatics using a model of insulin resistance. Bacterial translocation into mesenteric lymph was confirmed in approximately one third of lymph samples. The bacteria most commonly identified were *Enterococcus* and *Enterobacter,* which is with previous research, where translocation of *E. coli, Clostridium, Enterococcus* and *Enterobacter* were most common in Crohn's disease patients (Laffineur et al., 1992). Other typical gut bacteria such as *Clostridia* and *Lactobacillus* species were also identified in lymph samples. To our knowledge, our study is the only second to demonstrate the presence of bacteria in lymph draining from the intestine. Cole et al collected lymph from central thoracic duct under sterile conditions in 50 patients (Cole, Witte, & Witte, 1969), collected lymph from central thoracic duct under sterile conditions in 50 patients. At the same time, central venous blood from superior vena cava were collected from 30 of the 50 (60%) patients who had a wide variety of disorders. After incubation they found 20 of 50 patients (40%) of lymph and 6/30 or 20% of central venous blood cultures were culture positive. They detected a wide variety of microorganisms (pathogens and "non-pathogens") such as *Aerobacter aerogenes*, *Streptococcus, Clostridia sp*, *Corynebacterium sp*, *E. coli, Micrococcus sp*., *Mycobacterium tuberculosis*, and *Proteus sp.* (Cole et al., 1969).

3.6.3 Intestine as a source of bacterial translocation into mesenteric lymph

From our GFP tagged *E. coli* experiments, we were able to confirm evidence of direct translocation. However, contrary to our hypothesis we did not detect any evidence of increased translocation in the obese condition.

Kalischuk et. al showed *Campylobacter jejuni* promote translocation of non-invasive bacteria in IBD patients via lipid raft (Kalischuk et al., 2009). Interferon gamma is also known to induce translocation of commensal *E. coli* via a lipid raft mediated process (Clark, Hoare, Tanianis-Hughes, Carlson, & Warhurst, 2005). Other studies have reported that diets rich in lipid cause low-grade gut inflammation, reduced expression of tight junction coding genes and resulting in disruption of intestinal permeability (Ding et al., 2010; Lam et al., 2012). It was expected that altered intestinal integrity as well as increased lipid transport in the JCR:LA-cp rats would facilitate increased translocation either via lipid rafts or through disrupted intestinal barrier integrity. While no evidence was obtained that more live bacteria translocate perse, it remains possible that more bacteria may translocate, but are quickly filtered/removed by the immune system before arriving in the draining lymphatics. Some mechanism that are worthy of consideration given these observations are summarized in following figure **(see Figure 3.9)**.

To our knowledge, ours is the first study to observe the effect of HFD on gut microbiota in a rodent model of spontaneous insulin resistance (JCR:LA-cp rats). Our study documented effect of HFD on gut microbiota however, our finding do not support the impact of IR to microbiota alone. Futher we reveal evidence of bacterial translocation but neither obesity or HFD was found to exacerbate the condition. More research is needed to investigate the mechanisms behind our initial finding by exploring lipid/fat type,quality as well as their role for the regulation of bacterial translocation.

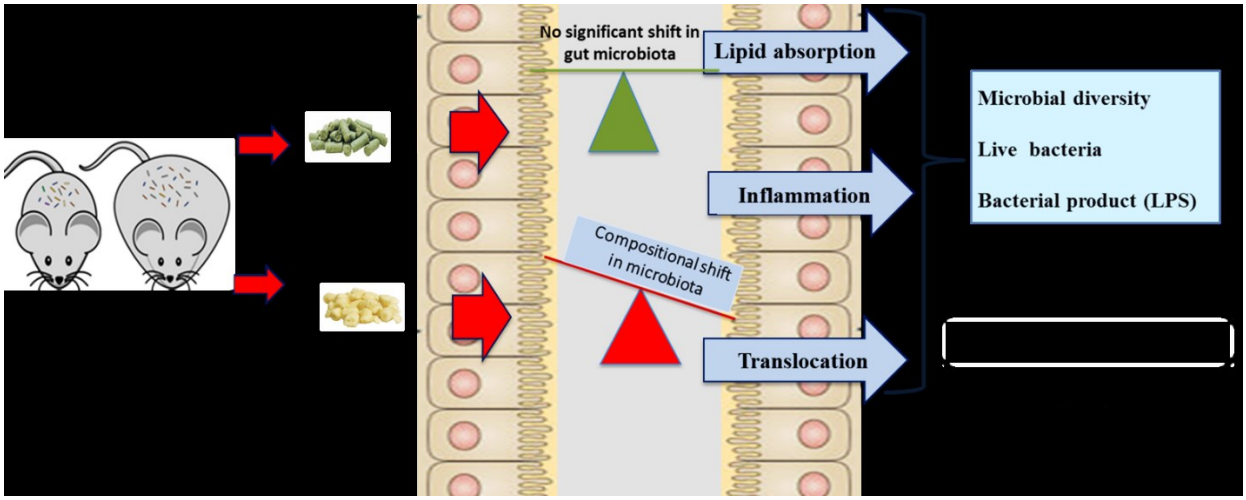


Figure 3.9 High fat diet induced dysbiosis and bacterial translocation in obese condition (overview and proposed mechanism)

Chapter 4: Investigation of the High fat high carbohydrate diet (HFHC) (with and without altering fatty acid composition) on intestinal microbiota in low birth weight swine:

4.1 Personal contribution for this Chapter

The first phase of experiments described in this chapter was led by a post-doctoral fellow. I assisted with the pilot phase of this work and took a lead role in collecting fecal sample and analyzing swine gut microbiota. For the follow up experiment (vaccenic acid intervention), I was equally involved with the assistance of a post-doctoral fellow, other laboratory staff and one MSc student. In this chapter my focus was to investigate gut microbiota profile in a newly established swine model of LBW and insulin resistance.

4.2 Introduction

Metabolic syndrome (MetS), defined by a cluster of conditions including hypertension, central obesity, dyslipidemia and hyperglycemia, is a major and growing health concern in both western and developing countries (Eckel, Grundy, & Zimmet, 2005; Grundy, 2004; Ogden, Lamb, Carroll, & Flegal, 2010; Suarez-Ortegon & Aguilar-de Plata, 2016). MetS is associated with risk factors such as type 2 diabetes and cardiovascular disease. Genetic background, excessive food consumption, sedentary life style, and decreased physical activity are the main predisposing factors for the disease. Epidemiological evidence from the 1944 Dutch Hunger Winter (Schulz, 2010) revealed a relationship between low birth weight (LBW) [a possible consequence of intrauterine growth restriction (IUGR)] and the predisposition to obesity and diabetes in adulthood (Barker, 2001; Gluckman & Hanson, 2004; McMillen et al., 2009; Visentin et al., 2014). These landmark

observations have underpinned the concept of the developmental origins of adult health and disease (Barker et al., 1993; van Steijn et al., 2009).

Globally, financial costs due to long-term metabolic disease risk associated with LBW offspring are inestimable (Hack et al., 1993; Schmidt et al., 2003). Several experimental studies have replicated IUGR (leading to LBW) using rodent models via a number of approaches: maternal diet restriction during gestation, administration of dexamethasone or by temporary uterine artery ligature to create placental insufficiency (McMillen & Robinson, 2005). These experimental approaches have enabled the field to investigate molecular and cellular mechanisms of fetal programming of chronic diseases in adulthood. While rodents are a useful model for multivariable experiments, they display several important differences in metabolism and physiology compared to humans (Davis, Cain, Banz, & Peterson, 2013). For example, in the normal mouse atheroprotective high-density lipoprotein (HDL) dominates the lipoprotein profile, whereas in humans low density lipoprotein (LDL) is most abundant (Kennedy, Ellacott, King, & Hasty, 2010; Spurlock & Gabler, 2008). Unlike rodents, swine possess many anatomical and physiological similarities to humans. Organ size, gastrointestinal physiology and metabolic features of swine are biologically more akin to humans (Guilloteau, Zabielski, Hammon, & Metges, 2010). Furthermore, swine exhibit naturally occurring IUGR, due to their ability to maintain large litter sizes that often exceed the utero-placental capacity (Wu, Bazer, Wallace, & Spencer, 2006). In the commercial sector, pork production trends have also increased litter size resulting in an increase in the average number of piglets born LBW, making it a readily accessible pre-clinical model.

Interestingly, previous work in the area has demonstrated that LBW per se is not the preceptor to adult metabolic disorders. Nutritional and environmental factors have also been shown to be particularly influential (Plagemann, Heidrich, Gotz, Rohde, & Dorner, 1992; Shepherd,

Crowther, Desai, Hales, & Ozanne, 1997). The "thrifty gene hypothesis" stipulates that a sub-optimal in utero environment leads to fetal adaptations to ensure short-term survival. Metabolic disorders such as obesity and type 2 diabetes manifest when the postnatal environment does not reflect that experienced in utero (Halliday, 2009; Vaag, Grunnet, Arora, & Brons, 2012; Varvarigou, 2010). These multifactorial lifestyle factors support the role of the postnatal environment (diet) contributing to development of adult chronic disease.

Postprandial hypertriglyceridemia (a consequence of increased intestinal lipoprotein secretion and reduced clearance of dietary-TG) has been linked to the development and exacerbation of cardiovascular diseases (Bansal et al., 2007; Hyson, Rutledge, & Berglund, 2003; Jackson, Poppitt, & Minihane, 2012). Further, evidence has demonstrated that insulin resistance (IR) is associated with overproduction and secretion of intestinal-derived lipids, contributing to postprandial atherogenic dyslipidemia (Adeli & Lewis, 2008; Duez et al., 2006; Vine et al., 2007). However, the effects of LBW and underlying mechanisms on intestinal lipid absorption leading to adult metabolic diseases are still not well understood.

4.4 High-fat, high-carbohydrate diet with *trans*-fat and low birth weight swine of insulin resistance

Trans fatty acids are a family of molecules with long hydrocarbon chain and a double bond in the trans configuration. Various forms of *trans* fats exist, that differ in their fatty acid length and structure. Some *trans* fats, used in industrial food production are produced synthetically via partial hydrogenation of vegetable oils. These industrially produced vegetable fats increase circulating total and LDL cholesterol, resulting in increase risk of coronary heart disease (Brouwer, Wanders, & Katan, 2010). By association, naturally occurring *trans* fats derived from ruminant animals have been affiliated with the same hypothesis. The structural differences between synthetic and natural

trans fats have led to research investigating the specific properties and health effects between the two types of molecules. The most abundant *trans* isomer in meat and dairy products is vaccenic acid (VA). It is produced through the incomplete biohydrogenation of the polyunsaturated fatty acids (PUFAs), a-linoleic acid (ALA) and linolenic acid (LA) in ruminant animal. Conjugated linoleic acid (CLA), also defined as a *trans* fat, has been shown to exhibit insulin sensitizing effects and improve lipid metabolism via the peroxisome proliferator-activated receptor alpha (PPARα) signaling pathway (Choi, Jung, Park, & Song, 2004; Wang et al., 2012).

My research group has shown that dietary VA supplementation, for both a short term (3 week) and long term (16 week) in the dyslipidemic JCR:LA-*cp* rodent model, to decreased plasma TG (Jacome-Sosa et al., 2016; Wang et al., 2009; Wang et al., 2008). Supplementation of VA activates peroxisome proliferator-activated receptor alpha (PPARα) (Jaudszus et al., 2012; Wang et al., 2012), which is present in high abundance in liver, intestine, muscle and heart tissues. PPARα works as a regulator of lipid oxidation (Yu, Correll, & Vanden Heuvel, 2002) and increased expression of CD36 as well as increase β-oxidation, resulting increase in FA uptake. For activation of this process peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) work in coordination and activate genes involved in the citric acid cycle and regulate transcription of genes involved in oxidative phosphorylation. Studies have shown that ingestion of diet rich in fat may reduce expression of PGC-1α, as a result, incomplete β-oxidation and leads to increase in IR (Nagatomo et al., 2012). However, the addition of VA in the diet can potentially counter the effect of high-fat diet and improve insulin sensitivity.

4.4.1 Association between dietary fat, gut microbiota and metabolic syndrome

Recently, it has been observed that the gut microbiota can also affect metabolic processes and contributes to obesity and its comorbidities (such as insulin resistance, diabetes and

cardiovascular disease). The advancement in new sequencing technology has improved our understanding about human gut microbiota. The quality of dietary fat is also known to affect gut microbiota composition. For example, saturated fat rich diet (palm oil) increases the ratio of *Firmicutes* to *Bacteroidetes* and reduces microbial diversity as compared to *n*-6 polyunsaturated fat (PUFA)) or monounsaturated fat (de Wit et al., 2012; Patterson et al., 2014). Due to the divergent nutritional requirements of various bacteria colonizing the gut, it is also well established that diet shapes the composition of the intestinal microbiota in mice and humans (Faith et al., 2011; Hildebrandt et al., 2009; Li et al., 2017b). Specifically, consumption of a western type diet has been shown to alter the composition of the gut microbiota (Carmody et al., 2015; J. J. Faith et al., 2011; Hildebrandt et al., 2009; Murphy et al., 2010), which in turn may promote the development of obesity and associated chronic diseases (Turnbaugh et al., 2008). In support of this notion, studies reported that in infancy, LBW individuals have a more dysbiotic gut microbiome compared to normal birth weight individuals (Costello, Carlisle, Bik, Morowitz, & Relman, 2013).

Based on the aforementioned findings, we hypothesized that LBW-induced changes in the gut microbiota would contribute to altered intestinal lipid absorption leading to the development of dyslipidemia and associated metabolic syndrome phenotype.

4.4.2 Hypothesis and objectives

Given findings from chapter 3, we hypothesized that LBW pigs fed a western (high fat, high fructose and cholesterol) [HFHC] diet would develop metabolic complications, contribute to impaired post-prandial dyslipidemia and induce dysbiosis in gut microbiota relative to their NBW littermates. We further hypothesized that supplementation of HFHC diet rich in VA fat would result in an reduced gut dysbiosis and improved lipid metabolism profile.

4.4.3 Materials and methods

4.4.3.1 Animals

Male Duroc boar and Large White/Landrace piglets were obtained and weaned at the Swine Research and Technology Center (SRTC), Department of Agriculture, Food and Nutritional Science, University of Alberta. All procedures were approved by the University's Animal Care and Use Committee– Livestock (ACUC) following guidelines from the Canadian Council on Animal Care (CCAC). All piglets were weighed weekly, house individually (from 4 week of age) and food intake measured daily. To ensure a low stress during handling and sampling, each piglet was socialized at minimum of twice weekly from 3 weeks of age.

4.4.3.2 Low and normal birth weight selection

A mean litter mean weight was determined to calculate a 95% confidence interval (CI), We categorized piglets as LBW (less than the 95% CI) or NBW (within or above the 95% CI). An unpaired t-test was used to ensure a significant difference in weight between the two groups. At birth, anthropometric measurements were taken, including crown-rump length (CR), snout-crown length (SC) and abdominal circumference (AC). Piglets suckled for 3 weeks before being weaned onto a standard control diet and moved into individually housed pens in a metabolic research room where they resided until study end.

