Effect of Consuming Dairy Fats on Obesity-Related Cardiometabolic Perturbations and Immune Function in Low Birthweight Swine

by

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ABSTRACT

Although dairy intake has been shown to have a neutral or some beneficial effect on major cardiometabolic risk factors, the impact of dairy, and especially dairy fat, on immune function remains to be investigated. To understand the effect of consuming dairy fat on cardiometabolic risk factors and immune function, we used an established low birthweight (LBW) swine model of diet-induced insulin resistance to compare high-fat and low-fat dairy products to a control high-fat diet (CHF). LBW piglets were randomized to consume one of the 3 experimental diets: 1) CHF, 2) CHF diet supplemented with 3 servings/day of high-fat dairy (HFDairy) and 3) CHF diet supplemented with 3 servings/day of low-fat dairy (LFDairy). As comparison groups, normal birthweight (NBW) piglets were fed a CHF (NBW-CHF) or standard pig grower diet (NBW-Chow). At 11 weeks of age, all piglets underwent an established modified oral glucose and fat tolerance test (MOGTT). At 12 weeks of age, piglets were euthanized. Fasting blood and tissue samples were collected. *Ex vivo* cytokine production by peripheral blood mononuclear cells (PBMCs) and isolated intestinal mesenteric lymph node (MLN) cells stimulated with mitogens were assessed. As expected, LBW-CHF piglets showed early signs of insulin resistance. Feeding high-fat dairy products improved (lowered) *fasting* plasma glucose concentrations more than low-fat dairy compared to LBW-CHF. Irrespective of fat content, dairy consumption had neutral effect on major cardiometabolic risk factors in both *fasting* and *postprandial* state. Following *ex vivo* mitogenic stimulation on PBMCs and MLN cells, we have for the first time confirmed that LBW-CHF piglets exhibited both impaired peripheral and intestinal immune function particularly T cell

function. While feeding high-fat dairy had relative minor effect, feeding low-fat dairy significantly improved the production of IL-2, TNF-α and/or IFN-γ from PBMCs and MLN cells respectively, suggesting improved peripheral and intestinal immune function. Overall, the research presented in this thesis has provided new mechanistic insight to support the role of dairy products, specifically milk, yogurt, and cheese, in counteracting some of the obesity-related cardiometabolic and immune perturbations.

PREFACE

Enclosed in this thesis is the original work of Yongbo She with contributions and support described below:

Chapter 1 is the literature review and portions of the content has been published as a review paper in Frontiers in Nutrition as "*She Y, Mangat R, Tsai S, Proctor SD, Richard C (2022) The Interplay of Obesity, Dyslipidemia and Immune Dysfunction: A Brief Overview on Pathophysiology, Animal Models, and Nutritional Modulation. Frontiers in Nutrition 9:840209. DOI: 10.3389/fnut.2022.840209*".

Assistance and guidance were appreciated and acknowledged from my supervisors: Dr. Caroline Richard and Dr. Spencer Proctor; supervisory committee member: Dr. Sue Tsai; research associate from Proctor's lab: Dr. Rabban Mangat.

Chapter 4 has been published in Frontiers in Nutrition as "*She Y, Wang K, Makarowski A, Mangat R, Tsai S, Willing BP, et al (2022) Effect of High-Fat and Low-Fat Dairy Products on Cardiometabolic Risk Factors and Immune Function in a Low Birthweight Swine Model of Diet-Induced Insulin Resistance. Frontiers in Nutrition 9:923120. DOI: 10.3389/fnut.2022.923120*".

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and immunology. Experiments, data collection, analyses and manuscript writing were performed by Yongbo She.

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Animal Ethics: The research experiments containing animal use described in this thesis were approved by the University of Alberta Animal Care and Use Committee for Livestock (AUCC) and the Canadian Council of Animal Care (CACC) with protocol number AUP0001184.

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"The greatest happiness of life is the conviction that we are loved -- loved for ourselves, or rather, loved in spite of ourselves."

--Victor Hugo

Dedication

To my beloved family, their endless love and support

To my Ph.D. supervisors, their guidance and encouragement

To the piglets, their sacrifice for pushing the human knowledge boundaries

To the glory of God

ACKNOWLEDGEMENT

This thesis was conducted partially during the COVID-19 pandemic. I would like to first express my deepest grateful to all the frontline workers in Canada and around the world. Thank you all for saving the lives and fighting against the virus.

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Last but not the least, I would like to thank my parents, friends, and my beloved ones for being my source of power. I have also dedicated my 10 years of youth (Year 2012 to 2022) to studying in Canada. Thank you, Canada.

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ACAT	Acyl-CoA:cholesterol acyltransferase
APC	Antigen presenting cell
Apo	Apolipoprotein
AUC	Area under curve
CD36	Cluster of differentiation 36
CE	Cholesterol ester
CETP	Cholesterol ester transfer protein
CHF	Control high-fat diet
CI	Confidence interval
CRP	C-reactive protein
CV	Coefficient variation
CVD	Cardiovascular disease
DAG	Di-acylglycerol
DASH	Dietary Approach to Stop Hypertension
DGAT	Diacylglycerol acyltransferases
DHA	Docosahexaenoic acid
DIO	Diet-induced obesity
EPA	Eicosapentaenoic acid
ER	Endoplasmic reticulum

ERK	Extracellular signal-regulated kinases
F6P	Fructose 6-phosphate
FA	Fatty acid
FABP	Fatty acid binding protein
FAS	Fatty acid synthase
FATP	Fatty acid transport protein
FFA	Free fatty acids
FPLC	Fast protein liquid chromatography
FSC	Forward scatter
G6P	Glucose 6-phosphate
GALT	Gut-associated lymphoid tissue
HDL	High-density lipoproteins
HFDairy	High-fat dairy diet
HL	Hepatic lipase
HOMA-IR	Homeostatic Model Assessment for Insulin Resistance
HPLC	High performance liquid chromatography
IDL	Intermediate-density lipoprotein
IEC	Intestinal epithelial cell
IFN-γ	Interferon gamma
Ig	Immunoglobin

IL	Interleukin
ILC	Innate-like lymphoid cell
ISI	Matsuda insulin sensitivity index
LBW	Low birthweight
LCFA	Long-chain fatty acids
LDL	Low-density lipoprotein
LDL-R	LDL receptor
LPL	Lipoprotein lipase
LPL	Lysophospholipid
LPS	Lipopolysaccharide
MAG	Monoacylglycerol
MCFA	Medium chain fatty acids
MCP-1	Monocyte chemoattractant protein-1
MF	Milk fat
MFGM	Milk fat globular membrane
MFI	Median fluorescence intensity
MGAT	Monoacylglycerol acyltransferase
MHC	Major histocompatibility complex
MHO	Metabolically healthy obesity
MLN	Mesenteric lymph node

MOGTT	Modified oral glucose and fat tolerance test
MTP	Microsomal triglyceride transfer protein
MUFA	Monounsaturated fatty acid
NBW	Normal birthweight
NPC1L1	Niemann-Pick C1-Like 1
PBMC	Peripheral blood mononuclear cell
PC	Phosphatidylcholine
PCTV	Pre-chylomicron transport vesicle
PE	Phosphatidylethanolamine
PEP	Phosphoenol pyruvate
PHA	Phytohemagglutinin
PI	Phosphatidylinositol
PL	Phospholipid
PMA-I	Phorbol myristate acetate-ionomycin
PRR	Pattern recognition receptor
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acid
PWM	Pokeweed mitogen
RCT	Randomized controlled trial
SCFA	Short chain fatty acid

sdLDL	Small dense low-density lipoprotein cholesterol
SEM	Standard error mean
SFA	Saturated fatty acid
SM	Sphingomyelin
SOP	Standard operating procedure
SR-B1	Scavenger Receptor Class B type 1
SREBP	Sterol regulatory element binding proteins
SSC	Side scatter
T2D	Type-2 diabetes
TC	Total cholesterol
TCA	The tricarboxylic acid
TE	Total energy
TG	Triglyceride
Th	Helper T cell
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-α
Treg	Regulatory T cell
VLDL	Very low-density lipoprotein

1 CHAPTER 1 Literature review

Some sections in Chapter 1 have been published in Frontiers in Nutrition as part of a review paper (She et al. 2022. DOI: 10.3389/fnut.2022.840209)

1.1 Introduction

Obesity is one of the most common non-communicable diseases globally and is often accompanied by several complications such as insulin resistance, diabetes, atherosclerosis, hypertension and cancer (de Heredia et al., 2012). According to the World Health Organization, the prevalence of obesity worldwide has tripled since 1975 with nearly 1.9 billion overweight adults. In Canada, over 30% of Canadian adults have obesity and may need medical intervention. Dyslipidemia including elevated levels of triglyceride (TG) and low-density lipoprotein cholesterol (LDL-C) are often seen in individuals with obesity (Klop et al., 2013), which underpins a concomitant increase in cardiovascular disease (CVD) risk. On the other hand, obesity is also widely recognized as being associated with a state of chronic low-grade systemic inflammation with elevated levels of circulating pro-inflammatory cytokines (de Heredia et al., 2012). More recent discoveries suggest that obesity and type-2 diabetes (T2D) are associated with marked changes in the immune system systemically and in metabolically relevant tissues (Luck et al., 2015), to an extent that impairs normal immune function (Richard et al., 2017). In that regard, individuals with obesity-related metabolic complications (i.e., T2D, hypertension) were also found to have an impaired immune

response to infection, including influenza and COVID-19 (Green & Beck, 2017; Kang et al., 2020). These findings suggest that obesity plays a key role in modulating immune function, development of metabolic complications, and related cardiometabolic diseases. Indeed, obesity is a heterogenous chronic disease that is influenced by multiple factors such as the environment and genetics. The notion of metabolically healthy obesity (MHO) which can be defined as individuals with obesity that do not have other comorbidities such as hyperglycemia, dyslipidemia or hypertension, has drawn substantial attention in the research field (Magkos, 2019). MHO individuals show improved insulin sensitivity, better adipose tissue function, a favorable adipokine secretion pattern, as well as less immune cell infiltrations into adipose tissue (Blüher, 2020). Yet, there is still a debate as to whether MHO individuals are truly healthy or if they will eventually develop metabolic perturbations associated with their obesity.

The pandemic of COVID-19 has drawn substantial interests in developing strategies to improve obesity-related immune dysfunction to protect our population. In this context, the importance of appropriate and adequate nutrition is often underscored. Nutrients are important since they provide fuel for the immune system, are substrates for immune metabolites and agents for anti-microbial peptides (Calder, 2013, 2020, 2022; Childs et al., 2019). Dairy is a unique and complex food matrix that consists of fats, proteins, vitamins and minerals. Still, many consumers tend to avoid consuming dairy foods mainly due to the perception that diets high in saturated fatty acids (SFA) may yield adverse health effects. However, emerging evidence have reported neutral to potentially

beneficial effect of dairy foods and risk of T2D and CVD (Sochol et al., 2019; Soedamah-Muthu & de Goede, 2018). Certain nutrients found in dairy fat have also been demonstrated to have a beneficial effect on the immune system (Azarcoya-Barrera et al., 2021; Blewett et al., 2009; Lewis et al., 2014; Ruth et al., 2010). Collectively, these findings have led to the hypothesis that dairy foods have the potential to improve obesity-related cardiometabolic and immune dysfunction. In this chapter, the interaction between obesity, insulin resistance, dyslipidemia and immune dysfunction will be presented. An overview of the current nutritional strategies to counteract some of the obesity-related cardiometabolic and immune perturbations, with a particular focus on dairy and dairy fat, will also be provided.

1.2 Overview of lipid metabolism

1.2.1 Intestinal lipid metabolism

The small intestine primarily absorbs dietary lipids but is also responsible for the endogenous *de novo* synthesis of lipids (Ko et al., 2020). Dietary lipids are first hydrolyzed by gastric and pancreatic lipase to form monoacylglycerol and free fatty acids prior to be absorbed by enterocytes. Cholesterol is hydrolyzed by carboxyl ester hydrolase to form free cholesterol and fatty acids, whereas phospholipids are hydrolyzed by the pancreatic phospholipase A-2 to release lysophospholipids and free fatty acids. Intestinal absorption of lipids mainly consists of two pathways, namely the protein-dependent transport mechanism and passive-diffusion. Lysophospholipids are typically absorbed by passive-diffusion. Proteins, including cluster of

differentiation 36 (CD36), fatty acid transport protein 4 (FATP-4), Niemann-Pick C1-like 1 (NPC1L1) as well as scavenger receptor class B type I (SR-BI) facilitate protein-dependent uptake for free fatty acids and cholesterol (Ko et al., 2020). An illustration of lipid uptake by enterocytes are shown in **Figure 1.1**.

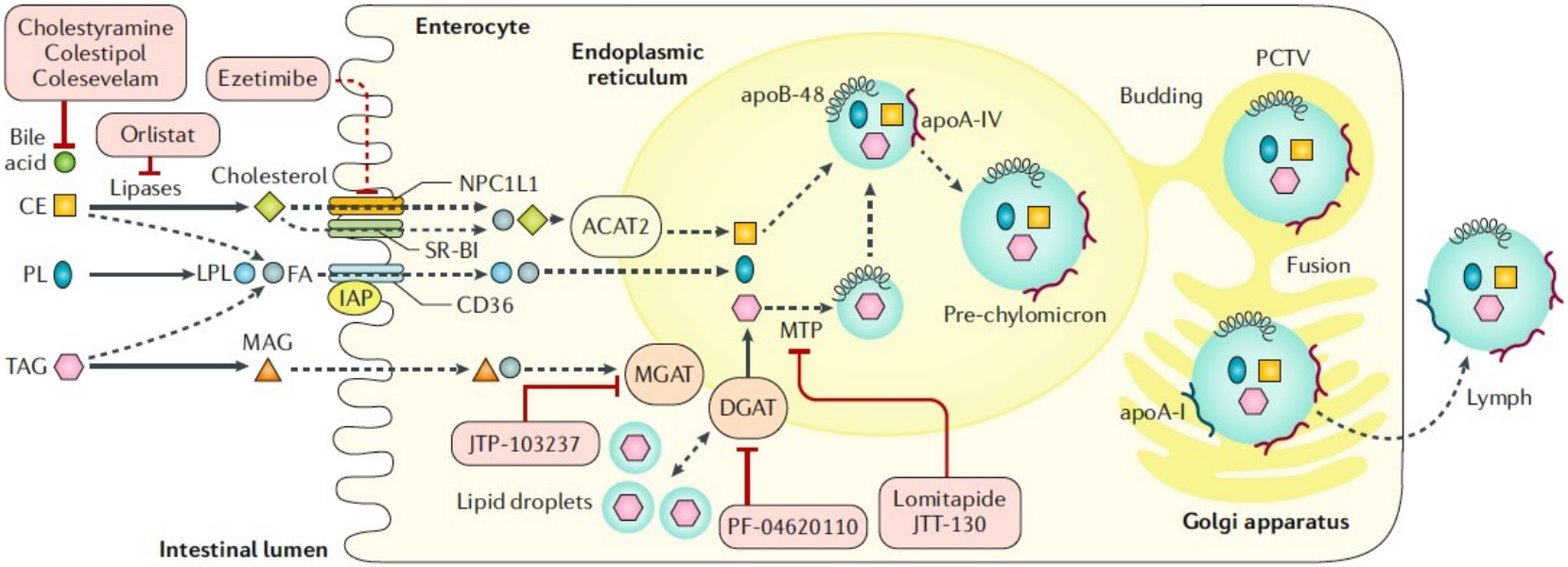

Figure 1.1 Summary of intestinal lipid uptake and transport

(Adapted from Ko et al., 2020)

ACAT, acyl-coenzyme A:cholesterol acyltransferases; CE, cholesterol ester; DGAT, diacylglycerol acyltransferases; FA, fatty acid; LPL, lysophospholipid; MAG, monoacylglycerol; MGAT, monoacylglycerol acyltransferase; MTP, microsomal triglyceride transfer protein; NPC1L1, Niemann-Pick C1-Like 1; PCTV, pre-chylomicron transport vesicle; PL, phospholipid; SR-B1, Scavenger Receptor Class B type 1; TAG, triglyceride. CE, PL and TAG from lumen are hydrolyzed to free FA, cholesterol, LPL and MAG and are transported to enterocyte by membrane transporters. Within enterocyte, they are re-esterified and further packed to apolipoprotein to form chylomicron.

Upon the uptake of lipid by enterocytes, they can be re-packaged to form chylomicron lipoprotein or stored as cytosolic droplets. These processes usually take place in the endoplasmic reticulum, where monoacylglycerols and free fatty acids are converted to diacylglycerides (DG) and then TG. DG can also be combined with choline to form phosphatidylcholine (PC). Cholesterol is esterified with free fatty acids to form cholesterol esters mediated by acyl-CoA:cholesterol acyltransferase (ACAT) (Hussain, 2014). Intriguingly, chylomicrons and cytosolic lipid droplets share similar structural features as they both contain TG and cholesterol ester in the core and are enveloped by a phospholipid monolayer along with free cholesterol and apolipoproteins. Cytosolic lipids are usually metabolized and/or secreted during fasting state when energy is needed. In contrast, the remining lipids are re-packaged into chylomicrons during the postprandial state (Ko et al., 2020). Assembly of chylomicron particles starts from the incorporation of apolipoprotein (Apo) B-48 into phospholipid-rich nascent chylomicron particles and is facilitated by microsomal triglyceride transfer protein (MTP). MTP prevents post-translational degradation of Apo B-48 and converts apo B-48 to be more ready for lipid incorporation (Hussain, 2014). Chylomicron particles also contain various other apolipoproteins including Apo A-I and A-IV. Pre-chylomicrons are then transported to the Golgi and eventually released from basolateral membrane to enter lymphatic circulation (Hussain, 2014). On the other hand, enterocytes also facilitate the secretion of high-density lipoproteins (HDL). It is thought that HDL particles compete for the same cholesterol pool as chylomicrons and are responsible for trans-intestinal cholesterol efflux (Ko et al., 2020; Mangat et al., 2018), yet the exact role and physiological functions of intestinal HDL remain for the most

part to be elucidated. Expression of SR-BI and LDL receptors on the basolateral membrane as well as ABCG5/8 on the apical membrane are also responsible for intestinal cholesterol efflux.

Intriguingly, emerging finding from our lab have demonstrated that HDL associated Apo A-I expression can be reduced in insulin-resistant state, suggesting reduced trans-intestinal cholesterol efflux (Mangat et al., 2018). On the other hand, the expression of CD36, NPCILI and FATP-4 have also been found to be upregulated in the context of insulin resistance, suggesting increased intestinal lipid absorption (Hsieh et al., 2008). Elevated basal levels of extracellular signal-regulated kinases (ERK-1/2) in enterocyte during insulin resistance were also found to contribute to the enhanced *de novo* lipogenesis (Federico et al., 2006). More importantly, up-regulated expression of MTP in the context of insulin resistance prevents Apo B-48 post-translational degradation and promotes overproduction of TG-rich chylomicrons (Adeli & Lewis, 2008). Previous findings from our lab and others have also showed that both fasting as well as postprandial concentrations of Apo B-48 can be significantly higher in obese/insulin resistant states in pre-clinical models as well as humans (Duez et al., 2006; Hayashi et al., 2011; Vine et al., 2007). In contrast, short-chain fatty acids (SCFA) and medium-chain fatty acids (MCFA) are absorbed by the enterocytes independently of protein transporters. Absorbed SCFA and MCFA are directly transported to the liver *via* portal vein (Schönfeld & Wojtczak, 2016). Intriguingly, feeding a diet rich in SCFA and MCFA to CD36 deficient mice can attenuate glucose uptake and hyperinsulinemia (Hajri et al., 2001). The benefits of MCFA on whole body fat oxidation and

weight management have also been studied (Papamandjaris et al., 1998). Of note, dairy is a good dietary source of SCFA and MCFA.

1.2.2 Hepatic lipid metabolism

In humans, the liver is the major site for fatty acid synthesis. The major sources of fatty acids in liver include free fatty acids (FFA) from circulation, TG recycled from remnant lipoproteins and fatty acids endogenously synthesized. FFA are primarily derived from adipocyte TG lipolysis and are taken up by the liver *via* proteins such as the FATP 2 & 5, fatty acid binding protein (FABP) as well as CD36 (Bechmann et al., 2012). Of note, the link between elevated free fatty acids, obesity and T2D has been well established (Boden, 1999). The elevation of FFA in the context of obesity, contributes to increasing fat deposition in liver and other tissues (i.e., skeletal muscles), causing lipotoxicity and insulin resistance (Sieber & Jehle, 2014).

Secondly, *de novo* fatty acid synthesis occurs in the cytosol of hepatocytes and starts with a molecule derived from tricarboxylic acid cycle, namely acetyl-CoA. The *de novo* synthesis of fatty acid is mainly catalyzed by acetyl-CoA carboxylase and fatty acid synthase (FAS) by adding the 2-carbon molecule (acetyl-CoA) into precursors until the formation of 16-carbon palmitic acid. In this context, insulin is known to modulate the expression of FAS and therefore, *de novo* lipogenesis. The expression of genes that encode FAS as well as other proteins involved in cholesterol synthesis are mediated by a "master controller", namely sterol regulatory element binding proteins (SREBP).

In turn, expression of SREBPs can also be modulated by insulin (Nguyen et al., 2008). Clear evidence has demonstrated that insulin resistance can up-regulate hepatic *de novo* lipogenesis (Smith et al., 2020). Of note, odd-chain fatty acids include pentadecanoic acid (15:0) and heptadecanoic acid (17:0) are primarily derived from bovine sources and cannot be endogenously synthesized in humans. On the other hand, fatty acid elongation and desaturation occurs following the *de novo* fatty acid synthesis in three main compartments including cytosol, mitochondria, and endoplasmic reticulum (Jump, 2009). For instance, palmitic acid can be further elongated and desaturated by Δ-9 desaturation to form oleic acid (18:1 n-9). In contrast, linoleic acid (18:2 n-6) and linolenic acid (18:3 n-3) cannot be synthesized in humans and therefore, they are essential fatty acids. However, they can undergo a series of elongation and desaturation to synthesis longer chain polyunsaturated fatty acids such as arachidonic acid (20:4 n-6), eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3). This process is mainly catalyzed by Δ-6, 5, 4 elongase and desaturase and often referred to "Sprecher Pathway" (Sprecher et al., 1995). Of note, the conversion rate from linolenic acid to EPA and DHA is relatively low in humans.

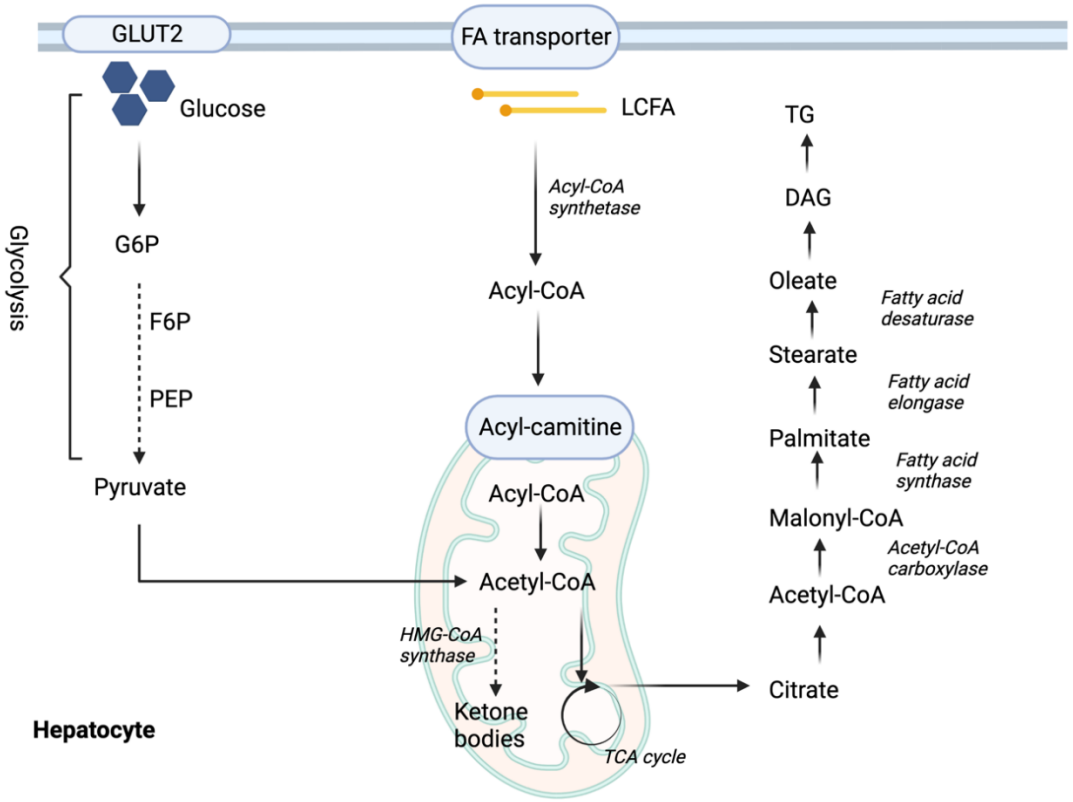

Figure 1.2 Metabolic pathway of hepatic *de novo* lipogenesis

(Adapted from Postic & Girard, 2008) Recreated with BioRender

DAG, di-acylglycerol; FA, fatty acid; F6P, fructose 6-phosphate; G6P, Glucose 6-phosphate; PEP, phosphoenol pyruvate; LCFA, long-chain fatty acids; TCA; the tricarboxylic acid; TG, triglyceride. The synthesis of TG in liver is nutritionally regulated. Enzymes involved in key metabolic pathways, including (i) glucokinase for glycolysis; (ii) Acyl-CoA synthetase, HMG-CoA synthetase, Acetyl-CoA carboxylase and fatty acid desaturase and enlongase for lipogenesis.

Similar to the assembly of chylomicrons in enterocytes, very low-density lipoprotein (VLDLs) formation is also facilitated by MTP. Under normal (healthy) conditions, insulin inhibits the expression of MTP, which favors the degradation of ApoB100 and therefore the secretion of VLDL. In the context of insulin resistance, insulin fails to inhibit FoxO1 and MTP expression (Haas et al., 2013), thereby increasing TG-rich VLDL particle secretion. It is well known that both postprandial and fasting levels of TG are significantly higher in the presence of insulin resistance (Robins et al., 2011; Søndergaard et al., 2017). A comprehensive review of hepatic lipid metabolism under normal and insulin resistant conditions can be found elsewhere (Crusio & Radeke, 2020; Feingold & Grunfeld, 2000; Hodson & Gunn, 2019).

1.2.3 Lipoproteins in circulation

In circulation, ApoA-I and IV on chylomicrons are replaced by ApoE and ApoC-II, which both play crucial roles in lipoprotein metabolism. ApoC-II is known to activate the lipoprotein lipase (LPL) and induce free fatty acid release from chylomicrons in order to be taken up by peripheral tissues such as muscle and adipocytes (Wolska et al., 2017). ApoE regulates chylomicron remnant uptake by the liver *via* LDL receptor (LDL-R) and SR-B1. Cholesterol esters, glycerol and fatty acids from chylomicron remnants are further metabolized in hepatocytes and packed into VLDL to re-enter circulation (Bechmann et al., 2012). TG within VLDL particles produced by the liver are hydrolyzed by LPL to form intermediate-density lipoprotein (IDL) and LDL particles.

However, overproduction of chylomicron and VLDL in the context of insulin resistance leads to hypertriglyceridemia which in turn has been associated with low levels of HDL and/or higher number of small/dense LDL particles in circulation. This phenotype is thought to develop, firstly, due to dysregulated LPL activity. In contrast to ApoC-II, increased levels of ApoC-III was found in the context of insulin resistance and T2D (Borén et al., 2020; Florez et al., 2006). ApoC-III is known to inhibit LPL activity while also preventing the binding of TG-rich lipoprotein remnants to LDL-R. Hence, accumulated TG-rich VLDL due to delayed clearance are hydrolyzed by hepatic lipase to form small/dense LDL particles (Borén et al., 2020). On the other hand, insulin resistance is also associated with increased cholesteryl ester transfer protein (CETP) activity (Rashid et al., 2003). This process results in an increased TG exchange between TG-rich lipoproteins and HDL and LDL and the dissociation of ApoA-I from HDL. TG-rich HDL and LDL particles become more susceptible to be hydrolyzed by hepatic lipase and generating even smaller and denser particles, which ultimately contributing to the progression of atherosclerosis (Higgins & Adeli, 2020).