4.4.3.3 Study design

Study 1 (Effect of HFHC diet on gut microbiota in LBW swine)

Newborn piglets (n=16) were selected and weighed within 24 hours of full-term birth from a total of 4 sows. At 5 weeks of age, LBW (n=8) and NBW (n=8) piglets were gradually switched onto either phase 2 control diet (control) or high-fat, high-carbohydrate diet (HFHC) and switched to phase 3 control and HFHC diet 1 week later. The block design yielded four experimental groups: LBW-Control, LBW-HFHC, NBW-Control and NBW-HFHC (n=4 per group). At 12 weeks of

age, pigs underwent an adapted 2-step modified oral glucose tolerance and fat challenge test (MOGTT) collecting samples via the ear vein using capillary tubes. Pigs were anesthetized at 13 weeks of age. Lymph has sampled prior to exsanguination and all other tissus collected at study end. **(Figure 4.0).**

Study 2 (Effect of HFHC diet rich with VA on gut microbiota in LBW swine)

Newborn piglets (n=6 NBW and n=16 LBW) were selected and weighed within 24 hours of full-term birth from a total of 6 sows. After weaning (at 4 week of age), pigs were switched onto their treatment group diets **(see Table 4.0 for diets)** normal birth weight control (NBW-Control, n=6), low birth weight control (LBW-Control, n=5), low birth weight high-fat, high-carbohydrate diet (LBW-HFHC, n=5) and low birth weight high-fat, high-carbohydrate diet with VA (LBW-HFHC+VA, n=6). At 9 weeks of age the pigs were fasted overnight, and jugular catheters were implanted. At week 10, a modified oral glucose tolerance and fat challenge test (MOGTT) was conducted. At 11 weeks of age pigs were anesthetized, lymph sampled, euthanizated, tissues and samples collected **(see Figure 4.1).**

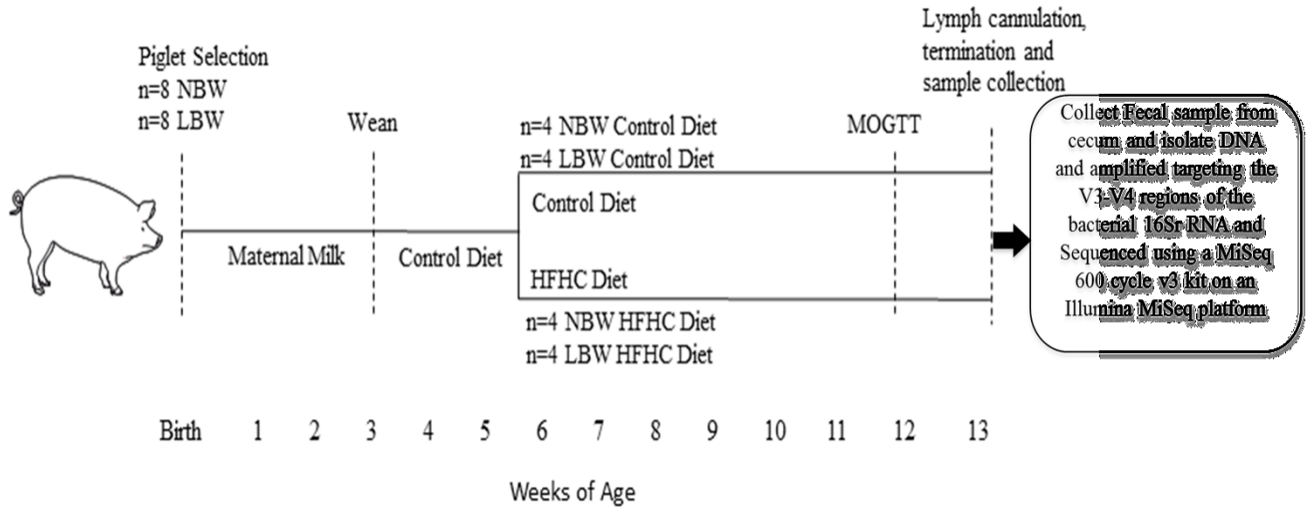


Figure 4.0 Study design to investigate the effect of HFHC diet on gut microbiota in LBW swine.

LBW, low birth weight; NBW normal birth weight; MOGTT, modified oral glucose tolerance test; HFHC, high-fat, high-carbohydrate; HFHC.

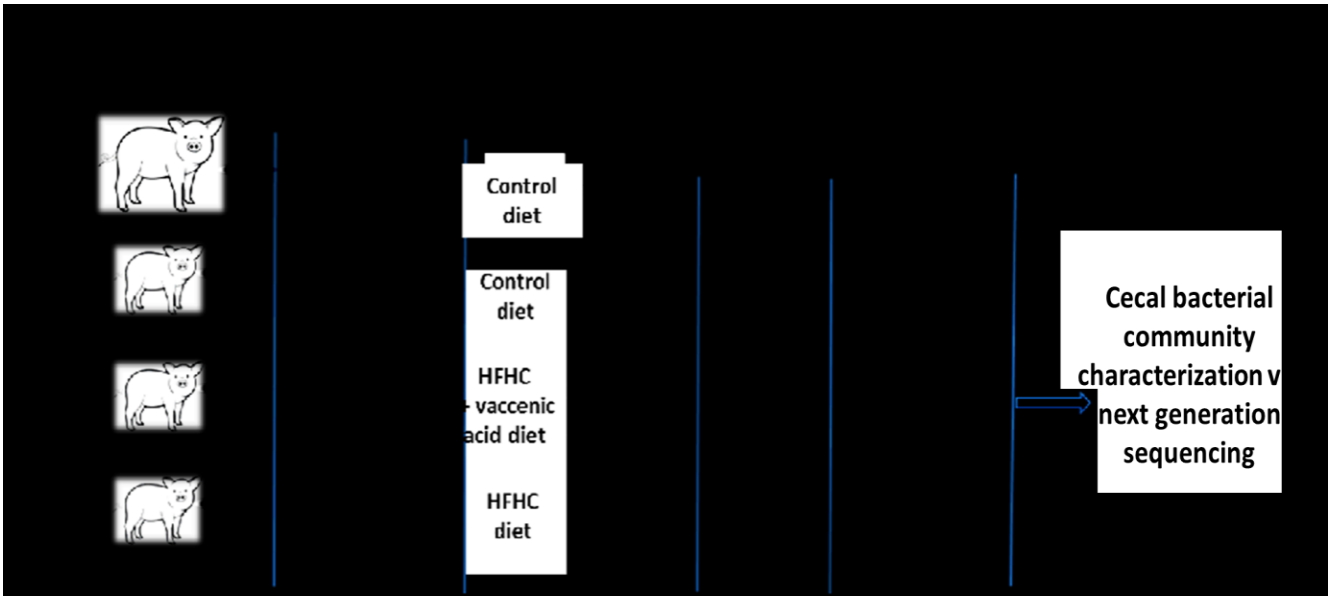


Figure 4.1 Study design schematic for low birth weight (LBW) and normal birth weight (NBW) piglets fed control, HFHC diet, or HFHC diet supplemented with VA

LBW, low birth weight; NBW normal birth weight; MOGTT, modified oral glucose tolerance test; HFHC, high-fat, high-carbohydrate; VA, vaccenic acid.

4.4.4 Diet

The diets (**detailed in Table 4.0)** were formulated to meet or exceed the nutrient requirements of growing-finishing pigs (National Research Council (U.S.), 2012). At 6 weeks of age, the pigs were housed individually in an environmentally controlled room and were given ad libitum access to both water and food.

For study 2, we used high-fat, high-carbohydrate diet by incorporating beef fat obtained from the Lacombe Research and Development Center, Lacombe, Alberta, Canada. The high VA enriched beef fat was collected from steers fed extruded flaxseed (25%) and hay (75%) sequentially, as previously described (Vahmani et al., 2017). Fat without VA was obtained from a commercial packing plant from steers fed a high barley-grain diet. Methods for fat collection and rendering are as previously described (Diane et al., 2016). Fatty acid composition **(Table 4.1)** was determined as previously described using an adapted method (Diane et al., 2016, Aldai et al., 2013). Diets were formulated to meet or exceed nutrient requirements of starter pigs (National Research Council (U.S.), 1998). All the experimental animals were given ad libitum access to feed and water.

Table 4.0. Ingredients of control and high-fat, high-carbohydrate diets.

Phase 2 diet was fed as creep-feed during suckling and post-weaning for 1 week. Phase 3 diet was fed ad libitum from completion of Phase 2 until termination. Feed was gradually changed between diet phases.

Ingredient, g/kg	**Control** Phase 2	**HFHC** Phase 2	**Control** Phase 3	**HFHC** Phase 3
Oats	100.00	.	59.0	.
Wheat	463.8	259.4	599.7	229.4
Wheat DDG	30.0	.	50.0	.
High lactose whey	125.0	.	.	.
Soybean meal	168.0	250.0	123.0	230.0
Corn	.	.	94.0	.
Fructose	.	178.0	.	178.0
Fishmeal	37.5	70.0	.	120.0
Fat (lard)	40.0	178.1	25.0	178.1
Flaxseed oil	.	1.9	.	1.9
Cholesterol	.	10.0	.	10.0
Limestone	15.0	8.6	15.0	8.6
Trace mineral swine pre-mix‡	.	6.0	.	6.0
Vitamin swine pre-mix§	.	6.0	.	6.0
Salt (NaCl)	0.5	5.0	4.7	5.0
L-Lysine	5.2	9.4	8.3	9.4
Methionine	2.0	5.0	2.7	5.0
L-Threonine	2.2	4.7	3.3	4.7
L-Tryptophan	0.1	0.9	0.4	0.9
Vitamin E – 5 KIU/kg premix [a]	1.0	.	0.5	.
Dicalcium phosphate	.	7.0	4.3	7.0
Copper Sulfate	.	.	0.4	.
Others$^{\phi}$	9.8	.	9.8	.

‡ Provided the following per kg of diet: Zn, 100 mg as ZnSO4; Fe, 80 mg as FeSO4; Cu, 50mg as CuSO4; Mn, 25 mg as MnSO4; I, 0.5 mg as Ca(IO3)2; and Se, 0.1 mg as Na2SeO.

§ Provided the following per kg of diet: vitamin A, 8,250 IU; vitamin D3, 825 IU; vitamin 666 E, 40 IU; niacin, 35 mg; D-pantothenic acid, 15 mg; riboflavin, 5 mg; menadione, 4 mg; folic acid, 2 mg; thiamine, 1 mg; d-biotin, 0.2 mg; and vitamin B12, 0.025 mg.

ϕ Provided the following per kg of diet: Vitamin mineral premix, 2.5 g; Bio-mos [b], 2.0 g; Bioplexzinc 15%, 1.70 g; Tetracid 500, 1.0 g; Water for enzyme application, 0.8 g; Choline, 0.7 g; Maxi-grow flavor [c], 0.5 g; Bioplus 2B [d], 0.40 g; Superzyme [c], 0.2 g.

[a] Viterra, Sherwood Park, AB, Canada

[b] Alltech, Nicholasville, KY, USA

[c] Canadian Biosystems, Calgary, AB, Canada

[d] Chr Hansen, Milwaukee, WI, USA

e DDG, dried distillers grain

Table 4.1. Fatty acid profiles for major fats in each HFHC diet using % FAME composition

Abbreviation	% in control diet	% in HFHC diet	% in HFHC with VA diet
C14:0	0.092	4.14	3.76
C16:0	15.688	30.41	22.54
C17:0	0.089	1.30	1.06
C18:0	1.672	25.62	21.00
c9-18:1	21.340	27.28	19.94
t11-18:1 (VA)	**n.d.**	**0.76**	**10.07**
c9-16:1	0.212	1.61	1.13
c11-18:1	1.765	0.74	0.71
c9, c12-18:2	52.873	0.95	1.13
c9, t11-18:2	n.d.	0.2	1.28
t11, c13- 18:2	n.d.	0.00	0.95
t11 ,c15-18:2	n.d.	0.09	2.74
t13-t14- 18:1	n.d.	0.41	1.58
C18:3n-3	4.200	0.20	1.12
Minor Fats	2.069	7.24	10.99
Total	**100**	**100**	**100**

n.d. is used when the percentage of the specific fat was too low to be determined

4.4.5 Modified oral glucose tolerance test

11-week-old piglets were fasted overnight and subject to an adapted 2-step MOGTT. Pigs were weighed and a fasted (time 0) sample of blood was collected. Pigs then consumed 25g of control diet mixed with 1g/kg body weight Devonshire cream (47% milk fat w/w) and 2g/kg body weight 50% glucose solution. Blood samples were taken at 15, 30, 60, and 120 min to assess the corresponding glucose response. A second meal was given at the 120 min timepoint containing only 25g control diet supplemented with Devonshire cream (1g/kg body weight). Blood was further collected at timed intervals at 180, 240, 300 min, in order to assess postprandial lipid response. Blood was collected into EDTA coated tubes. Plasma was isolated by centrifugation at 3500rpm for 10min at 4^{o}C and aliquots stored at -80^{o}C.

4.4.5.1 Mesenteric lymph duct cannulation and nascent lymph collection

At 13 weeks of age (7 weeks of diet), pigs were fasted overnight, weighed and general anesthetic was induced. Anthropometric measurements were taken as was done at birth. Under anesthesia, a cannula was implanted into the superior mesenteric lymph duct and lymph was collected into an EDTA-coated Vacutainer^{TM} as previously described (Uwiera, Mangat, Kelly, Uwiera, & Proctor, 2016). Total lymph volume as well as lymph flow rate (lymph volume/hr) was recorded and the animal was euthanized via exsanguination under anesthetic.

4.4.5.2 Sample collection and processing

Fasted blood was collected along with tissue samples, including heart, liver, kidney, adipose, muscle, intestine (jejunum and ileum), intestinal scrapings (jejunum and ileum) as well as fecal samples from proximal distal colon and cecum and all snap frozen in liquid nitrogen prior to storage at -80^{o}C. Prior to freezing, all tissue samples were flushed with ice-cold sterile saline. Blood and lymph were placed on ice until centrifugation at 3500g for 10 min at 4^{o}C, aliquoted and

stored at -80ºC. Fresh lymph was used to determine particle size by an automated laser particle sizer (Zetasizer Nano-ZS, Malvern Instruments Ltd., Malvern, UK) to assess size based on Brownian motion using Dynamic Light Scattering (DLS).

4.4.5.3 Plasma and lymph biochemical analysis

Plasma and lymph were assessed for lipid profiles using commercially available enzymatic colorimetric kits, including triglyceride (TG) (WAKO, Chemicals USA Inc., Richman, VA, USA, Cat#461-08992), glucose (WAKO, Cat#439-90901), LDL-cholesterol (WAKO, Cat#993-00404/999-00504), HDL-cholesterol (WAKO, Cat#993-72593/993-72693) and total cholesterol (TC, WAKO, Cat#439-17501). Plasma insulin levels were assessed using a commercially available porcine-specific enzyme-linked immunosorbent assay (ELISA) with a detection limit of 0.007ng/ml and intra-assay coefficient of variation (CV%) of 4.0% at 0.255ng/ml (ALPCO, Salem, New Hampshire, USA). For lipoprotein cholesterol and TG analysis by fast protein liquid chromatography (FPLC) gel filtration technique, fresh plasma was transferred to Agilent autosample vials and sent for analysis to the University of Alberta Lipidomic core facility with post-column detection of both cholesterol and TG.

4.4.5.4 Protein analysis

Apolipoprotein B48 (apoB48) quantification

An adapted western blot procedure was used to determine the concentration of intestinally derived chylomicrons (CM) (D. F. Vine et al., 2007). SDS-PAGE on a 3-8% Tris-acetate polyacrylamide NuPage gel was used to separate lymph and plasma proteins (Invitrogen, Camarillo, CA, USA). Separated proteins were transferred to a polyvinylidene fluoride membrane (0.45um, ImmobilonTM, Millipore, Billerica, MA, USA). A goat polyclonal antibody specific for apoB (Santa Cruz Biotech, CA) was incubated with the membrane, which has specificity for both

apoB100 and apoB48 isoforms. An anti-goat secondary antibody tagged with hydrogen peroxidase (Santa Cruz Biotech, CA) was used for detection purposes and Enhanced Chemiluminescence (ECL) (Amersham Biosciences Little Chalfont, Bucks, UK) intensity was used to quantify and compare with a known mass of purified rodent apoB48 protein (Vine et al., 2007).

4.4.6 Caecal bacterial community characterization

Cecal digesta samples were collected at the end of the study and total DNA was extracted using a QIAamp DNA Stool Mini Kit (Qiagen, Inc.), according to the manufacturer's instructions, with the addition of a bead-beating step (FastPrep instrument, MP Biomedicals, Solon OH). DNA concentration was measured using a Quant-iT™ PicoGreen® dsDNA Assay Kit (Thermo Fischer Scientific). Extracted DNA was amplified targeting the V3-V4 regions of the bacterial 16Sr RNA genes using KAPA HiFidelity Hot Start Polymerase (Kapa Biosystems, Inc. Wilmington, MA, USA) with the following conditions: 95°C for 3 min, followed by 25 cycles of 95°C for 30 seconds, 55°C for 30 second, and 72°C for 30 second, and 72°C for 5 min with universal primers (Forward 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3'and Reverse 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3') (Klindworth et al., 2013). After amplification, AMPure XP beads were used to purify the 16S amplicon to remove primers and primer dimer species. Dual indices and Illumina sequencing adapters were attached using the Nextera XT Index Kit, followed by a second PCR cleanup and quantification. Samples were diluted to 4nM and 5 µL aliquots of each diluted DNA sample were pooled and size-selected and denatured with NaOH, diluted to 4 pM in Illumina HT1 buffer, spiked with 2- PhiX and heat denatured at 96°C for 2 min. The library was sequenced using a MiSeq 600 cycle v3 kit on an Illumina MiSeq platform (Illumina USA), according to the manufacturer's instructions.

Sequence data processing

I analyzed sequenced by using qiime pipeline (MacQIIMW 1.8.0 OS10.10) (Caporaso JG, et al. 2010). For quality filtering PANDAseq was used to assemble paired end reads into contigs with miscalled or uncalled bases discarded. UCHIME and UPARSE workflows were used to clear chimeras and singletons from resulting sequences and subsequently clustered into operational taxonomic units (OTUs) having >97% similarity with USEARCH (Edgar RC, 2010). QIIME default setting and Ribosomal Database Project classifier V2 were used to assigned taxonomy (Wang Q, et al, 2007). Alpha-diversity and beta-diversity estimations were conducted using the QIIME workflowcore_diversity_analysis.py (Seda A, et al 2015). Chao-1, simpson and shannon indices were used to estimate the alpha diversity (Colwell RK, et al 2012). Differences in microbial communities were investigated using Bray-Curtis dissimilarity. One-way analysis of similarity (ANOSIM) was applied to compare the differences of bacterial community structure between control and HFHC diets and birth weight. Diversity and abundance were compared using Kruskal-Wallis test.

4.4.6.1 Circulating endotoxin (LPS) Assay

Serum LPS concentrations were measured using PYROGENT-5000 kit as per manufacturer's instructions (Lonza, Mississauga, Canada). The absorbance was measured at 340 nm per minute for 1h at 37°C using a SpectraMax® M3 Microplate Reader (Molecular Devices, LLC. Sunnyvale, CA, USA). Reaction time was defined as the time required for the absorbance to increase 0.03 absorbance units.

4.4.6.2 Statistical analysis

Analyses were performed using GraphPad Prism version 7.02 for Windows, GraphPad Software, La Jolla California USA. Prior to analysis, data was estimated to be normally distributed using the Shapiro-Wilk test due to its ability to test normality with a lower sample size. Tabular

results are presented as mean ± SD while graphs are presented as mean ± SEM. Data presented was analyzed by two-way ANOVA (birth weight x diet) with repeated measures and multiple comparisons tested for using Fisher's LSD test. All tests and comparisons with $p<0.05$ were considered statistically significant. Differences in microbial communities by diet and birth weight were investigated using Bray-Curtis dissimilarity metrics. One-way analysis of similarity (ANOSIM) was applied to compare the differences of bacterial community structure between control and HFHC diets and birth weight. Diversity and abundance were compared using Kruskal-Wallis test.

4.7 Results

4.7.1 Body weight gain and food intake

At birth, LBW offspring were significantly lower in weight compared to those selected as NBW (birth weight effect, $p<0.0001$) as shown in **Table 4.2**. At 13 weeks of age, ending weights for LBW pigs were still significantly lower (birth weight effect, $p<0.0001$). Additionally, a diet effect was observed with NBW swine on a HFHC diet showing increased ending weight compared to NBW on control diet ($p=0.0089$). LBW pigs continued to consume less feed for the duration of the study ($p=0.0007$). Both a diet and birth weight effect were observed for the average daily weight gain (ADG), with NBW swine gaining more weight per day than LBW with HFHC diet (diet effect, $p=0.0143$, birth weight effect, $p<0.0001$). NBW swine ate more food per day on average compared to LBW groups ($p=0.0017$). The HFHC diet resulted in a lower feed conversion ratio (FCR) suggesting less feed was necessary to gain each kilogram of weight (diet effect, $p=0.037$). Additionally, LBW swine had an increased fractional growth rate (FGR) (diet effect, $p=0.0021$, birth weight effect, $p=0.0002$).

In study 2, I found no statistical difference in food intake across all the group, however LBW groups all had a greater fractional growth rate compared to NBW **(Table 4.3).** Normal birth weight piglets did gain more weight over the course of the study. Low birth weight piglets on both HFHC diets resulted in less crown-rump growth (body length) than the control diet groups (**Table 4.3).**

Table 4.2. Growth and feed consumption of LBW and NBW swine fed either control or high-fat, high-carbohydrate diet for 6 weeks after weaning.

	NBW-Control	LBW-Control	NBW-HFHC	LBW-HFHC	P		
					BW	Diet	Interaction
n	4	4	4	4			
BW, kg	1.65 ±0.13^{a}	1.13 ±0.10^{b}	1.5 ±0.08^{a}	0.92 ±0.15^{c}	<0.0001***	0.0114*	0.6774
FW, kg	57 ±2.3^{a}	49 ±17^{b}	62.9 ±1.7^{c}	50.1 ±3.0^{b}	<0.0001***	0.0089**	0.0545
ADG, g/d	621 ±10^{a}	525 ±22^{b}	672 ±20^{c}	541 ±34^{b}	<0.0001***	0.0143*	0.1572
ADF, kg/d	1.7 ±0.1^{a}	1.37 ±0.1^{b}	1.69 ±0.1^{a}	1.39 ±0.1^{b}	0.0017**	0.9256	0.8449
FCR, kg	2.06 ±0.18^{a}	1.94±0.32^{ab}	1.78 ±0.21^{b}	1.69 ±0.16^{ab}	0.3544	0.0368*	0.8800
FGR, g	378 ±34^{a}	470 ±59^{b}	441 ±11^{ab}	592 ±64^{c}	0.0002***	0.0021**	0.2319

Values are means ± SD. NBW, normal birth weight; LBW, low birth weight. Control, control diet; HFHC, high-fat, high-carbohydrate diet; ADG, average daily weight gain; ADF, average daily feed intake; FCR, feed conversion ratio; FGR, fractional growth rate ((fw-bw)/(d*bw)); bw, birth weight; fw, final weight; d, days alive.

a,b,c refers to statistical difference with a p-value <0.05 between different letters. (*) refers to significance p<0.05, (**) refers to significance p<0.01, (***) refers to significance p<0.001

Table 4.3. Growth, feed consumption and anthropometric measurements of piglets fed control, HFHC and HFHC+VA diets until 10 weeks of age

	NBW-Control	LBW-Control	LBW-Control	LBW-HFHC+VA	p
n	6	5	5	5	
BW, kg	1.6 ±0.2a	1.12±0.4b	1.14±0.2b	1.2 ±0.2b	0.0110*
FW, kg	39.5±3.2a	37.8±5.3ab	32.9±4.8b	35.3±2.1ab	0.061
ADG, g/d	506 ±8a	495 ±47ab	443 ±67b	471 ±27ab	0.1285
ADF, g/d	724 ±29[a]	718 ±125[a]	638 ±93[a]	619 ±58[a]	0.198
FCR, kg	1.25±0.15a	1.28±0.03a	1.09±0.05a	1.04±0.09a	0.0033**
FGR, g (bw-fw)/(d*bw)	324 ±28[a]	387 ±62[b]	393 ±49[b]	397 ±40[b]	0.0413*
Anthropometric Measurements					
Birth SC, cm	10.6±1.0a	10.3±0.7ab	9.4 ±0.6[b]	9.95±0.5ab	0.0689
Birth CR, cm	25.5±1.4a	24.7±1.4a	24.1±2.0a	24.3±1.0a	0.4339
Birth AC, cm	26.8±1.2a	24.6±1.1b	24.2±1.4b	24.2±0.4b	0.0032**
End SC, cm	22.8±1.0a	22.3±1.3ab	21.5±0.4b	21.5±0.5b	0.0584
End CR, cm	83.6±2.7a	83 ±2.3[a]	78.7±3.0b	78.5±1.8b	0.0031**
End AC, cm	72.1±2.8a	70.8±2.4ab	67.9±4.0b	70.7±0.7ab	0.1299
Change: SC, cm	13.7±3.5a	12 ±1.9[a]	12.1±0.7a	11.6±0.6a	0.3677
Change: CR, cm	58.1±3.0a	58.3±2.0a	54.6±4.1b	54.2±±1.9b	0.0451*
Change: AC,cm	45.3±2.5a	46.2±1.9a	43.7±47a	45.1±2.6a	0.632

Values are means ± SD. NBW, normal birth weight; LBW, low birth weight. Control, control diet; HFHC, high-fat, high-carbohydrate; VA, vaccenic acid; ADG, average daily weight gain; ADF, average daily feed intake; FCR, feed conversion ratio; FGR, fractional growth rate; SC, snout to crown; CR, crown to rump; AC, abdominal circumference; bw, birth weight; fw, final weight. Change refers to measurement difference from birth to ending.

a,b,c refers to statistical difference with a p-value<0.05 between different letters. (*) refers to significance p<0.05, (**) refers to significance p<0.01, (***) refers to significance p<0.001

4.7.2 Fasting plasma glucose, insulin and lipid concentrations

Fasting plasma lipid, glucose and insulin levels are presented in **Table 4.4.** There were no differences observed due to birth weight in any fasting parameter. In contrast LBW pigs fed a HFHC diet showed an increase in fasting TG concentration relative to NBW-HFHC pigs (p-value=0.03). There was an additional effect of diet in glucose (p-value=0.0008), cholesterol (p-value=0.01) and LDL (p-value=0.0003) in both LBW and NBW pigs fed a high-fat diet.

In study 2, we observed that LBW pigs on HFHC diet had significantly greater concentrations of plasma total cholesterol and LDL-cholesterol as compared to LBW on a control diet. The addition of VA resulted in an increase in HDL (p-value=0.032) **Table 4.5**.

Study 1:

Table 4.4. Fasting plasma lipid, glucose and insulin levels in LBW and NBW swine fed control and HFHC diet for 6 weeks post-weaning

	NBW-Control	LBW-Control	NBW-HFHC	LBW-HFHC	p		
					BW	Diet	Interaction
Insulin, uI U/ml	1.86±0.8[a]	1.65±0.5[a]	1.25±0.5[a]	1.93±0.9[a]	0.5118	0.6290	0.2157
Glucose, mmol/l	3.8 ±0.2[a]	4.3 ±0.7[a]	5.33±0.5[b]	5.1 ±0.5[b]	0.6110	0.0008***	0.1937
Cholesterol mg/dl	106 ±5.4[a]	95.5±37.5[a]	370±54.6[ab]	369±141[b]	0.4676	0.0131*	0.4209
LDL, mg/ml	45.6±24.8[a]	59.1±24.9[a]	253 ±120[b]	385±176[b]	0.2031	0.0003***	0.2949
TG, mg/dl	19.3 ±7.6[a]	23.9±15.5[a]	20.7 ±2.2[a]	36.4±6.0[b]	0.0766	0.1124	0.1137

Values are means ± SD. NBW, normal birth weight; LBW, low birth weight. Control, control diet; HFHC, high-fat, high-carbohydrate diet; BW, birth weight

[a,b,c] refer to statistical difference with a p-value <0.05 between different letters. (*) refers to significance p<0.05, (**) refers to significance p<0.01, (***) refers to significance p<0.001

Study 2:

Table 4.5. Fasting plasma lipid, glucose and insulin concentrations piglets fed control, HFHC and HFHC+VA diets until 10 weeks of age

	NBW-Control	LBW-Control	LBW-HFHC	LBW-HFHC-+VA	1-way ANOVA
n	6	5	5	6	
Insulin, uU/ml	0.32±0.03[1a]	0.33±0.04[1a]	0.32±0.04[1a]	0.31±0.02[a]	0.5747
Glucose,mmol/l	2.71±1.68[a]	2.70±0.5[a]	2.14±0.5[a]	2.73±0.2[a]	0.7247
Cholesterol, mg/dl	111 ±14.3[a]	104 ±19.3[a]	239 ±83.3[b]	278 ±93.5[b]	0.0003***
LDL, mg/ml	44.4±3.0[a]	43 ±8.5[a]	89.5±25[b]	98.9±25[b]	<0.0001***
TG, mg/dl	21.2±5.5[ab]	19.0±2.7[1a]	31.2±8.4[ab]	31.4±13.5[b]	0.0781
HDL, mg/ml	26.6±6.5[a]	37.2±6.5[ab]	45.3±14[b]	58.1±7.5[c]	0.0001***

Values are means ± SD. NBW, normal birth weight; LBW, low birth weight; control, control diet; HFHC, high-fat, high-carbohydrate; VA, vaccenic acid. Sample size is smaller where indicated due to irregularity in analysis and removal using the ROUT method outlier test with a Q value of 10%.

a,b,c refer to statistical difference with a p-value<0.05 between different letters. (*) refers to significance p<0.05, (**) refers to significance p<0.01, (***) refers to significance p<0.001

4.7.3 Postprandial glucose and insulin concentrations

In response to an adapted 2-step MOGTT, both NBW and LBW swine fed HFHC had higher postprandial glucose compared to control diet **(Figure 4.2).** This was further emphasized by the glucose area under the curve (AUC) (diet effect, $p=0.002$). LBW pigs fed a HFHC diet had a higher plasma insulin excursion ($p<0.01$) at the 15 min time point and greater glucose in circulation ($p<0.05$) at time points 15, 30 and 60 min compared to NBW pigs on the same diet. Additionally, when we compared the modified HOMA-IR score (Newell-Fugate et al., 2015), LBW pigs on HFHC diet had a greater ($p<0.05$) HOMA-IR score at 15 and 60 minutes compared to both LBW pigs as well as NBW pigs on control diet. Results suggest LBW pigs fed a HFHC diet were showing signs of early insulin resistance **(Figure 4.3).**

Postprandial lipid responses from study 2 showed a similar pattern. High-fat, high-carbohydrate diet showed increased postprandial glucose concentrations, as shown in **Figure 4.4** (p-value<0.05). Both HFHC diet groups appear to also have delayed clearance of glucose as can be observed in the postprandial curve in the later glucose peak. **Figure 4.5** shows that birth weight did not appear to have an impact on insulin response (p-value=0.99), but postprandial insulin was significantly increased by HFHC diet (p-value=0.04). Interestingly, the LBW pigs fed a HFHC+VA diet showed no change in glucose AUC (p-value=0.93) and insulin was not statistically different from either LBW control or LBW-HFHC (p-value=0.14). We calculated the slope of the glucose and insulin response between 0-30 min to represent the initial (appearance) phase of the postprandial period and between 30-120 min to represent the second (clearance) phase of the postprandial period. We observed that the slope in the initial appearance phase of glucose and the postprandial insulin response were significantly increased in the LBW-HFHC group compared to LBW-control but it was decreased in the LBW-HFHC+VA group compared to the LBW-HFHC

group. During the second (clearance) phase the slope was more vertical in the LBW-HFHC group compared to LBW-control as well as less steep in the LBW-HFHC+VA group compared to the LBW-HFHC group.

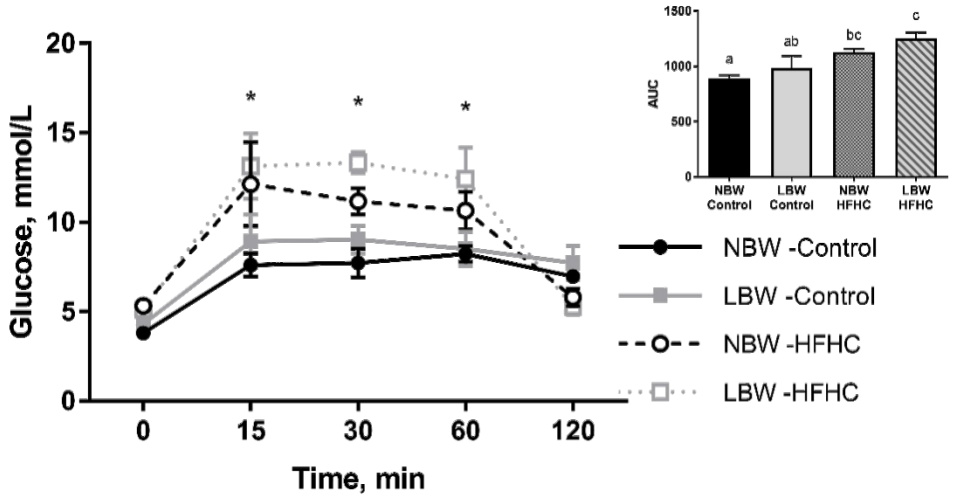


Figure 4.2 Postprandial glucose response following a modified oral glucose challenge (Study 1).