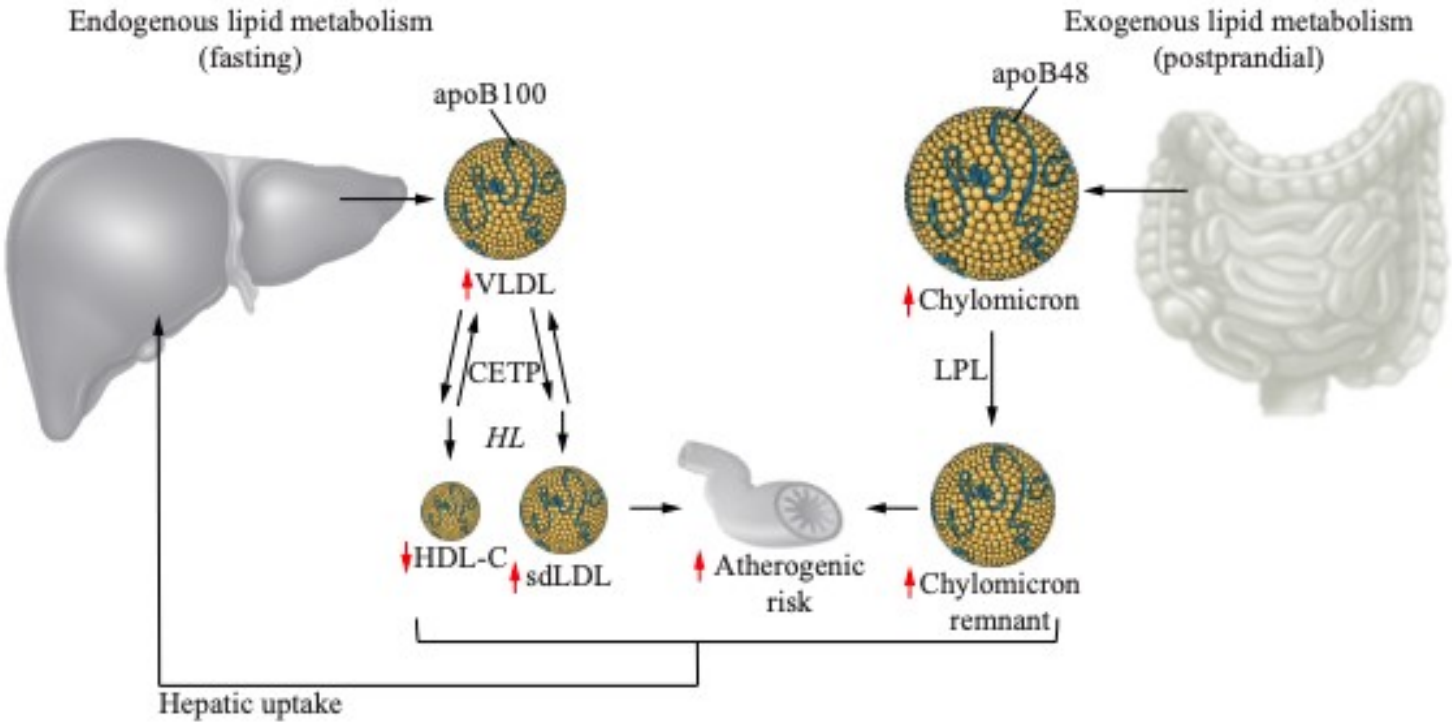

Figure 1.3 Overview of fasting and postprandial dyslipidemia in the context of insulin resistance

(Adapted from Higgins & Adeli, 2020)

CETP, cholesterol ester transfer protein; HDL-C, high-density lipoprotein cholesterol; HL, hepatic lipase; LPL, lipoprotein lipase; sdLDL, small dense low-density lipoprotein cholesterol. In the fasting state, VLDL particles are overproduced from the liver. Accumulation of VLDL and enhanced CETP and HL activity contribute to the formation of sdLDL particles and reduced HDL-C. In the postprandial state, chylomicrons are overproduced from intestine, contributing to an accumulation of chylomicron remnants through the action of LPL. Both sdLDL and chylomicron remnants contribute to risk of atherosclerosis.

1.3 Overview of the immune system

Our immune system becomes activated due to infection and/or injury, yet the type of immune responses will depend on the type of infection. Fundamentally, there are two major arms to the immune system, the innate and adaptive (T and B lymphocytes) immune systems. There are also four general functions of the immune system: 1) preventing pathogen breach, 2) identifying the pathogen, 3) eliminating the pathogen and 4) generating immune memory towards the pathogen (Calder, 2020).

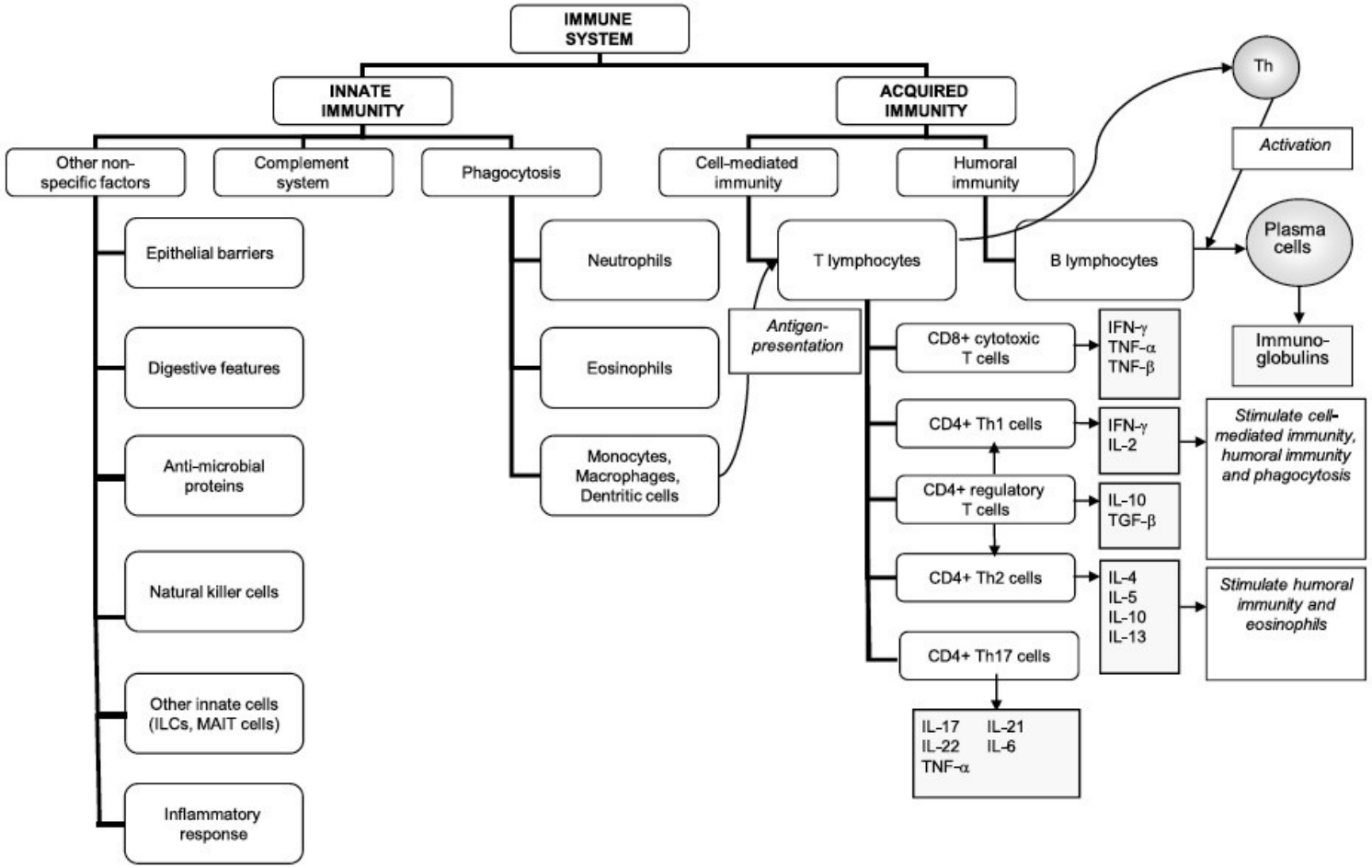

Figure 1.4 Summary of innate and adaptive immune system

(Adapted from Calder, 2022)

IFN, interferon; ILCs, innate lymphoid cells; IL, interleukin; MAIT, mucosal associated invariant T cells; TGF, transforming growth factor; Th, T helper cells; TNF, tumor necrosis factor.

1.3.1 Innate immune system

Barriers including skin, mucosal layers, chemical as well as biological barriers (i.e., stomach acid, commensal organisms) are the first line of defense against pathogen breaching (Calder, 2020). Pathogens can be recognized by cells of the innate immune system such as macrophages and dendritic cells through the presence of pattern recognition receptors (PRR) including toll-like receptors (TLR). To date, 10 functional TLRs have been identified in mammals and each of them has a distinct role (Calder, 2020). The most well-studied TLR is the TLR-4 that recognizes lipopolysaccharide (LPS) from gram-negative bacteria (Molteni et al., 2016). Extracellular pathogens (i.e., bacteria) are endocytosed by phagocytes and the peptide fragments of bacteria are presented by antigen presenting cells (APC, macrophage, dendritic cell, B cell) with major histocompatibility complex (MHC) class II molecules. In contrast, intracellular pathogens (i.e., virus) are small enough to penetrate into cells and can be presented by MHC class I molecules in nearly all nucleated cells (Wieczorek et al., 2017). Upon activation of TLRs, a series of intracellular signaling cascades *via* pathways such as MyD-88 and NF-kB trigger the production of cytokines and influences T cell differentiation (**Figure 1.5**). Of note, professional APCs such as dendritic cells are capable of cross-presentation of MHC class I and II molecules.

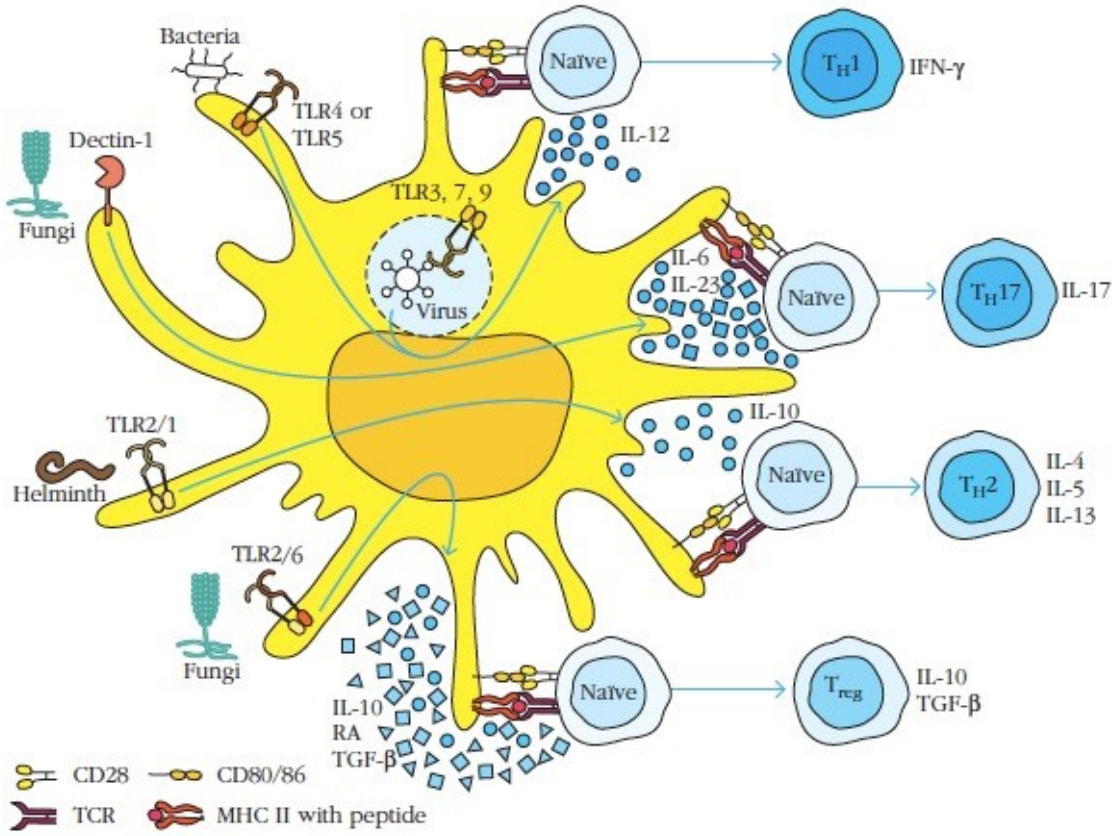

Figure 1.5 Differential signaling through dendritic cell PRRs influences helper T cell functions

(Adapted from Judith A. Owen, Jenni Punt, 2017)

IFN, interferon; ILCs, innate lymphoid cells; IL, interleukin; MAIT, mucosal associated invariant T cells; MHC, major histocompatibility complex; TGF, transforming growth factor; TCR, T cell receptor; Th, T helper cells; TLR, toll-like receptor; TNF, tumor necrosis factor; Treg, T regulatory cells. Dendritic cells are professional antigen presenting cells. To date, there are 10 TLRs identified. Upon activation, dendritic cells can activate naive T cells through MHC class II and TCR engagement and induce T cell proliferation and differentiation with the signaling of cytokines.

1.3.2 Adaptive immune system

The adaptive immune system is primarily regulated by T and B cells. T cells are considered to be the master-controller of the adaptive immune system. There are three major subsets of T cells, including helper T cells (Th, CD4+), cytotoxic T cells (CD8+) and regulatory T cells (Treg). CD8+ T cells recognize MHC class I molecules and secret perforin, granzymes, and granulysin to induce cell apoptosis and cell death. In contrast, MHC class II molecules presented by APCs are recognized by CD4+ T cells (Gaudino & Kumar, 2019). Activated naive CD4+ T cells can undergo differentiation and proliferation into Th1 and Th2 among others. Generally speaking, Th1 cells regulate cell-mediated immunity, whereas Th2 cells regulate humoral immunity. Th1 cells produce cytokines such as tumor necrosis factor-α (TNF-α) and interferon gamma (IFN-γ) that enhances APC function and cytotoxic activity. Th2 cells produce cytokines such as interleukin (IL)-4, IL-5 and IL-13 and regulate B cell activity and antibody production. In contrast, Treg cells act to suppress immune responses and maintain homeostasis (Kondělková et al., 2010). This sophisticated but highly organized immune system works closely together to mount an appropriate and effective immune response to foreign stimuli. However, factors including age, smoking, alcohol consumption, physical activity, stress, obesity as well as poor nutrition, can all significantly impact the efficacy and magnitude of immune responses (Calder, 2022). A detailed review of the major components of the immune system and their respective functions can be found in (Judith A. Owen, Jenni Punt, 2017).

1.3.3 Intestinal immune system

The intestinal immune system is the largest secondary immune organ in the body termed as gut-associated lymphoid tissue (GALT) shown in **Figure 1.6**. The GALT is a collective of organized immune tissues that include mesenteric lymph node (MLN), Peyer's patches and lamina propria where lymphocytes are diffusely scattered (Forchielli & Walker, 2005). Intestinal epithelial cells (IEC) are a population normally not recognized as immune cells; however, they are the barrier between bacteria from intestinal lumen and the underlying sterile environment. Intriguingly, IECs also express MHC class II and TLR similarly to some immune cells (Heuberger et al., 2021; Purchiaroni et al., 2013). Evidence has shown a marked difference on TLR expression particularly the TLR-4 between healthy and individuals with inflammatory-bowl disease, highlighting the immunomodulatory effect of IECs (Cario & Podolsky, 2000). Peyer's patches are located beneath a group of epithelial cells called follicle-associated epithelium with the presence of M cell that can directly uptake antigens from intestinal lumen (Tezuka & Ohteki, 2019). Hence, antigen-loaded dendritic cells can then interact with T cells and circulated to MLN through lymphatic vessels to generate subsequent immune responses.

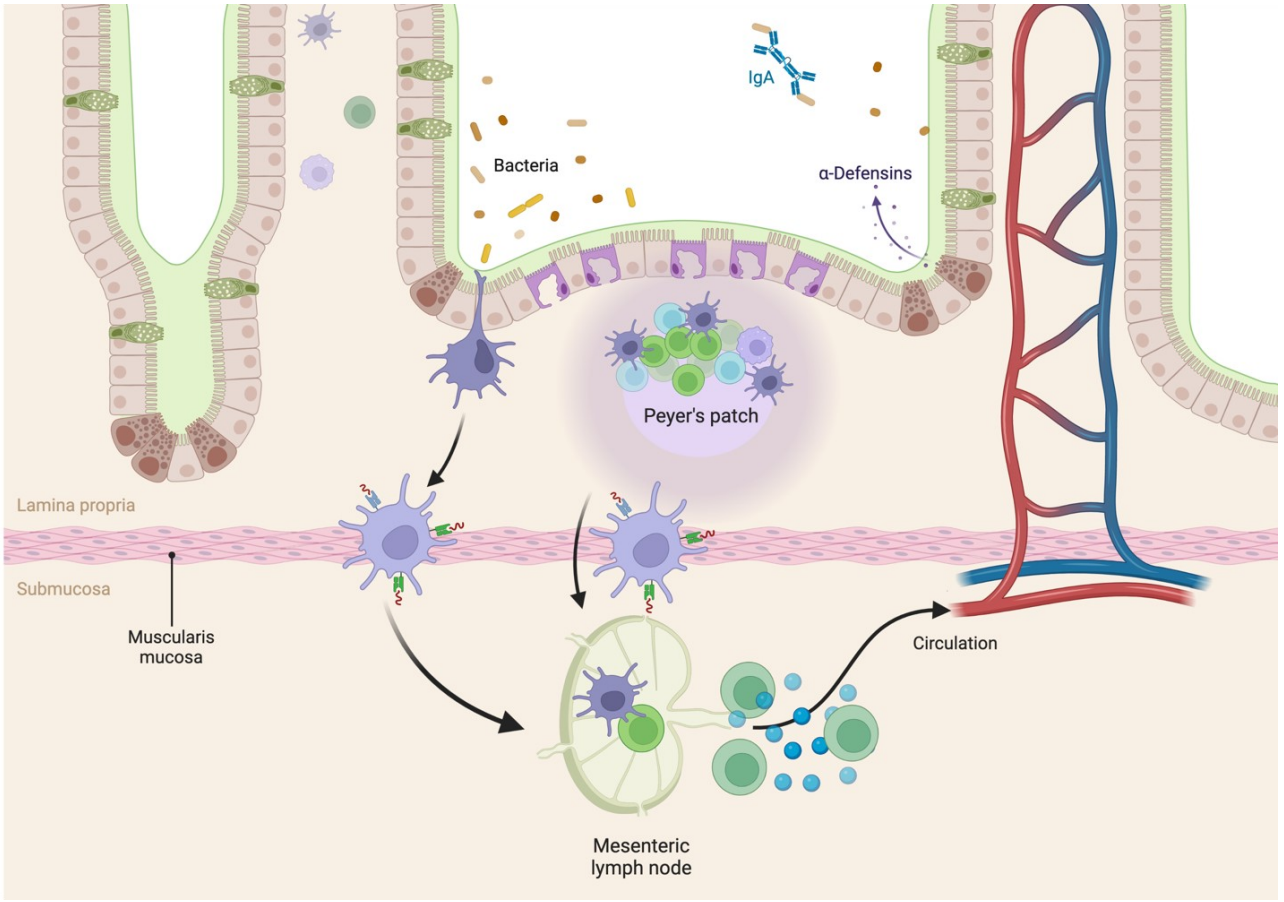

Figure 1.6 Illustration of gut-associated immune system

(Adapted from Calder, 2013) Recreated with BioRender

Gut-associated immune system consists of mucosal barriers, IgA and defensins as well as various immune cells that reside in lamina propria, Payer's Patch and mesenteric lymph node. Of note, mesenteric lymph node serves as an intermediate between intestinal and peripheral immune systems. Antigen loaded dendritic cells move to mesenteric lymph node and initiate subsequent immune responses.

When introducing the GALT, it is worth acknowledging the discovery of secretory immunoglobin (Ig) A which is a hallmark for understanding the mucosal immunity against pathogens. Nearly 80% of IgA+ producing plasma cells are residing in the intestine in humans and constantly secret IgA that prevents the binding of pathogens to intestinal epithelium (Tezuka & Ohteki, 2019). There are also a diverse population of both innate and adaptive immune cells residing in the GALT. Intestinal dendritic cells are one of the crucial populations that regulate the tolerogenic response to food antigens, homing of lymphocytes to gut and the differentiation of T cells (Gaudino & Kumar, 2019). Dendritic cells also express tight junction protein that can penetrate epithelial membrane and uptake pathogens without compromising the epithelial barrier (Purchiaroni et al., 2013). Apart from this, accumulation of T cells such as CD4+ T cell, CD8+ T cells, Treg cells as well as innate-like lymphoid cells (ILC) in GALT (mostly in lamina propria) can each have a distinct role and contributing to maintain intestinal immune homeostasis and normal function (Gaudino & Kumar, 2019; Wu & Wu, 2012).

1.3.4 Interaction of gut-microbiota and immune system

There is also a growing interest in understanding the physiological roles of the gut microbiota and its interactions with the immune system. Since it is beyond the scope of this thesis, the importance of the gut microbiota in modulating both local (i.e., in the intestine) and systemic immune responses have been appreciated in the field as reviewed in (Gaudino & Kumar, 2019; Soderholm & Pedicord, 2019). Of note, dysbiosis of gut microbiota has been linked to inflammatory bowel

disease, obesity and T2D (Levy et al., 2017; Yoo et al., 2020).

1.3.5 Importance of inflammation in immunity

It is worth to note that acute inflammation is one of the most crucial and normal immunological responses to infection and injury. There are five classic signs of inflammation, including heat, swelling, pain, redness and loss of function. These responses facilitate the movement of circulating immune cells to the local site of infection/inflammation, eliminate the pathogen and repair the damage. However, failure to resolve acute inflammation will lead to chronic exposure to pro-inflammatory cytokines which in turn will lead to the development of a number of metabolic complications including insulin resistance (Calder, 2003). Obesity is characterized by chronic low-grade systemic inflammation and has been identified as the underlying cause for developing chronic diseases such as T2D as reviewed in following sections.

1.4 The interplay of obesity, dyslipidemia and immune dysfunction

1.4.1 Obesity causes systemic inflammation

Studies have demonstrated that subjects with obesity have elevated circulating pro-inflammatory cytokine levels, including TNF-α, IL-6 and C-reactive protein (CRP) (Festa et al., 2001; Halle et al., 2004; Park et al., 2005). There is the notion that adipose tissue is a main contributor to systemic inflammation by producing substantial amounts of TNF-α and IL-6 (Coppack, 2001). It has also been demonstrated that these pro-inflammatory cytokines impair normal insulin signaling

pathways and therefore can lead to insulin resistance as well as dyslipidemia. However, the role of IL-6 *per se* may depend on the target organ. For example, IL-6 has been shown to promote glucose uptake by skeletal muscles therefore improving whole body glucose homeostasis (Glund et al., 2007). Nevertheless, how inflammation is initiated in the context of obesity and further leads to a state of chronic low-grade systemic inflammation still remains to be fully elucidated.

Bornstein et al. were the first to report the involvement of macrophages in adipose tissue leading to inflammation (Bornstein et al., 2000). This discovery has led to an emergent hypothesis that defines the relationship between obesity-related inflammation and impaired immunity. Later studies found that macrophage infiltration in adipose tissue is increased in obesity (Weisberg et al., 2003; Xu et al., 2003). Infiltrating macrophages appear as aggregated crown-like structures and tend to shift from M2 (anti-inflammatory) to M1 (pro-inflammatory) phenotypes (de Heredia et al., 2012). However, recent findings have challenged this initial simplified notion of M1/M2 phenotypes in adipose tissue. The population of macrophages in adipose tissue appear to have more diverse phenotypes (i.e., metabolic activated or oxidized), with distinct surface markers activated by a wide range of stimuli such as free fatty acids and/or glucose (Russo & Lumeng, 2018). The mechanisms of macrophage recruitment into adipose tissue are still not fully understood but appear to be partially due to adipose tissue expansion, leptin resistance and adipocyte death (Surmi & Hasty, 2008). M1 macrophages are pro-inflammatory and secrete substantial amounts of TNF-α and IL-6, while also secreting other pro-inflammatory mediators

such as monocyte chemoattractant protein-1 (MCP-1) that can recruit additional circulating monocytes (Makki et al., 2013; Surmi & Hasty, 2008). The understanding of macrophage infiltration is still evolving; however, we also know that other pro-inflammatory immune cells including CD8+ cytotoxic T cells, CD4+ Th 1 cells, neutrophils as well as B cells (particularly the B2 subtype that promote inflammation) have been observed during the progression of obesity in adipose tissue (Makki et al., 2013; Srikakulapu & Mcnamara, 2020).

On the other hand, obesity is often associated with gut microbiota dysbiosis featured as loss of the diversity (Turnbaugh et al., 2006). One of the major consequences of this dysbiosis is a compromised intestinal barrier integrity and increased leakage of LPS that further contribute to systemic inflammation. Reduced expression of epithelial tight junction proteins such as zonula occludens 1 and occludin has been proposed as one of the mechanisms to explain this reduced intestinal barrier integrity in obesity (Cani et al., 2008). Due to the fact that we are in a non-fasting state for most of the day, it is important to recognize that consumption of a high-fat meal is also known to directly induce LPS translocation and postprandial inflammation (Herieka & Erridge, 2014). Hence, these local (adipose tissue and intestine) perturbations can all contribute to systemic inflammation in the context of obesity.

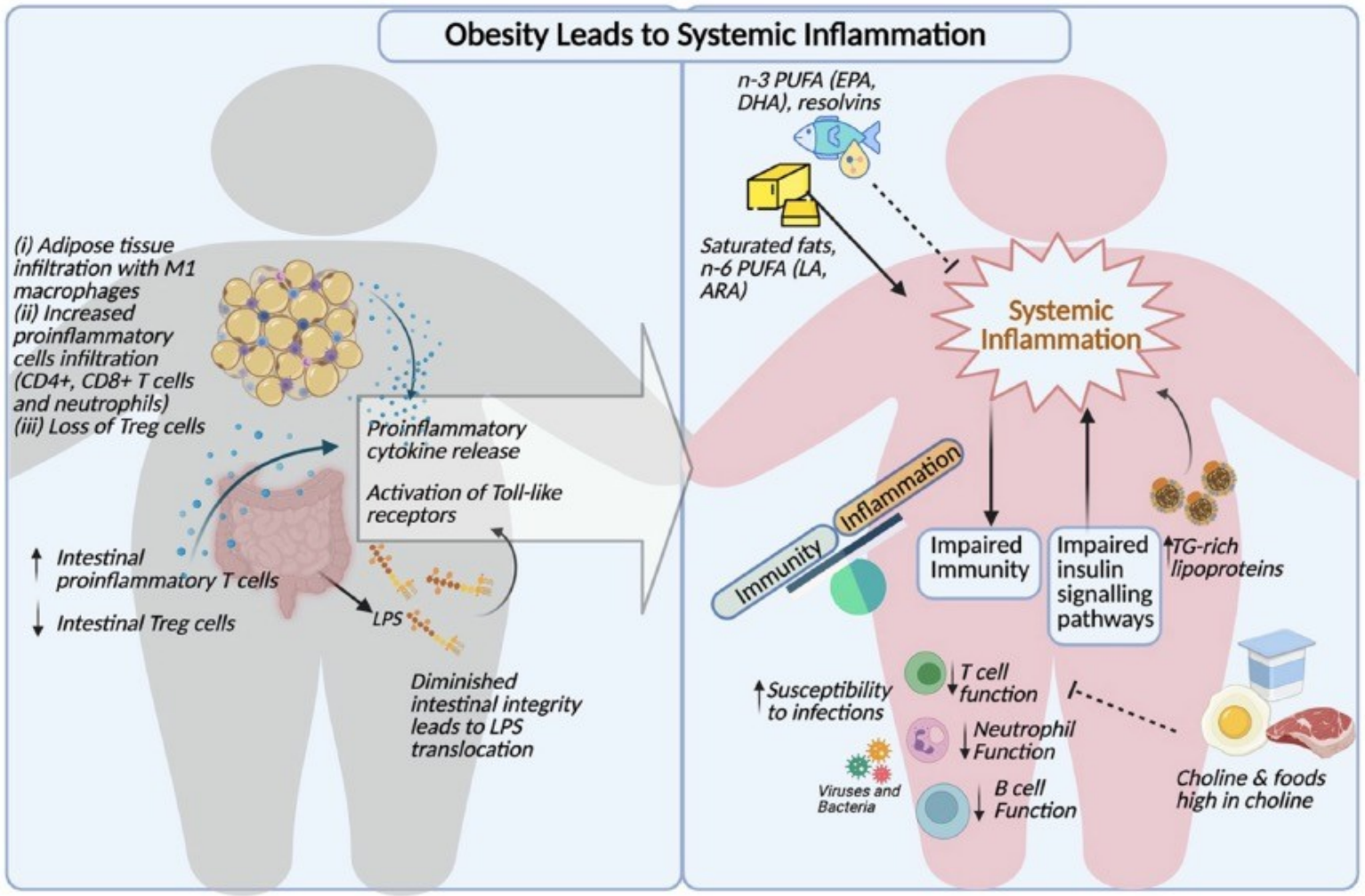

Figure 1.7 Comprehensive summary of obesity induced inflammation, dyslipidemia and impaired immunity
(Adapted from She et al., 2022)

1.4.2 Obesity causes immune dysfunction

There is a clear shift from anti- to pro-inflammatory immune cells in adipose tissue in the context of obesity (SantaCruz-Calvo et al., 2022), which leads to a constant exposure to higher circulating levels of pro-inflammatory cytokines. How these physiological changes in turn impair the magnitude of their response to immune challenges remains unclear. IL-2 is a crucial cytokine produced by T cells that regulates both cell proliferation and differentiation. In 2002, Lamas et al. assessed immune responses to foreign stimuli by *ex vivo* mitogen stimulation. Cafeteria diet-induced obesity (DIO) in Wistar rats lowered the production of IL-2 from splenocytes after mitogen stimulation compared to lean controls which suggests impair T cell function (Lamas et al., 2002). Consistent with this, Richard et al. compared the immune function between subjects with obesity (stage 0; i.e. MHO) and subjects with obesity and T2D (stage 2; i.e. with T2D). Peripheral blood mononuclear cells (PBMC) from stage 2 patients produced significantly lower amounts of IL-2, IL-6 and TNF-α from after T cell mitogen stimulation, suggesting impaired T cell function when compared to stage 0 subjects. In the same study, stage 2 patients also produced lower levels of IL-6 after LPS stimulation, suggesting impaired antigen presenting cell function compared to stage 0 subjects (Richard et al., 2017). Therefore, both obesity and/or hyperglycemia negatively affect T cell responses.

Alterations of T cell homeostasis

More recently, Li et al. observed a striking loss of Treg cells in the visceral adipose tissue from obese mice, driven by IFN-γ producing dendritic cells (C. Li et al., 2021). The loss of circulating Treg cells has also been previously observed in humans in the context of obesity (Wagner et al., 2013). Conversely, van der Weerd et al. found that morbidly obese subjects (BMI > 40) had higher absolute counts of Treg as well as naïve and memory T cells in PBMC compared to lean subjects (van der Weerd et al., 2012). Higher proportions of CD4+ Th cells in PBMC were also observed in obese subjects (BMI > 35) compared to lean controls (O'Rourke et al., 2005). In addition to obesity, factors such as hyperglycemia, dyslipidemia and insulin resistance have been shown to further modulate circulating T cell subtypes by increasing the ratio of Th1 to Th2 cells (Matia-Garcia et al., 2021). Recent findings from our own group demonstrated that hyperglycemia in obesity and T2D leads to an increased proportion of activated Th cells (expressing CD278), cytotoxic T cells as well as inflammatory monocytes (CD14+CRTh2+) in PBMC (Richard et al., 2017). Hence, obesity and/or hyperglycemia not only affect immune cells response to stimuli, but also the proportions of circulating T cell subsets.

Alterations of B cell homeostasis

In another study, splenic B cells from DIO mice poorly responded to CpG+anti IgM stimulation *ex vivo* compared to lean controls, suggesting that obesity also impairs peripheral B cells function, particularly the ability to produce IgM and IgG antibodies. In the same study, B cells from PBMC of subjects with obesity produced less IL-6 upon *ex vivo* stimulation despite having elevated

proportions of total B cells compared to lean subjects (Kosaraju et al., 2017). DIO has also been shown to lead to impair antibody production upon influenza virus infection (Milner et al., 2013). Interestingly, vaccinated individuals with obesity still displayed a remarkably higher risk of developing influenza compared to vaccinated lean subjects (Neidich et al., 2017), suggesting that the response to vaccination in individuals with obesity is lower.

Alterations of GALT and its function

It is now known that obesity is associated with changes in innate as well as adaptive immunity within the GALT (Winer et al., 2016). An accumulation of Th1 cells and cytotoxic T cells, innate lymphoid cells and a reduction of Treg cells has been observed in the small and large intestine of DIO mice and individuals with obesity (Luck et al., 2015). Luck et al. also found that obesity significantly impairs intestinal B cell homeostasis in a manner that reduces IgA producing plasma cells in MLN and secretory IgA antibody concentrations in the colon of DIO mice (Luck et al., 2019). Collectively, these findings demonstrate a proinflammatory shift in intestinal immune cell population in the context of obesity (**Figure 1.8**).

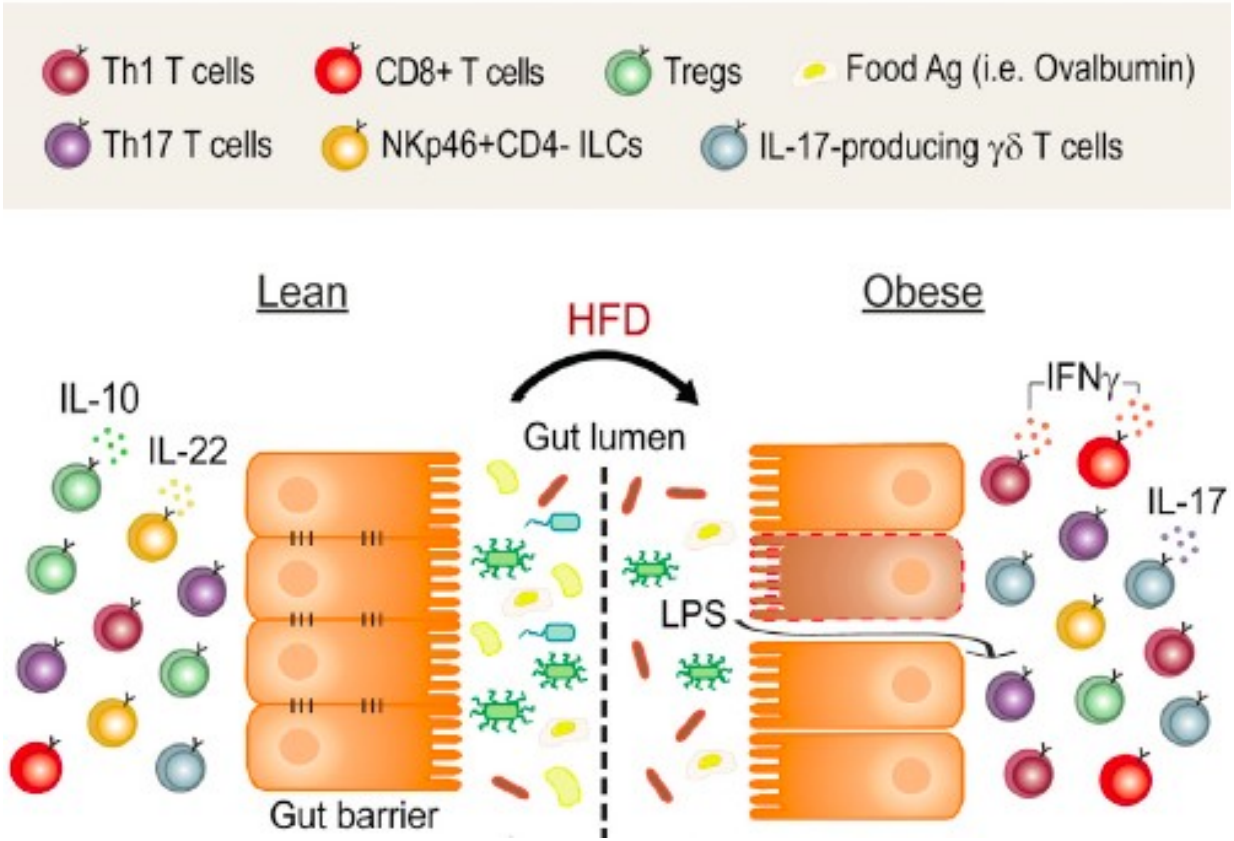

Figure 1.8 Proinflammatory shift of immune cell population in intestine in the context of obesity

(Adapted from Luck et al., 2015)

Ag, antigen; HFD, high-fat diet; IL, interleukin; ILCs, innate lymphoid cells; IFN, interferon; LPS, lipopolysaccharide; Th, T helper cells; Treg, T regulatory cells. Under normal (lean) conditions, the gut barrier maintains an intact integrity. Under HFD-fed and/or obese conditions, the gut barrier integrity is compromised and leading to increased endotoxin (LPS) translocation. There is also observation of striking loss of Treg cells and accumulation of pro-inflammatory (Th1 and CD8+T) cells.

Few studies have also reported that obesity negatively affects intestinal immune cell function characterized by abnormal *ex vivo* IL-2, IL-4 and TNF-a production from MLN in obese rats (Blewett et al., 2009, Ruth et al., 2009). In another study, a lower IL-10 production from MLN was also reported in obese rat when compared to their lean counterpart, suggesting a reduced suppressive ability of the intestinal immune system (Molina et al., 2015). Despite some evidence demonstrating that obesity negatively affects the intestinal immune system, the etiology underpinning this dysregulation remains to be fully elucidated.

1.4.3 Interplay of dyslipidemia and immune system in the context of obesity

In *vitro* studies have demonstrated that TG-rich lipoproteins activate human aortic endothelium cells to express vascular cell adhesion molecules (de Vries et al., 2014). These adhesion molecules are in turn able to bind circulating monocytes and facilitate their migration into the subendothelial space. LDL and remnants of TG-rich lipoproteins are also small enough to penetrate the subendothelial space and offer additional sources of plaque-derived cholesterol (Duran & Pradhan, 2021; Proctor et al., 2002). Internalized lipoprotein-cholesterol can subsequently activate numerous inflammatory signaling pathways and initiate the production of pro-inflammatory cytokines and chemokines (de Vries et al., 2014).

Also of note are studies that report postprandial activation of the immune system including increases of neutrophils, monocytes as well as total leukocyte count (reflected by expression of

CD11b and CD 66b) in humans after consumption of a high fat meal (Alipour et al., 2008; van Oostrom, Sijmonsma, Rabelink, et al., 2003; van Oostrom, Sijmonsma, Verseyden, et al., 2003). Similar to postprandial TG dynamics, postprandial total leukocyte, lymphocyte as well as neutrophil counts are dramatically increased following a standard oral fat challenge (van Oostrom, Sijmonsma, Rabelink, et al., 2003; van Oostrom, Sijmonsma, Verseyden, et al., 2003). Of note, neutrophil counts were found to reach a peak value at 4-h post challenge meal, whereas lymphocyte counts reached the maximum at 8-h. A study in which an oral fat load was conducted in eleven subjects using stable isotope tracers (^{13}C-palmitate) demonstrated that postprandial leukocytes became enriched with meal-derived ^{13}C-palmitate (Alipour et al., 2008). This finding revealed a putative direct interaction between postprandial lipids and circulating leukocytes in that postprandial TG-rich lipoproteins may be directly phagocytosed or postprandial free fatty acid up-taken by leukocytes. These important findings suggest that hypertriglyceridemia can not only induce a proinflammatory response in the postprandial state, but also serves as an activator of circulating leukocytes. The TG-mediated immune cell activation may ultimately contribute to the progression of atherosclerosis and dysregulated immune function in the context of obesity.

1.5 Dairy and obesity-related cardiometabolic and immune perturbations

1.5.1 Dairy consumption and weight management

It has been postulated that consumption of dairy foods may be beneficial to weight loss and body composition due to the presence of certain bioactive nutrients. Mechanisms of dairy intake on

weight loss are largely attributed to increased fecal fat excretion facilitated by the binding of calcium to bile acids in intestine (Christensen et al., 2009). Indeed, a recent randomized controlled trial (RCT) reported that consumption of 3 servings per day of fat-free milk (1200 mg calcium) promoted weight loss and improved body composition in 14 individuals with T2D, compared to a low-calcium control group (Gomes et al., 2019). Similarly, IIich et al. reported that daily intake of 4-5 servings of low-fat dairy products led to favorable changes in body composition in 135 individuals with overweight and obesity (Ilich et al., 2019). Of note, these RCTs were energy restricted. In contrast, findings from meta-analysis of studies without energy restriction reported no effect of increasing dairy intake on weight/body fat loss (Abargouei et al., 2012; Chen et al., 2012).

Since most studies that have investigated dairy and weight loss have typically focused on low-fat dairy intake, the questions surrounding the role of low- *vs* regular-fat dairy on body weight still remain unclear. Indeed, low-fat dairy is a major component in the "Dietary Approach to Stop Hypertension (DASH)" diet portfolio, which has been shown to improve body mass index (Azadi-Yazdi et al., 2017). However, there are no remarkable differences in calcium content between regular- and low-fat dairy products based on USDA nutrient database. Dairy fat is also a rich source of SCFA/MCFAs, which have been shown to improve fatty acid oxidation and energy expenditure (Amer et al., 2017). Observational studies suggest that consumption of regular-fat dairy is associated with less weight gain (Rautiainen et al., 2016), particularly less than meats or processed

snacks (Mozaffarian et al., 2011). Collectively, current findings on the role of low- *vs* regular-fat dairy on body weight remain inconclusive.

1.5.2 Dairy consumption, insulin resistance and T2D

According to recommendations from National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK, USA), a healthy diet pattern to manage T2D should include protein, vegetables, good portions of whole grains as well as non- or low-fat dairy products. Indeed, observational studies have revealed that low-fat dairy intake is associated with reduced risk of developing T2D (Mitri et al., 2019). A meta-analysis of 30 RCTs has also demonstrated that total dairy intake (particularly low-fat dairy) was associated with a potential and beneficial effect on reduction of HOMA-IR (mean difference of -1.21) (Sochol et al., 2019). Interestingly, there does not appear to have any direct evidence indicating the detrimental effect of full/high-fat dairy on the risk of T2D. A systematic review conducted by Drouin-Chartier et al reported no association between high-fat dairy and risk of T2D (Drouin-Chartier, Brassard, et al., 2016). A meta-analysis of 8 cohort studies has also reported that whole-fat dairy intake has associated with a slight (but non-significant) improvement on insulin resistance as assessed by HOMA-IR (Benatar et al., 2013). In contrast, the unique dairy fatty acid, specifically the C15:0 has been recently proposed to be an essential fatty acid due to a number of established cardiometabolic health benefits, including attenuation of glucose concentrations (Venn-Watson et al., 2020). Measuring the circulating concentrations of C15:0, C17:0 and *trans*16:1n-7 is an emerging and reliable approach to reflect the dietary intake

of dairy fat. In a pooled analysis of 16 prospective cohort studies from 7 countries, higher blood levels of C15:0, C17:0 and t16:1n-7 were associated with lower risk of T2D (Imamura et al., 2018).

On the other hand, examining the association between dairy fat intake and cardiovascular risk factors is challenging for many reasons. In most studies, products such as skimmed milk (0–1% fat), non-fat yogurt (0% fat), and low-fat cheese (15–20% fat) are often categorized in the same group as low-fat dairy and *vice versa* for their high-fat compartments. However, there are also studies that compare milk (low-fat, 1%) to cheese (regular-fat, 31%) or dairy to non-dairy controls (Abdullah et al., 2015; Rancourt-Bouchard et al., 2020). Clearly, these comparisons do not lead to a clear understanding on the role of dairy fat *per se*. We have to recognize the importance of considering the food matrix when comparing low-fat to high-fat dairy for their effects on cardiovascular risk factors. More recently, Schmidt et al. compared the effect of 3.3 servings of either low- or high-fat dairy products from similar food matrix to a limited dairy diet group on glucose tolerance and insulin sensitivity in 72 individuals with metabolic syndrome. This study has underscored the current research gap to understand the role of dairy fat *per se*; both the low-fat and full-fat dairy diets in that study decreased the Matsuda insulin sensitivity index (ISI) and increased HOMA-IR relative to the limited dairy diet (Schmidt et al., 2021b).

Therefore, controversies still exist around the beneficial effect of dairy foods and dairy fat on health. Indeed, differences in the type and number of dairy products being tested, study approach,

the population being studied as well as the length of the intervention, all contribute to the heterogeneity and discordance in the literature. In this thesis, the role of dairy and dairy fat *per se* considering the food matrix will be assessed.

1.5.3 Dairy consumption and dyslipidemia

Along with American Heart Association and other dietary guidelines, limiting the intake of saturated fatty acids (SFA) to less than 10% of total energy (TE) has been recommended, owing to the deleterious actions of SFA on LDL-C levels. Indeed, dairy products are rich in dietary SFA (up to 70% of total fat in dairy) and therefore, consumers often tend to avoid regular fat dairy products. However, in a food-level substitution modeling assessment from the 2015 Canadian Community Health Survey, it was determined that the population intake of SFA would only decrease from 10.8% to 10% of total energy, if all regular-fat dairy products were replaced with the low-fat counterparts (Harrison et al., 2021). In this same analysis, it was revealed that reducing consumption of ultra-processed foods at the population level would yield greater reduction in total SFA intake. Therefore, dairy fat in the Canadian population is not a major contributor to total SFA intake when compared to other important dietary sources.

Meta-analysis and comprehensive reviews of RCTs have shown that dairy consumption, irrespective of the fat content, exert neutral effects on lipid profile including TG, TC, LDL-C and HDL-C (Drouin-Chartier et al., 2016; J.R. et al., 2013). Consistent with this meta-analysis, the

most recent RCT conducted by Schmidt et al. demonstrated that consuming 3.3 servings per day of dairy products (milk, yogurt and cheese), irrespective of fat content, had no impact on fasting TG, TC and LDL-C (Schmidt et al., 2021a). In the RCT conducted by Engel et al., consumption of whole-milk did not affect fasting blood TG and LDL-C but increased HDL-C concentrations compared to skim-milk consumption (Engel, Elhauge, et al., 2018). In contrast, Abdullah et al. reported that 3 servings per day of dairy intake (1% milk, 1.5% yogurt and 34% cheese) resulted in a slight but significant rise in LDL-C concentrations, compared to isocaloric non-dairy controls (Abdullah et al., 2015). However, the clinical relevance of the increase on LDL-C level (+0.08 mmol/L, *vs* Control) remains questionable. In contrast, there is lack of high-quality evidence on the effect of dairy foods and/or dairy fat *per se* on postprandial lipidemia. Despite this, Thorning et al. demonstrated that consumption of a diet rich in high-fat cheese had no impact on postprandial (3h post of a challenge meal) TC, LDL-C, HDL-C and TG concentrations, compared to diets rich in meat or carbohydrates (Thorning et al., 2015). Collectively, current findings support the fact that dairy consumption, irrespective of fat content, may exert neutral effects on blood lipid levels.

It is also worth noting that the food matrix can affect clinical observations in lipid metabolism. For instance, the RCT from my own master's thesis compared the effects of consuming a diet rich in SFA from cheese and butter relative to a diet high in carbohydrate and low-fat on cardiometabolic risk factors (Brassard et al., 2017). Interestingly, when 7% of total energy from carbohydrates in the diet was replaced by SFA from cheese, (but not from butter), it resulted in a significant lower

serum LDL-C and HDL-C concentration than predicted based on pre-established equation (Mensink et al., 2003). This evidence highlights the importance of considering the food matrix when assessing lipid responses.

1.5.4 Dairy consumption and inflammation

Earlier evidence from meta-analyses of RCTs have reported no associations between either low- or regular-fat dairy products and circulating levels of CRP, suggesting a neutral effect on systemic inflammation (Benatar et al., 2013). Later, Labonté et al. confirmed that dairy consumption (3 servings/day of low-fat milk, low-fat yogurt and regular cheese) had no impact on CRP levels compared to an energy-matched control in 112 individuals with low-grade systemic inflammation (Labonté et al., 2014). Consistently, Rancourt-Bouchard et al. reported that there was no difference between a no-dairy diet, a diet supplemented with 3 servings/day of low-fat milk and a diet supplemented with 1 serving/day of 31% cheese, on serum concentrations of CRP in 55 healthy individuals (Rancourt-Bouchard et al., 2020). Still, the role of dairy fat *per se* remains unclear as these studies did not directly compare low- and high-fat dairy foods using similar food matrix. To fill this gap, Schmidt et al. revealed that daily intake of 3.3 servings of either low-fat or high-fat dairy products (specifically milk, yogurt and cheese) did not modulate circulating CRP, IL-6, nor adiponectin concentrations compared to limited dairy intake in 72 participants (Schmidt et al., 2021a). Collectively, current findings suggest that dairy consumption, regardless of fat content, has a neutral effect on systemic inflammation. Intriguingly, the unique odd-chain fatty acid derived

from dairy fat, specifically pentadecanoic acid (C15:0) has been reported to exert anti-inflammatory properties. Oral supplementation of C15:0 to obese mice fed a high-fat diet was found to attenuate MCP-1 and IL-6 concentrations compared to unsupplemented groups (Venn-Watson et al., 2020).

On the other hand, people are more commonly in the non-fasting (postprandial) state for most of the day. We know that levels of neutrophils, lymphocytes and total leukocytes can become elevated acutely (1-6 hrs) in response to a fat meal (van Oostrom, Sijmonsma, Rabelink, et al., 2003). There is limited evidence on the effect of dairy on postprandial inflammation *per se*. Schmid et al. reported that consumption of full-fat milk, cheese or butter in a context of high-fat meal have no impact on the postprandial inflammatory response (CRP, IL-6 and TNF-α) when compared to non-dairy high-fat meal in a randomized crossover acute study (Schmid et al., 2015). In contrast, Pei et al. demonstrated that premeal low-fat yogurt consumption attenuated postprandial plasma IL-6 concentrations and LPS binding protein to soluble-CD14 ratio in both obese and non-obese subjects after a high-fat high-carbohydrate meal (Pei et al., 2018). Similarly, another randomized crossover study reported that both probiotic yogurt and acidified milk intake significantly reduced postprandial IL-6, TNF-α and chemokine ligand 5 concentrations in response to a high-fat meal challenge in 14 healthy men (Burton et al., 2017). Collectively, these findings suggest that consumption of dairy foods and/or dairy fat have neutral effect on postprandial inflammation, whereas fermented dairy products may be potentially beneficial to postprandial inflammation.

1.5.5 Dairy and emerging benefits on immune function

Dairy is a highly complex food matrix and contains over 400 fatty acids (only about 15 fatty acids are at level of 1% or higher) (Lindmark Månsson, 2008). The exact fatty acid composition of dairy fat varies due to breed genetics, options of feed and gastrointestinal fermentation status in the dairy cows. Generally speaking, SFA present in milk accounts for approximately 70% of total fatty acids; however, about 11% are SCFA and MCFA (C4:0-C10:0) (Lindmark Månsson, 2008). In addition, dairy fat also contains natural *trans* fatty acids like vaccenic acid (*trans* C18:1) that accounts for approximately 2.7% of total fatty acids in dairy (Lindmark Månsson, 2008). Few studies have assessed certain bioactive fatty acids found in dairy and their impact on immune function including vaccenic acid (*trans* C18:1). Consistent to findings in humans, obese and insulin resistant JCR:LA-cp rats exhibited impaired splenocyte T cell function compared to lean controls (reflected by lower *ex vivo* IL-2 production). Supplementing dietary vaccenic acid in obese rats normalized the IL-2 production, suggesting that vaccenic acid can improve T cell function in the context of obesity (Ruth et al., 2010). SCFA such as propionate (C3:0) and butyrate (C4:0) have also been shown to promote intestinal barrier integrity and induce colonic Treg differentiation (Colonic et al., 2013; Peng et al., 2009), suggesting a beneficial effect on intestinal immune function.

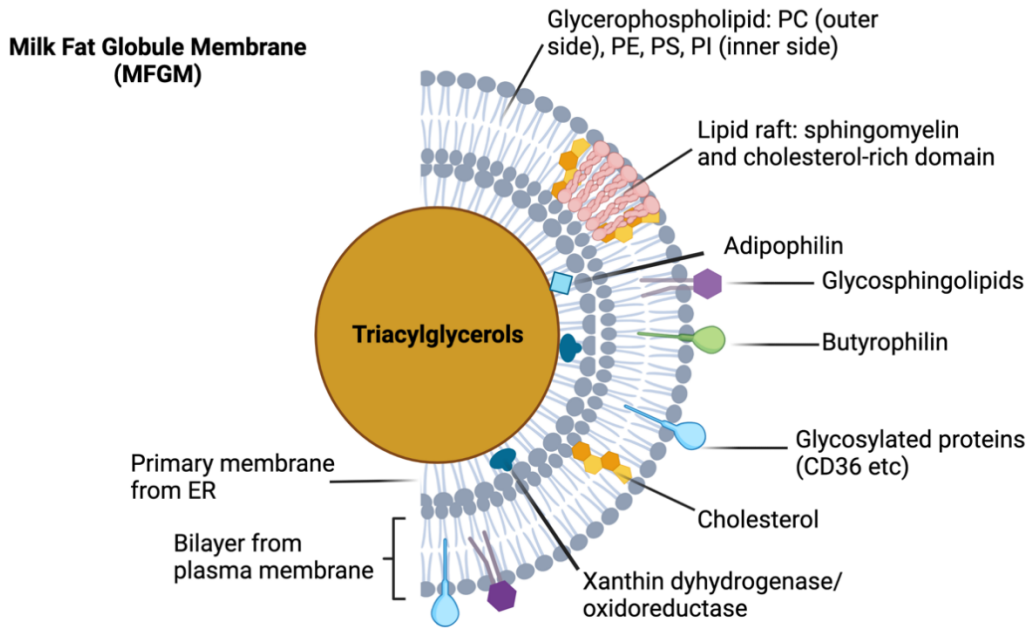

Figure 1.9 Structure of milk fat globular membrane

(Adapted from Kwak et al., 2011) Recreated with BioRender

ER, endoplasmic reticulum; MFGM, milk fat globular membrane; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol. The structure of MFGM is similar to lipoprotein; however, MFGM contains a triacylglycerol core and surrounded by a phospholipid tri-layer. The phospholipid tri-layer is embedded with cholesterol, proteins etc. The outer membrane is primarily formed by PC, whereas the inner membrane has PE, PS and PI.

We also know that dairy products are a major source of dietary choline (Lewis et al., 2014). Polar lipids (in the form of phosphatidylcholine (PC) and sphingomyelin (SM)), are naturally present in the milk fat globular membrane (MFGM, **Figure 1.9**). Azarcoya-Barrera et al. have demonstrated that consumption of lipid soluble forms of choline (PC and SM) beneficially affect the development of the immune system in suckled offspring as well as maternal immune function during the lactating period by enhancing T cell function and the production of Th1 cytokines in Sprague-Dawley rats (Azarcoya-Barrera et al., 2020, 2021, 2022). They also demonstrated that feeding lipid-soluble forms of choline enhanced the development of the gut-associated immune system with marked improvement in cytokine production from MLN early in life (Barrera et al., 2021). Supplementation of MFGM and dairy polar lipids have also been shown to attenuate intestinal bacteria translocation and serum LPS in rodents (Y. Li et al., 2019; Norris et al., 2016).

Collectively, these results suggest several plausible mechanisms by which dairy intake or dairy fat may be beneficial to both peripheral and intestinal immune function. Yet, how these findings would manifest clinically remains to be investigated. It is also unclear whether dairy consumed as a whole considering the complex food matrix would exert the same beneficial effects. In this thesis, the effect of low-fat and high-fat dairy using similar food matrix will be investigated for their effect on both local (intestine) and peripheral immune function.

2 CHAPTER 2 Rationale and objectives

2.1 Rationale

Obesity is characterized by chronic low-grade systemic inflammation that is associated with many cardiometabolic (i.e., insulin resistance, dyslipidemia) and immune (both local and peripheral) perturbations. Currently, scientific evidence suggests an overall neutral effect of dairy foods, regardless of their fat content, on inflammation and other cardiometabolic risk factors (i.e., TG, TC, LDL-C). Yet, there remains a lack of knowledge on the contribution of dairy fat *per se* on overall cardiometabolic health. One of the major reasons for the lack of data is that most studies to date have overlooked the food matrix when comparing low-fat to high-fat dairy products (i.e., milk *vs* cheese). In addition to the fact that high-fat dairy contains SFA, fatty acids specific to dairy such as C15:0 and C17:0 have been inversely associated with variables of insulin resistance. Some studies have also documented the anti-inflammatory properties of C15:0. Dairy fat contains significant amount of short and medium chain fatty acids, which may be beneficial to postprandial lipid metabolism since they are absorbed and metabolized differently than long chain fatty acids. However, the effect of dairy foods and dairy fat *per se* on postprandial insulinemic, lipidemic and inflammatory responses remain under studied.

Another significant issue to consider is that dairy foods are one of the major dietary sources of choline. Lipid soluble forms of choline, including PC and SM are naturally present in MFGM. Studies from our group have demonstrated a beneficial effect of buttermilk-derived choline forms

(mainly PC and SM) on immune function in both lactating rats and their offspring. MFGM and their polar lipids (PC and SM) have also been found to be beneficial for the maintenance of intestinal barrier integrity and attenuate endotoxin translocation. However, to date, very few studies have investigated the effects of dairy and dairy fat consumed as a whole within a complex food matrix on local (intestinal) as well as peripheral immune function.

Consequently, the approach for this thesis was to consider a diet containing whole dairy food (either low- or high-fat dairy) rather than isolated nutrients, in order to better translate to the human condition. Based on the recommendation of Canada's Food Guide 2007, 3 servings of dairy products per day for a 2000 Kcal diet (250ml milk, 175g yogurt and 50g cheese) using commercially available high-fat dairy products were compared to their low-fat counterpart. We chose to employ a recently established swine model of insulin resistance with a preventative approach (i.e., feeding from an early age). Ultimately, outcomes from this study will provide an advanced understanding and novel insights into the role of dairy foods and dairy fat *per se* on cardiometabolic and immune health that can be used by both health professionals and educators.

2.2 Objectives and hypothesis

The overall objective of this thesis was to determine the impact of consuming low- and high-fat dairy products on obesity-related cardiometabolic and immune perturbations in a swine model of

insulin resistance. To address this overall objective, the following specific objectives and their respective hypothesis were established:

1. To determine the effect of consuming 3 servings per day of either low- or high-fat dairy products on obesity-related cardiometabolic and immune perturbations in the *fasting state*. This objective was investigated by dividing into the following specific objectives and hypotheses:

a. Determine the effect of consuming 3 servings per day of either low- or high-fat dairy products on obesity-related cardiometabolic risk factors in the *fasting state*, including concentrations of glucose, insulin, inflammatory markers and lipids profile. We hypothesized that consumption of 3 servings per day of dairy products, irrespective of the fat content, would exert neutral effect on the lipid profile. We also proposed that feeding high-fat dairy may provide greater benefits on glucose metabolism and inflammation.

b. Determine the impact of consuming 3 servings per day of either low- or high-fat dairy products on peripheral immune function in the *fasting state*. We hypothesized that feeding high-fat dairy may provide greater benefits on the immune function due to the presence of unique bioactive nutrients in dairy fat.