Values are means ± SEM, n=4 NBW-control, n=4 LBW-control, n=4 NBW-HFHC, n=4 LBW-HFHC. AUC is shown (inset). (*) denotes statistical difference between LBW-control and LBW-HFHC (p-value<0.05)

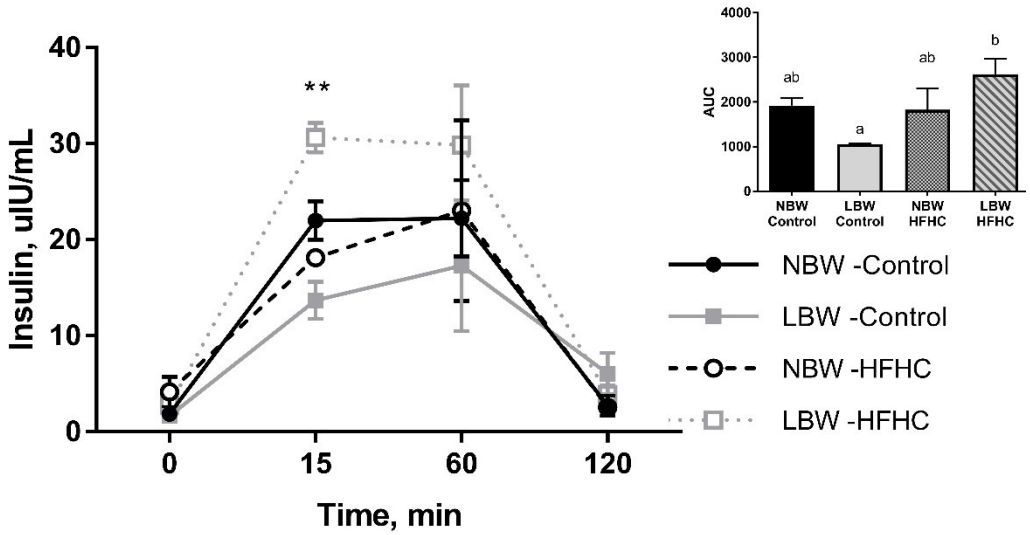


Figure 4.3 Postprandial insulin response following a modified oral glucose challenge (Study 1).

Values are means ± SEM, n=4 NBW-control, n=4 LBW-control, n=4 NBW-HFHC, n=4 LBW-HFHC. AUC is shown (inset). (**) denotes statistical difference between LBW-control and LBW-HFHC (p-value<0.01).

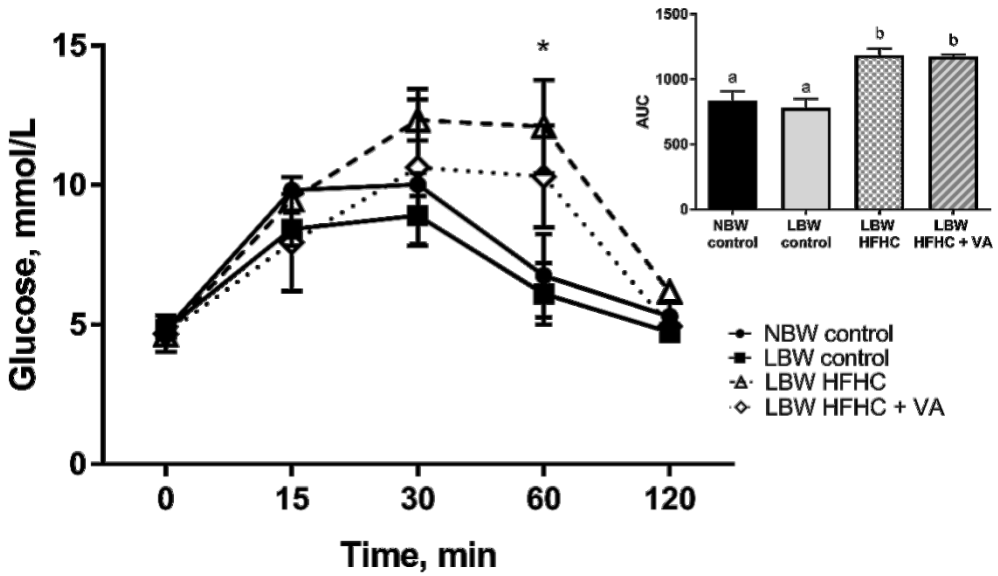


Figure 4.4 Postprandial glucose response following a modified oral glucose challenge (Study 2).

Values are means ± SEM, n=6 NBW-control (black circles), n=4 LBW-control (black squares), n=3 LBW-HFHC (open triangles), n=4 LBW-HFHC +VA (open diamonds). AUC is shown (inset). (*) denotes statistical difference between LBW-control and LBW-HFHC (p-value<0.05).

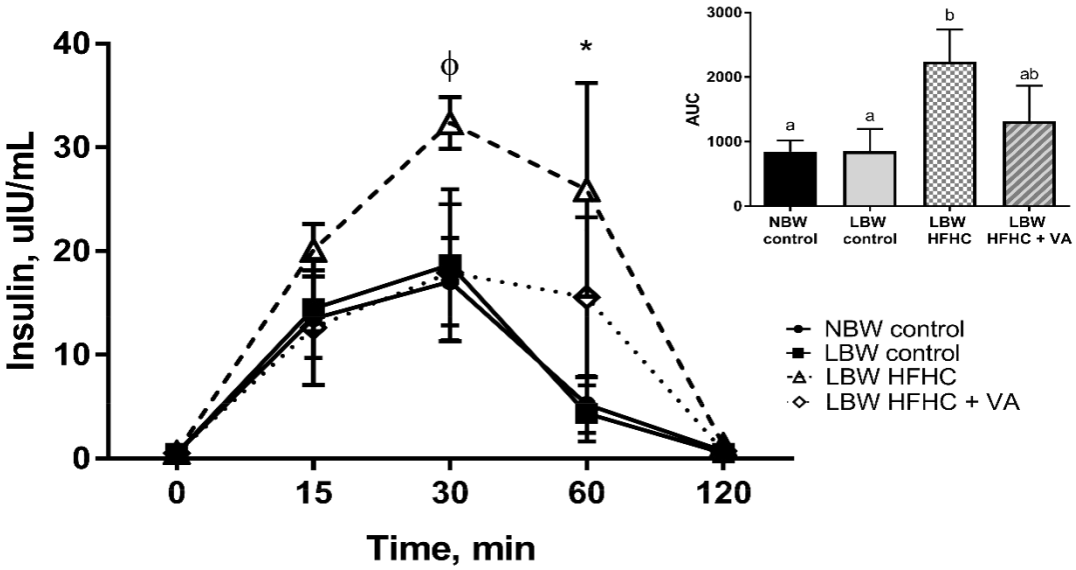


Figure 4.5 Postprandial insulin response following a modified oral glucose challenge (Study 2).

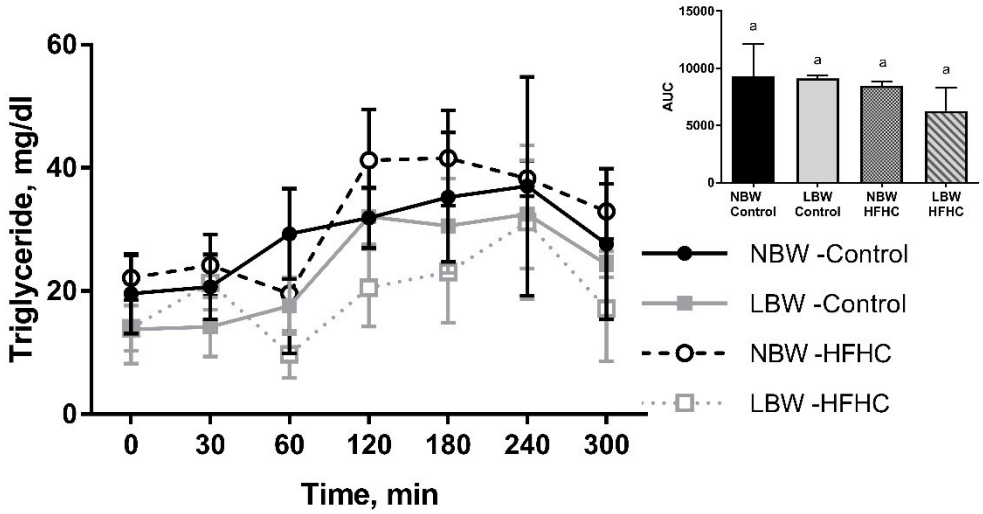
Values are means ± SEM, n=6 NBW-control (black circles), n=4 LBW-control (black squares), n=3 LBW-HFHC (open triangles), n=4 LBW-HFHC+VA (open diamonds). AUC is shown (inset). (*) denotes statistical difference between LBW-control and LBW-HFHC (p-value<0.05) and (ϕ) denotes trend between LBW-HFHC and LBW-HFHC+VA (p-value=0.09).

4.7.4 Postprandial lipid concentrations

There were no differences in postprandial TG AUC **(Figure 4.6A and 4.6 B)** in plasma following a fat tolerance test in either birth weight or diet groups. Using incremental values, analysis of the TG response showed a significant increase at the 240 min timepoint only in LBW-HFHC piglets relative to all three other groups (p-value<0.05).

Data collected from study 2 showed a trend towards a slight increase in postprandial TG in the HFHC diet (p-value=0.19) and a trend towards a decrease of TG in the LBW-HFHC+VA diet (p-value=0.09) **(Figure 4.6.1A and 4.6.1B).** Incremental curve analysis showed that there were no significant different in iAUC between control diets or HFHC diet, but LBW pigs fed a HFHC diet enriched in VA showed a substantial decrease in the TG response (LBW-HFHC= 1450 ±643; LBW-HFHC+VA= 255 ±217, p-value=0.005).

A



B

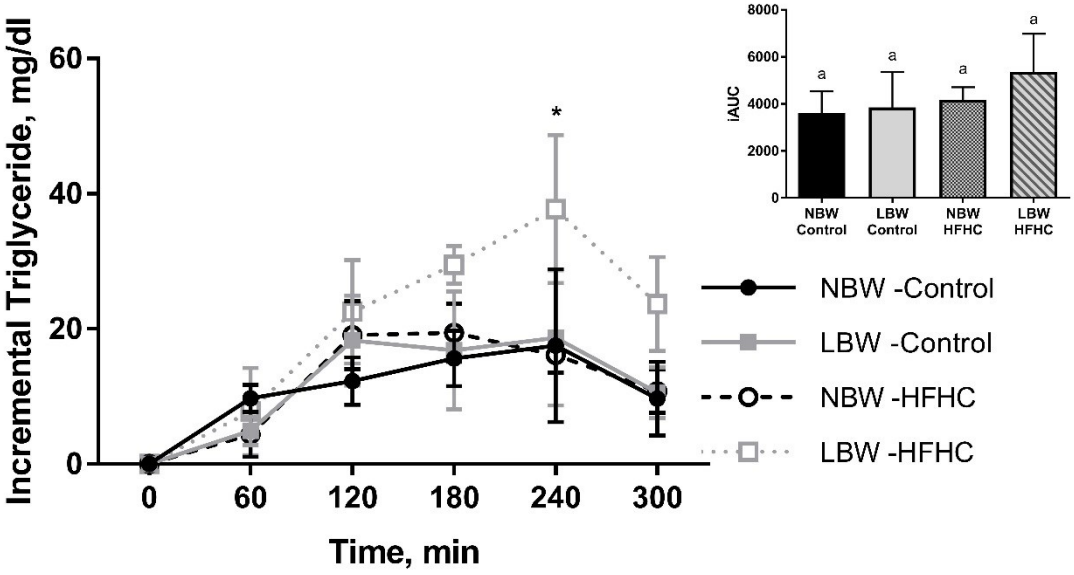
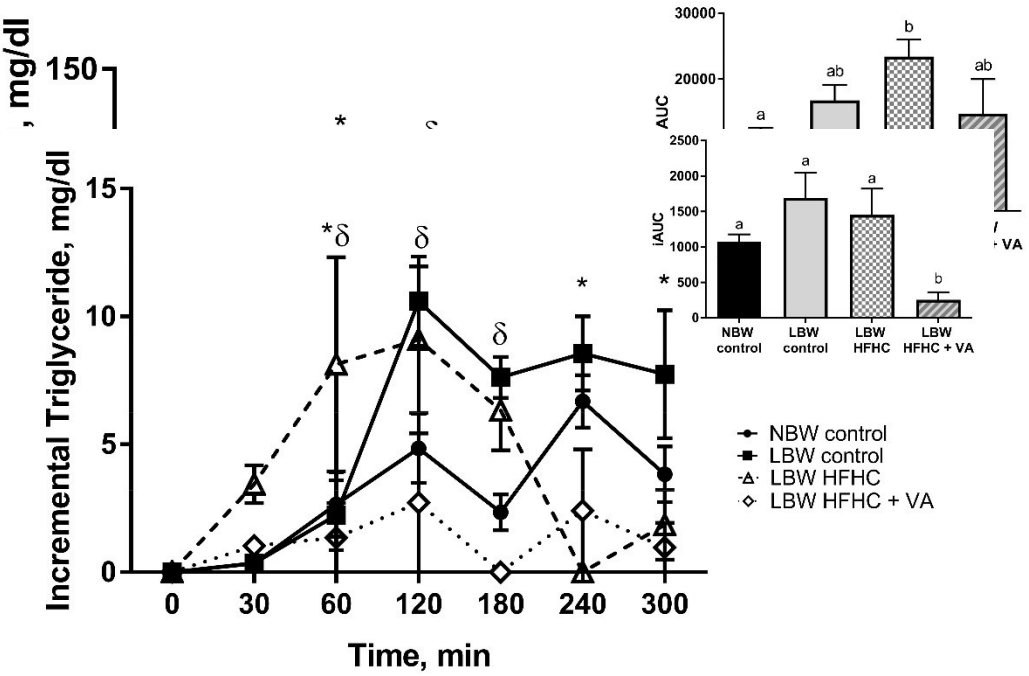


Figure 4.6 Postprandial triglyceride (A) and incremental triglyceride (B) response following a modified oral glucose and fat challenge (Study 1).

Values are means ± SEM, n=4 NBW-control (black circles), n=4 LBW-control (black squares), n=4 NBW-HFHC (open circles), n=4 LBW-HFHC (open squares). AUC is shown (inset). Different letters denote statistical difference (p-value<0.05). (*) refers to statistical significance (p-value<0.05) between the LBW-HFHC group and all three other groups at that timepoint.

A



B

Figure 4.6.1 Postprandial triglyceride (A) and incremental (B) response following a modified oral glucose and fat challenge (MOGTT) (Study 2).

Values are means ± SEM, n=6 NBW-control (black circles), n=4 LBW-control (black squares), n=3 LBW-HFHC (open triangles), n=4 LBW-HFHC+VA (open diamonds). AUC is shown (inset). (*) denotes statistical difference between LBW-control and LBW-HFHC (p-value<0.05) and (δ) denotes statistical difference between LBW-HFHC and LBW-HFHC+VA (p-value<0.05).

4.7.5 Lymph lipoprotein and lipid concentrations

LBW pigs exhibited a trend towards greater lymph flow rate than NBW pigs (birth weight effect, p=0.055). Additionally, HFD feeding was decreased lymph flow rate (diet effect, p=0.011), as shown in **Table 4.6**. Interestingly, regardless of the diet, lymph TG was significantly greater in LBW swine (birth weight effect, p=0.013, LBW-control= 534±225 mg/dl, p=0.063, NBW-HFHC= 226±69.6 mg/dl; LBW-control= 652±281mg/dl, p=0.040). No differences were found using multiple comparisons testing between groups for lymph cholesterol, however a diet effect was observed (diet effect, p=0.0331) with increased lymph cholesterol in the HFHC diets using two-way ANOVA. Consistant with TG results, apoB48 in lymph was also higher in LBW swine (birth weight effect, p=0.013) compared to NBW control (p=0.016). No differences were observed in CM secretion rate (apoB48/hr) between birth weights; however, a significant increase was observed when fed a HFD (diet effect, p=0.04).