2. The second objective was to determine the effect of consuming 3 servings per day of either low- or high-fat dairy products on obesity-related cardiometabolic perturbations in the *postprandial state* as well as on the intestinal immune function. This objective was investigated by dividing into the following specific objectives and hypotheses:

a. Determine the effect of consuming 3 servings per day of either low- or high-fat dairy products

on obesity-related cardiometabolic risk factors in the *postprandial state* in response to a modified oral glucose and fat tolerance test (MOGTT). Including concentrations of glucose, insulin, inflammatory markers and lipid profile. We hypothesized that consumption of 3 servings per day of dairy products, irrespective of fat content, would have a neutral effect on postprandial responses.

b. Determine the effect of consuming 3 servings per day of either low- or high-fat dairy products on intestinal immune function. We proposed that feeding high-fat dairy would provide greater benefits than low-fat dairy on intestinal immune function due to the presence of unique bioactive nutrients in dairy fat.

2.3 Chapter format

The objectives and hypotheses stated above were tested in a series of analyses as part of a larger study. These analyses were organized into thesis chapters and have been published and/or submitted for publication as individual manuscripts.

Chapter 3 introduces the animal model, experimental design and analytical methods used in the present thesis.

Chapter 4 examines the impact of consuming 3 servings of low- or high-fat dairy products on obesity-related cardiometabolic and immune perturbations in the *fasting state*. The findings of chapter 4 have been published in Frontiers in Nutrition (DOI: 10.3389/fnut.2022.923120). We found that consumption of 3 servings per day of high-fat dairy products lowered fasting glucose

more than low-fat dairy, whereas low-fat dairy improved immune function particularly T cell function to a greater extent than high-fat dairy. Irrespective of fat content, consumption of 3 servings of dairy products had a neutral effect on the lipid profile and concentrations of inflammatory markers in this swine model of insulin resistance.

Chapter 5 examines the impact of consuming 3 servings of low- or high-fat dairy products on obesity-related cardiometabolic and immune perturbations in the *postprandial state* as well as on intestinal immune function. The findings of chapter 5 have been published in European Journal of Nutrition (DOI: 10.1007/s00394-022-03013-8). We found that consumption of 3 servings per day of dairy products, irrespective of fat content, has a neutral effect on postprandial lipidemia, inflammation and glucose tolerance. Consuming low-fat dairy products improved intestinal T cell function to a greater extent than high-fat dairy in this swine model of obesity and insulin resistance.

Chapter 6 provides an overall discussion that includes a summary of the main finding from each chapter along with limitation, and future directions and perspectives.

Appendix Chapter includes the review manuscript published in Frontiers in Nutrition (DOI: 10.3389/fnut.2022.840209), the manuscript which is coauthored and submitted for publication in Frontiers in Veterinarian Science (revision in progress as of October 2022), and other related study protocols.

3 CHAPTER 3 Experimental design and materials

3.1 Introduction

Large animals such as pigs share tremendous similarities with humans in terms of anatomy, genetics and physiology. To highlight the similarities, pig-to-primate organ transplantation was successfully achieved over a decade ago (Meurens et al., 2012). Of relevance to this thesis, a number of swine models with different strains have also been introduced to study obesity-related metabolic complications and pathologies, including the Yucutan miniature pigs and Ossabaw pigs (Myrie et al., 2011, 2017; Spurlock & Gabler, 2008). These models are capable of recapitulating criteria of metabolic syndrome in humans, including insulin resistance, hypertriglyceridemia, hypertension, and visceral adiposity when fed with a high fat diet. The immune system composition of the pig also closely resembles that of humans, whereas mice only share about 10% similarity (Meurens et al., 2012; Spurlock & Gabler, 2008). Pawar et al. has also found increased expression of TNF-α in abdominal subcutaneous adipose tissue of obese pigs fed with high-fat and high-fructose diet, along with macrophage infiltration in both abdominal and pericardial adipose tissues, which paralleled the observations made in humans with obesity (Pawar et al., 2015).

Since the introduction of the concept of "developmental origin of adult disease" by David Barker, the link between low birthweight (LBW) and numerous metabolic complications later in life has been established (Malhotra et al., 2014). LBW is primarily due to malnutrition during pregnancy in humans. In contrast, pigs often give birth to a large litter size and therefore, LBW can occur

naturally even with adequate nutrition. Researchers have also referred to this phenomenon as intrauterine growth restriction and found impaired glucose and lipid metabolism when fed with high-fat diet (McKnight et al., 2012; Myrie et al., 2011, 2017). Similarly, our research group has also worked extensively during the past few years to establish a swine model of insulin resistance with a strain of Duroc cross Large White-Landrace using LBW piglets. Previous work by Fontaine et al. has successfully demonstrated that this specific strain of LBW piglets can develop early signs of insulin resistance, impaired lipid metabolism leading to hepatic fibrosis when fed with a diet high in fat, cholesterol and fructose (Fontaine et al., 2019; Singh et al., 2021). In the present thesis, we continued to use this pre-clinical model and aimed to understand the effect of dairy foods, particularly dairy fat, in modulating obesity-related cardiometabolic and immune perturbations.

3.2 Experimental design

3.2.1 Animals and housing

All piglets were born and raised at the Swine Research Technology Centre (SRTC), Department of Agricultural, Food and Nutritional Science, University of Alberta, Canada. Piglets used in the present study were offspring of the cross of Duroc boar and Large White-Landrace sow. Only male piglets were used in this study, as they were more susceptible to develop early insulin resistance. To characterize LBW and normal birthweight (NBW), a mean litter weight and standard deviation was calculated to determine the 95% confidence interval (CI), categorizing piglets as LBW (less than the 95% CI) or NBW (within or above the 95% CI). From birth, all piglets were weighed

weekly. Echoing the principle of environmental enrichment, piglets were initially housed with their litter mates. Piglets were co-housed (2~3 animals per pen) from 5 weeks of age to 9 weeks of age, then were single housed from 9 weeks of age to 12 weeks of age due to their larger size. Even when they were single-housed, pigs were socialized by facility staff or researchers on a daily basis. All piglets were provided with a known amount of feed in the hopper following the industry standard and adjusted weekly. Water, feed, room temperature and health condition were checked daily by trained staff at the SRTC. All study protocols were approved by University of Alberta Animal Care and Use Committee and in accordance with regulations of Canadian Council of Animal Care (Protocol AUP00001184).

3.2.2 Study design

All piglets were weaned at 3 weeks of age with a SRTC pig grower diet (Chow) until 5 weeks of age. At 5 weeks of age, LBW piglets were randomized to consume one of the 3 experimental diets: (i) Control High-Fat (CHF), (ii) CHF supplemented with 3 servings per day of high-fat dairy products (HFDairy) or (iii) CHF supplemented with 3 servings per day of low-fat dairy products (LFDairy). As comparison groups, NBW piglets were fed a Chow (NBW-Chow) or CHF diet (NBW-CHF), and served as controls for the "metabolic phenotype" to confirm that the LBW-CHF group developed similar cardiometabolic perturbations as seen in obesity with insulin resistance. A total of 35 pigs (LBW-CHF n=8, LBW-HFDairy n=8, LBW-LFDairy n=8, NBW-CHF n=6, NBW-Chow n=5) completed the study and were fed for a total of 7 weeks, including 1 week of

CHF transition diet (**Figure 3.1**). At 10 weeks of age, all pigs were fasted overnight and underwent a jugular catheter surgery. At 11 weeks of age, all pigs underwent an established modified oral glucose and fat tolerance test (MOGTT) where fasting and postprandial blood were collected. At 12 weeks of age, pigs were terminated, fasting blood and tissue samples were collected.

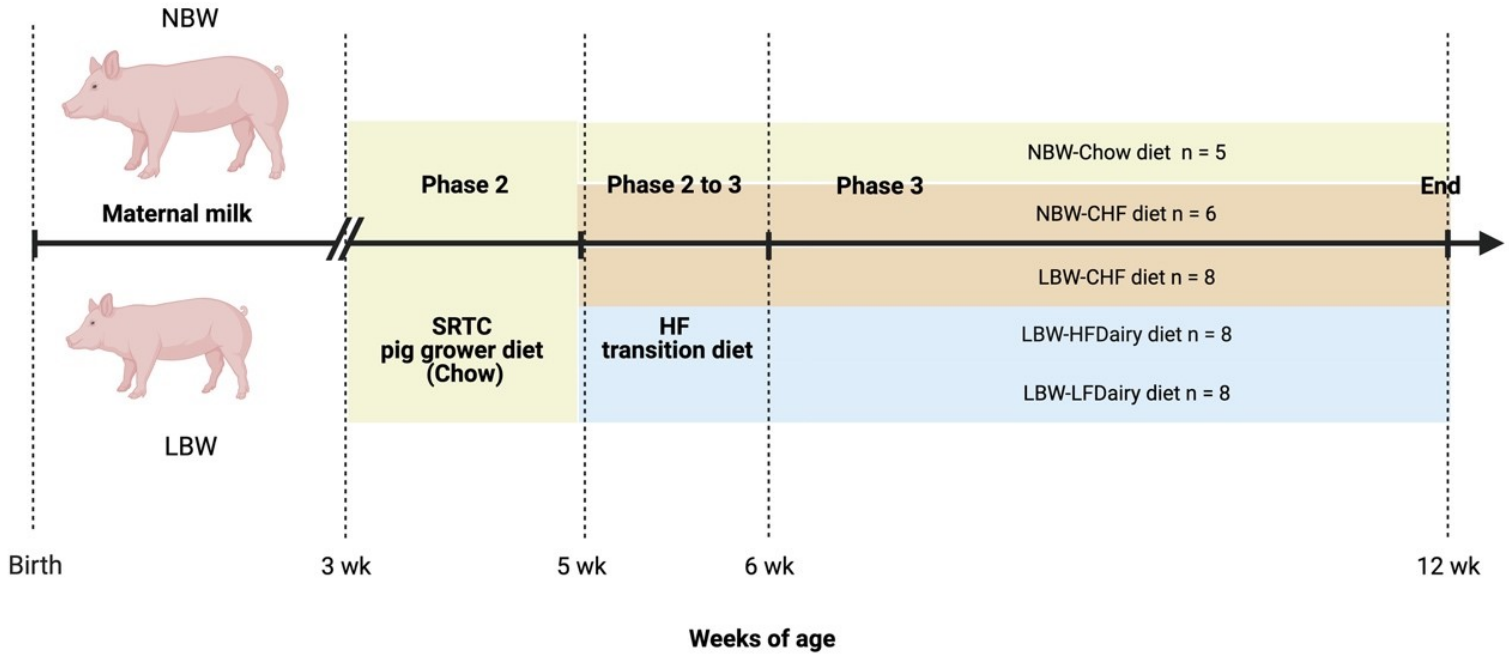

Figure 3.1 Schematic illustration of study design and randomization

3.2.3 Diet

The diets were formulated to meet or exceed the nutrient requirements of growing-finishing pigs. The detailed nutrient composition of the experimental Chow and CHF diet is shown in **Table 3.1**. In the CHF diet, the respective percentages of energy (Kcal) derived from fat, carbohydrate and protein were 46% (mainly lard), 33% (with 17% calories from fructose specifically) and 21%. 1% w/w cholesterol was also added. In contrast, the Chow diet consisted of 14% total energy from fat, 69% total energy from carbohydrate and 17% total energy from protein.

Table 3.1 Nutrient composition of Chow and CHF diets

(Adapted and published in Fontaine et al., 2019; Singh et al., 2021)

Ingredient, g/kg	Chow Phase 2	CHF Phase 2	Chow Phase 3	CHF 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	1.0	.	0.5	.
Dicalcium phosphate	.	7.0	4.3	7.0
Copper Sulfate	.	.	0.4	.
Others*	9.8	.	9.8	.

Trace mineral swine pre-mix was 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; vitamin swine pre-mix was 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; *others were 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. DDG, dried distillers grain

To understand the effect of dairy products and more specifically dairy fat, the CHF diet was supplemented with 3 servings for every 2000 Kcal of either low- or high-fat dairy products. In the HFDairy diet group, one serving of whole milk powder (3.25% fat; Bulk Barn, Canada, **Figure 3.2A**), plain yogurt (10% fat; Liberté, Canada, **Figure 3.3A**) and mozzarella cheese (28% fat; No Name®, Canada, **Figure 3.4A**) was fed to each pig daily contributing to approximatively 28% of total energy intake in that group. In the LFDairy diet, one serving of skimmed milk powder (0% fat; No Name®, Canada, **Figure 3.2B**), plain yogurt (1% fat; Foremost Farms, Canada, **Figure 3.3B**) and mozzarella cheese (18% fat; No Name®, Canada, **Figure 3.4B**) was fed to each pig daily contributing to approximatively 16% of total energy intake in that group (**Figure 3.5**).

Since all dairy products were purchased from retail outlets and from different brands, therefore their ingredients and nutrient composition may vary. The serving size for each dairy product was based on the previous Canada's food guide (version 2007) which consisted of 250 ml of milk or powdered milk (24 g low-fat milk powder or 33.5 g high-fat milk powder), 175 ml of yogurt and 50 g of cheese regardless of their fat content (**Table 3.2**). The macronutrient distribution of each experimental diets is presented in **Table 3.4**

A

Ingredients:

Whole milk powder, vitamin D3.

Directions for Use:

To reconstitute, always add milk powder to cold water. For smoothness and flavour, mix at night for use the next day. For 1 glass of milk, mix 3 tablespoons (30 g) of powder with 1 cup (250 ml) of cold water. For 1 litre (4 cups) of milk, mix 13 tablespoons (125 g) of powder with 1 litre (4 cups) of cold water.

Allergens:

Contains milk. May contain soy and sulphites.

Nutrition Facts

Per 100 g

Calories 499	% Daily Value*
Fat 26.5 g	35 %
Saturated 16.9 g + Trans 0.7 g	88 %
Carbohydrate 38 g	
Fibre 0 g	0 %
Sugars 37 g	37 %
Protein 26.9 g	
Cholesterol 94 mg	
Sodium 395 mg	17 %
Potassium 1266 mg	37 %
Calcium 960 mg	74 %
Iron 0.3 mg	2 %

*5% or less is **a little**, 15% or more is **a lot**

B

NUTRITION FACTS & INGREDIENTS

Serving Size grams **(40 g)**

Amount Per Serving	% DV
Calories 150 cal	
Total Fat 0.3 g	1 %
Saturated Fat 0.2 g	
Saturated Trans Fat	1 %
Trans. Fat 0.0 g	1 %
Monounsaturated Fat 0.1 g	
Cholesterol 10 mg	
Sodium 210 mg	9 %
Total Carbohydrate 21 g	7 %
Sugars 21 g	
Dietary Fiber 0 g	0 %
Protein 15 g	
Potassium 700 mg	21 %

Amount Per Serving	% DV
Vitamin A	0 %
Vitamin C	4 %
Calcium	45 %
Iron	0 %
Thiamine	15 %
Riboflavin	40 %
Vitamin B6	8 %
Vitamin B12	80 %
Pantothenate	20 %
Phosphorus	35 %
Magnesium	20 %
Zinc	20 %
Selenium	20 %

Ingredients

Skim Milk Powder, Vitamin A Palmitate, Vitamin D3.

Figure 3.2 Nutrition facts and ingredients of whole (A) and skimmed (B) milk powder

Information as of December 2022 available at
https://www.bulkbarn.ca/en/products/all/whole-milk-powder-non-instant-1224
https://www.nofrills.ca/pasteurized-instant-skim-milk-powder/p/20052038_EA

A

Nutrition Facts

Per 3/4 cup (175g)

Amount	% Daily Value
Calories 200	
Fat 18g	24%
Saturated 10g	53%
Trans 0,5g	
Cholesterol 60mg	
Sodium 80mg	3%
Carbohydrate 5g	
Fibre 0g	0%
Sugars 5g	5%
Protein 6g	
Potassium 250mg	5%
Calcium 225mg	17%
Iron 9,1	1%

Made with

Whole milk, cream, milk protein, bacterial cultures.

LAIT-DAIRY MK 591

B

NUTRITION FACTS & INGREDIENTS

Serving Size grams (175 g)

Amount Per Serving	% DV	Amount Per Serving	% DV
Calories 100 cal		Vitamin A	0 %
Total Fat 1.5 g	2 %	Vitamin C	0 %
Saturated Fat 1.0 g		Calcium	20 %
Saturated Trans Fat	5 %		
Trans. Fat 0.0 g	2 %	Iron	0 %
Cholesterol 10 mg			
Sodium 100 mg	4 %		
Total Carbohydrate 14 g	5 %		
Sugars 7 g			
Dietary Fiber 0 g	0 %		
Protein 8 g			

Ingredients

Milk Ingredients, Modified Corn Starch, Carrageenan, Pectin, Bacterial Culture

Figure 3.3 Nutrition fact and ingredients of high- (A) and low-fat (B) Greek yogurt

Information as of December 2022 available at
https://liberte.ca/en/mediterranee/plain-10
https://www.loblaws.ca/plain-stirred-1-m-f-yogurt/p/20044542_EA

A

NUTRITION FACTS & INGREDIENTS

Serving Size grams (30 g)

Amount Per Serving	% DV	Amount Per Serving	% DV
Calories 110 cal		Vitamin A	10 %
Total Fat 8 g	12 %	Vitamin C	0 %
Saturated Fat 5 g		Calcium	20 %
Saturated Trans Fat	27 %		
Trans. Fat 0.3 g		Iron	0 %
Cholesterol 30 mg			
Sodium 230 mg	10 %		
Total Carbohydrate 1 g	1 %		
Sugars 0 g			
Dietary Fiber 0 g	0 %		
Protein 7 g			
Potassium 0 mg	0 %		

Ingredients

Pasteurized Milk, Modified Milk Ingredients, Bacterial Culture, Salt, Calcium Chloride, Microbial Enzyme.

B

NUTRITION FACTS & INGREDIENTS

Serving Size grams (30 g)

Amount Per Serving	% DV	Amount Per Serving	% DV
Calories 90 cal		Vitamin A	4 %
Total Fat 5 g	8 %	Vitamin C	0 %
Saturated Fat 3.5 g		Calcium	20 %
Saturated Trans Fat	19 %		
Trans. Fat 0.2 g	8 %	Iron	0 %
Cholesterol 20 mg			
Sodium 240 mg	10 %		
Total Carbohydrate 1 g	1 %		
Sugars 0 g			
Dietary Fiber 0 g	0 %		
Protein 9 g			

Ingredients

Partly Skimmed Milk, Modified Milk Ingredients, Bacterial Culture, Salt, Calcium Chloride, Microbial Enzyme.

Figure 3.4 Nutrition fact and ingredients of high- (A) and low-fat (B) Mozzarella cheese

Information as of December 2022 available at
https://www.loblaws.ca/pizza-mozzarella-cheese-28-mf/p/20975887_EA
https://www.loblaws.ca/pizza-mozzarella-cheese-light-18-mf/p/20975784_E

Table 3.2 Nutrient composition of dairy products per serving

	Amount per serving (g)	Energy (kcal)	Protein (g)	Fat (g)	Carbohydrate (g)
Milk powder, 3.25% MF	33	174.9	8.91	8.91	13.2
Greek yogurt, 10% MF	175	200	6	17.5	5
Mozzarella cheese, 28% MF	50	183.3	11.7	13.3	1.7
Milk powder, 0% MF	24	90	9	0.18	12.6
Greek yogurt, 1% MF	175	100	8	1.5	14
Mozzarella cheese, 18% MF	50	150	15	8.3	1.7

Table 3.3 Serving per day/energy intake (Kcal) of dairy products

	Week of age					
Serving per day/energy intake (Kcal)	6	7	8	9	10	11
Energy intake (Kcal)	4245.6	4774.7	5762.6	6858.4	6842.7	8998.0
Yogurt (175g/2000 Kcal)	371.5	417.8	504.2	600.1	598.7	787.3
Cheese (50g/2000 Kcal)	106.1	119.4	144.1	171.5	171.1	225.0
Powdered milk skim (24g/2000 Kcal)	50.9	57.3	69.2	82.3	82.1	108.0
Powdered milk whole (33g/2000 Kcal)	70.1	78.8	95.1	113.2	112.9	148.5

Nutrient composition was calculated based on serving size of Canada's Food Guide (2007) in a 2000 Kcal diet. All dairy products were purchased from local distributor. MF, milk fat; Greek yogurt (1% MF), Foremost Farms, Canada; Greek yogurt (10% MF), Liberté, Canada; Milk powder (0% MF), No Name®, Canada; Milk powder (3.25% MF), Bulk Barn, Canada; Mozzarella cheese (18% MF); No Name®, Canada; Mozzarella cheese (28% MF), No Name®, Canada.

The number of servings provided per day was adjusted weekly proportionally to their increasing energy intake to maintain a constant ratio of 3 servings per 2000 Kcal intake (**Table 3.3**). All products were available and purchased *via* retail outlets fresh every 10 days. Since pigs in both dairy groups did not lower their consumption of the experimental CHF diet, these two groups were pair-fed to the group receiving the experimental CHF diet only. Therefore, less experimental CHF diet was added to the feeder to compensate for the number of calories coming from dairy products so that energy intake would be consistent across all three LBW groups. Body weight was measured weekly and food intake was recorded daily.

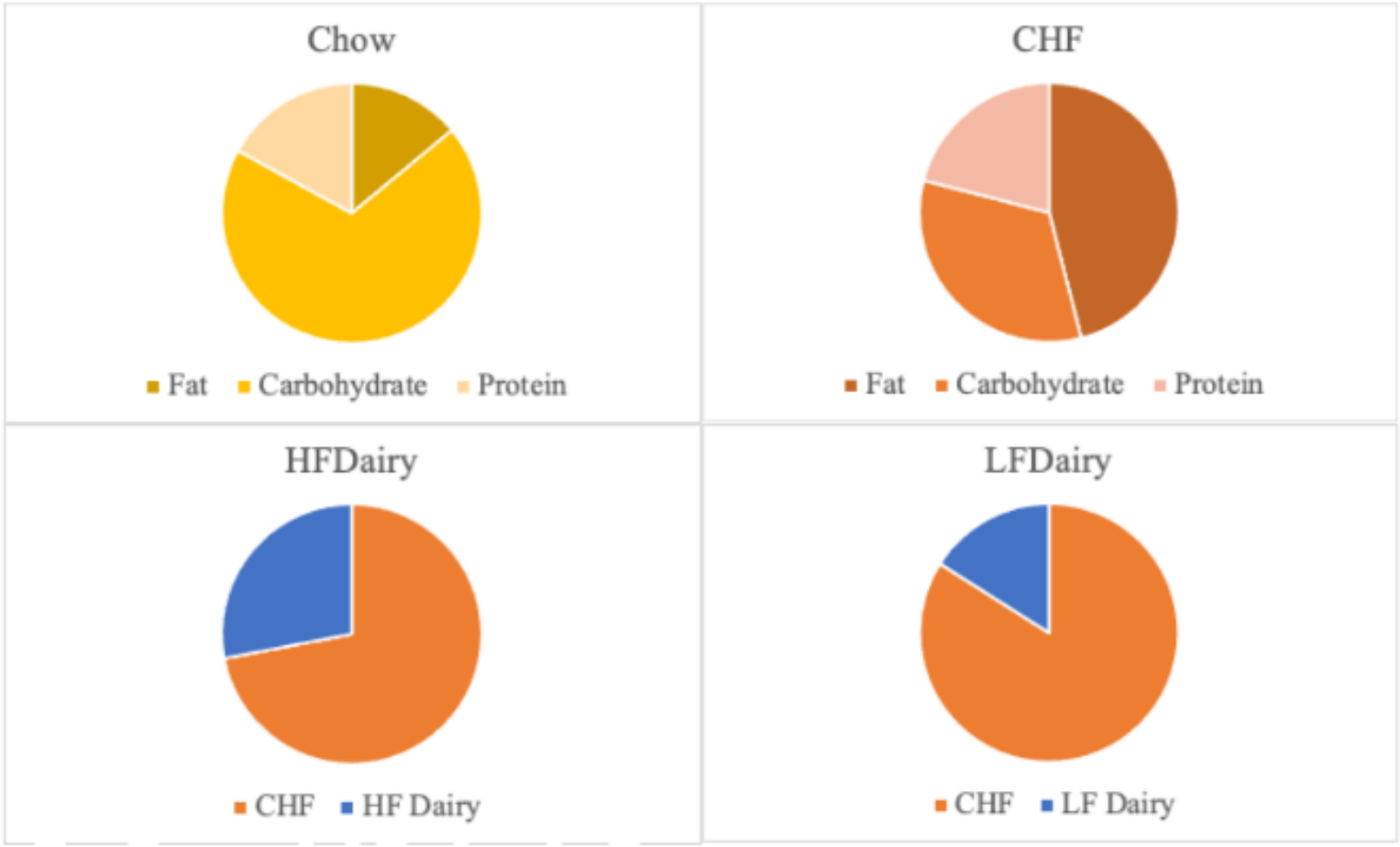

Figure 3.5 Pie chart of macronutrient composition and energy contribution from dairy products in experimental diets

Table 3.4 Macronutrient distribution of experimental diets per 2000 Kcal

% of energy	Chow	CHF	HFDairy	LFDairy
Carbohydrate	69	33	28	33
main source	*wheat* *soybean* *oat*	*wheat* *soybean* *fructose*	*wheat* *soybean* *fructose*	*wheat* *soybean* *fructose*
Fat	14	46	51	43
main source	*lard*	*lard*	*lard* *dairy*[a]	*lard* *dairy*[b]
Protein	17	21	21	24
main source	*fishmeal*	*fishmeal*	*fishmeal* *dairy*[a]	*fishmeal* *dairy*[b]

Energy value was calculated based on serving size of Canada's Food Guide (2007) in a 2000 Kcal diet. All dairy products were purchased from local distributor. CHF, control high-fat; HFDairy, high-fat dairy diet; LFDairy, low-fat dairy diet; MF, milk fat.

[a]In the HFDairy group, 33 g milk powder (3.25% MF), Bulk Barn, Canada; 175 g Greek yogurt (10% MF), Liberté, Canada; 50 g mozzarella cheese (28% MF), No Name®, Canada were given and contributing approximately 28% of total energy in the group per 2000 Kcal diet.

[b]In the LFDairy group, 24 g milk powder (0% MF), No Name®, Canada, 175 g Greek yogurt (1% MF), Foremost Farms, Canada; 50 g mozzarella cheese (18% MF); No Name®, Canada were given and contributing approximately 16% of total energy in the group per 2000 Kcal diet.

3.2.4 Jugular catheter surgery and MOGTT

At 10 weeks of age, all piglets were fasted overnight, and anesthesia was induced with isoflurane. Jugular catheter was implanted into the left jugular vein under sterile condition, allowing for blood collection through a pouch on the back of each piglets containing the catheter tubing (**Figure 3.6**). Post-surgical recovery of each pig was monitored and recorded on a daily basis. Treatments including buprenorphine (0.1 mg/kg body weight, every 4-8 h intramuscular for the 12 h post-surgical period) to reduce pain and trimethoprim-sulpha were given if there were post-surgical complications. Each catheter was flushed daily with 3 ml (100 IU/ml) heparin in saline solution to prevent clotting. Piglets were fasted overnight at 11 weeks of age and underwent an established MOGTT procedure (**Figure 3.7**). A fasting blood sample was collected *via* jugular catheter prior to consuming the first challenge meal (25 g Chow diet with 1 g/kg body weight Devonshire cream (40% milk fat w/w) and 2 g/kg body weight 50% glucose solution). The second challenge meal (25 g Chow diet with 1 g/kg body weight Devonshire cream) was given at 120 min. Postprandial blood samplings were collected into EDTA vacutainers at timed intervals (15 min, 30 min, 60 min, 120 min, 180 min, 240 min and 300 min). Samples were transferred on-ice to laboratory for further processing

Figure 3.6 Piglets were individually housed in metabolic pen post jugular catheter surgery

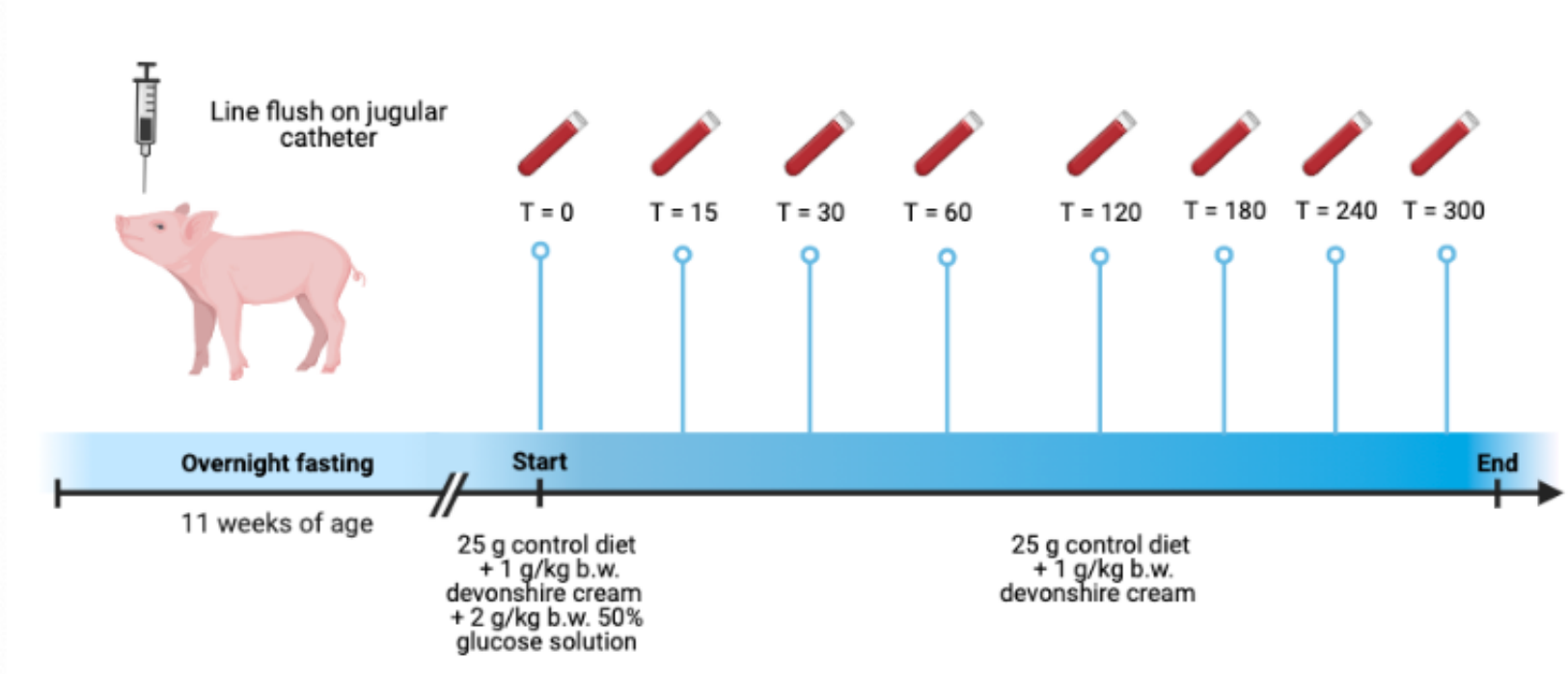

Figure 3.7 Schematic procedure of modified oral glucose and fat tolerance test

Our laboratory and the staff from SRTC have invested significant time to ensure each piglet in this thesis was taken care of with a high quality of life. We made sure each piglet was socialized daily to minimize stress response during subsequent experimental procedures. Piglets that did not successfully adapt to the research environment such as exhibiting aggression, anxiety or anorexia during the intervention were removed from the study to alleviate the distress. Animal surgery was handled by senior veterinarians and skilled personnel to ensure high standards of animal welfare.

3.2.5 Fasting blood and tissue collection

At 12 weeks of age, all piglets were fasted overnight (12h). The fasting blood were collected. Tissue samples including liver, intestine, subcutaneous muscle and fat were collected after piglets were terminated. All samples were treated with ice-cold saline solution first and followed by snap frozen in liquid nitrogen prior to be stored at -80°C until further processing.

3.2.6 Plasma and PBMCs isolation

Fasting and postprandial blood samples were collected in tubes containing EDTA, dipeptidyl peptidase 4 inhibitor (EMD Millipore, MA), and Complete® general protease inhibitor (Sigma-Aldrich, USA) before being centrifuged at 2000 x g for 10 min to obtain plasma. Plasma was transferred into 500 ul aliquots and stored at -80°C until further analysis.

To isolate PBMCs (peripheral blood mononuclear cells), 1% bovine serum albumin in phosphate-buffered saline was added to dilute and resuspend the buffy coat. Cell suspension was then layered over 5 ml histopaque and followed by centrifugation. PBMCs were recovered from the gradient interface, washed with 1% bovine serum albumin in phosphate-buffered saline, and reconstituted with 5% complete culture medium (RPMI 1640 media; Life Technologies, Burlington, ON, Canada).

3.2.7 MLN immune cell isolation

MLN were processed to isolate immune cells under sterilized conditions (all instruments and nylon mesh were soaked overnight in 70% ethanol). MLN and nylon mesh were first flushed with buffer (1% bovine serum albumin in KRH) to keep a wet condition. MLN were then trimmed to remove excess fat tissue and cut into smaller pieces and pushed through the nylon mesh using the barrel of sterile syringe along with buffer flush (1% bovine serum albumin in KRH). Buffer (1% bovine serum albumin in KRH) was used to keep tissue wet and to rinse nylon mesh after. Collected samples were then centrifugated at 500 x g for 10 min to pellet cells. Supernatant was discarded and cells reconstituted with 5 ml 5% complete culture medium (RPMI 1640 media; Life Technologies, Burlington, ON, Canada).

3.2.8 PBMCs and MLN immune cell count

Prior to *ex vivo* analyses, a hemocytometer was used to count cells using trypan blue dye exclusion. 20 µl trypan blue was added 1:1 to 20 µl cell suspension and load onto hemocytometer and counted with a 5 by 5 square. Of note, lymphocytes have a sphere-round structure (**Figure 3.8**). All cell suspensions were then adjusted to 1.25×10^6 cells/ml. Additional cells were frozen in 10% complete culture media (RPMI 1640 media; Life Technologies, Burlington, ON, Canada) with 10% DMSO at -80°C before transferred to liquid nitrogen.

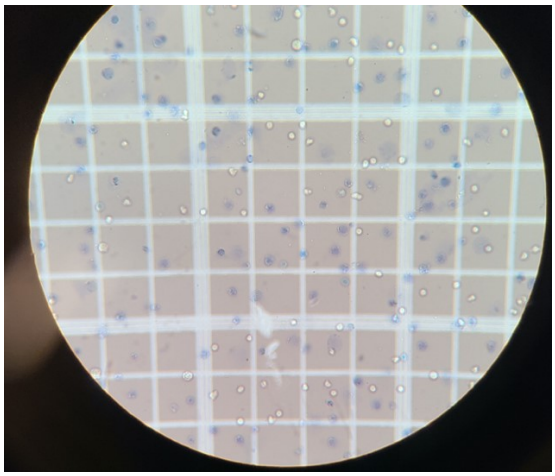

Figure 3.8 PBMCs under microscopy of a hemocytometer

For complete standard operating procedures (SOP) on tissue processing and cell isolation, refer to ***Appendix C****.*

3.3 Analytical materials and methods

3.3.1 Fasting plasma biochemical analysis

Fasting plasma concentrations of TG, TC, LDL-C, HDL-C and glucose were assessed using commercial enzymatic colorimetric kits (Wako Pure Chemicals, Tokyo, Japan) and as described previously (Fontaine et al., 2019; Singh et al., 2021). Plasma insulin concentration was measured using commercial porcine-specific ELISA kits as per manufacturer's instructions (Mercodia, USA). Additional aliquots of plasma within the same group were mixed and measured for lipoprotein associated plasma TG and cholesterol fractions by fast protein liquid chromatography (FPLC) at the University of Alberta lipidomic core facility as described elsewhere (H. Wang et al., 2007). Fasting plasma inflammatory marker concentrations were assessed by porcine

cytokine/chemokine 13-Plex Discovery Assay® Array at Eve Technologies (Calgary, Canada).

3.3.2 Postprandial plasma biochemical analysis

Postprandial plasma TG concentrations were measured at the following timepoints: 0 min, 15 min, 30 min, 60 min, 120 min, 180 min, 240 min and 300 min using commercial enzymatic colorimetric kits (Wako Pure Chemicals, Tokyo, Japan). Postprandial insulin concentrations were measured at 0 min, 15 min, 30 min, 60 min, 120 min with porcine-specific ELISA kits (Mercodia, USA) as per manufacturer's instructions. Postprandial glucose concentrations were measure immediately onsite following MOGTT by a glucometer (Accu-Chek, Roche, Switzerland) in whole blood. Postprandial plasma inflammatory marker concentrations were assessed at 0 min, 60 min, 120 min, 180 min and 240 min by a porcine cytokine/chemokine 13-Plex Discovery Assay® Array provided by Eve Technologies (Calgary, Canada)

3.3.3 Liver fatty acids analysis

Liver fatty acid profile was assessed by gas chromatography equipped with a flame ionization detector (Agilent 8890). Briefly, 100 mg of liver tissue in 1 ml phosphate-buffered saline were treated with tissue homogenizer, and the homogenates were collected for analysis. 200 µl homogenate was pipetted into glass tube and followed by conventional Folch extraction for total lipids. After overnight incubation at 4°C, the bottom layer of the lipid phase was dried under nitrogen gas. Methanolic KOH (1.5 ml) was then added followed by saponification at 110°C for 1

hour. Methylation of fatty acids was conducted by adding 1.5 ml boron trifluoride and 1.5 ml hexane followed by incubation at 110°C for 1 hour. After cooling, 1mL of deionized water was added. The top layer was then collected, dried, reconstituted with hexane and then stored at -80°C until further analysis. All fatty acids in liver were expressed as percentage of total identified fatty acids.

3.3.4 PBMC membrane phospholipid analysis

Phospholipids in PBMC membranes were analyzed by high performance liquid chromatography (HPLC) at University of Alberta Lipidomic Core Facility. Briefly, isolated cells were resuspended in 200 µl phosphate-buffered saline and sonicated to homogenize and disrupt cell membranes. Cell homogenate was then measured for protein content by standard bicinchoninic acid assay and followed by Folch extraction. Internal standards were added to quantify phospholipids. After overnight incubation at 4°C, the bottom layer of the lipid phase was dried under nitrogen gas and redissolved with chloroform:isooctane (1:1) and transferred to HPLC vial for further analysis. The proportion of PC and phosphatidylethanolamine (PE) was determined, and PC:PE ratio was calculated.

Since the development of flow cytometry by Herzenbergs and colleagues, this technique has been extensively used in immunological studies. Briefly, the instrument is equipped with a multi-laser light beam device and signal detector (Gao et al., 2018). *It detects fluorescence parameters and*

visible light scatters in two directions, namely forward scatter (FSC, reflect cell size) and 90º side scatter (SSC, reflect cell graduality) (McKinnon, 2018). *The expression of cell surface and intracellular antigens is detected with fluorophore-conjugated antibodies. The fluorescence signals are then measured on a flow cytometer. Cells in each sample can be tagged by several antibodies with unique fluorescence color simultaneously.*

3.3.5 PBMCs phenotype analysis

Two to four multicolor flow cytometry panels were designed and PBMCs were stained with different antibodies to the following markers: T cell panel: CD3 (PerCP), CD4 (FITC), CD8 (PE), CD8 (AF647), CD25 (STAR PE), CD80 (APC), and CD45RA (PE); Antigen-presenting cell (APC) panel: CD14 (FITC), CD11c (PE), CD284 (AF647), SLAII (FITC) and CD21 (PE). Briefly, 100 µl of whole blood was added to pre-treated 96 well plates and incubated twice with lysis buffer. Lysed cells were then washed with buffer (5% fetal calf serum in phosphate-buffered saline, pH=7.2) and spun down. Cells were then stained with fluorescently tagged antibodies (all purchased from BD Biosciences, BioLegend, Bio-Rad Laborarories, USA) at 4°C for 30 minutes, followed by repeated wash with phosphate-buffered saline and fixation in paraformaldehyde (10 g/l, Thermo Fisher Scientific) until further analysis. All samples were acquired within 72 hours of preparation by BD LSR Fortessa flow cytometer at University of Alberta.

3.3.6 Postprandial PBMCs phenotype analysis

To understand postprandial immune cell phenotype changes, we also determined PBMCs phenotype following the MOGTT in the subgroup of LBW piglets at timed intervals (0 min, 60 min, 120 min, 180 min and 240 min) as described in 3.3.5. Cells were stained with different antibodies for the following markers: T cell panel: CD3 (PerCP), CD4 (FITC), CD8 (AF647), and CD25 (STAR PE); Antigen-presenting cell (APC) panel: CD14 (FITC), CD11c (PE), CD284 (AF647), SLAII (FITC) and CD21 (PE).

3.3.7 MLN immune cells phenotype analysis

To determine the MLN immune cells phenotype, 100 µl of isolated MLN cell suspensions were added to pre-treated 96 well v-bottom plates followed by repeated washes with buffer (5% fetal calf serum in phosphate-buffered saline, pH=7.2) as described in 3.3.5. Cells were stained with different antibodies for the following markers: T cell panel: CD3 (PerCP), CD4 (FITC), CD8 (PE), CD8 (AF647), CD25 (STAR PE), CD80 (APC), and CD45RA (PE); Antigen-presenting cell (APC) panel: CD14 (FITC), CD11c (PE), CD284 (AF647), SLAII (FITC) and CD21 (PE). The median fluorescence intensity (MFI) of total CD25+, CD25+ in CD4+ and CD8+, MFI of total CD284+ and MFI of CD284+ in SLAII+ in MLN cells was determined.

3.3.8 FlowJo gating strategy

All data acquired by flow cytometer was analyzed using FlowJo v10 (USA). Regarding the gating

strategy, lymphocyte and monocyte/macrophage populations were first gated based on FSC-A *vs* SSC-A and excluding debris and granulocytes. Doublets were then excluded based on FSC-A *vs* FSC-Height, and viable cells were identified in all stained panels based on FSC-A for each detector channel. For live lymphocytes, total T cells were defined as CD3+ and within CD3+ cells, cytotoxic T cells were identified by gating on CD8+. In contrast, Th cells were identified as CD3+CD8- due to the poor staining of CD4+ antibodies. Th and cytotoxic T cells expressing IL-2 receptor were then identified as CD3+CD8-CD25+ and CD3+CD8+CD25+. Naive Th and cytotoxic T cells were identified as CD3+CD8-CD45RA+ and CD3+CD8+CD45RA+. Of note, CD3+CD4+ instead of CD3+CD8- was used to represent the Th cells to avoid confusion. B cells were identified as CD21+. CD14+ (LPS receptor) and CD11c+ were used to reflect the population of monocytes/macrophages. TLR-4 was identified as CD284+. MHC class II was identified as SLA-II. An example of gating strategy used in FlowJo is shown in **Figure 3.9**.

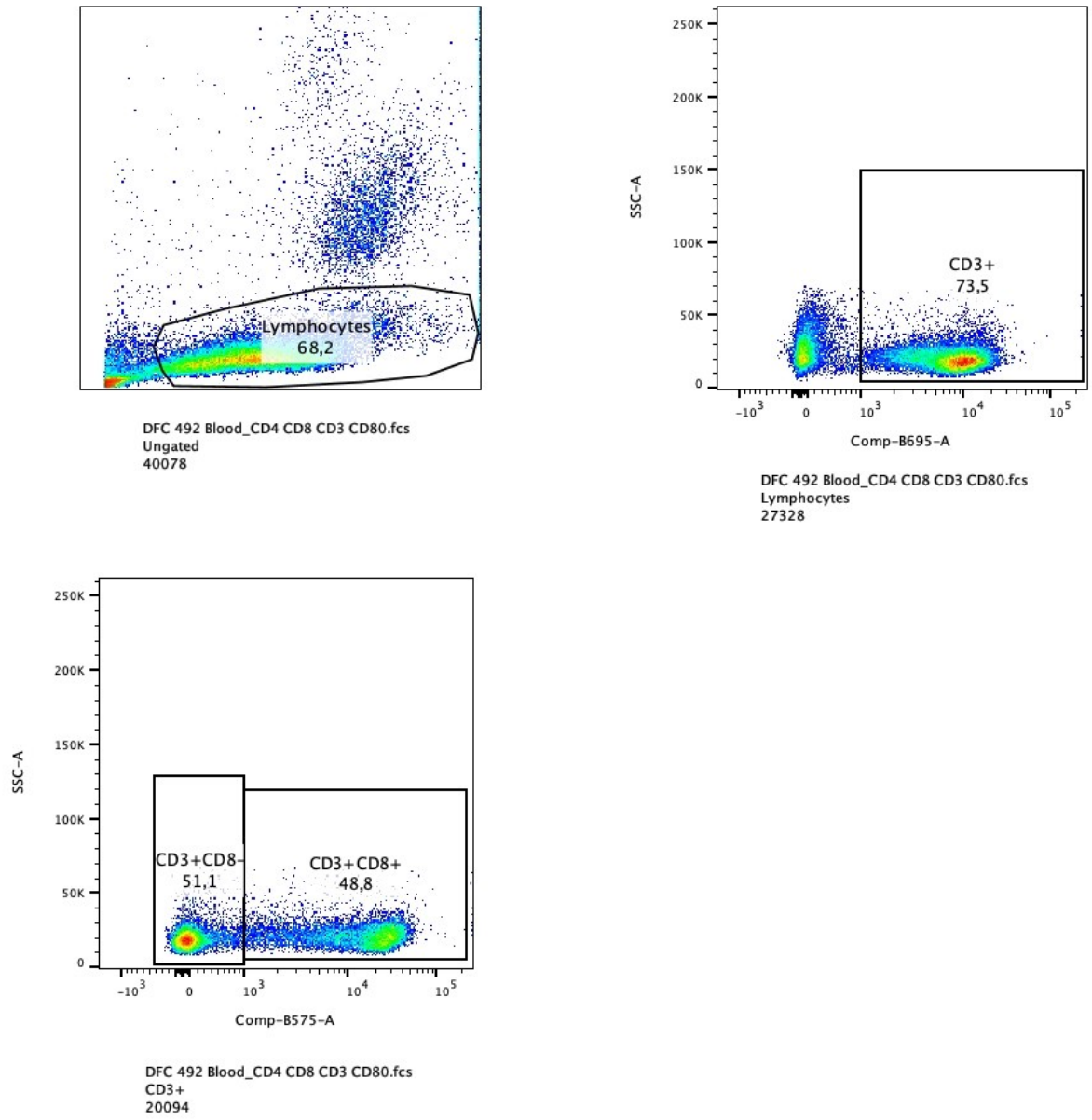

Figure 3.9 Illustration of gating strategy of PBMCs in FlowJo v10 (USA)

Ex vivo mitogen stimulation is a powerful technique that gauges the immune responsiveness at the cellular level under controlled conditions in culture. Isolated immune cells are often incubated with mitogens (known to stimulate immune cells), and the cytokines produced from the immune cells are measured in the supernatant.

3.3.9 Ex vivo cytokine production from mitogen stimulated PBMCs

Isolated PBMCs (1.25 x 10^6 cells/ml) were cultured without mitogens (unstimulated) or with mitogens: phorbol myristate acetate-ionomycin (PMA-I, T cell mitogen, Fisher Scientific, 2 2 µg/ml), phytohemagglutinin (PHA, T cell mitogen, Sigma-Aldrich, 25 µg/ml), pokeweed mitogen (PWM, T and antigen presenting cells mitogen, Sigma-Aldrich, 55 µg/ml). After 48 hours incubation, samples were centrifuged at 1500 rpm for 10 minutes to pellet cells. Supernatant was collected and stored at -80°C. Concentrations of IL-1β, IL-2, IL-6, IL-10, IFN-γ and TNF-α produced from PBMCs were measured by commercial porcine specific ELISA kits (R&D systems, Minnesota) following manufacturer's instructions and were quantified on a microplate reader (wavelength at 450 nm to 570 nm, SpectraMax 190, Molecular Devices). Samples were assayed in duplicate with CV <1 0%.

3.3.10 Ex vivo cytokine production from mitogen stimulated MLN immune cells

Isolated MLN immune cells (1.25 x 10^6 cells/ml) were cultured without mitogens (unstimulated) or with mitogens: phorbol myristate acetate-ionomycin (PMA-I, T cell mitogen, Fisher Scientific,

2 μg/ml), pokeweed mitogen (PWM, T cell and APC mitogen, Sigma-Aldrich, 55 μg/ml), lipopolysaccharide (LPS, APC mitogen, 5 μg/ml). After 48 hours incubation, samples were centrifuged at 1500 rpm for 10 minutes to pellet cells. Supernatant was collected and stored at -80°C. Concentrations of and IL-2, IL-6, IL-10, IFN-γ and TNF-α produced from MLN immune cells were measured by commercial porcine specific ELISA kits (R&D systems, Minnesota) following manufacturer's instructions and were quantified on a microplate reader (wavelength at 450 nm to 570 nm, SpectraMax 190, Molecular Devices). Samples were assayed in duplicate with CV < 10%.

3.4 Statistical analysis

All results were expressed as means ± standard error mean (SEM) unless otherwise stated. Statistics were performed by using one-way ANOVA procedure with Tukey adjustment for multiple comparisons between groups (Prism 8). All tests and comparisons were considered significant at P value < 0.05. Based on previous findings (Fontaine et al., 2019), we have been able to detect 20% differences on average with a 95% CI with n = 4-5 piglets per group when comparing BW differences (15-20% difference; LBW *vs* NBW) and diet induced differences (20-30% difference; CHF *vs* Chow) on the lipid profile, insulin and glucose concentrations. As this was our first time assessing immune function in this pre-established model, we aimed to increase our sample size to n = 8 piglets per group in order to detect significant differences in peripheral immune

outcomes. The determination of postprandial and intestinal immune responses was exploratory and a secondary objective. Area under the curve (AUC) of each postprandial parameter of each pig was individually calculated and then tested for differences in grouped analysis with one-way ANOVA.

Bridge to Chapters 4 & 5

Findings with more detailed explanation of data collection were organized into Chapters 4 and 5. Chapter 4 consists of experimental result and the respective discussion of findings on the impact of consuming low- or high-fat dairy products on cardiometabolic and immune perturbations in the fasting state. Chapter 6 consists of experimental result and the respective discussion of findings on the impact of consuming low- or high-fat dairy products on cardiometabolic and immune perturbations in the postprandial state and on intestinal immune function.

4 CHAPTER 4 Effect of high-fat and low-fat dairy products on cardiometabolic risk factors and immune function in a low birthweight swine model of diet-induced insulin resistance

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4.1 Introduction

The immune system consists of two major arms, namely innate (e.g., skin, mucosal, myeloid immune cells) and adaptive (T and B lymphocytes) immunity, that together mount a regulated protective immune response to foreign stimuli. A proper immune response to infections is influenced by genetics, age, sex, smoking, alcohol, as well as the presence of obesity and nutrition (Calder, 2020; Zimmermann & Curtis, 2019). In the context of obesity, alterations in the secretory output from adipose tissue is characterized by an increased release of cytokines and chemokines resulting in an excessive recruitment, infiltration and polarization of macrophages towards a pro-inflammatory phenotype (i.e. M1-like macrophages) (Zatterale et al., 2020). On the other hand, obesity-related metabolic complications are also associated with an increased intestinal permeability that further promotes systemic inflammation through the translocation of endotoxin into circulation (Luck et al., 2015; Nagpal et al., 2018). Obesity-induced inflammation is also known to impair insulin signaling which contributes to insulin resistance and dyslipidemia and ultimately to the progression of CVD (Powell-Wiley et al., 2021). We have previously demonstrated that individuals with obesity and T2D have an impaired T cell and neutrophil response following mitogen stimulation (Richard et al., 2017) compared to metabolically healthy individuals with obesity. This suggests that regardless of the obesity status, hyperglycemia and/or insulin resistance further impairs immune function. More recently, obesity and T2D have also been identified as major risk factors for severe COVID-19 outcomes (Sjögren et al., 2021) contributing to the body of evidence demonstrating that obesity impairs immune function.

Nutrients play a critical role in modulating obesity-related cardiometabolic perturbations and immune dysfunction. Fatty acids are key components in modulating blood lipid profile, while also exerting pro/anti-inflammatory activities (Fernandez & West, 2005; Fritsche, 2015). In this context, SFA are generally considered pro-inflammatory, yet not all SFA exert the same pro-inflammatory properties (Fritsche, 2015). Medium-chain SFA may be beneficial for weight loss and major CVD risk factors (Bohl et al., 2017; Kris-Etherton & Fleming, 2015). Pentadecanoic acid (C15:0), the ruminant-derived odd-chain SFA, has been shown to exert anti-inflammatory activity (Venn-Watson et al., 2020) and be inversely associated with variables of insulin resistance and T2D (Imamura et al., 2018). Additionally, the natural *trans*-fat vaccenic acid (18:1 *trans*-11) has been found to be beneficial to immune function (Blewett et al., 2009). Recent work from our group has also demonstrated the promising role of dietary choline, particularly in the forms of PC and SM, at improving the immune system development and function in rats (Azarcoya-Barrera et al., 2020, 2021). Dairy products are unique and possess a complex food matrix that contains significant amounts of choline and medium-chain SFA along with other important nutrients. Still, many consumers are avoiding dairy products due to the perception that diets high in SFA may lead to adverse effects on health. In contrast, emerging evidence now point towards a neutral to potentially beneficial effect of total dairy and dairy fat intake on most CVD risk factors including blood pressure, inflammation, T2D and dyslipidemia (Drouin-Chartier, Brassard, et al., 2016; Drouin-Chartier, Côté, et al., 2016; Drouin-Chartier et al., 2021; Hirahatake et al., 2020; Liang et al., 2018).

The debate continues on the contribution of dairy fat *per se* on overall cardiometabolic health. One of the major reasons for this is that most studies to date have not considered the complex food matrix when comparing high-fat vs. low-fat dairy products (i.e., comparing milk to cheese or milk to butter) (Brassard et al., 2017; Rancourt-Bouchard et al., 2020). Few studies have assessed the effect of dairy foods and dairy fat, considering the importance of the food matrix on immune function. Given the glucose, insulin and immune modulatory effects attributed to certain nutrients found in dairy foods and dairy fat, the overall aim of this study was to determine the effect of consuming 3 servings/day of high-fat vs. low-fat products on obesity-related cardiometabolic perturbations and immune function in a pre-established swine model of insulin resistance. Commercially available high-fat and low-fat dairy products of similar food matrix were chosen. We hypothesized that consumption of 3 servings/day of dairy products, irrespective of the fat content, would exert a neutral effect on the lipid profile. We also proposed that feeding high-fat dairy may provide greater benefits on glucose metabolism, inflammation and immune function due to the presence of unique bioactive nutrients in dairy fat.

4.2 Materials and Methods

4.2.1 Animals and housing

Refer to Chapter 3.2.1

4.2.2 Study design

Refer to Chapter 3.2.2

4.2.3 Diet

Refer to Chapter 3.2.3

4.2.4 Tissues, blood processing and PBMC isolation

Refer to Chapter 3.2.5 & 3.3.6

4.2.5 Plasma biochemical and liver fatty acid analysis

Refer to Chapter 3.3.1 & 3.3.3

4.2.6 PBMC membrane phospholipid analysis

Refer to Chapter 3.3.4

4.2.7 PBMC phenotype analysis

Refer to Chapter 3.3.5 & 3.3.8

4.2.8 Ex vivo cytokine production from mitogen stimulated PBMCs

Refer to Chapter 3.2.8 & 3.3.9

4.2.9 Statistical analyses

Refer to Chapter 3.4

4.3 Results

4.3.1 Pig growth and daily food intake parameters

As expected, the birthweights of piglets in all three LBW groups were lower than piglets in NBW groups (all, $P < 0.05$, **Supplementary Table 4.1**). The NBW-Chow group had a lower average energy intake compared to all high-fat diet fed groups (all, $P < 0.05$). The energy to weight gain ratio of NBW-Chow piglets were also lower than all other groups (all, $P < 0.001$); however, there were no differences in average daily growth. There were also no differences on final bodyweight among all NBW and LBW groups. LBW piglets caught up to the NBW piglets starting from 5 weeks of age as shown in **Supplementary Figure 4.1.**

4.3.2 Fatty acid profile in liver tissue

The analysis was conducted in all LBW groups only, as CHF is considered the control group for comparing the effect of dairy in this model and are presented in **Supplementary Table 4.2**. In liver tissue, the proportion of myristic acid (C14:0) was higher in LBW-HFDairy compared to both LBW-CHF and LBW-LFDairy (both $P = 0.001$). Similarly, the unique dairy-derived odd-chain fatty acid, pentadecanoic acid (C15:0), was also higher in LBW-HFDairy compared to both LBW-CHF and LBW-LFDairy (both $P < 0.004$). The proportion of EPA (C20:5 n3) was higher in LBW-

CHF compared to LBW-HFDairy only ($P < 0.01$). There were no differences in the proportion of total SFA, mono- (MUFA) and polyunsaturated fatty acids (PUFA) across all LBW groups.

4.3.3 Fasting plasma lipids, glucose, insulin, HOMA-IR and inflammatory marker profiles

Fasting plasma lipid profile, glucose, insulin and calculated HOMA-IR are shown in **Figure 4.1**. Fasting glucose in NBW-Chow was lower than in NBW-CHF and LBW-CHF (both $P < 0.01$). The LBW-HFDairy had lower plasma glucose concentrations compared to the LBW-CHF group ($P < 0.05$). Feeding low-fat dairy products also improved (lowered) fasting glucose concentrations in that they were no longer different to NBW-Chow, but to a lesser extent than high-fat dairy products. Although no statistical differences were observed in fasting insulin concentrations and HOMA-IR among all groups, there was a trend toward higher HOMA-IR in the LBW-CHF group when compared to all other groups (P-model = 0.08) suggesting early signs of insulin resistance.

There were no differences in plasma TG and HDL-C concentrations across all groups. However, plasma TC concentrations in NBW-Chow were lower than in all other high-fat diet fed groups (all, $P < 0.001$). Similarly, LDL-C concentrations in NBW-Chow were lower compared to all other groups (all, $P < 0.05$). Consistent with this, FPLC results showed that the NBW-Chow had the lowest amount of cholesterol in VLDL, IDL and LDL particles compared to all other high-fat diet fed groups (**Figure 4.2**). Although no significant difference was observed in fasting plasma TG levels among groups, the three LBW groups had increased TG in VLDL particles compared to

both NBW groups.

Full results on fasting circulating inflammatory marker concentrations are shown in **Supplementary Table 4.3**. IL-1α and IL-1β in LBW-HFDairy were higher than in NBW-CHF (both $P < 0.05$). IL-4 in LBW-HFDairy was higher than in both NBW-Chow and NBW-CHF (both $P < 0.05$). The ratio of IL-4/IFN-γ in LBW-HFDairy was higher than in NBW-Chow, NBW-CHF and LBW-CHF (**Figure 4.1**, all, $P < 0.05$). There were no differences in IL-1Ra, IL-2, IL-6, IL-8, IL-10, IL-12. IL-18, IFN-γ and TNF-α concentrations across all the groups.