In study 2, LBW swine fed a control diet had lower lymph flow rate compared to NBW swine on control diet. There were no differences in lymph particle size across all groups. Irrespective of VA content, the HFHC diet increased lymphatic cholesterol levels (p-value<0.05). Interestingly, no statistical difference was observed in lymph TG content or apoB48 with dietary intervention. However, LBW swine fed a HFHC diet did have a trend towards an increased lymphatic apoB48 particle secretion rate (apoB48/hr) compared to control diet. **(Table 4.6.1)**

Study 1:

Table 4.6. Fasting lymph lipoprotein and lipid concentrations

	NBW-Control	LBW-Control	NBW-HFHC	LBW-HFHC	P value BW	Diet	Interaction
TG, mg/dl	162 ±50.2[1a]	534 ±225[ab]	226 ±69.9[a]	652 ±281[1b]	0.0135*	0.5757	0.4995
Cholesterol, mg/dl	128 ±32.7[a]	134 ±33.7[a]	251 ±66.8[a]	255 ±144[a]	0.9068	0.0331*	0.9733
ApoB48, ug/ml	888 ±273[a]	1786 ±586[b]	992 ±136[a]	1436 ±446[a]	0.0127*	0.5741	0.3107
ApoB48/hr, mg/hr	15.9±10.7[a]	16.6 ±3.69[a]	51.4 ±22.8[a]	36.2 ±26.2[a]	0.519	0.0372*	0.4801
TG/ApoB48 ratio	0.28±0.12[a]	0.33 ±0.22[a]	0.23 ±0.06[a]	0.38 ±0.26[a]	0.9846	0.9656	0.4491
Flow rate, ml/hr	68.3±32.5ab	107 ±12.1a	21.5±6.36b	51.3 ±29b	0.0548	0.0108*	0.7752

Values are means ± SD. N=3 for each group due to only 3 pigs undergoing lymph cannulation in each group for this study. NBW, normal birth weight; LBW, low birth weight; control, control diet; HFHC, high-fat, high-carbohydrate

[a,b,c] refer to statistical difference with a p-value <0.05 between different letters. (*) refers to significance p<0.05, (**) refers to significance p<0.01, (***) refers to significance p<0.001, (****) refers to significance p<0.0001

[1]n=2 due to lymph contaminated with blood.

Study 2:

Table 4.6.1. Fasting lymph lipoprotein and lipid concentrations

	NBW-Control	LBW Control	LBW-HFHC	LBW-HFHC +VA	1-way ANOVA
N	**6**	**5**	**5**	**6**	
TG, mg/dl	373 ±113[a]	168 ±48[b]	290 ±154[ab]	272 ±106[ab]	0.0716
Cholesterol, mg/dl	42.1±8.5[a]	40.8±2.8[1a]	68.6±21.3[b]	84.7±13.2[b]	<0.0001***
ApoB48, ug/ml	273 ±93[1a]	261 ±88[1a]	431 ±140[a]	425 ±179[2a]	0.1379
ApoB48/hr, mg/hr	19.79±7.3[1ab]	11.10±5.2[1a]	29.16±11.4[bc]	36.2±11.1[1c]	0.0048**
TG/ApoB48 ratio	1.19±0.7[2a]	0.60±0.1[a]	0.95±0.5[a]	0.78±0.2[a]	0.3606
Particle size, nm	83.5±2.3[1a]	81.3±14.4[a]	93.7±27[a]	87.9±12[a]	0.6667
Flow rate, ml/hr	71.7±6.5[a]	52.5±10.0[1b]	63.2±14.4[1ab]	62.4±14.7[2ab]	0.1336

Values are means ± SD. NBW, normal birth weight; LBW, low birth weight; control, control diet; HFHC, high-fat, high-carbohydrate; VA, vaccenic acid. Sample size is smaller where indicated due to irregularity in analysis and removal using the ROUT method outlier test with a Q value of 10%. [1]n=4, 2 n=5

a,b,c refers to statistical difference with a p-value <0.05 between different letters. (*) refers to significance p<0.05, (**) refers to significance p<0.01, (***) refers to significance p<0.001, (****) refers to significance p<0.0001

4.7.6 Cecal digesta microbiome was affected by diet but not by birth weight

To determine whether birth weight or diet affected the gut microbial composition in pigs, the V3-V4 region of 16S rRNA gene was sequenced on an Illumina MiSeq platform in the cecal samples. Principal Coordinate Analysis (PCoA) of cecal microbiota composition using Bray Curtis metrics (**Figure 4.7A)** showed clustering by diet (ADONIS, p=0.003), but not by birth weight (ADONIS, p=0.792). This difference was confirmed with microbial community analysis using weighted UniFrac metrics (which takes taxonomy into further consideration). The four most abundant phyla in cecal digesta were identified as *Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Spirochetes*, constituting 96.6% of all phyla. Bacterial communities in cecal digesta changed in response to HFHC diet with an increase in *Firmicutes*, *Proteobacteria* and *Spirochaetes* ($p<0.05$) and a decrease *Bacteroidetes* and *Tenericutes* ($p<0.05$) as compared to control diet fed pigs (**Table 4.7A**). At the class level, *Bacilli, Clostridia* and *Deltaproteobacteria* ($p<0.05$) were more abundant in HFHC diet fed pigs, whereas *Bacteroidia, Spirochaetes,* and *Mollicutes* ($p<0.05$) were less. At the order level *Lactobacillales, Clostridiales, Desulfovibrionales* and *Enterobacteriales* ($p<0.05$) were significantly higher in HFHC diet fed pigs compared to Control diet fed pigs. At the Family level, *Lactobacillaceae*, *Streptococcaceae*, *Clostridiaceae*, and *Desulfovibrionaceae,* were more abundant ($p<0.05$). Unclassified families; *PF16, p-2534-18B5*, (affiliated to *Bacteroidales*) and *Porphyromonadaceae, Sphaerochaetaceae, Spirochaetaceae*, were less ($p<0.05$) in HFHC fed pigs compared to control. Increased *Clostridium, Streptococcus, Desulfovibrio* and *Lactobacillus* genera was consistent with the increase in *Firmicutes* in HFHC fed pigs. Genera including *Parabacteroides, Sphaerochaeta, Treponema* and unclassified *RF16* and *p-2534-18B5* were ($p<0.05$) increased in control diet fed pigs compare to HFHC diet fed pigs.

Study 1:

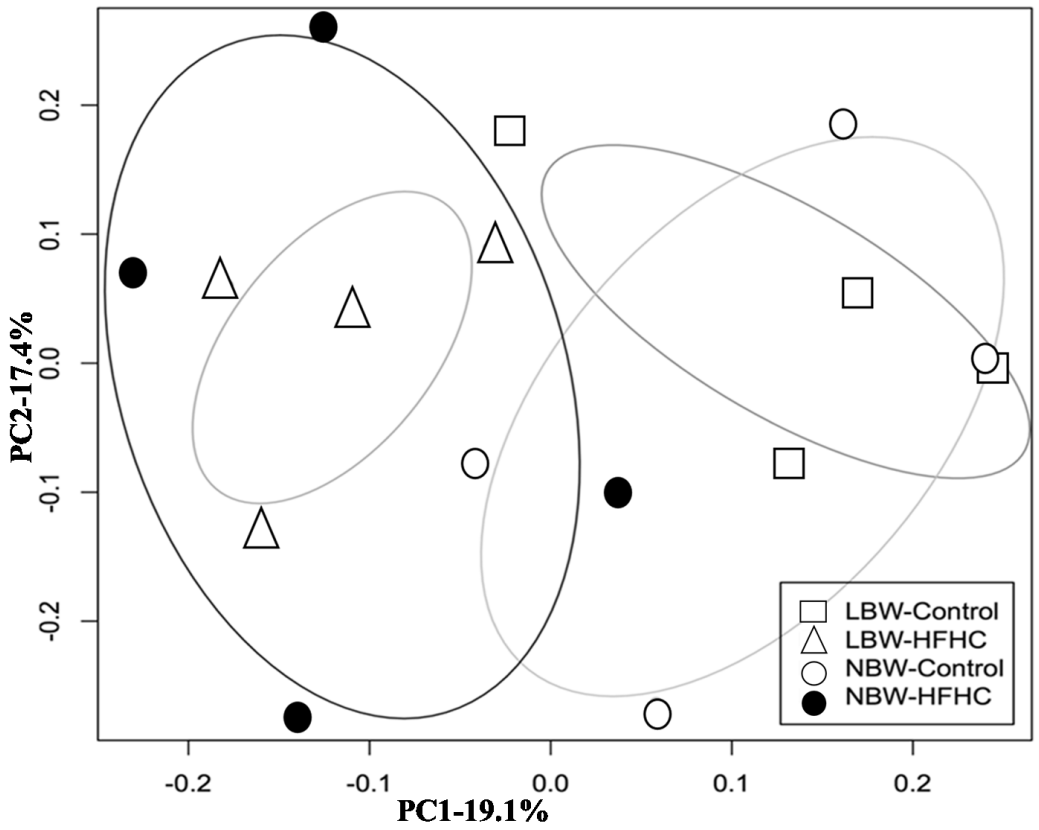


Figure 4.7A Principle coordinate analysis of cecal bacterial community composition using Bray-Curtis distance metrics (Study 1).

Cecal microbiota was significantly affected by high fat high carbohydrate (HFHC) diet (ADONIS, p=0.003) but no effect of birth weight (ADONIS, p=0.792) or by individual treatment (p=0.171; n=4) was observed.

Study 1:

Table 4.7A. Relative abundance of gut bacteria from phylum to genus in cecal digesta.

Phylum	NBW-Control	LBW-Control	NBW-HFHC	LBW-HFHC	p value	
					Diet	BW
Bacteroidetes	56.4±7.5	49.4±3.5	42.6±4.6	38.8±12.0	**0.02***	0.34
Firmicutes	25.5±8.6	29.7±3.9	37.8±1.3	42.0±16.4	**0.02***	0.83
Spirochaetes	8.8±8.5	13.1±6.6	1.2±1.3	2.3±2.5	**0.01***	0.29
Proteobacteria	6.3±9.3	3.8±0.97	15.2±6.0	14.0±7.03	**0.03***	0.67
Tenericutes	0.09±0.10	0.07±0.03	0.006±0.01	0.0±00	**0.00***	1.00
Fibrobacteres	0.118±0.1	0.01±0.02	0.03±0.08	0.0±0.01	0.16	0.16
Actinobacteria	0.08±0.09	0.10±0.07	0.06±0.01	0.09±0.03	0.92	0.34
Verrucomicrobia	0.01±0.02	0.22±0.38	0.12±0.2	0.03±0.02	0.75	0.29
Class						
Bacteroidetes						
Bacteroidia	56.4±9.30	49.4±6.49	42.6±4.59	38.8±11.9	**0.02***	0.29
Firmicutes						
Bacilli	0.30±0.22	088±0.23	2.48±1.49	4.23±5.51	**0.04***	0.34
Clostridia	24.4±8.21	28.0±5.06	34.4±1.55	36.9±20.16	0.06	0.34
Spirochaetes						
Spirochaetes	8.86±8.55	13.1±9.06	1.21±1.16	2.32±2.30	**0.00***	0.19
Proteobacteria						
Deltaproteobacteria	0.16±0.16	0.19±0.15	0.50±0.23	0.55±0.428	**0.00***	0.83
Tenericutes						
Mollicutes	0.1±0.10	0.07±0.04	0.00	0.00	**0.00***	0.14
Fibrobacteres						
Fibrobacteria	0.12±0.09	0.02±0.02	0.04±0.07	0.00	0.14	0.40
Order						
Bacteroidia						
Bacteroidales	56.41±9.37	49.4±6.4	42.6±9.17	38.8±11.91	**0.04***	0.29
Bacilli						
Lactobacillales	0.00	0.77±0.09	1.76±1.12	3.61±5.32	**0.00***	0.14
Clostridia						
Clostridiales	24.7±8.21	28.04±5.07	34.3±1.5	36.9±29.16	0.06	0.34
Spirochaetes						
Sphaerochaetales	0.75±0.27	1.00±0.20	0.08±0.09	0.08±0.11	**0.00***	0.06
Spirochaetales	8.10±8.55	12.14±8.95	1.12±1.18	2.24±2.24	**0.02***	0.22
Deltaproteobacteria						
Desulfovibrionales	0.12±0.17	0.12±0.13	0.43±0.22	0.54±0.43	**0.01***	0.19
Enterobacteriales	0.00	0.00	0.30±0.29	0.31±0.27	**0.00***	0.06
Mollicutes						
RF39	0.07±0.088	0.03±0.01	0.00	0.00	**0.00***	0.06
Fibrobacteria						
Fibrobacterales	0.01±0.94	0.01±0.02	0.37±0.74	00±0.01	0.16	0.46
Family						
Bacteroidales						
Bacteroidaceae	3.48±3.76	4.49±4.25	0.52±0.62	3.72±6.41	0.14	0.45
Porphyromonadaceae	3.96±0.79	2.8±1.00	1.34±0.35	2.31±2.31	**0.03***	0.29
RF16	1.06±0.41	1.58±1.49	0.10±0.18	0.16±0.18	**0.01***	0.19

p-2534-18B5	0.30±0.51	0.29±0.49	0.02±0.041	0.00	0.06	0.34
Lactobacillales						
Lactobacillaceae	0.00	0.08±0.07	1.60±1.10	2.09±2.58	**0.00***	0.14
Streptococcaceae	0.00	0.00	0.02±0.036	1.51±2.9	**0.00***	0.19
Clostridiales						
Clostridiaceae	3.46	4.79±3.69	9.06±3.2	11.9±6.82	**0.02***	0.20
Sphaerochaetales						
Sphaerochaetaceae	0.75±0.27	0.51±0.33	0.08±0.09	0.85±0.11	**0.00***	0.06
Spirochaetales						
Spirochaetaceae	8.10±8.5	8.33±0.31	1.12±1.18	2.24±24	**0.02***	0.19
Desulfovibrionales						
Desulfovibrionaceae	0.16±0.15	0.13±0.04	0.49±0.22	0.54±0.43	**0.00***	0.17
Enterobacteriales						
Enterobacteriaceae	0.00	0.00	0.30±0.29	0.31±0.27	0.44	0.70
RF39						
RF39; f	0.07±0.08	0.07±0.01	0.00	0.00	**0.00***	0.16
Fibrobacterales						
Fibrobacteraceae	0.11±0.09	0.01±0.01	0.03±0.04	0.00	0.16	0.46
Genus						
Bacteroidaceae						
Bacteroides	3.48±3.76	4.49±4.25	0.52±0.62	0.52±0.62	0.14	0.44
Porphyromonadaceae						
Parabacteroides	3.96±0.79	2.82±1.00	1.34±0.35	1.34±0.35	**0.02***	0.22
f__RF16						
RF16; g	1.06±1.01	1.58±1.49	0.10±0.18	0.16±0.18	**0.01***	0.19
f__p-2534-18B5						
p-2534-18B5; g	0.03±0.51	0.29±0.49	0.02±0.04	0.00	0.06	0.30
Lactobacillaceae						
Lactobacillus	0.00	0.00	1.73±1.10	2.00±1.10	**0.00***	0.14
Streptococcaceae						
Streptococcus	0.0	0.00	0.00	0.02±0.03	**0.01***	0.19
Clostridiaceae						
Clostridium	0.19±0.20	0.36±0.27	0.95±0.93	1.91±1.82	**0.00***	0.09
SMB53	0.24±0.177	0.26±0.23	0.10±0.12	0.95±0.93	0.15	0.49
Sphaerochaetaceae						
Sphaerochaeta	0.75±0.27	1.00±0.20	0.08±0.09	0.08±0.09	**0.00***	0.06
Spirochaetaceae						
Treponema	8.11±8.55	12.14±8.95	1.12±1.18	2.24±2.42	**0.01***	0.20
Desulfovibrionaceae						
Desulfovibrio	0.00±0.00	0.044±0.04	0.38±1.10	0.39±0.29	**0.00***	0.06
Enterobacteriaceae						
Enterobacteriaceae;g	0.00	0.00	0.23±0.26	0.29±0.29	**0.00***	**0.06***
RF39; f__						
RF39; f_;g__	0.07±0.08	0.03±0.01	0.00	0.00	**0.00***	0.14
Fibrobacteraceae						
Fibrobacter	0.11±0.09	0.01±0.02`	0.03±0.07	0.03±0.07	0.14	0.48

Values are means ± SD. BW; birth weight, NBW, normal birth weight; LBW, low birth weight; Control, control diet; HFHC, high-fat, high-carbohydrate diet, HFHC+VA, high-fat, high-carbohydrate diet enriched with vaccenic acid.