4.3.4 PBMC membrane phospholipid classes

No differences were observed in the total amount of the different phospholipid classes in PBMC membranes except for PE and PC. PE and PC were found to be higher and lower, respectively, in the NBW-Chow and LBW-LFDairy groups compared to the LBW-CHF group (both $P < 0.05$, data not shown). In **Figure 4.3**, the ratio of PC:PE was found to be lower in NBW-Chow and LFDairy groups compared with both the NBW-CHF and LBW-CHF groups ($P < 0.001$).

4.3.5 Ex vivo cytokine production from mitogen stimulated PBMCs

Ex vivo cytokine production by PBMCs stimulated with mitogens is presented in **Table 4.1**. Following PWM stimulation, IL-2, TNF-α and IFN-γ production were significantly lower in LBW-CHF than in NBW-Chow (all, $P < 0.001$). While feeding high-fat dairy products had minimal

effect on Th 1 cytokine production, feeding low-fat dairy products significantly improved IL-2, TNF-α and IFN-γ production compared to LBW-CHF (all, $P < 0.05$). Additionally, LBW-CHF piglets were also found to produce less IL-1β compared to NBW-Chow ($P < 0.01$) and less IL-10 compared to both NBW-Chow ($P < 0.001$) and NBW-CHF ($P < 0.001$). Dairy products normalized the IL-1β production in that it was no longer different from the NBW-Chow group but did not normalize IL-10 production.

Following PHA stimulation, IL-2 production was significantly lower in LBW-CHF than in NBW-Chow and NBW-CHF (all, $P < 0.05$). While Feeding high-fat dairy products had minimal effects on IL-2 production, feeding low-fat dairy products significantly improved IL-2 production compared to LBW-CHF ($P < 0.01$). TNF-α and IFN-γ production also tended to be lower in LBW-CHF than in NBW-Chow and NBW-CHF (all, P model < 0.05). Similarly, while feeding high-fat dairy products had minimal effects on TNF-α and IFN-γ production, feeding low-fat dairy products significantly improved IFN-γ production compared to LBW-CHF ($P < 0.05$). Additionally, LBW-CHF was found to produce less IL-10 compared to NBW-Chow ($P < 0.01$); however, dairy products had minimal effect.

Following PMA-I stimulation, there was an effect of treatment (P model < 0.05) on TNF-α production and tended to be lower in LBW-CHF than in NBW-Chow and NBW-CHF. While feeding high-fat dairy products had minimal effect on TNF-α production, feeding low-fat dairy

products significantly improved TNF-α production compared to LBW-CHF ($P < 0.01$). IL-6 and IL-10 production were also lower in LBW-CHF than in NBW-Chow (all, $P < 0.05$), and the production of IL-10 was no longer different compared to NBW-Chow in both LBW-HFDairy and LBW-LFDairy groups.

4.3.6 PBMC immune cell phenotypes

As presented in **Table 4.2**, the proportion of T cells (CD3+) were similar among all groups except for the LBW-LFDairy, which had a slightly higher proportion compared to the NBW-CHF ($P < 0.05$). No change in the overall proportion of helper T cells and cytotoxic T cells were observed among groups. No other differences were observed among groups for any other T cell markers including naïve T cells (CD3+CD45RA+) and helper and cytotoxic T cells expressing the IL-2 receptor (CD3+CD4+CD25+ and CD3+CD8+CD25+). We were unable to precisely identify B cell population (CD21+) due to the insufficient antibody staining and therefore, results not shown.

The proportion of monocytes (CD14+) was lower in LBW-HFDairy compared to NBW-Chow, NBW-CHF and LBW-CHF, and was lower in LBW-LFDairy compared to NBW-Chow and NBW-CHF (all, $P < 0.05$). No differences were observed among groups for activated monocytes (CD14+CD11c+). The proportion of APCs expressing toll-like receptor 4 (TLR-4, CD284+) was lower in both the LBW-CHF and LBW-HFDairy but not in the LBW-LFDairy compared with the NBW-Chow (all, $P < 0.05$). However, the proportion of monocytes expressing the TLR-4

(CD14+CD284+) and APCs expressing both major histocompatibility complex (MHC) class II and TLR-4 (SLAII+CD284+) were lower in all high-fat diet fed groups than in NBW-Chow (all, $P < 0.05$).

4.4 Discussion

In the present study we assessed the intake of 3 servings/day of either low- or high-fat commercially available dairy products, that included milk, yogurt and cheese, on obesity-related cardiometabolic perturbations and immune function. We have confirmed that LBW piglets fed a high-fat diet exhibited early signs of insulin resistance and also demonstrated peripheral immune dysfunction. Feeding dairy products, regardless of fat content, was associated with improvements in fasting plasma glucose levels. Feeding low-fat dairy products improved peripheral immune function to a greater extent than high-fat dairy. These findings provide novel insights into the role of dairy products and dairy fat in modulating obesity-related cardiometabolic perturbations and immune dysfunction (**Figure 4.4**).

4.4.1 Dairy fat and its effect on choline moieties in PBMC's membrane and systemic inflammation

Intriguingly, our findings contradicted part of our initial hypothesis that high-fat dairy would potentially be more effective than low-fat dairy intake at improving immune function. This is likely in part due to the differences in choline content (and the forms of choline) between low- and high-

fat dairy products. Unlike the structure of other dietary fats, milk fat droplets are normally enveloped by an amphiphilic membrane, namely MFGM. Polar lipids including PC, PE and SM are naturally found in dairy and specifically in the MFGM. During industrial processes such as making butter from raw milk, the MFGM together with polar lipids are disassociated from fat globules and preferentially enriched in the aqueous phase such as skimmed milk and buttermilk (Rombaut et al., 2006). Indeed, we have previously compared the choline content in 48 commercial dairy products in Canada and reported that total as well as both water- and lipid-soluble forms of choline were negatively associated with total dairy fat content (Richard et al., 2016). Our group has also recently demonstrated that buttermilk, as an important source of lipid soluble forms of choline (i.e., PC and SM), exhibited promising ability to support the immune system development and T cell function (Azarcoya-Barrera et al., 2020, 2021). Therefore, the higher choline content in low-fat dairy products might explain, at least in part, the ameliorated immune function seen in the LBW-LFDairy group. We also assessed the two major phospholipid classes (PE and PC) in PBMCs membrane as an indirect marker of dietary PC intake. In rodents, we have previously shown that feeding a diet containing 100% PC as compared to a diet containing 100% free choline increased the PC content in splenocyte cell membranes which was associated with enhanced T cell function (Lewis et al., 2016). Here, we demonstrated that the PC:PE ratio in PBMCs increases in the context of high-fat diet feeding, which can be normalized by dairy consumption with the most beneficial effect observed with low-fat dairy. Although there is limited evidence regarding the importance of the PC:PE ratio on immune function, a higher ratio has been linked to the progression of several

metabolic diseases such as liver steatosis in *ob/ob* mice and insulin resistance in skeletal muscle (van der Veen et al., 2017). These data suggest that part of the greater beneficial effect that low-fat dairy has on immune function may be attributable to the higher amount of lipid soluble forms of choline normalizing the PC:PE ratio in the context of a high-fat diet.

The fact that high-fat dairy led to fewer changes in immune function than low-fat dairy in this model could also be due to the overall higher proportion of fat coming from the diet in this group. The proportion of fat coming from the 3 servings of high-fat dairy is roughly 64% whereas the proportion of fat in the low-fat dairy is about 26%. Therefore, by substituting some of the CHF diet that contains 46% fat by 3 servings of high-fat dairy, the overall proportion of fat in the diet was increased to roughly 51%. It has been demonstrated that high-fat diet and obesity can lead to an increased gut permeability and LPS translocation which in turn would trigger an inflammatory response (Duan et al., 2018; Massier et al., 2021; Mohammad & Thiemermann, 2021). Similarly, we demonstrated that feeding a high-fat diet in this swine model tended to increase circulating levels of inflammatory markers. Therefore, in the context of a high-fat diet, providing a diet that contains a higher proportion of fat may have led to a higher LPS translocation and greater systemic inflammation as observed in the LBW-HFDairy group. Indeed, we have observed higher concentrations of circulating IL-1α and IL-1β in the LBW-HFDairy group. In addition, LBW-HFDairy group also had a higher Th2 cytokine profile (reflected by the ratio of IL-4/IFN-γ), which is known to suppress Th1 responses. Collectively, this could explain, at least in part, the lower

immune function observed in that group when compared to LBW-LFDairy.

4.4.2 Dairy fat and its impact on glucose metabolism and insulin resistance

Although the proportions of total SFA, MUFA and PUFA in liver tissue were similar among all LBW groups, the proportion of C14:0 and C15:0 were higher in the LBW-HFDairy group. These SFAs have been used as biomarkers of dairy fat intake in humans (Abdullah et al., 2015; Golley & Hendrie, 2014; Liang et al., 2018; Lund-Blix et al., 2016; Pranger et al., 2019). Particularly, C15:0 and C17:0, are odd-chain SFAs that cannot be endogenously synthesized and are specific to dairy and ruminant meat (Abdullah et al., 2015). However, it has been previously reported that these two odd-chain SFAs are present in a variety of marine fish species (Özogul et al., 2009). Hence, it is not surprising that we have also detected C15:0 and C17:0 and higher proportion of EPA in liver tissue in the group that received the CHF diet since fish meal is added to the experimental diet. Both dairy groups consumed less of the experimental CHF diet to account for the energy intake coming from dairy consumption and therefore accumulated less EPA. However, we confirmed that the biomarkers for dairy fat intake in humans are also relevant for this large animal swine model.

Meta-analyses of cohort studies have previously reported an inverse relationship between biomarkers of dairy fat intake, particularly the C15:0 and C17:0, and the incident of T2D in humans (Huang et al., 2019; Imamura et al., 2018). *In vitro* studies have also reported that C15:0 play a

direct role in glucose metabolism *via* promoting GLUT4 translocation to plasma membrane in myotubes (Fu et al., 2021). C15:0 has also recently been proposed to be an essential fatty acid due to a number of established cardiometabolic benefits including attenuation of glucose concentrations in mice fed with high-fat diet (Venn-Watson et al., 2020). Importantly, our findings in LBW swine model are in agreement with previous findings, suggesting that dairy fat may be responsible, at least to some extent, for the improvement in glucose metabolism in this model.

4.4.3 Dairy fat and its impact on dyslipidemia

Dairy fat is still a subject of controversy as it relates to its beneficial effect on cardiometabolic health due to its high content in SFA. Yet, emerging evidence from RCTs mostly point to a neutral effect of dairy, irrespective of their fat content, on several cardiometabolic risk factors (Drouin-Chartier, Côté, et al., 2016). For instance, a recent RCT in 72 subjects with metabolic syndrome reported that consumption of 3.3 servings/day of either low- or high-fat dairy from the same food matrix, specifically milk, yogurt and cheese for 12 weeks, did not modulate serum concentrations of TC, LDL-C, HDL-C and TG (Schmidt et al., 2021a). We also reported a neutral effect of dairy consumption, regardless of their fat content, on TC, LDL-C, HDL-C and TG when compared to the LBW-CHF group. Altogether, our results suggest that feeding dairy products in the context of a high-fat diet has little effect on the lipid profile.

4.4.4 Dairy and their impact on peripheral blood T cell function

Previous findings in humans and rodents have demonstrated an impaired immune response to mitogen stimulation in the context of obesity and T2D (Azarcoya-Barrera et al., 2022; Lamas et al., 2002; Richard et al., 2017). Here, we found that consuming a high-fat diet in the context of a LBW swine model of insulin resistance also led to impaired immune function, particularly impaired T cell function. IL-2 is a cytokine that induces T cell proliferation and differentiation (Ross & Cantrell, 2018). Lower IL-2 production after PHA and PWM stimulation in the LBW-CHF suggests an impaired T cell proliferation and differentiation. Additionally, IFN-γ and TNF-α are two crucial cytokines produced by Th1 cells and in turn, also being a major element to induce T cell proliferation (Davignon et al., 2018). Therefore, the lower IFN-γ and TNF-α production after PWM stimulation in LBW-CHF could explain, to some extent, the lower IL-2 production in this group. On the other hand, we have demonstrated that feeding low-fat dairy in the context of a high-fat diet exerted a greater ability to normalize IL-2, IFN-γ and TNF-α production after stimulation relative to the NBW-Chow group, suggesting that low-fat dairy may be more effective than high-fat dairy. Remarkably, we still observed that feeding high-fat dairy improved IL-2 after PHA, and TNF-α after PMA-I stimulation even though it was to a lesser extent than the low-fat dairy. Previous studies in humans have reported that individuals with obesity and T2D (when compared to individuals with obesity but metabolically healthy (MHO)) have higher proportions of naïve T cells (CD3+CD45RA+) and cytotoxic T cells despite having similar proportions of total T cells (Richard et al., 2017). Moreover, individuals with obesity and T2D had a higher proportion

of immune cells expressing activation markers such as CD80, and T cells expressing CD278, which play an important role in IL-2 production and T cell proliferation (Richard et al., 2017). In the current study, we did not observe any significant changes on the major T cell subsets or activation markers. A possible explanation is that although our model was characterized by early signs of insulin resistance, it did not lead to a state of frank T2D. Altogether, our data suggest that a high-fat diet impairs T cell function in LBW swine and that feeding low-fat dairy can counteract some of the obesity-related T cell dysfunction.

4.4.5 Dairy and their impact on peripheral blood APC function

IFN-γ and TNF-α can also be produced by APCs such as monocytes and dendritic cells. IL-1β is another crucial cytokine that can be induced by nearly all microbial substances and causes inflammation (Dinarello, 2018). Therefore, lower levels of IFN-γ, TNF-α and IL-1β produced from LBW-CHF after PWM stimulation may also suggest an impaired innate immune response. Similarly, feeding low-fat dairy normalized the production of these cytokines in that they were no longer differ from the NBW-Chow. Feeding high-fat dairy also normalized the production of IL-1β, but not the other cytokines measured. This could be explained, at least in part, by the differences in total cells expressing CD284+ (TLR-4) in PBMCs. TLR-4 is known to be one of the first lines of defense for the innate immune system by recognizing bacterial endotoxins and inducing subsequent immune responses (Molteni et al., 2016). Indeed, we reported that the expression of TLR-4 was lower in both the LBW-CHF and LBW-HFDairy groups while the LBW-

LFDairy was similar to NBW-Chow. Decreased expression of TLR-4 has been previously reported in elderly patients who have a higher prevalence of infection than younger patients (Schaenman et al., 2019). In contrast, B cell proportions were previously found to be similar in individuals with obesity and T2D compared to MHO (Richard et al., 2017). This is consistent with the current finding that the proportions of B cells and macrophages remained unchanged across all groups. Overall, our data suggest that a high-fat diet may impair APCs function in LBW swine and that feeding low-fat dairy can improve APC function by partially normalizing the expression of TLR-4.

As much as the acute inflammatory response to stimuli is important in mounting an adequate immune response, the resolution phase of inflammation is as important to prevent tissue damage. IL-10 is a key regulatory cytokine with anti-inflammatory properties and can be produced by a number of myeloid and lymphoid cells including Th2, regulatory T cells (Treg), dendritic cells and macrophages (Saraiva & O'Garra, 2010). The action of IL-10 on promoting the resolution phase of inflammation has also been previously reported (Takeda et al., 2015). In the present study, lower production of IL-10 in LBW-CHF after both T cell mitogen and APC mitogen stimulations is suggestive of an impaired anti-inflammatory cytokine production and overall diminished immune-suppressive ability. Indeed, serum concentrations of IL-10 have been found to be significantly reduced in obese subjects and correlated with hyperinsulinemia and insulin resistance (Leon-Cabrera et al., 2015). IL-10 has also been found to be less effective at inhibiting inflammation in

T2D patients, and failed to attenuate TNF-α production upon LPS stimulation (Barry et al., 2016). On the other hand, we demonstrated that feeding dairy, regardless of fat content, normalized to some extent the IL-10 production after PMA-I stimulation. Although this suggests that dairy may improve the resolution phase of inflammation, the enhanced IL-10 production cannot be attributable to a specific cell type (i.e., APCs, Th2 or Treg cells) nor inflammatory phase. Thus, future studies are warranted to validate which cell type is responsible for the IL-10 production post-stimulation with more detailed laboratory assessment such as intra-cellular cytokine staining.

4.4.6 Strength and limitations

To the best of our knowledge, this is the first study using a swine model of obesity and insulin resistance to investigate the effect of low- *vs* high-fat dairy products using similar food matrices on cardiometabolic risk factors and immune function. This is important since most studies in the field trying to understand the effect of dairy fat have compared milk or yogurt to cheese and butter which do not take into consideration the importance of the food matrix. The higher-than-expected inter-individual variations in some outcomes (i.e., insulin) suggest that future studies should increase the sample size and perhaps the duration of the intervention to induce a stronger insulin-resistant phenotype. As in any nutritional study, there is a replacement effect in that by adding dairy products to the diet, other nutrients were consumed in lower amounts. In this study, we aimed to match the overall energy intake among the high-fat diet treatment groups to compare the effect of adding dairy and dairy fat *per se* in the context of an isoenergetic diet. However, the high-fat

dairy products contained more fat and slightly less protein compared to the low-fat dairy products, which led to an overall higher fat intake and a slight reciprocal reduction in protein and carbohydrate intake. Therefore, it is virtually impossible to attribute the effect observed in our study solely to adding dairy products. We have to stress however, that this type of nutritional study also reflects the real-life scenario in humans. Indeed, people consume whole foods and not isolated nutrients. Therefore, when incorporating dairy in our diet, most people will reduce their intake of other foods leading to differences in protein, carbohydrate and fat intake.

4.5 Conclusion

In conclusion, findings from the present study provide new mechanistic evidence that support the role of dairy products, specifically milk, yogurt and cheese, in counteracting some of the cardiometabolic and immune dysfunction associated with obesity. Consumption of 3 servings per day of high-fat dairy products lowered fasting glucose more than low-fat dairy, whereas low-fat dairy improved immune function particularly T cell function to a greater extent than high-fat dairy. Irrespective of fat content, consumption of 3 servings of dairy products had a neutral effect on the lipid profile in this swine model of insulin resistance.

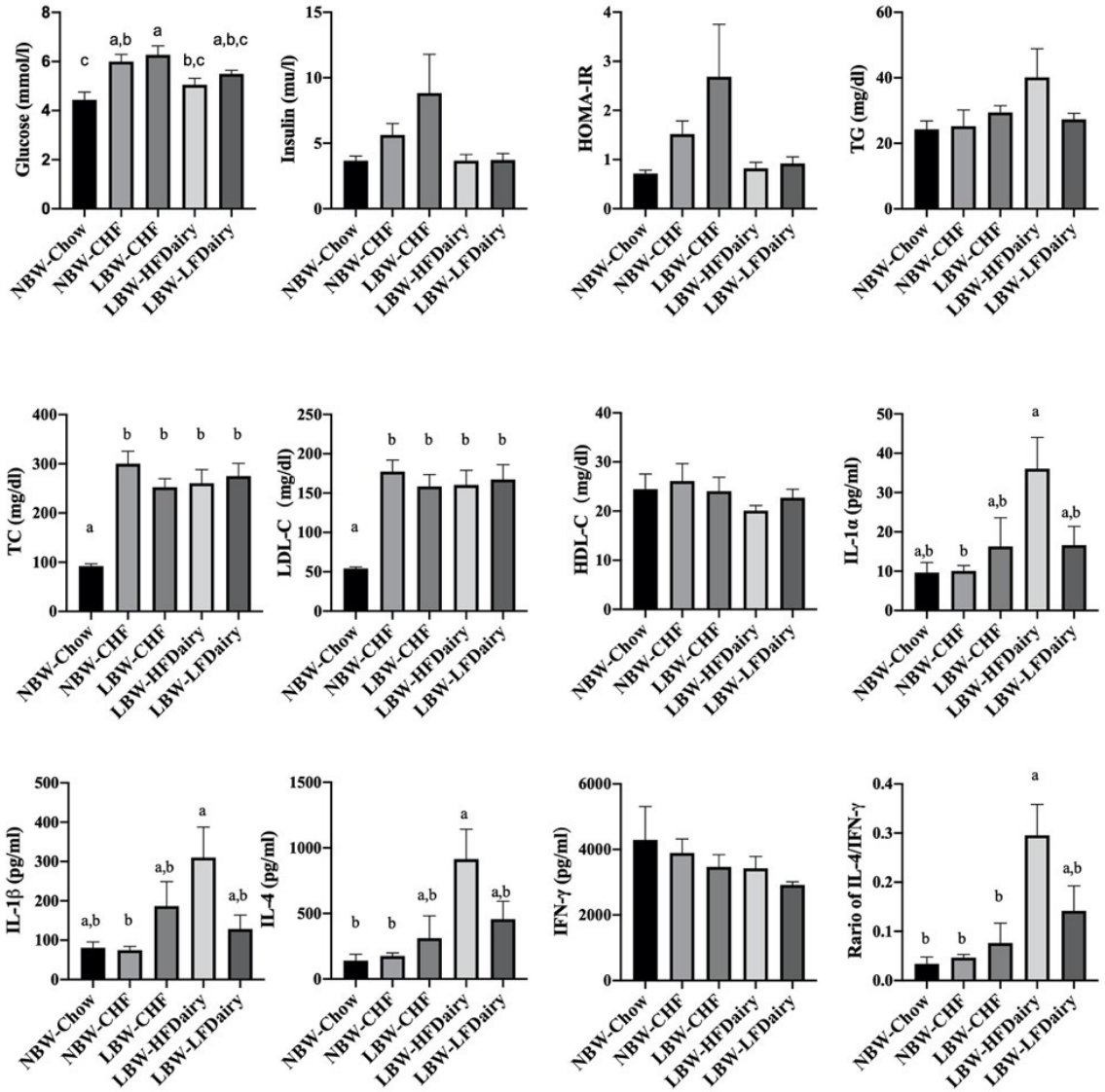


Figure 4.1 Changes on fasting plasma biochemical parameters and inflammatory cytokines in LBW and NBW swine fed different

experimental diets

P-model for insulin = 0.094; *P*-model for HOMA-IR = 0.08. CHF, control high-fat diet; HDL-C, high-density lipoprotein cholesterol; HFDairy, high-fat dairy diet; HOMA-IR, homeostatic model assessment for insulin resistance; IFN-γ, interferon-gamma; IL, interleukin; LBW, low birthweight; LDL-C, low-density lipoprotein cholesterol; LFDairy, low-fat dairy diet; NBW, normal birthweight; TC, total cholesterol; TG, triglycerides; TNF-α, tumor necrosis factor-alpha. All values are expressed as means ± SEM. A p value was considered as statistically significant when <0.05. Means sharing the same letter were not significantly different from each other.

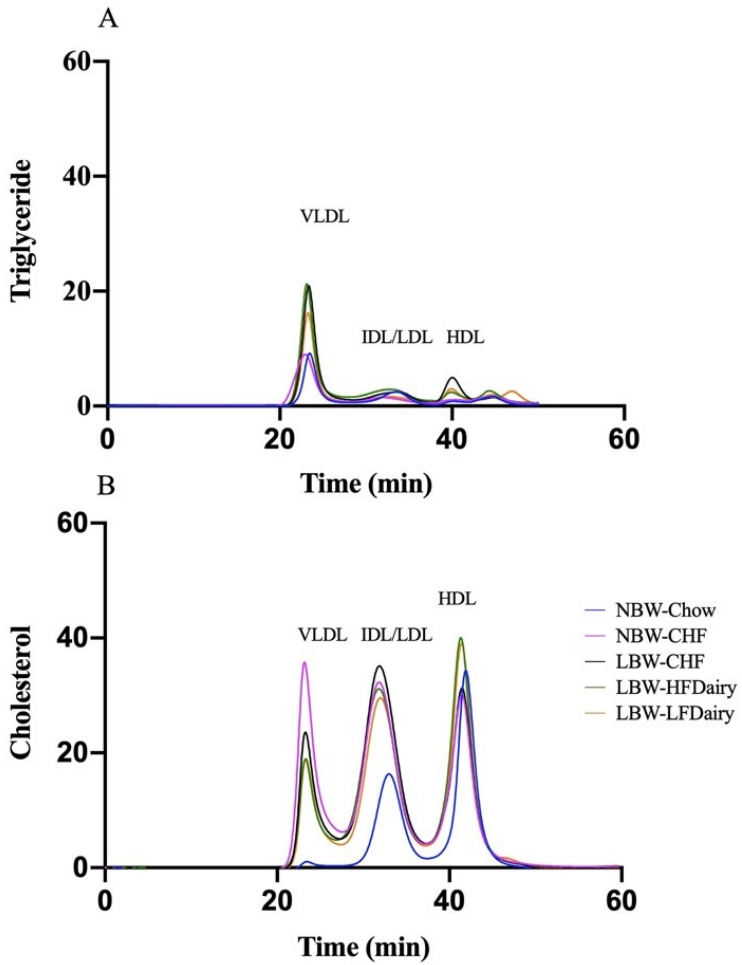


Figure 4.2 Fasting plasma triglyceride (A) and cholesterol (B) in lipoprotein subfractions measured by FPLC

CHF, control high-fat diet; FPLC, fast protein liquid chromatography; HDL, high-density lipoprotein; HFDairy, high-fat dairy diet; IDL, intermediate-density lipoprotein; LBW, low birthweight; LDL, low-density lipoprotein; LFDairy, low-fat dairy diet; NBW, normal birthweight; VLDL, very low-density lipoprotein.

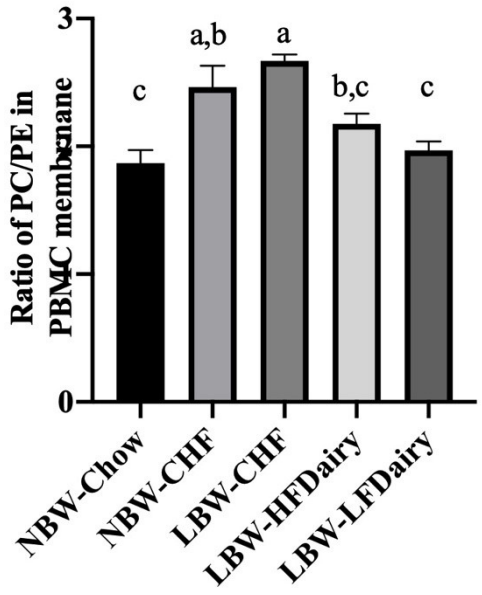


Figure 4.3 The phosphatidylcholine to phosphatidylethanolamine ratio in PBMC membrane

CHF, control high-fat diet; HFDairy, high-fat dairy diet; LBW, low birthweight; LFDairy, low-fat dairy diet; NBW, normal birthweight; PBMC, peripheral blood mononuclear cell; PC, phosphatidylcholine; PE, phosphatidylethanolamine. All values are expressed as means ± SEM. A p value was considered as statistically significant when <0.05. Means sharing the same letter were not significantly different from each other.