4.7.7 Vaccenic acid enriched diet attenuate HFHC diet induced dysbiosis in LBW pigs

The V3-V4 region of 16S rRNA gene was sequenced on an Illumina MiSeq platform in the cecal samples of NBW and LBW pigs fed on Control, HFHC and HFHC+VA diets. A total of 1,149,290 reads, with an average of 52240.4±63475 reads per sample were obtained. Sequences were clustered into operational taxonomic units (OTU) with USEARCH resulting in a total of 1454 OTUs. Principal Coordinates Analysis (PCoA) of cecal microbiota composition using Bray Curtis metrics **(Figure. 4.7B)** showed LBW pigs fed on HFHC showed significant shift in intestinal microbiota (ADONIS, $p = 0.019$ between LBW HFHC and LBW Control), while addition of VA in HFHC normalized from high fat diet induced shift.

In study 2, the four most abundant phyla in cecal digesta were identified as *Firmicutes*, *Bacteroidetes*, Proteobacteria and Spirochetes, constituting 97.6% of all phyla. (**Table 4.7B)** HFHC diet made an impact on bacterial communities in cecal digesta in LBW pigs. HFHC diet intake in LBW pigs showed an increase in *Firmicutes* ($p<0.05$) and a decrease *Bacteroidetes*, *Proteobacteria*, and *Spirochaetes* ($p<0.05$) as compared to pigs fed on control diet. Addition of VA in HFHC diet showed increases in *Bacteroidetes* and *Proteobacteria* in LBW pigs. At the class level, we found the similar impact of HFHC diet in LBW pigs with significant increase in *Bacilli, Clostridia*, and reduced *Bacteroidia*. Addition of VA in HFHC diet showed non-significant increase in *Bacteroidia* in LBW pigs. At the order level *Lactobacillales* and *Clostridiales* were more abundant in LBW pig fed on HFHC diet, whereas *Bacteroidales* ($p<0.05$) were less. While LBW animal fed on HFHC diet with VA increase abundance of *Bacteroidales* and *Clostridiales* with reduced *Lactobacillales* compare to LBW pig with HFHC diet. The family *Porphyromonadaceae* (affiliated to *Bacteroidales*) decreased but *Lactobacillaceae, Streptococcaceae*, and *Clostridiaceae* (affiliated to *Lactobacillales* and *Clostridiales*

respectively), was higher after HFHC introduction in LBW swine. Family *Porphyromonadaceae* ($p<0.05$) was only significantly higher in HFHC diet fed pigs compared to controls. Increased *Clostridium, Lactobacillus, Desulfovibrio* and *unclassified RF16* genera was consistent with the increase in *Firmicutes* in HFHC fed pigs. Genera including *Parabacteroides, Sphaerochaeta, Treponema* and were increased in control diet fed pigs compare to HFHC diet fed pigs.

Study 2:

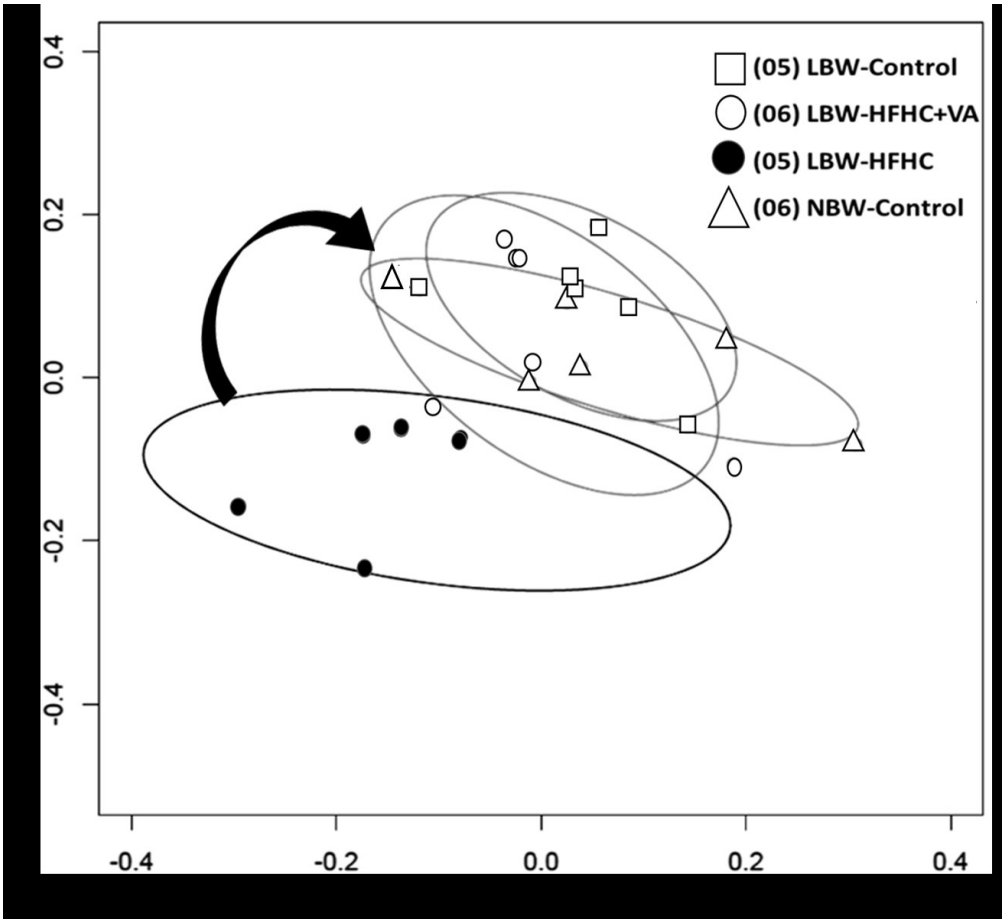


Figure 4.7B Principle coordinate analysis of cecal bacterial community composition using Bray-Curtis distance metrics (Study 2).

Cecal microbiota was significantly affected by high fat high carbohydrate (HFHC) diet (ADONIS, p=0.015) but HFHC+ VA diet don have effect on gut microbiota.

Study 2:

Table 4.7B. Differences in relative proportion of bacteria from class to genus in cecal digesta of low birth weight (LBW) and normal birth weight pigs (NBW) pigs.

consuming control, high-fat, high-carbohydrate diet (HFHC) and, high-fat, high-carbohydrate diet enriched with vaccenic acid (HFHC+VA).

Phylum	LBW Control	NBW Control	LBW-HFHC	LBW-HFHC+VA	p Value	
					BW	Diet
Bacteroidetes	55.8±10.51	56.4±12.6	37.9*±7.20	44.6±6.80	0.07	**0.03***
Firmicutes	26.8±4.00	28.2±8.3	45.7*±8.91	29.2±6.60	0.33	**0.02***
Spirochaetes	2.9±2.60	2.7±2.20	1.7±1.30	1.7±1.40	0.55	0.75
Proteobacteria	8.9±5.50	8.5±6.51	8.0±3.90	15.6±5.20	0.33	0.16
Fibrobacteres	0.4±0.70	0.1±0.20	0.0±0.00	0.0±0.00	0.26	0.30
Actinobacteria	0.0±0.00	0.1±0.11	0.1±0.00	0.0±0.00	0.41	0.14
Verrucomicrobia	0.1±0.10	0.2±0.21	0.3±0.60	0.1±0.11	0.15	0.24
Class						
Bacteroidetes						
Bacteroidia	55.8 ±10.59	56.6 ±12.96	37.9±7.10	44.6±6.75	0.08	**0.03***
Firmicutes						
Bacilli	0.4±0.13	1.25±0.75	4.22±1.34	1.73±1.35	0.56	**0.00***
Clostridia	24.9±1.85	25.6±8.50	40.2±7.78	26.1±5.60	0.24	**0.03***
Spirochaetes						
Spirochaetes	2.91±2.68	2.74±2.23	1.71±1.36	1.66±1.40	0.56	0.76
Proteobacteria						
Deltaproteobacteria	0.57±0.43	0.27±0.11	0.43±0.20	0.63±0.29	0.06	0.18
Tenericutes						
Mollicutes	0.20±0.27	0.11±0.06	0.13±0.05	0.06±0.10	0.69	0.31
Fibrobacteres						
Fibrobacteria	0.37±0.65	0.17±0.15	0.06±0.07	0.02±0.03	0.26	0.30
Order						
Bacteroidia						
Bacteroidales	55.8±10.59	56.6±12.96	37.9±7.20	44.6±13.02	0.08	**0.03***
Bacilli						
Lactobacillales	0.23±0.30	0.85±0.54	4.08±1.26	1.70±0.62	0.34	**0.00***
Clostridia						
Clostridiales	24.91±4.15	25.66±8.50	40.2±7.78	26.15±5.60	0.24	**0.03***
Spirochaetes						
Sphaerochaetales	0.29±0.20	0.5±0.58	0.05±0.03	0.09 ±0.08	0.42	0.14
Spirochaetales	2.63 ±2.87	2.25±1.98	1.67±1.35	1.57±1.35	0.66	0.92
Deltaproteobacteria						
Desulfovibrionales	0.35±0.25	0.24±0.10	0.41±0.20	0.56±0.20	0.07	0.12
Enterobacteriales	0.32±0.36	0.57±0.69	3.57 ±4.36	0.71±0.69	0.38	0.20
Mollicutes						
RF39	0.08±0.06	0.08±0.59	0.11±0.03	0.06±0.10	1.00	0.29
Fibrobacteria						
Fibrobacterales	0.37±0.65	0.17±0.15	0.06±0.06	0.02±0.03	0.26	0.31
Family						
Bacteroidales						
Bacteroidaceae	0.57±0.87	0.29±0.31	0.78±1.16	0.50±0.39	0.34	0.55
Porphyromonadaceae	4.73±2.53	2.49±1.87	0.52±0.36	1.73±40	0.42	**0.00***

RF16	0.12±0.12	0.63±0.67	0.40±0.83	0.10±0.11	0.12	0.47
p-2534-18B5	0.10±0.20	1.10±2.68	0.08±0.12	0.01±0.01	0.18	0.56
Lactobacillales						
Lactobacillaceae	0.17±0.20	0.78±0.54	4.01±1.22	1.64±1.33	0.42	0.14
Streptococcaceae	0.06±0.10	0.07±0.13	0.02±0.01	0.06±0.08	0.61	0.91
Clostridiales						
Clostridiaceae	2.74±1.34	1.76±1.31	5.90±4.24	3.26±1.54	0.04	0.15
Sphaerochaetales						
Sphaerochaetaceae	0.29±0.20	0.49±0.58	0.05±0.02	0.09±0.08	0.42	0.14
Spirochaetales						
Spirochaetaceae	2.63±2.87	2.25±0.2.00	1.67±1.35	1.57±1.35	0.66	0.92
Desulfovibrionales						
Desulfovibrionaceae	0.31±0.24	0.22±0.10	0.39±0.18	0.45±0.17	0.24	0.12
Enterobacteriales						
Enterobacteriaceae	0.32±0.36	0.57±0.69	3.57±4.36	0.71±0.61	0.38	0.20
RF39						
RF39;f__	0.08±0.06	0.08±0.05	0.11±0.03	0.06±0.10	1.00	0.29
Fibrobacterales						
Fibrobacteraceae	0.37±0.65	0.17±0.15	0.06±0.07	0.02±0.04	0.26	0.30
Genus						
Bacteroidaceae						
Bacteroides	0.57±0.87	0.29±0.31	0.78±1.16	0.50±0.38	0.14	0.55
Porphyromonadaceae						
Parabacteroides	4.73±2.50	1.87±0.97	0.42±0.33	1.73±1.40	0.66	**0.00***
f__RF16						
RF16;g__	0.12±0.13	0.63±0.67	0.40±0.83	0.10±0.11	0.12	0.47
f__p-2534-18B5						
p-2534-18B5;g	0.10±0.20	1.10±2.68	0.08±0.11	0.01±0.01	0.18	0.56
Lactobacillaceae						
Lactobacillus	0.17±0.20	0.74±0.53	4.00±1.25	1.62±1.27	0.42	**0.00***
Streptococcaceae						
Streptococcus	0.06±0.10	0.07±0.13	0.01±0.01	0.06±0.08	0.71	0.91
Clostridiaceae						
Clostridium	0.12±0.06	0.24±0.15	1.98±2.46	0.29±0.27	0.71	0.06
Sphaerochaetaceae						
Sphaerochaeta	0.29±0.20	0.49±0.58	0.05±0.02	0.09±0.08	0.42	0.14
Spirochaetaceae						
Treponema	2.63±2.87	2.25±1.98	1.67±1.35	1.57±1.35	0.66	0.92
Desulfovibrionaceae						
Desulfovibrio	0.15±0.13	0.10±0.06	0.27±0.18	0.25±0.18	0.10	0.28
Enterobacteriaceae						
*Enterobacteriaceae;g_*	0.32±0.36	0.57±0.69	3.57±4.30	0.71±0.61	0.38	0.20
RF39;f__						
RF39;f__;g__	0.08±0.06	0.08±0.06	0.11±0.03	0.06±0.10	1.00	0.29
Fibrobacteraceae						
Fibrobacter	0.37±0.65	0.17±0.15`	0.06±0.07	0.02±0.04	0.26	0.30

Values are means ± SD, . BW; birth weight, NBW, normal birth weight; LBW, low birth weight; Control, control diet; HFHC, high-fat, high-carbohydrate diet

4.7.7.1 Species richness and evenness of gut microbiota by chao-1 diversity and shannon diversity index

In Study 1 alpha diversity analysis indicated significanct difference in richness and evenness via chao-1, simpson and shannon diversity index based on diet.

In study 2 alpha diversity showed no significanct difference in richness and evenness via chao-1, shannon and simpson diversity index based on diet. **(Table 4.8A and Figure 4.8.1A)**

Table 4.8. alpha diversity based on treatment and diet by chao-1 and Shannon diversity index

Study 1:

A

Diet			
	Control diet (LBW+NBW)	**HFHC diet (LBW+NBW)**	**p**
Shannon	6.2±0.28	5.6±0.33	0.015*
Chao-1	273.4±37.14	231.1±17.1	0.029*
Simpson	0.970±0.008	0.958±0.01	0.05*

Study 2:

B

Diet				
	Control diet (LBW+NBW)	**HFHC diet (LBW+NBW)**	**HFHC+VAdiet (LBW+NBW)**	**p**
Shannon	6.0±0.35	6.1±0.35	5.9±0.0.14	0.556
Chao-1	289.3±54.4	305.1±49.7	248.2±14.8	0.105
Simpson	0.97±0.00	0.97±0.00	0.96±0.00	0.091

Values are means ± SD. NBW, normal birth weight; LBW, low birth weight; Control, control diet; HFHC, high-fat, high-carbohydrate diet, HFHC+VA, high-fat, high-carbohydrate diet enriched with vaccenic acid.