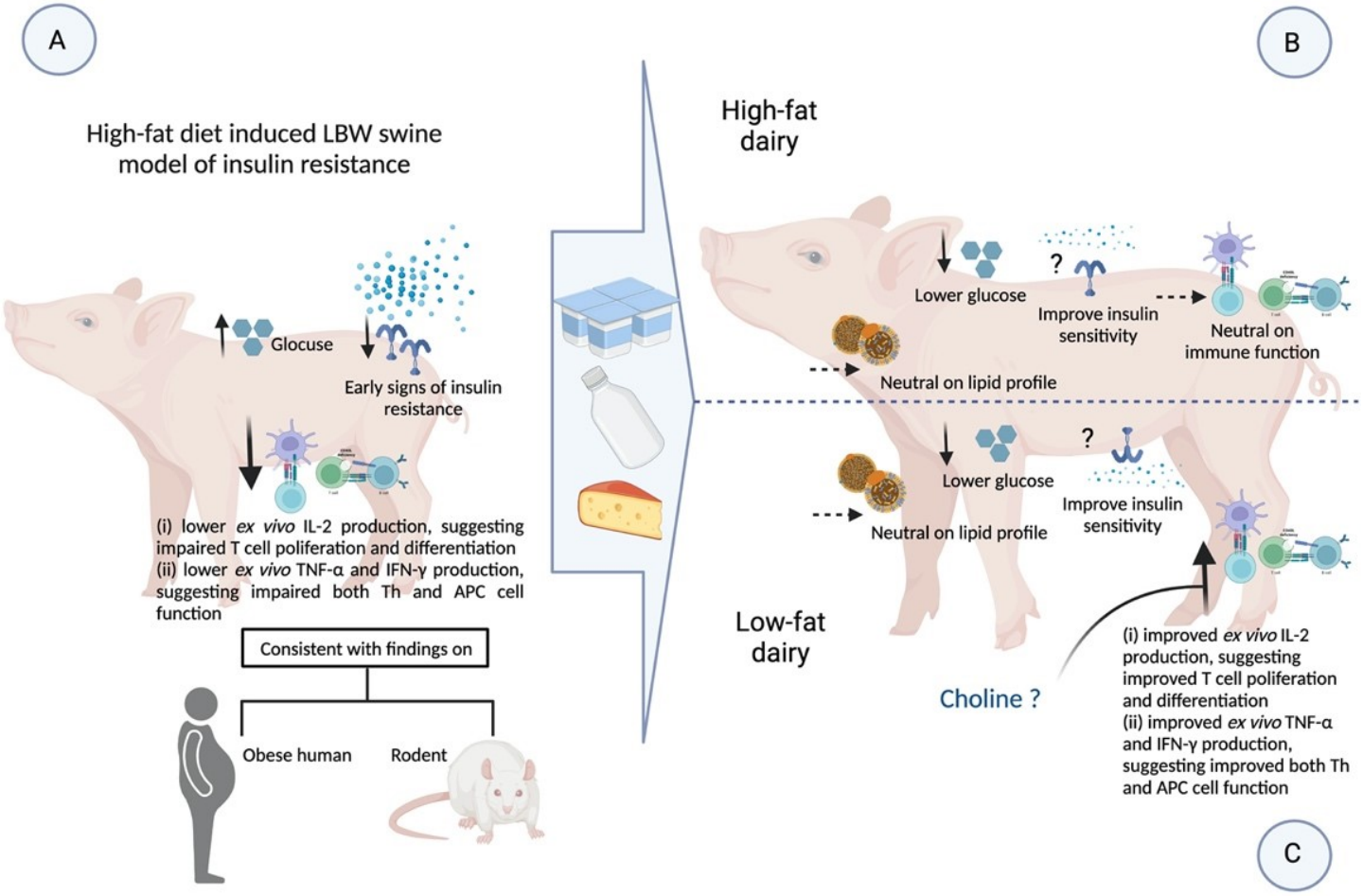


Figure 4.4 Summary of impact of consuming 3 servings high- and low-fat dairy products on obesity-related cardiometabolic and immune perturbations

Figure created with BioRender. APC, antigen presenting cell; CHF, control high-fat diet; HFDairy, high-fat dairy diet; IFN-γ, interferon-gamma; IL, interleukin; LBW, low birthweight; LDL-C, low-density lipoprotein cholesterol; LFDairy, low-fat dairy diet; NBW, normal birthweight; TC, total

cholesterol; Th, T helper cell; TG, triglycerides; TNF-α, tumor necrosis factor-alpha. (A) LBW piglets fed with a high-fat diet (LBW-CHF) developed elevated fasting glucose concentrations and early signs of insulin resistance. Following *ex vivo* immune cell stimulation, poor IL-2, TNF-α and IFN-γ productions was observed suggesting impaired T and APC cell function compared to NBW control groups. This is also consistent with findings in individuals with obesity and rodent models. (B) Feeding high-fat dairy products (LBW-HFDairy) improved (lowered) fasting glucose while had minor effects on immune function compared to LBW-CHF. (C) Feeding low-fat dairy products (LBW-LFDairy) also improved (lowered) fasting glucose while significantly improved IL-2, TNF-α and IFN-γ productions following *ex vivo* stimulation, suggesting improved T and APC function compared to LBW-CHF. Feeding dairy products, regardless of fat content, had neutral effects on lipid profile.

Table 4.1 *Ex vivo* cytokine production by mitogen stimulated PBMCs in LBW and NBW swine fed different experimental diets

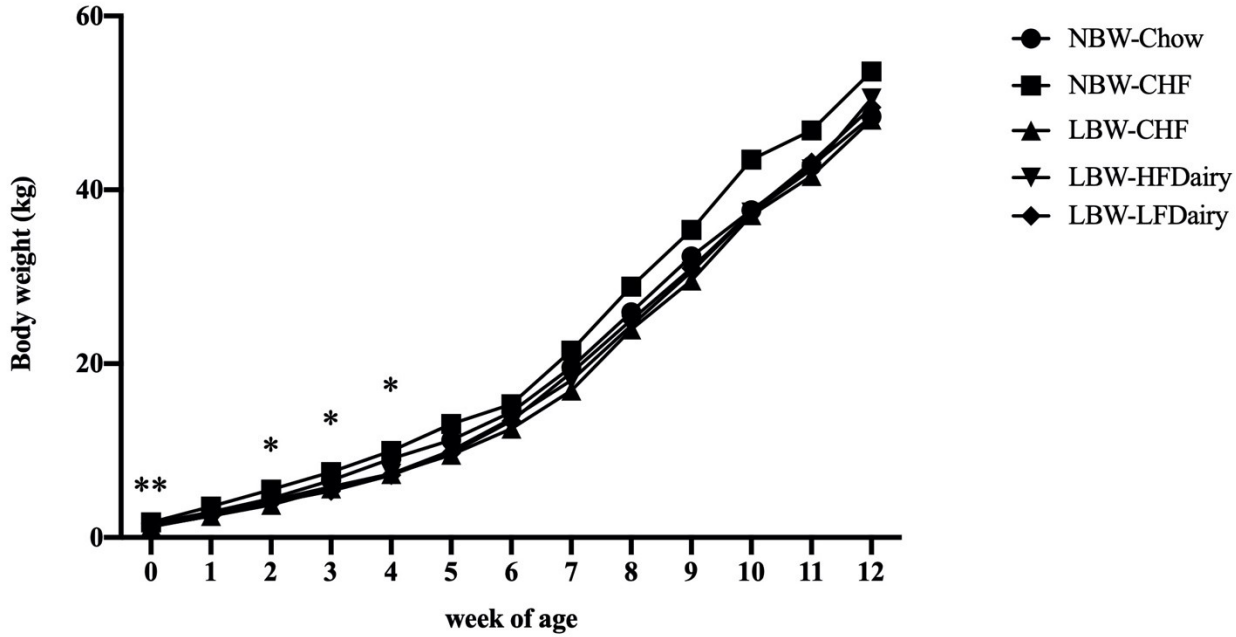
	NBW-Chow	NBW-CHF	LBW-CHF	LBW-HFDairy	LBW-LFDairy	*P*
PWM (T and APC cell mitogen)						
IL-2, pg/ml	494.7±76.6^{a}	297.0±39.8^{b,c}	130.7±26.0^{c}	148.9±16.1^{c}	313.8±55.5^{a,b}	<0.001
TNF-α, pg/ml	1385±195.5^{a}	567.8±57.8^{c}	686.3±82.2^{c}	691.3±56.1^{c}	1105±73.2^{a}	<0.001
IFN-γ, pg/ml	494.7±76.6^{a}	297.0±39.8^{b,c}	130.7±26.0^{c}	148.9±16.1^{c}	313.8±55.5^{a,b}	<0.001
IL-1β, pg/ml	3754±348.3^{a}	2187±136.8^{a,b}	2125±306.1^{b}	2960±292.5^{a,b}	2918±139.1^{a,b}	0.007
IL-6, pg/ml	258.3±51.5	156.9±62.4	161.6±38.5	214.4±27.3	163.9±28.6	0.411
IL-10, pg/ml	3014±428.1^{a}	2455±194.2^{a}	1121±103.0^{b}	998.4±102.4^{b}	1622±167.9^{b}	<0.001
PHA (T cell mitogen)						
IL-2, pg/ml	234.0±25.2^{a}	227.4±28.6^{a}	116.3±12.5^{b}	176.5±15.3^{a,b}	220.3±20.3^{a}	0.002
TNF-α, pg/ml	365.1±61.9	378.3±40.6	232.2±45.4	242.2±26.9	370.5±41.6	0.031
IFN-γ, pg/ml	77.6±17.7^{a,b}	52.3±15.1^{a,b}	37.7±10.2^{b}	29.3±5.5^{b}	102.2±19.7^{a}	0.006
IL-6, pg/ml	160.2±21.3	121.7±25.7	93.9±40.8	94.4±21.4	190.5±24.2	0.059
IL-10, pg/ml	2422±448.3^{a}	1693±235.5^{a,b}	1052±183.0^{b}	958.3±137.7^{b}	1326±171.8^{b}	0.001
PMA-I (T cell mitogen)						
IL-2, pg/ml	1343±282.4	1675±184.1	1056±197.4	1685±195.6	1686±207.2	0.138
TNF-α, pg/ml	3611±882.4^{a,b}	3242±229.4^{a,b}	1915±250.5^{b}	3514±357.5^{a,b}	4139±437.1^{a}	0.013
IFN-γ, pg/ml	4207±491.4	3587±152.2	2366±744.0	3537±657.5	4383±261.3	0.178
IL-6, pg/ml	615.1±167.0^{a}	279.6±66.8^{a,b}	147.9±47.8^{b}	114.2±30.2^{b}	237.4±45.5^{b}	0.009
IL-10, pg/ml	2185±437.8^{a}	2035±84.8^{a,b}	1095±181.8^{b}	1319±142.6^{a,b}	1652±152.3^{a,b}	0.012

APC, antigen presenting cell; CHF, control high-fat diet; HFDairy, high-fat dairy diet; IFN-γ, interferon-gamma; IL, interleukin; LBW, low birthweight; LFDairy, low-fat dairy diet; NBW, normal birthweight; PBMC, peripheral blood mononuclear cell; PHA, phytohemagglutinin; PMA-I, phorbol 12-myristate 13-acetate plus ionomycin; PWM, pokeweed mitogen; TNF-α, tumor necrosis factor-alpha. All values are expressed as means ± SEM. A p value was considered as statistically significant when <0.05. Means sharing the same letter were not significantly different from each other.

Table 4.2 PBMCs population of NBW and LBW swine fed different experimental diets

Phenotype	NBW-Chow	NBW-CHF	LBW-CHF	LBW-HFDairy	LBW-LFDairy	*P*
% of gated cells						
Total CD3+ (T cell)	61.3±4.1^{a,b}	59.6±1.7^{b}	63.3±1.7^{a,b}	65.5±2.3^{a,b}	69.5±1.2^{a}	0.032
CD3+CD4+ (Th cell)	62.4±2.4	61.0±2.9	64.8±2.0	59.3±2.9	59.2±2.5	0.519
CD3+CD8+ (cytotoxic T cell)	37.7±2.5	38.8±2.9	34.4±2.4	40.6±2.9	41.2±2.5	0.383
CD3+CD45RA+ (naïve T cell)	16.2±2.4	16.9±0.6	18.4±1.8	14.1±2.7	16.0±2.4	0.712
Total CD25+ (IL-2 receptor)	28.2±1.3	29.4±2.8	29.8±1.9	29.5±1.3	26.2±2.5	0.676
CD3+CD4+CD25+	30.5±1.1	28.2±4.2	36.1±4.5	40.0±4.9	30.0±3.7	0.257
CD3+CD8+CD25+	22.2±2.0	20.7±2.2	20.1±0.7	20.8±1.4	19.7±1.9	0.875
Total CD80+ (co-stimulator of T cell)	14.6±0.9	10.0±1.2	8.3±2.2	8.1±1.5	8.8±1.4	0.207
Total CD14+	7.2±0.7^{a}	6.5±1.1^{a}	5.4±0.7^{a,b}	2.8±0.3^{c}	3.5±0.5^{b,c}	<0.001
CD14+CD11c+	8.7±2.4	8.2±3.4	7.7±2.3	5.9±2.1	3.6±1.1	0.502
Total CD284+ (TLR-4)	3.9±0.4^{a}	3.0±1.1^{a,b}	1.2±0.3^{b}	1.3±0.4^{b}	2.5±0.6^{a,b}	0.021
CD14+CD284+	25.2±3.6^{a}	11.6±4.8^{b}	9.8±2.5^{b}	7.3±1.3^{b}	9.8±1.6^{b}	0.002
SLAII+CD284+	11.0±0.8^{a}	2.9±0.7^{b}	2.7±0.8^{b}	2.2±0.7^{b}	3.0±0.8^{b}	<0.001

B cell population (CD21+) was not shown due to the insufficient antibody staining; CHF, control high-fat diet; HFDairy, high-fat dairy diet; IL, interleukin; LBW, low birthweight; LFDairy, low-fat dairy diet; NBW, normal birthweight; PBMC, peripheral blood mononuclear cell; Th, T helper cell; TLR-4, toll-like receptor 4. All values are expressed as means ± SEM. A p value was considered as statistically significant when <0.05. Means sharing the same letter were not significantly different from each other.



Supplementary Figure 4.1 Pig growth parameters during the intervention for NBW and LBW piglets fed the experimental diets

CHF, control high-fat diet; HFDairy, high-fat dairy diet; LBW, low birthweight; LFDairy, low-fat dairy diet; NBW, normal birthweight. A *p* value was considered as statistically significant when < 0.05. ** $P < 0.001$, *$P < 0.05$. At birth (week 0), body weight of all LBW groups were lower than NBW groups. At 2 weeks of age, body weight of LBW-CHF was lower than NBW-CHF. At 3 weeks of age, body weight of LBW-CHF and LBW-LFDairy were lower than NBW-CHF. At 4 weeks of age, body weight of all LBW groups were lower than NBW-CHF. Starting at the 5 weeks of age and until 12 weeks of age, there were no difference in body weight among groups.

Supplementary Table 4.1 Growth and feed consumption of LBW and NBW swine fed different experimental diets

	NBW-Chow	NBW-CHF	LBW-CHF	LBW-HFDairy	LBW-LFDairy	*P*
Birthweight (kg)	1.62±0.07[a]	1.73±0.10[a]	1.29±0.08[b]	1.23±0.06[b]	1.23±0.09[b]	<0.001
Final body weight (kg)	48.44±3.16	53.62±1.97	48.10±2.77	50.53±2.81	49.49±1.88	0.618
Average daily growth (g/day)	779.20±47.34	854.00±15.80	794.70±41.00	824.10±40.47	815.40±27.78	0.723
Average energy intake (Kcal/day)	4077±290.10[a]	5717±340.30[b]	5314±237.90[b]	5716±250.60[b]	5678±172.20[b]	0.001
Energy to gain ratio	5.23±0.14[a]	6.67±0.29[b]	6.72±0.15[b]	6.96±0.14[b]	6.97±0.10[b]	<0.001

CHF, control high-fat diet; HFDairy, high-fat dairy diet; LBW, low birthweight; LFDairy, low-fat dairy diet; NBW, normal birthweight. All values are expressed as means ± SEM. A p value was considered as statistically significant when <0.05. Means sharing the same letter were not significantly different from each other.

Supplementary Table 4.2 Liver total fatty acids profile in LBW swine fed experimental diets at 12 weeks of age

	LBW-CHF	LBW-LFDairy	LBW-HFDairy	*P*
Total SFA	45.60±0.61	45.86±0.41	47.02±0.38	0.565
Total MUFA	17.35±0.60	17.30±0.93	16.63±0.70	0.795
Total PUFA	36.47±0.40	36.23±0.61	35.72±0.55	0.907
C14:0	0.31±0.02^{b}	0.37±0.03^{b}	0.56±0.04^{a}	<0.001
C15:0	0.24±0.02^{b}	0.25±0.01^{b}	0.32±0.01^{a}	<0.001
C16:0	15.12±0.43	15.63±0.46	16.57±0.68	0.179
C16:1	0.66±0.05	0.69±0.06	0.63±0.04	0.744
C17:0	0.58±0.03	0.61±0.03	0.62±0.05	0.756
C18:0	29.23±0.70	28.95±0.79	28.71±0.90	0.902
C18:1	16.29±0.56	16.21±0.88	15.67±0.66	0.801
C18:2n6	15.41±0.47	15.30±0.36	14.88±0.27	0.581
C18:3n6	0.17±0.03	0.15±0.01	0.17±0.02	0.701
C18:3n3	0.19±0.01	0.17±0.01	0.17±0.01	0.249
C20:0	0.46±0.06	0.41±0.03	0.41±0.03	0.581
C20:1	0.23±0.01^{a,b}	0.23±0.01^{a}	0.19±0.01^{b}	0.018
C20:2n6	0.48±0.01^{a}	0.49±0.01^{a}	0.41±0.02^{b}	<0.001
C20:3n6	1.57±0.11	1.86±0.09	1.52±0.12	0.072
C20:4n6	10.08±0.29	9.98±0.48	10.69±0.35	0.373
C20:5n3	2.25±0.12^{a}	1.83±0.10^{a,b}	1.51±0.22^{b}	0.011
C24:0	0.24±0.01	0.24±0.02	0.46±0.20	0.336
C24:1	0.17±0.01	0.17±0.01	0.14±0.01	0.116
C22:5n3	1.28±0.07	1.31±0.10	1.37±0.07	0.711
C22:6n3	5.05±0.34	5.15±0.43	5.01±0.37	0.965

CHF, control high-fat diet; HFDairy, high-fat dairy diet; LBW, low birthweight; LFDairy, low-fat dairy diet; MUFA, monounsaturated fatty acid; NBW, normal birthweight; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid. All values are expressed as means±SEM. A p value was considered as statistically significant when <0.05. Means sharing the same letter were not significantly different from each other.

Supplementary Table 4.3 Fasting plasma inflammatory markers in LBW and NBW swine fed different experimental diets

	NBW-Chow	NBW-CHF	LBW-CHF	LBW-HFDairy	LBW-LFDairy	*P*
IL-1α, pg/ml	9.7±2.6^{a,b}	10.1±1.4^{b}	16.3±7.3^{a,b}	36.1±7.9^{a}	16.6±4.8^{a,b}	0.029
IL-1β, pg/ml	80.8±15.0^{a,b}	74.7±9.7^{b}	186.7±62.4^{a,b}	310.4±77.2^{a}	128.4±35.5^{a,b}	0.031
IL-1Ra, pg/ml	207.5±56.3	200.6±25.6	307.6±34.8	378.5±47.9	314.0±48.9	0.051
IL-2, pg/ml	56.4±12.9	61.9±14.2	122.7±53.9	224.7±57.4	107.4±36.6	0.087
IL-4, pg/ml	140.2±48.9^{b}	175.3±23.8^{b}	312.3±169.1^{a,b}	914.9±227.3^{a}	456.4±137.0^{a,b}	0.013
IL-6, pg/ml	34.9±7.2	31.0±4.9	60.7±26.5	103.8±27.7	46.7±13.7	0.135
IL-8, pg/ml	52.8±2.8	55.0±3.1	61.2±6.9	58.4±3.6	55.1±1.1	0.682
IL-10, pg/ml	160.9±35.4	149.7±39.1	356.8±155.7	604.5±173.7	392.9±124.2	0.157
IL-12, pg/ml	575.7±156.8	669.3±72.8	757.3±134.9	666.8±87.9	519.3±61.7	0.548
IL-18, pg/ml	628.0±73.0	582.3±94.2	1039±295.8	1472±310.7	866.7±220.2	0.110
TNF-α, pg/ml	59.7±12.5	76.6±6.6	72.7±8.9	90.6±16.2	80.6±10.7	0.518
IFN-γ, pg/ml	4290±1022	3893±429	3469±375.9	3421±368.7	2920±93.5	0.423
IL4/IFN-γ ratio	0.03±0.01^{b}	0.05±0.01^{b}	0.08±0.04^{b}	0.30±0.06^{a}	0.14±0.05^{a,b}	0.001

CHF, control high-fat diet; HFDairy, high-fat dairy diet; IFN-γ, interferon-gamma; IL, interleukin; LBW, low birthweight; LFDairy, low-fat dairy diet; NBW, normal birthweight; TNF-α, tumor necrosis factor-alpha. All values are expressed as means ± SEM. A p value was considered as statistically significant when <0.05. Means sharing the same letter were not significantly different from each other.

5 CHAPTER 5 Low-fat dairy consumption improves intestinal immune function more than high-fat dairy in a diet induced swine model of insulin resistance

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5.1 Introduction

The gastrointestinal immune system is the largest secondary immune organ in the body and is often referred to as the GALT. This extensive and organized immune system consists of physical barriers, a mucosal coating as well as innate and adaptive immune cells that reside in the lamina propria, Peyer's patches and MLN (Calder, 2013). The crosstalk between intestinal immune cells and IEC has been appreciated and regulates several physiological responses including secretion of antimicrobial peptides, tolerance to food antigens and tight junction protein formation (Soderholm & Pedicord, 2019).

However, obesity often leads to increased intestinal permeability and alterations in intestinal immune cells (Winer et al., 2016). One of the major consequences is increased bacterial endotoxin (LPS) translocation, which triggers the activation of TLR-4 and is thought to contribute to systemic inflammation and insulin resistance. Intestinal immune alterations in the context of obesity have been documented and include a reduction in a collective of physiologically important immune cells such as Treg cells and IgA producing plasma cells along with increased accumulation of pro-inflammatory T cells in the lamina propria (Luck et al., 2015; Winer et al., 2016). The extent to which these cellular changes lead to alterations in the GALT associated immune function remains to be investigated. In rodents, few studies have demonstrated altered MLN immune cell function in the context of obesity (Blewett et al., 2009; Ruth et al., 2009). On the other hand, obesity induced insulin resistance also promotes the overproduction of TG-rich lipoproteins and induces

postprandial hypertriglyceridemia (Bozzetto et al., 2020). Remnants of TG-rich lipoproteins are known to be a risk factor for atherosclerosis (Mangat et al., 2012). Intriguingly, postprandial activation of immune cells and phagocytosis of lipoproteins by phagocytes along with a transient elevation in systemic inflammation after a high fat meal have been documented (Alipour et al., 2008; van Oostrom, Sijmonsma, Rabelink, et al., 2003).

In recent years, understanding the functional roles of dairy foods has gained substantial interest. Certain nutrients such as S/MCFAs found in dairy are metabolized differently, in terms of intestinal absorption and transportation than long-chain saturated fatty acids (Kris-Etherton & Fleming, 2015), hence they may be beneficial to postprandial lipemia. Butyrate and propionate have been shown to promote the development of intestinal barrier integrity and induce colonic Treg differentiation (Colonic et al., 2013; Peng et al., 2009). Supplementation of MFGM and polar lipids have been shown to attenuate bacterial translocation and serum LPS activity in rodents (Y. Li et al., 2019; Norris et al., 2016). Polar lipids contribute approximately 1 % of total lipids in milk fat and include PC and SM, the two lipid-soluble forms of choline in the diet. Preliminary findings from our own group have also demonstrated that dietary choline, particularly the lipid-soluble forms of choline (PC and SM), improved *ex vivo* cytokine production by MLN cells, suggesting a beneficial effect to intestinal immune function in Sprague Dawley rat offspring (Barrera et al., 2021). Biomarkers of dairy fat, including C15:0 and C17:0 have also been found to be inversely associated with variables of insulin resistance (Imamura et al., 2018). Collectively, these findings

suggest the potential beneficial effects of dairy foods and dairy fat on GALT development and function. However, little is known on the role of dairy and dairy fat *per se* when consumed as a whole within a complex food matrix.

Pigs share tremendous similarity with humans in terms of physiology, anatomy, metabolism and immunity (She, Mangat, et al., 2022). We have previously established a diet-induced swine model of insulin resistance (Fontaine et al., 2019) and were able to compare the effect of high- and low-fat dairy products on peripheral immune function (She, Wang, et al., 2022). In the present study, we aim to determine the effect of consuming 3 servings per day of high- and low-fat dairy products on intestinal immune function and postprandial lipidemia in this established swine model. We hypothesized that consumption of 3 servings per day of dairy products, irrespective of fat content, would have a neutral effect on postprandial lipidemia. We also proposed that feeding high-fat dairy would provide greater benefits than low-fat dairy on intestinal immune function due to the beneficial lipids.

5.2 Materials and Methods

5.2.1 Animals and ethics

Refer to Chapter 3.2.1

5.2.2 Study design

Refer to Chapter 3.2.2

5.2.3 Diet

Refer to Chapter 3.2.3

5.2.4 Modified oral glucose and fat tolerance test (MOGTT)

Refer to Chapter 3.2.4

5.2.5 Blood processing and MLN immune cell isolation

Refer to Chapter 3.2.6 & 3.2.7

5.2.6 Postprandial plasma biochemical analysis

Refer to Chapter 3.3.2

5.2.7 MLN cells and postprandial peripheral blood mononuclear cells (PBMCs) phenotype analysis

Refer to Chapter 3.3.6, 3.3.7 & 3.3.8

5.2.8 Ex vivo cytokine production from mitogen stimulated MLN cells

Refer to Chapter 3.2.8 & 3.3.10

5.2.9 Statistical analysis

Refer to Chapter 3.4

5.3 Results

5.3.1 Pig growth parameters

The pig growth and daily food intake data have been previously reported elsewhere and are not duplicated here (She, Wang, et al., 2022). Importantly, all LBW piglets' growth caught up to the NBW piglets at 5 weeks of age. There were no differences in average daily growth nor final bodyweight among groups.

5.3.2 Postprandial lipid, glucose, insulin and inflammatory marker profiles

Postprandial plasma lipids, glucose and insulin are shown in **Figure 5.1**. Glucose at 0 min in NBW-Chow was lower than in LBW-CHF, LBW-HFDairy and LBW-LFDairy groups (all, $P < 0.05$). Postprandial insulin concentrations were not different at any timepoint across all groups. TG concentrations in LBW-LFDairy were lower than in NBW-CHF at 15 minutes ($P < 0.05$). There were no differences in incremental TG at any timepoint across all groups. TC and LDL-C in NBW-Chow were lower than in all other high-fat diet fed groups at all timepoints (all, $P < 0.05$). AUCs of TC and LDL-C were lower in NBW-Chow than in all other groups (both $P < 0.01$). AUCs of TG, incremental TG, glucose, and insulin were not different between groups (**Table 5.1**). There

were no differences at any timepoint in IL-1α, IL-1β, IL-1Ra, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-18, IFN-γ and TNF-α (**Supplementary Table 5.1**), indicating no differences on postprandial inflammation.

5.3.3 Postprandial PBMC phenotypes

Postprandial PBMC phenotypes were assessed in LBW groups only. No differences were observed among groups (**Supplementary Table 5.2**) for T cell markers including total T cells (CD3+), CD8+ T cells (CD3+CD8+), Th cells (CD3+CD4+) and the expression of IL-2 receptor (CD25+). Interestingly, postprandial proportions of PBMCs expressing TLR-4 (CD284) in LBW-CHF was lower than in both dairy feeding groups, particularly the LBW-LFDairy (**Figure 5.2**). AUC of total CD284+ was lower in LBW-CHF than in both LBW-LFDairy and LBW-HFDairy groups ($P < 0.05$).

5.3.4 MLN cell phenotypes

MLN cell phenotypes are shown in **Table 5.2**. No differences were observed in the overall proportion of total T cells (CD3+), Th cells (CD3+CD4+), cytotoxic T cells (CD3+CD8+) and naïve T cells (CD3+CD45RA+) among groups. However, the proportion of T cells expressing CD25 (IL-2 receptor) was lower in LBW-LFDairy than in LBW-HFDairy ($P < 0.05$). The proportion of Th cells expressing IL-2 receptor (CD3+CD4+CD25+) was also lower in LBW-LFDairy relative to LBW-CHF ($P < 0.05$). MFI of total CD25+ and CD25+ in CD4+ and CD8+

cells were not different among groups. Furthermore, the proportion of T cell co-stimulator (CD80+) was lower in LBW-LFDairy compared to the NBW-CHF ($P < 0.05$). There were no differences in APC phenotypes including total APCs (SLAII+), B cells (CD21+), monocytes (CD14+), macrophages (CD14+CD11c+) and monocytes expressing TLR-4 (CD14+CD284+). However, the proportion of APCs expressing both MHC class II and TLR-4 (SLAII+CD284+) was lower in all groups fed with high-fat diet than in NBW-Chow (all, $P < 0.05$, **Figure 5.3**). MFI of total TLR-4 (CD284+) was higher in LBW-CHF compared to NBW-Chow and LBW-LFDairy ($P < 0.05$). A similar trend was observed for MFI of TLR-4 in MHC class II expressing cells (SLAII+CD284+, *P*-model = 0.07)

5.3.5 Ex vivo mitogen stimulation on MLN cells

Ex vivo cytokine production by stimulated MLN immune cells is presented in **Table 5.3**. Following PWM stimulation, IL-2 and TNF-α production were significantly lower in LBW-CHF than in NBW-Chow (all, $P < 0.05$). While feeding high-fat dairy products had minimal effect, feeding low-fat dairy products significantly improved IL-2 and TNF-α production compared to LBW-CHF (all, $P < 0.05$). Following PMA-I stimulation, IL-6 production was lower in LBW-CHF than in NBW-CHF ($P < 0.05$). Feeding both low- and high-fat dairy products appeared to normalize IL-6 production. There were no differences in *ex vivo* cytokine production across all groups following LPS stimulation.

5.4 Discussion

To our knowledge, this is the first study using a swine model of obesity and insulin resistance to understand the effects of consuming 3 servings/day of low- *vs* high-fat dairy products within the same food matrix on gut associated immune function. In the present study, we confirmed that LBW piglets fed with a high-fat diet exhibited impaired MLN immune function, particularly T cell function. Consistent with our previous findings on peripheral immune function (She, Wang, et al., 2022), feeding low-fat dairy products was associated with greater improvements in MLN immune function when comparing to high-fat dairy intake. We have also demonstrated a neutral effect of dairy fat *per se* on postprandial lipidemia, inflammation and glucose tolerance. A summary of main findings is illustrated in **Figure 5.4**.

Interestingly, our results did not support part of our original hypothesis that high-fat dairy would potentially be more effective than low-fat dairy intake to improve intestinal immune function. One of the likely explanations could be due to the overall higher proportion of choline and MFGM in low-fat dairy products *vs* high-fat dairy. Previous studies have demonstrated that low-fat dairy products are often a rich source of choline (both water-soluble and lipid-soluble) and more so than high-fat dairy (Richard et al., 2016). Lipid soluble forms of choline, including PC and SM are predominantly present in MFGM, and have been found to support immune function in rats (Azarcoya-Barrera et al., 2020, 2021). Consistent with this, we have previously reported that low-fat dairy products are beneficial to peripheral immune function in LBW piglets (She, Wang, et al.,

2022). Le Huerou-Luron et al. also assessed the role of MFGM in supporting the GALT development and maturation and demonstrated that feeding infant formula supplemented with MFGM to pig neonates normalized IFN-γ production from *ex vivo* stimulated MLN cells to a similar level of sow-fed neonates (le Huërou-Luron et al., 2018). On the other hand, dairy fat is also a rich source of palmitic acid, which has been found to reduce intestinal barrier integrity and induce intestinal inflammation (Ghezzal et al., 2020). Hence, it is also plausible that the improved immune function seen in the LBW-LFDairy group is attributable to, at least in part, the lower dietary intake of palmitic acid. Collectively, our findings support the notion that intestinal immune function can be improved by increasing low-fat dairy intake.