4.7.8 High-fat, high-carbohydrate diet enriched with vaccenic acid did not affect the serum LPS

We also wanted to address if there was an increase in serum LPS in LBW piglets, however we didnot detect any evidence for difference between groups. **(Figure 4.9)**

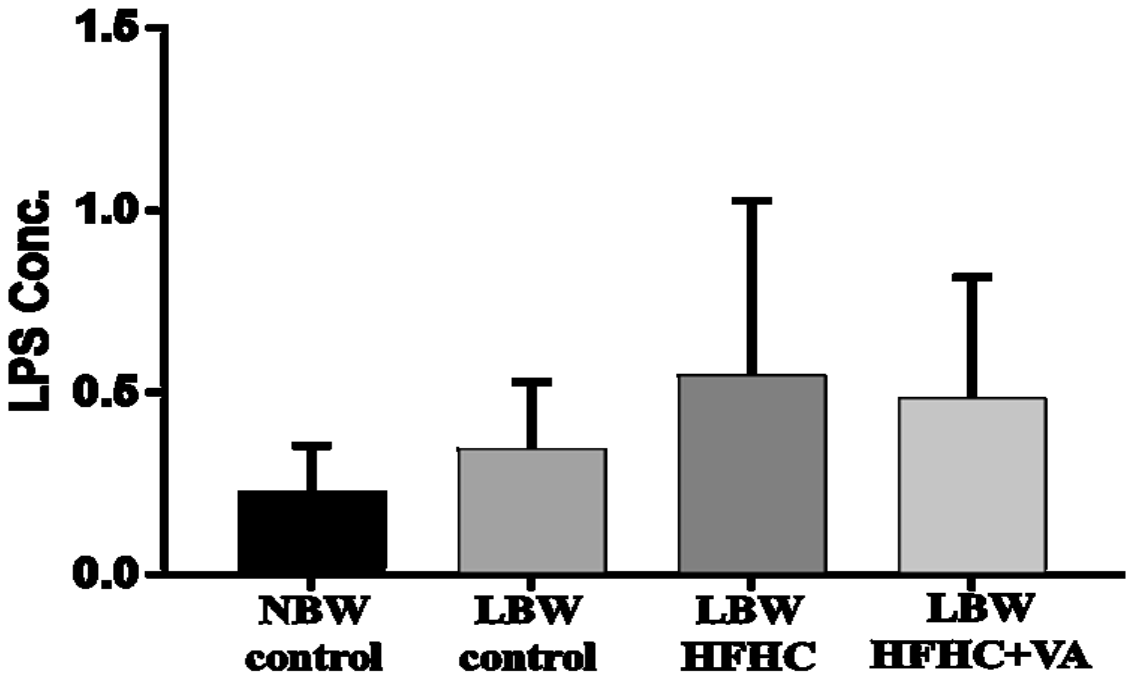


Figure 4.9 Concentration of serum lipopolysaccharide (LPS) in LBW, NBW pigs on control diet and LBW pigs on HFHC as well as on HFHC+VA diet.

HFHC+VA did not affect LPS in LBW swine. Values are means ± SD. NBW, normal birth weight; LBW, low birth weight; Control, control diet; HFHC, high-fat, high-carbohydrate diet, HFHC+VA, high-fat, high-carbohydrate diet enriched with vaccenic acid, (p=0.852).

4.8 Discussion

4.8.1 Development of insulin resistance swine on energy dense diet *(largely a focus for collaboration with another MSc student)*

Low birth weight remains a worldwide public health problem, especially in the context of its predisposition to chronic diseases in adulthood (Blanc & Wardlaw, 2005). Strategies to manage metabolic complications associated with LBW require a better understanding of the underlying mechanisms using translational animal models (K. Ferenc et al., 2014; Guilloteau et al., 2010). Of interest to the development of metabolic complications, sows spontaneously produce LBW offspring due to production trends increasing litter sizes over the natural placental capacity (G. Wu et al., 2006). Low birth weight Landrace-Large White x Duroc swine used in this study presented with lower daily body weight gain (ADG) and food intake (ADF) compared to their NBW counterparts. These observations are consistent with a recent study reporting that LBW pigs exhibit a reduced postnatal growth rate and do not return to normal weight even after 25 weeks (Yan et al., 2017). Despite this, it was notable that LBW piglets from the current study had significantly greater fractional growth rate (FGR). Previous literature have also demonstrated that LBW pigs fed standard diet show increased FGR yet remain significantly lower in weight until 7-months of age (K. R. Poore & A. L. Fowden, 2002). Findings suggest that IUGR leading to low birth weight may allow pigs to physiologically and metabolically adapt when fed an "*obesogenic"* diet, preserving an excessive weight gain at least until young adulthood. Our data also underscore that consumption of an energy-dense western type diet can significantly increase ADG in NBW pigs, and increased FGR in LBW pigs. In contrast, *Yan H et al.* reported increased daily weight gain only in LBW pigs (Yan et al., 2017). Of note, *Yan et al*. fed pigs a high-fat diet (replacing cornstarch for 10% lard) for 20 weeks. Whereas the higher fat content (17.9% lard) and additional

fructose (17.8%) used in our study may have stimulated weight gain in both LBW and NBW offspring.

For study 2, during the 10-week timeframe, LBW swine did not gain enough weight to catch-up with their NBW littermates. In the LBW groups, no difference was observed in ADG or ADF between diets. The VA enriched HFHC diet did not influence weight gain nor feed intake. Yan *et al.* found that feeding high-fat diet to LBW and NBW piglets for 20 weeks showed that LBW piglets had a higher ADG compared to NBW pigs fed the same diet (Yan et al., 2017). In this study, we hypothesize that if we had continued to feed the HFHC diet to our study piglets for a longer period of time we would observe greater weight gain in the LBW pigs, suggestive of catch-up growth. Our results did find that LBW groups had increased fractional growth rate compared to NBW littermates. An increased FGR in LBW offspring is consistent with literature (K. R. Poore & a L. Fowden, 2002), corroborating our hypothesis that a longer study would result in catch-up growth with a HFHC diet. Interestingly, a significant difference was observed in body length growth (crown-rump length) between diet interventions. We also observed that regardless of VA content the HFHC diet was associated with a decreased 5.5% of body length from birth. This is similar to study published by *Bielohuby et al.,* where rats were fed a low carbohydrate, high-fat diet, and showed significantly decreased in body length (nose-rump) (Bielohuby et al., 2011).

4.8.2 Impact of an energy dense diet to circulating and intestinal lipid metabolism (*largely a focus for collaboration with another MSc student*)

During the fasting period, plasma lipid levels primarily reflect hepatic VLDL metabolism. Recent data have reported both diet and birth weight are major factors impacting lipid metabolism (Liu et al., 2012). Our results showed that LBW pigs have increased fasting plasma VLDL-TG as

well as LDL-cholesterol compared with NBW pigs. From a cardiovascular risk viewpoint, increased cholesterol in the LDL fraction suggests increased risk of atherosclerosis by its contribution to atherosclerotic plaque development (A. Ravandi, A. Kuksis, & N. A. Shaikh, 2000). Our results showed that LBW pigs on western diet had increased incremental TG at 240 minutes post MOGTT. Similarly, results from a study in Yucatan mini pigs (on standard diet) showed that LBW pigs had increased triglycerides in response to an oral fat tolerance test and they concluded that it was potentially due to delayed plasma chylomicron clearance (Myrie et al., 2017). However, the strength of our current study is that we were also able to analyze mesenteric lymphatic lipids to determine if intestinal secretion/production was also affected. One of the most striking findings of this study was that LBW was significantly associated with increased lymphatic TG as well as CM number (apoB48), irrespective of diet or insulin status. Postprandial atherogenic dyslipidemia (a consequence of the intestinal lipoprotein secretion and reduced clearance of dietary-TG) has been linked to the development and exacerbation of cardiovascular diseases (Bansal et al., 2007; Hyson et al., 2003; Jackson et al., 2012). This is the first study to show that the LBW phenotype is associated with early intestinal adaptation for maximizing lipid secretion that then transforms into an obesogenic phenotype under energy excess.

For study 2, i observed that LBW pigs fed HFHC diet have higher fasting plasma total cholesterol, LDL and TG levels. Contrary to previous reports, i did not find any difference in cholesterol, LDL or TG concentrations in the plasma in LBW swine fed a HFHC diet enriched in VA compared to those on a HFHC diet with low VA content. The most likely cause for these different findings is the different models or different type of VA fed to the models. In previous rodent studies, pure forms of VA supplemented were applied to the diet, whereas, our LBW swine

were fed with beef fat. Enriching the diet with VA via beef fat consider fatty acid intake from the food matrix, as oppsed to simply consuming as a supplement.

Interestingly, intake of HFHC diet enriched with VA diet did result in an increase in fasting plasma HDL. Raising plasma HDL may have a beneficial health effect by reducing cardiovascular event risk and improving glycemic control (Barter, 2013). The VA enriched HFHC diet had a high percentage of cis-9, *trans*-11 CLA (HFHC=0.2%, HFHC+VA=1.28%) which may also responsible for the increased plasma HDL. Additionally, oleic acid (cis-9 C18:0) has also been known to increase plasma HDL, and yet was substantially lower in our HFHC+VA diet (HFHC=27.28%, HFHC+VA=19.94%) (Bermudez et al., 2011). On the other hand, in a human randomized trial, intake of VA enriched diet for 5 weeks resulted in lower total and HDL cholesterol in healthy young men (Tholstrup et al., 2006). While in another clinical trial, feeding both a CLA and VA supplemented diet found no changes in plasma lipids, insulin or glucose in healthy young men (Tricon et al., 2006). Our current swine model may be an excellent animal model to further study the mechanisms surrounding these findings for translation to humans. The present findings suggest that feeding LBW swine a HFHC diet with beef fat enriched in VA can increase plasma HDL cholesterol, potentially decreasing cardiometabolic risk.

In terms of lymph flow, we found LBW swine fed a control diet had lower lymph flow rate, decreased total lymph TG than NBW littermates on the same diet. Interestingly, there was no difference in number of CM particles (determined by apoB48 concentration) between birth weight groups on a control diet. Our findings suggest that LBW swine fed a control diet secreted less TG in other lipoproteins (such as HDL). There was also no altered lymph flow rate, apoB48 or TG in both HFHC diet intervention (with low or high VA content). Current results are contradictory to previous rodent studies showing decreased TG and CM particle number in lymph (apoB48

concentration) after intake of diet enriched with VA (1% *w/w*) (Y. Wang et al., 2009). Current results may be different due to the fact the diet was previously supplemented with VA in a pure form in the intestine. In contrast, the current trial used beef fat enriched in VA. There may be a difference in the bioavailability of each approach.

4.8.3 Development of insulin resistance in swine *(largely a focus for collaboration with another MSc student)*

Epidemiological and animal studies have shown that accelerated postnatal growth, or "catch-up" growth increases chances of LBW individuals to develop "obesogenic" diet-induced obesity, IR and abnormal lipid metabolism in adulthood (Eriksson et al., 1999; Ong et al., 2004; Rueda-Clausen et al., 2011; J. Wang et al., 2016). In our study, we found that LBW offspring showed a possible trend towards becoming more insulin sensitive than their NBW littermates. Our results suggest that insulin and glucose metabolism may be impacted during the period of catch-up growth, prior to undistinguishable weight differences between groups. According to Poore and Fowden the post-natal diet influenced the development of IR through increased weight gain in LBW offspring compared to NBW. Our results support this hypothesis in that a HFHC diet further increases weight gain postnatally and can possibly induce IR in a younger model. Our current study demonstrates that LBW leads to the development of early IR in Landrace-Large White x Duroc swine following HFHC feeding from weaning into young adulthood.

In study 2, LBW pigs fed on HFHC diet had increased plasma glucose and insulin following an MOGTT as compared to LBW swine fed a control diet. In addition, our results found a trend towards a decrease in insulin AUC in low birth weight swine fed a HFHC diet enriched in VA. Based on the linear regression, we observed the slope of the glucose and insulin between 0-30min (represent the initial phase of the postprandial period). During 30-120min, which represent

the second (clearance) phase of the postprandial period, we observed that the slope was less steep in the LBW-HFHC+VA group compared to the LBW-HFHC group.

The VA enriched HFHC diet resulted in a possible insulin sensitizing effect. VA has been shown to activate peroxisome proliferator-activated receptors (PPARs) and showed insulin sensitizing effects (Parodi, 2016). In this study we used less number of animals in each treatment group, so further investigation is needed to confirm the insulin sensitizing effects. In our VA diet, we had 7.87% reduction in palmitic acid (C16:0) content. It has been shown that, a saturated fatty acid (SFA) (palmitic acid) are associated with increase IR (Musso et al., 2003). So, lower palmitic acid in the VA enriched diet may also contribute to the increased insulin sensitivity.

4.8.4 Birth weight and diet impacting gut microbiota

I hypothesized that the underlying mechanisms of LBW-induced increased intestinal lipid absorption may involve dysbiosis in gut microbiota (Costello et al., 2013). However, the gut microbiota data did not support this hypothesis, as an effect of birthweight was not observed at the community, or taxonomic level. In agreement with the previous reports on microbiota of pigs, *Firmicutes* increased in abundance in response to high fat diet **(See Figure 4.10 for summary),** indicating that postnatal diet rather than birth weight predominantly shaped the gut microbiota composition (Panasevich et al., 2018; Pedersen, Andersen, Hermann-Bank, Stagsted, & Boye, 2013; Pedersen, Andersen, Molbak, Stagsted, & Boye, 2013). My findings are in accordance with a recent study in low and normal birth weight guinea pigs (Al et al., 2017), which showed significant effect of Western diet on the gut microbiota but no effect of birthweight.

Consistant with the study 1 and other reports in the study 2, we found the HFHC diet increased the ratio of *Firmicutes*: *Bacteroidetes* in LBW pigs. This finding is consistent with the

previous literature, that repoted high fat diet shaped the gut microbiota (Panasevich et al., 2018; Pedersen, Andersen, Hermann-Bank, et al., 2013; Pedersen, Andersen, Molbak, et al., 2013).

Interestingly addition of VA in HFHC diet, we observed decrease in *Firmicutes*, which is consistent with literature (Li et al., 2017a). Principal coordinates analysis (PCoA) of microbiota composition via bray curtis metrics showed HFHC showed separate clustering and significant shift in intestinal microbiota than control group but addition of VA in HFHC normalizing the HFHC diet induced shift. It is possible that diet enriched with VA may have reduced HFHC induced dysbiosis by reducing the expression of inflammatory marker as well as other regulatory pathway in LBW pigs. Previous literatures have shown blood lipid-lowering and anti-inflammatory effects of VA is partially associated with activation of PPAR dependent pathway (Y. Wang et al., 2012). Our research group demonstrated that VA affects the tissue endocannabinoids (ECs) in JCR:LA-cp rat (Jacome-Sosa et al., 2016). Addition of VA increased the concentration of jejunal anandamide and the noncannabinoid signaling molecules, oleoylethanolamide and palmitoylethanolamide. VA treatment also showed decreased expression of two key inflammatory markers, TNFα and IL1β in the intestine (Jacome-Sosa et al., 2016), but research is needed to understand the effect of VA on gut microbiota during insulin resistance and related mechanism.

In conclusion, in our first study LBW pigs on western diet show upregulated lymphatic TG absorption/secretion, insulin resistance, and dyslipidemia. This study does not provide evidence of increased dysbiosis in LBW-pigs contributing to increased severity of metabolic phenotypes. We propose that the LBW metabolic milieu leads to intestinal perturbations that under an obesogenic environment exacerbate the metabolic phenotype and production swine offer a useful model to study these pathways in human disease. While in study 2, intervention of beef fat enriched with VA had a mild insulin sensitizing effect with high plasma HDL cholesterol. Supplementation of

VA may reduce postprandial plasma TG with unaltered lymphatic TG, CM particle number (apoB48 concentration) and particle size. In term of gut microbiota, addition of VA showed protection from dysbiosis in pigs. But still more research is needed to confirm the effect of VA on insulin sensitivity and gut microbiota shift.

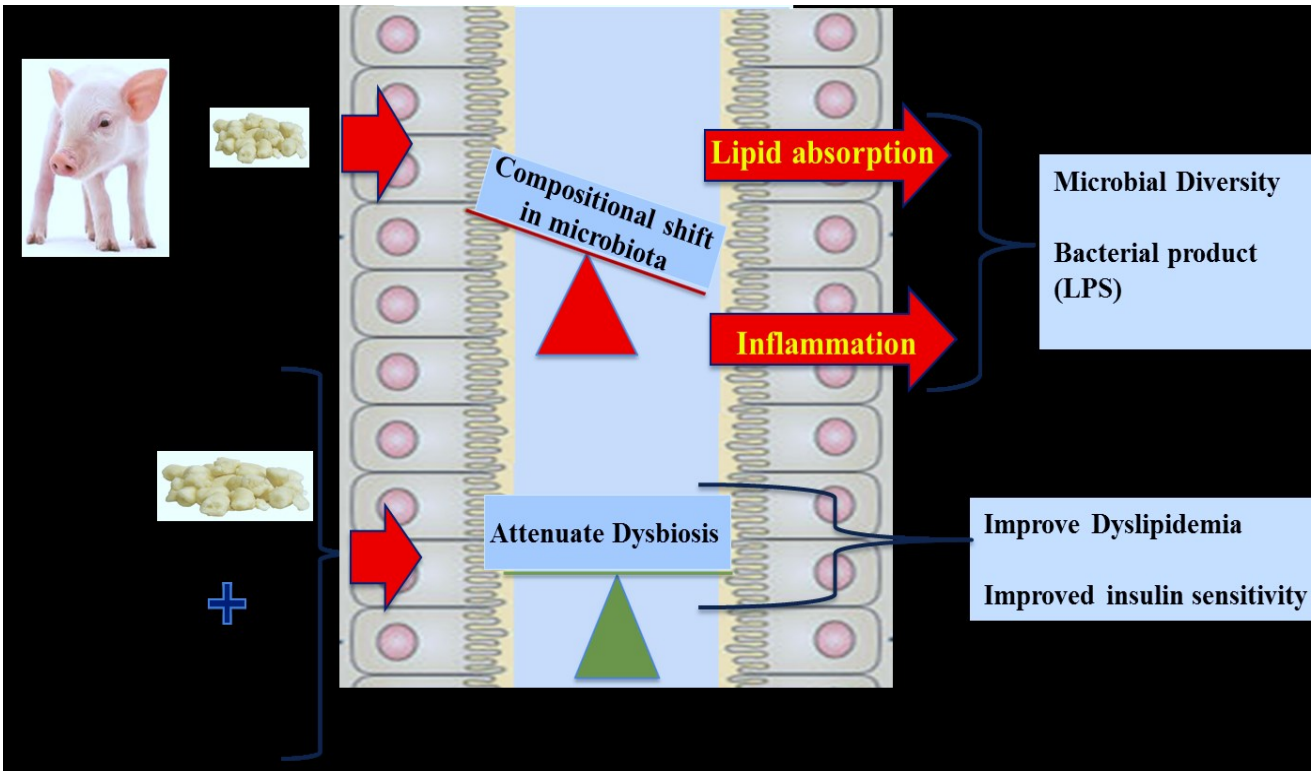


Figure 4.10 High fat high carbohydrates diet induced dysbiosis and addition of Vaccenic acid enriched improved dysbiosis, dyslipidemia and Insulin sensitivity in LBW pigs
(overview and proposed mechanism)

Chapter 5: General discussion:

5.1 Discussion of findings and future directions

In this thesis i sought to utilize JCR:LA-cp rat (a model of spontaneous IR) as well as LBW IR pigs to understand the association between high fat diet and gut microbiota composition during IR condition. **Figure 5.1** provides a summary of the observation from this thesis.

5.2 Insulin resistance did not affect the gut microbiota in both JCR:LA-cp rats and LBW pigs

To our knowledge this is the first study to utilize next generation sequencing to characterize intestinal microbiota in the JCR:LA-cp rat. In this thesis i did expect to observe differential modification to the intestinal microbial profile in both animal models of insulin resistance (JCR:LA-cp rat and LBW pigs). In both studies (Chapter 3 and Chapter 4), we observed that IR did not have impact on gut microbiota composition. In Chapter 3 we observed that lean and obese rats on chow did not show any shift in gut microbiota. Moreover, findings from Chapter 4 also appeared to reproduce this finding in LBW pigs (**Figure 5.1**).

Contradictory to our hypothesis, our results are not consistent with previous findings, where obese JCR:LA-cp rats fed a control diet for 10 weeks displayed an increased *Firmicutes*:*Bacteroidetes* ratio compared to their lean counterpart (Ellekilde et al., 2014; Hakkak, Korourian, Foley, & Erickson, 2017; Parnell & Reimer, 2012). However in this study, Parnell et. al chose to select a few genera including *Clostridium spp. and Lactobacillus spp.* (*Firmicutes*) as well *Bacteroides and Prevotella (Bacteroidetes*) phylum.

In this thesis, i used two different animal models of MetS (12 weeks old JCR:LA-cp rat and 5 weeks old LBW pig) that were fed for a shorter duration (6 weeks only) than most other published studies. The longer intervention period and older age of animals used by Parnell et. al

and Pederson et. al may explain the differential gut microbiota composition which is contradictory to our current findings.

5.2.1 High fat diet altered the gut microbiota during insulin resistance (JCR:LA-cp rats and LBW pigs)

In Chapter 3, we demonstrated that a HFD can increase the ratio of *Firmicutes:Bacteroidetes* in the JCR:LA-cp rat. Moreover, findings from Chapter 4 also appeared to reproduce this finding in LBW pigs. Though the ratio of the *Firmicutes*:*Bacteroidetes* does not seem to be consistant in human studies (Sze & Schloss, 2016), our findings appear concordant with other studies where a high-fat diet increases *Firmicutes:Bacteroidetes* ratio (Turnbaugh et al., 2008).

Findings from Chapter 4 also showed that a HFD can increase the postprandial insulin response, circulating and intestinal lipids. However, intake of control diet showed lower postprandial insulin secretion in LBW swine. We have also noticed that addition of VA showed modest insulin sensitizing effects. From our findings, we may conclude that HFD is major factor impacting intestinal microbiota composition. Interestingly, we noticed increased in *Lactobacillus* genus in our swine study. This increased *Lactobacillus* may have been due to the addition of fructose in our HFD. Fructose is known to facilitate the growth Lactobacillus not only as a carbon source but also as an electron acceptor and help in acetate production with extra ATP synthesis via the mannitol pathway (Kang, Korber, & Tanaka, 2013; Poolman, 1993). Tomas et. al observed that HFD reduced the expression of anti-microbial peptides, lysozyme and α-defensins, which resulted in disruption of the mucus layer and increase epithelial permeability by down-regulating claudin-7 levels (Tomas et al., 2016). During obesity, a higher yields of SCFAs with other metabolic end products, such as amines, phenols, thiols, and hydrogen sulfide and facilitate the

growth of specific gut bacteria (Araujo, Tomas, Brenner, & Sansonetti, 2017; Turnbaugh et al., 2006). For example, a HFD can increase the propotion of gram negative bacteria (source of LPS), resulting in translocation of LPS by incorporation into chylomicron during lipid absorption (Cani et al., 2007; Ghoshal, Witta, Zhong, de Villiers, & Eckhardt, 2009).

5.2.2 High fat diet did not exacerbate bacterial translocation during insulin resistance

To our knowledge this is the first study to characterize bacterial translocation into the lymph draining the mesenteric lymph during the condition of insulin resistance. Given the impact of HFD on systemic LPS, we expected that HFD would exacerbate bacterial translocation in JCR:LA-cp rats. Results from Chapter 3 showed that bacterial translocation was present in approximately one third of the samples from all treatment group, including lean chow-fed rats. Our analysis is consistent with the other findings that have identified translocation of microorganisms into the lyphatics in patients with wide variety of disorders (Cole et al., 1969).

Introduction of green fluorescent protein (GFP) labeled *E. coli* provided some evidence that live bacteria do translocate. However, contrary to my hypothesis i did not detect any evidence of increased translocation in the obese condition. In our study we didn not collet lymph node samples. We acknowledge that anatomically, the messenteric duct is downstream of node(s) and therefore it is possible that live gut bacteria could be present in upstream mesenteric lymph nodes (Garcia-Tsao, Lee, Barden, Cartun, & West, 1995; Ikeda et al., 2018; J. Zhang et al., 2017). We also speculate that it is still plausible to have greater bacterial translocation, but potentially filtered/removed by the immune system before arriving in the draining lymphatics. Further investigation in this area is necessary.

A very recent publication by *Chasssaing et al* reported that insulin resistance–associated dysglycemia can facilitate gut bacteria encroachment. *Chasssaing et al* enrolled 42 pateints and

collected mucosal biopsies. The authors observed that the distance of gut bacteria from the epithelial wall is inversely correlated with body mass index, fasting glucose levels, and hemoglobin A_{1C} (Chassaing, Raja, Lewis, Srinivasan, & Gewirtz, 2017).

5.3 Vaccenic acid reduced dysbiosis in the gut microbiota in LBW swine

To our knowledge, this is the first study to observe the effect of HFHC diet rich in VA (a *trans* fat) on gut microbiota and lipid metabolism in LBW swine. Our own swine study showed that HFHC diet intake in LBW pigs caused metabolic complications, impaired post-prandial dyslipidemia and induced dysbiosis. We expected that supplementation of HFHC diet rich in VA would improve gut dysbiosis and lipid metabolism in LBW swine. PCoA data (Chapter 4) showed HFHC showed separate clustering and significant shift in intestinal microbiota than control group but addition of VA in HFHC normalizing the HFHC diet induced shift **(Figure. 4.7B)**. It may be possible that VA enriched diet may be reducing the dysbiosis induced inflammation, affecting the expression of inflammatory marker as well as other regulatory pathway in LBW pigs. Studies has shown that the gut microbiota regulates the endocannabinoids CB1 expression, anandamide (AEA) content and its degrading enzyme FAAH. Our own research group shown that vaccenic acid associated with activation of PPAR and affect tissue endocannabinoids (ECs), arachidonoylglycerol (2-AG), and increased the concentration of anandamide. Moreover, the noncannabinoid signaling molecules, oleoylethanolamide and palmitoylethanolamide in intestine with reduce expression of FAAH and two key inflammatory markers, TNFα and IL1β in the intestine. It has also been discovered, *in vivo* and *in vitro*, that the gut barrier function is controlled by CB1 through the distribution and localization of tight junction proteins (ZO-1 and occludin). But still need more research to understand the effect of VA on gut microbiota during insulin resistance and related mechanism.

5.4 Factors impacting results and interpretation

5.4.1 Variability and genetic diversity in animal models

In Chapter 3 we used a rodent model, which are a convenient biomedical tool for the study biomedical due to their ease of reproduction, surgical and genetic manipulation. The gene knock-out methods are often used to recapitulate numerous human diseases in mice. In rodents, inbreeding in established colonies help to reduce genetic variability within the model, which may be beneficial in research studies. Though some studies such as toxicology studies prefered out bred animal models, where the study focus to assess responses throughout a population of heterogenous individuals. However, rodent research has shown substantial significance and phenotypic effects from certain treatments without translating to any difference in human trials. The use of a swine model in Chapter 4 was considered for this thesis due to a potentially higher translatability to humans. Although, individual pigs can display high genetic variability compared to rodent models, the swine research and technology center (SRTC) at university of alberta uses very few boars (adult male pigs) for reproduction.

In this thesis, significance was difficult to achieve due to small samples sizes. For example our LPS results from both chapter showed the same pattern as in other studies but did not find the significant difference. In a few instances we discussed changes between groups that were not statistically significant, but rather trends towards a significant change. Studies should be repeated to confirm the changes we observed and to add power to the results provided in this thesis.

5.5 Limitation and future direction

A limitation of the pilot study described in Chapter 3 is the relatively low number of animals in each groups. The study should be repeated with larger numbers in each group to confirm our results related to diet, gut microbiota and bacterial translocation during insulin resistance. For

the study assessing bacterial translocation described in Chapter 3, we had difficulty in maintaining sterile conditions during the lymph collection procedure. We did not measure bacterial translocation in LBW pigs. It might be easier to maintain sterility in future experiments if lymph was collected directly from the mesenteric artery with a sterile syringe or vacutainer instead of a catheter. For the study assessing bacterial translocation described in Chapter 3, we were focused on mesenteric lymph and live GFP tagged *E. coli* in lymph but unable to detect the *E coli* LPS in serum. Other possible aspects could be investigated using labeled LPS, bacterial products or bacterial DNA. The measurement of important inflammatory markers such as TLR 9 and TLR2 as well as incorporating samples from lymphnodes may help to confirm the presence of live bacteria or bacterial products.

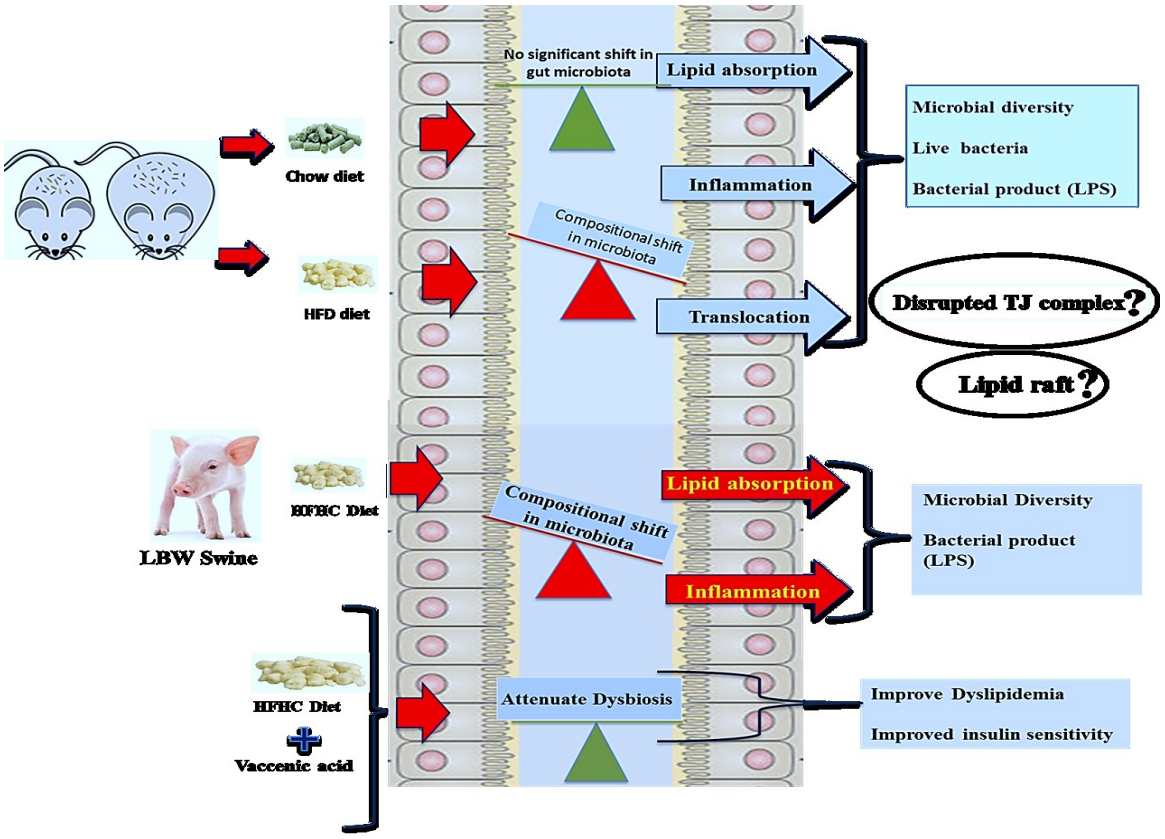
Overall, to overcome the limitations in future experiments we can alter the gut microbiota by using antibiotics or using the defined mock community of gut microbiota in animals. We can feed them with chow, HFD as well as HFD modified with specific dietary fatty acid for longer time periods. Later, by measuring the composition of the gut microbiota, inflammation, bacterial translocation as well as expression of tight junction barrier of all groups of animals will help to observe the impact of diet, phenotype on gut microbiota as well as their effect on bacterial translocation.

5.6 Conclusion

The data presented in this thesis gives experimental evidence to suggest that increased energy intake and obesity does not affect the gut microbiota composition during the condition of IR. However, the addition of a HFD produced substantial changes in intestinal microbiota. Our observations are consistent with both rodent and human studies and suggest that LBW swine fed a HFHC diet can be utilized as a model for human translational research.

In our study, we fed two different animal models of MetS for a shorter duration (6 weeks only) than most other published literatures. The longer intervention period and older age of animals may help to understand the impact of diet (with or without specific dietary fatty acid modification) on gut microbiota during the condition of insulin resistance.

Figure 5.1 Over all summary of both studies (Rodent as well as LBW swine)



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