5.4.1 Dairy and its impact on postprandial glucose tolerance, lipemia and inflammation

Consistent with our previous findings (She, Wang, et al., 2022), fasting glucose was higher in LBW-CHF than NBW-Chow, suggesting an early sign of insulin resistance. However, contrary to another previous report using a similar LBW swine model (Fontaine et al., 2019), LBW-CHF piglets in our study did not exhibit impaired postprandial glucose and insulin responses. We attribute this discrepancy (at least in part), to the higher birthweight of piglets (particularly in LBW-CHF group), compared to the previous study, which may have reduced the susceptibility to developing a strong insulin resistant phenotype. Intriguingly, neither the low- nor the high-fat dairy consumption had an effect on postprandial glucose levels. This is consistent with the most recent randomized control trial conducted by Schmidt et al. where 3.3 servings/day of dairy products had

no effect on glucose tolerance in individuals with metabolic syndrome (Schmidt et al., 2021b). Accumulating evidence have also suggested a neutral effect of dairy consumption on insulin sensitivity following an oral glucose tolerance test (Engel, Tholstrup, et al., 2018; O'Connor et al., 2019).

As expected, postprandial TC and LDL-C concentrations were the lowest in NBW-Chow compared to all other high-fat fed groups. This is mostly due to the additional 1% w/w cholesterol added in the CHF diets. Neither high- nor low-fat dairy products modulated postprandial TG, TC and LDL-C concentrations in the context of a high-fat diet. Although there is limited evidence on the effect of dairy fat *per se* on postprandial lipidemia, some acute, as well as longer term intervention studies have reported an overall neutral effect (Bohl et al., 2017; Clemente et al., 2003; Thorning et al., 2015). Studies have also shown higher fecal fat excretion and attenuated gene expression of fatty acid transporter (CD36) associated with dairy or nutrients found in dairy (i.e., casein and MCFA) (Bohl et al., 2015; Thorning et al., 2015). The mechanisms by which dairy modulate fecal fat excretion and CD36 expression remain to be fully elucidated. Intriguingly, preliminary findings from our group also demonstrated higher CD36 expression in intestinal mucosal scraping of LBW-CHF piglets compared to NBW piglets (K. Wang et al., 2021), despite no differences found in TG levels. Nevertheless, our data in LBW swine are in agreement with those from human findings, suggesting a neutral effect of dairy and dairy fat on postprandial lipidemia.

Our data also suggest that feeding dairy products (irrespective of the fat content) had a neutral effect on postprandial inflammation. Despite there being limited evidence investigating the effects of long-term consumption of dairy products on postprandial inflammation, an acute study has reported that neither cheese, butter nor milk supplemented high-fat meals modulated postprandial inflammatory marker concentrations compared to a non-dairy high-fat meal (Schmid et al., 2015). Of note, although it is generally well accepted that high-fat meal consumption induces postprandial inflammation (through endotoxin translocation) (Erridge et al., 2007; Herieka & Erridge, 2014), not all studies have reported increases in inflammatory markers postprandially (Herieka & Erridge, 2014). Several factors including the total energy load of the meal, the fat composition and the food matrix can all affect the magnitude of the postprandial responses. On the other hand, studies have also suggested that postprandial hypertriglyceridemia is another main contributor to postprandial inflammation by activating circulating leukocytes (van Oostrom, Sijmonsma, Rabelink, et al., 2003; van Oostrom, Sijmonsma, Verseyden, et al., 2003). It has also been proposed that plasma inflammatory markers are not consistently raised, but more often detected from leukocytes (Herieka & Erridge, 2014). Similarly in our study, we observed postprandial changes of TLR-4 expression in response to a high-fat meal challenge.

5.4.2 Dairy and its impact on TLR-4 expression

It is well known that high-fat meal consumption induces LPS translocation and triggers subsequent

immune responses (Fritsche, 2015). Obesity and insulin resistance are also known to be associated with increased gut permeability (Mkumbuzi et al., 2020), further enhancing the translocation of LPS which is recognized by TLR-4. In humans, individuals with obesity were found to have higher cell surface expression of TLR-4 (reflected by MFI) compared to lean individuals (de Loera-Rodriguez et al., 2014). Although there were no differences in the proportion of MLN cells expressing TLR-4, we have demonstrated that the MFI of total TLR-4 was higher in LBW-CHF compared to NBW-Chow and LBW-LFDairy. A similar trend was also found for MFI of TLR-4 in MHC class II expressing cells (SLAII+CD284+), despite the proportion of SLAII+CD284+ cells being lower in all high-fat diet fed groups compared to NBW-Chow. Feeding dairy products, particularly low-fat dairy, appeared to normalize TLR-4 expression in the context of a high-fat diet.

Intriguingly, we have also found that the proportion of PBMCs expressing TLR-4 in response to a high-fat meal challenge (MOGTT) was significantly lower in LBW-CHF compared to dairy-fed groups. We attribute this discrepancy, in part, to a potential compensatory mechanism to prevent chronic activation and inflammation during excessive LPS exposure. In previous studies, down-regulation of TLR-4 expression was observed following exposure to LPS (Akashi et al., 2000) and it is often referred to as "endotoxin tolerance" (Vergadi et al., 2018). However, this notion requires further understanding and validation. Although the LBW-CHF have lower proportions of cells expressing the TLR-4 in the postprandial state relative to the dairy feeding groups, these data should be interpreted with caution as phenotypes of NBW groups and LPS concentrations in

circulation were not assessed. Hence, the etiology underpinning this phenomenon cannot be fully elucidated.

5.4.3 Dairy and its impact on MLN immune cell function

Previous studies have demonstrated impaired peripheral immune function in the context of obesity and T2D in humans, rodents and swine (Lamas et al., 2002; Richard et al., 2017; She, Wang, et al., 2022). Here, we found that consuming a high-fat diet in the context of a LBW swine model can also lead to impaired intestinal immune function, particularly T cell function. IL-2 is a cytokine that induces T cell proliferation and activation (Ross & Cantrell, 2018). Consistent with our previous findings in PBMCs (She, Wang, et al., 2022), lower IL-2 production from MLN cells after PWM stimulation in LBW-CHF suggests impaired T cell proliferation and differentiation. Lower production of TNF-α, a major Th1 cytokine, in LBW-CHF after PWM stimulation also suggests an impaired Th1 response. Th1 cytokines are also known to increase IL-2 production and therefore, may partly explain the lower IL-2 production. On the other hand, TNF-α can also be produced by APCs such as macrophages (Zelová & Hošek, 2013). A bacterial challenge with LPS (the major component of gram-negative bacteria) is another stimulant to activate APCs *via* the activation of TLR-4 (Tucureanu et al., 2018). However, we did not observe any differences on TNF-α production after a LPS challenge among groups. This finding suggests, at least in part, that the TNF-α produced after PWM stimulation was likely mainly from Th1 cells.

We have also demonstrated that feeding low-fat dairy compared to high-fat dairy in the context of a high-fat diet had a greater ability to normalize the production of IL-2 and TNF-α after PWM stimulation and IL-6 after PMA-I stimulation relative to NBW-Chow. Feeding high-fat dairy also improved IL-2 production after PWM stimulation and IL-6 after PMA-I stimulation in that the production was no longer different from NBW-Chow, even though to a lesser extent than low-fat dairy products. Previous studies in humans have shown that individuals with obesity and type-2 diabetes have higher proportions of PBMCs expressing activation markers such as CD80 (Richard et al., 2017). Preliminary findings from our group have also demonstrated higher proportions of PBMCs expressing CD25 (IL-2 receptor) in individuals with obesity (irrespective of glycemic condition) compared to lean individuals (Tibaes et al., 2021). In our current study, we observed a reduction in the proportions of total MLN cells expressing CD80, CD25, and the proportions of Th cells expressing CD25 in LBW-LFDairy. The lower proportion of activation markers including the IL-2 receptor along with a better Th1 response and T cell proliferation in the LBW-LFDairy group suggests that feeding low-fat dairy as part of a high fat diet improves intestinal immune cell responsiveness to mitogenic stimuli.

5.4.4 Strength and limitations

The present study has provided novel insights into the role of commercial dairy foods and particularly dairy fat on postprandial obesity-related cardiometabolic risk factors. Indeed, we acknowledge that there is an absence of frank (post-prandial) insulin resistance which may have

limited our potential to detect statistical differences for some parameters (i.e., glucose tolerance, inflammatory markers). However, we have been able to demonstrate the nutritional modulation of intestinal immune function by dairy. Another limitation is that our diets were not all perfectly matched for the macronutrient distribution. By pair-feeding the pigs to ensure similar energy intake across groups, the proportion of fats, carbohydrates and protein were slightly different between diets. We would like to stress however, that this is more representative of a real-life scenario in humans where incorporating dairy products in a diet would lead to changes in macro- and micronutrient intake. Measurements of gut microbiota, gut permeability, circulating concentrations of LPS as well as immune function of Payer's Patch tissue are recommended for future research, in order to fully underpin the effects of dairy consumption on modulation of GALT associated immune function.

5.5 Conclusion

In conclusion, we provide novel insights into the role of dairy foods in modulating obesity-related cardiometabolic perturbations and gut-associated immune dysfunction. Consumption of 3 servings per day of dairy products, irrespective of fat content, had a neutral effect on postprandial lipidemia, inflammation and glucose tolerance. Consuming low-fat dairy products improved intestinal T cell function to a greater extent than high-fat dairy in this swine model of obesity and insulin resistance.

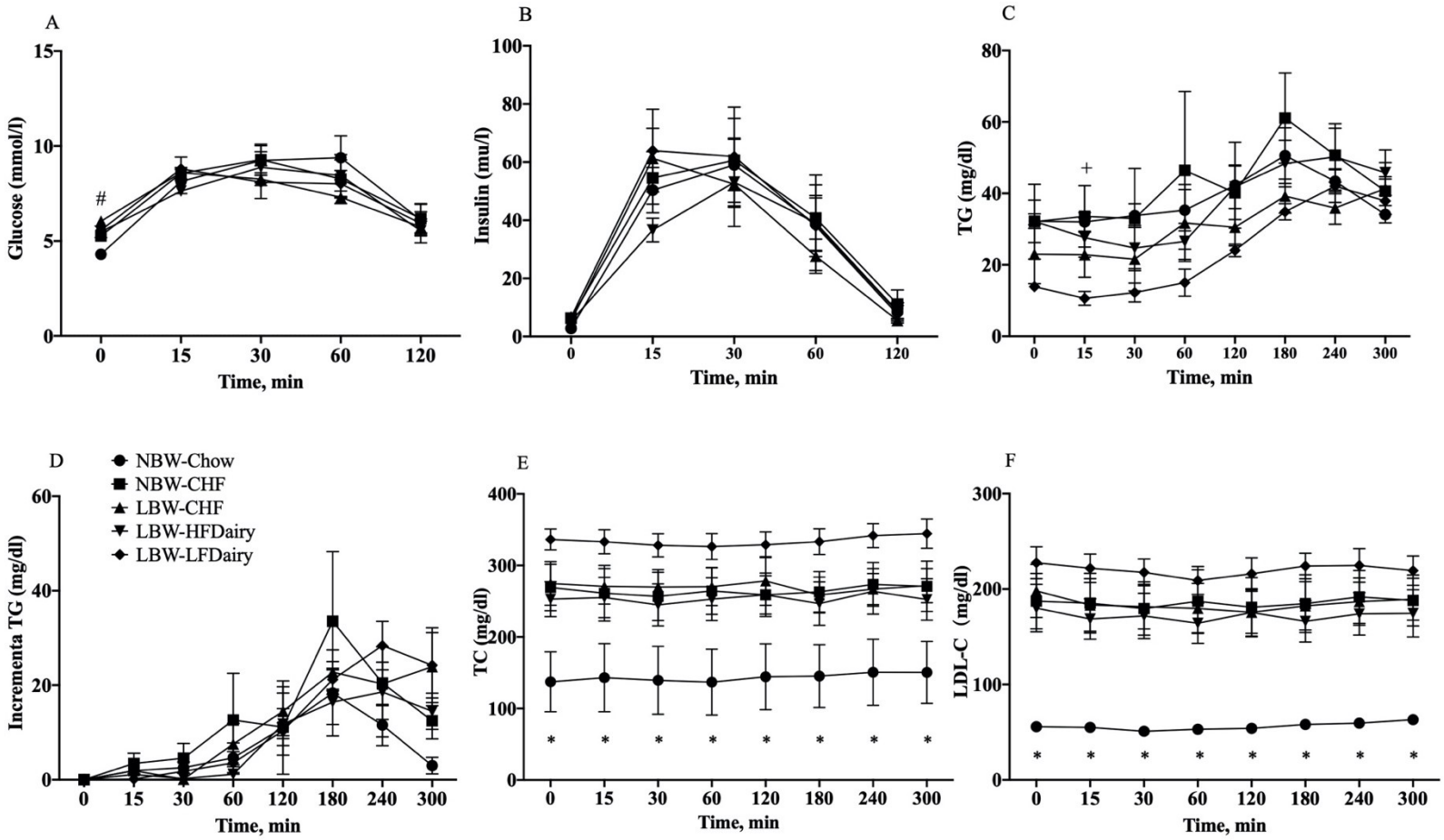

Figure 5.1 Postprandial changes of plasma biochemical parameters in LBW and NBW swine fed different experimental diets

CHF, control high-fat diet; HFDairy, high-fat dairy diet; LBW, low birthweight; LDL-C, low-density lipoprotein cholesterol; LFDairy, low-fat dairy diet; NBW, normal birthweight; TC, total cholesterol; TG, triglyceride. All values are expressed as means ± SEM. A p value was considered as statistically different when < 0.05. (A) postprandial glucose, # NBW-Chow is lower than LBW-CHF, LBW-HFDairy and LBW-LFDairy; (B), postprandial insulin; (C) postprandial TG, + NBW-CHF is higher than LBW-LFDairy; (D) incremental TG; (E) postprandial TC, *NBW-Chow is lower than all other groups; (F) postprandial LDL-C, *NBW-Chow is lower than all other groups.

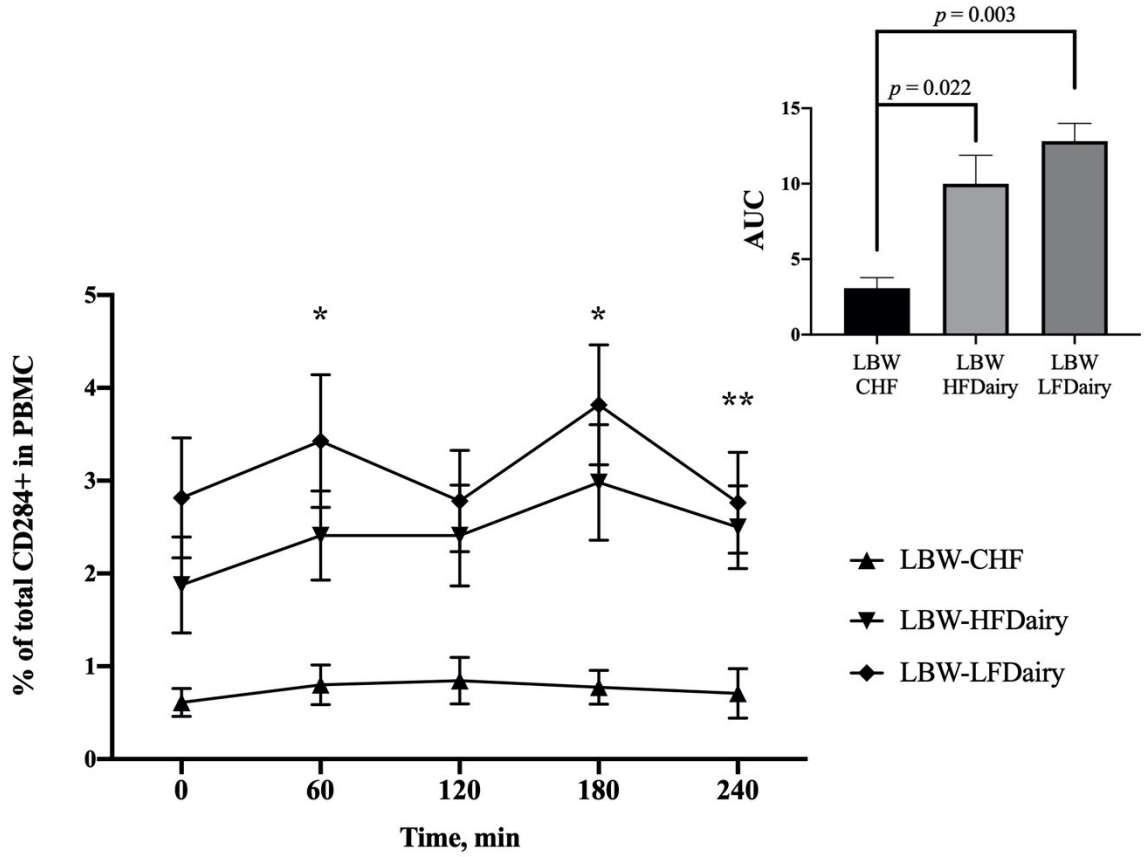

Figure 5.2 Proportion of PBMCs expressing TLR-4 (CD284+) in response to MOGTT of LBW swine fed different experimental diets

AUC, area under the curve; CHF, control high-fat diet; HFDairy, high-fat dairy diet; LBW, low birthweight; LFDairy, low-fat dairy diet; PBMC, peripheral blood mononuclear cells. All values are expressed as means ± SEM. A p value was considered as statistically different when < 0.05. * LBW-LFDairy is higher than LBW-CHF, ** Both LBW-LFDairy and LBW-HFDairy are higher than LBW-CHF.

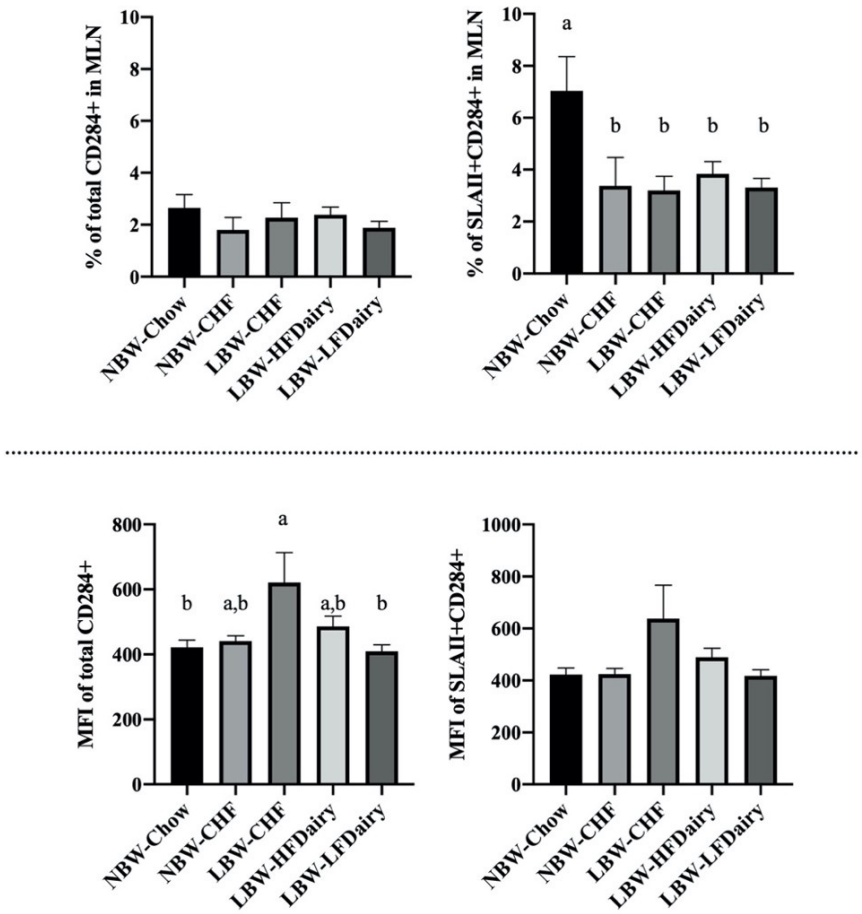

Figure 5.3 Proportion of MLN cells expressing TLR-4 (CD284+) and MHC class II (SLAII+) and MFI of NBW and LBW swine fed different experimental diets

CHF, control high-fat diet; HFDairy, high-fat dairy diet; LBW, low birthweight; LFDairy, low-fat dairy diet; MHC-II, major histocompatibility complex class II; MFI, median fluorescence intensity; MLN, mesenteric lymph node; NBW, normal birthweight; TLR-4, toll-like receptor 4. All values are expressed as means ± SEM. A p value was considered as statistically different when < 0.05. Means sharing the same letter were not significantly different from each other. *P*-model of MFI of SLAII+CD284+ = 0.07.

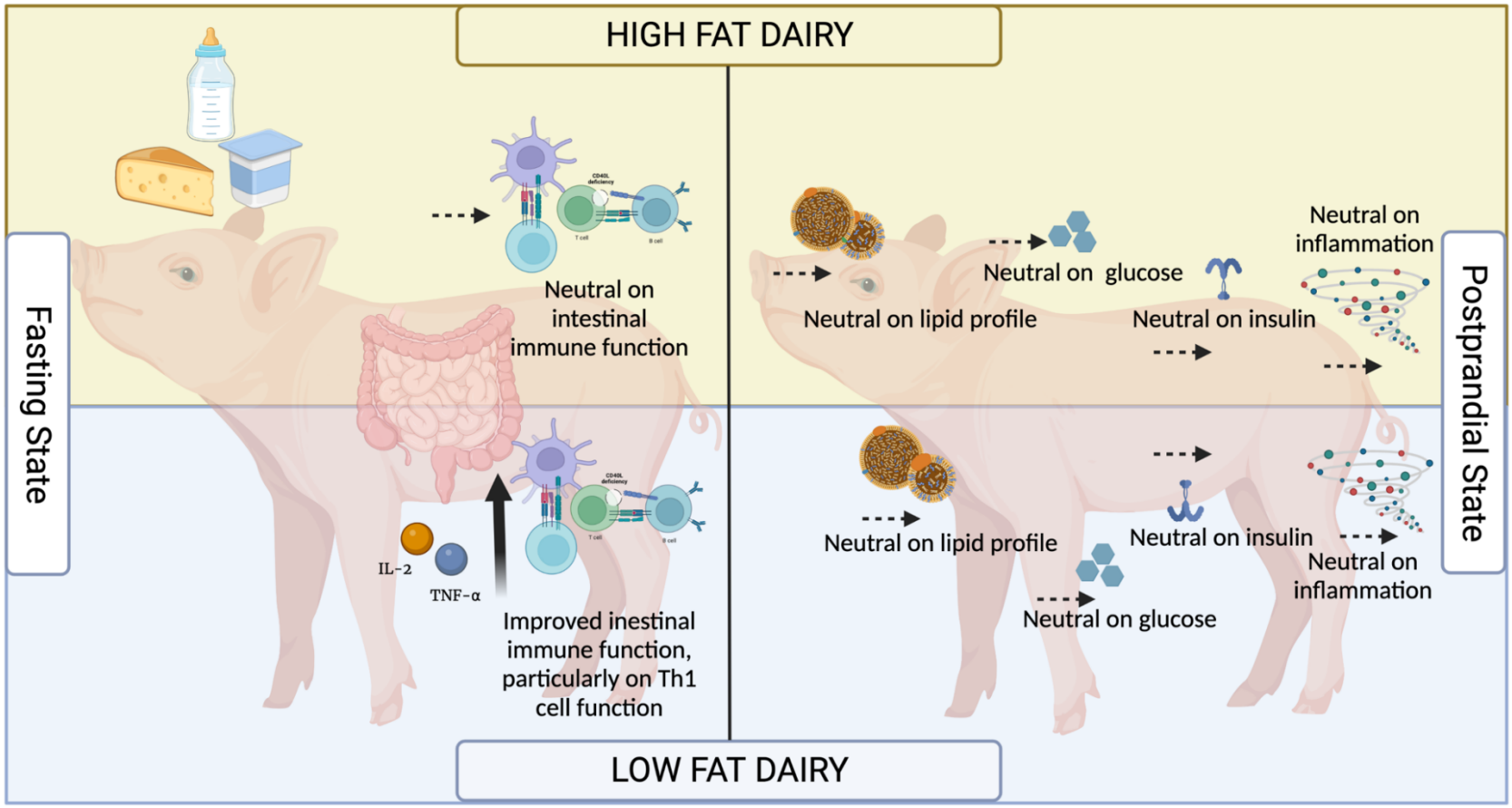

Figure 5.4 Summary of impact of consuming 3 servings high- and low-fat dairy products on intestinal immune function in *fasting state* and obesity-related cardiometabolic perturbations in *postprandial state* in high-fat diet induced LBW swine model of insulin resistance

Figure created with BioRender. IL, interleukin; LBW, low birthweight; Th, T helper cell; TNF-a, tumor necrosis factor-alpha. Feeding low-fat dairy products (LBW-LFDairy) improved IL-2, TNF-α production following *ex vivo* stimulation on mesenteric lymph node cells in *fasting state*, suggesting improved intestinal immune function particularly on Th1 cell. Feeding dairy products, regardless of fat content, had neutral effects on obesity-related cardiometabolic perturbations in *postprandial state*.

Table 5.1 AUC of postprandial plasma triglycerides, glucose and insulin in LBW and NBW swine fed different experimental diets

AUC	NBW-Chow	NBW-CHF	LBW-CHF	LBW-HFDairy	LBW-LFDairy	*P*
TC	29809±1109[a]	78898±9030[b]	80741±7737[b]	76360±8964[b]	88265±12707[b]	0.005
LDL-C	16975±1106[a]	55499±8649[b]	54783±7270[b]	51321±6808[b]	57976±8715[b]	0.008
TG	12270±1518	13784±2244	8917±1447	12204±1449	7046±1415	0.048
iTG	2842±1011	5000±1223	4526±1190	3315±1296	3774±834.2	0.672
Glucose	967.4±89.5	934.4±58.1	860.9±17.1	901.8±72.6	798.9±114.5	0.662
Insulin	4097±649.6	4532±792.8	3547±398.8	3809±1104	3915±1007	0.960

AUC, area under the curve; CHF, control high-fat diet; HFDairy, high-fat dairy diet; iTG, incremental triglyceride, LBW, low birthweight; LFDairy, low-fat dairy diet; NBW, normal birthweight; TG, triglyceride. All values are expressed as means ± SEM. A p value was considered as statistically different when < 0.05. Means sharing the same letter were not significantly different from each other.

Table 5.2 MLN cell phenotypes of LBW and NBW swine fed different experimental diets

Phenotype	NBW-Chow	NBW-CHF	LBW-CHF	LBW-HFDairy	LBW-LFDairy	*P*
% of gated cells						
Total CD3+ (T cells)	65.4±3.4	58.6±4.4	61.3±3.1	55.9±1.6	64.1±2.6	0.178
CD3+CD4+ (Th cell)	55.6±2.6	51.6±2.1	52.9±3.3	54.3±2.9	56.1±3.1	0.831
CD3+CD8+ (cytotoxic T cell)	44.1±2.7	47.9±2.1	47.1±3.3	43.9±3.5	42.6±3.3	0.738
CD3+CD45RA+ (naïve T cell)	20.9±1.4	23.8±2.6	24.1±1.4	21.2±1.6	19.4±2.1	0.332
Total CD25+ (IL-2 receptor)	41.3±2.9^{a,b}	41.4±3.9^{a,b}	47.1±3.6^{a,b}	50.2±2.7^{a}	35.4±3.9^{b}	0.037
CD3+CD4+CD25+	44.5±4.5^{a,b}	32.6±2.9^{a,b}	47.5±6.3^{a}	46.2±9.0^{a,b}	24.3±2.1^{b}	0.031
CD3+CD8+CD25+	39.0±2.0	32.5±3.7	37.9±2.4	36.5±3.5	27.2±2.8	0.054
Total CD80+ (co-stimulator of T cell)	4.9±1.8^{a,b}	6.7±1.3^{a}	4.2±0.3^{a,b}	4.0±0.2^{a,b}	3.3±0.4^{b}	0.036
Total CD21+ (B cell)	34.8±3.6	34.7±4.2	28.7±3.2	34.3±1.0	29.4±2.5	0.361
Total CD14+	7.9±1.8	6.1±1.0	6.4±1.5	8.2±0.7	5.7±0.7	0.386
CD14+CD11c+	17.1±1.9	13.4±2.6	21.5±3.2	21.5±2.7	17.5±1.6	0.189
CD14+CD284+	15.9±2.5	14.8±1.7	21.04±4.0	18.7±1.4	13.7±1.3	0.212
Total SLAII (MHC-II)	47.0±5.2	55.1±6.3	49.5±3.5	49.4±1.5	47.4±2.5	0.624

CHF, control high-fat diet; HFDairy, high-fat dairy diet; IL, interleukin; LBW, low birthweight; LFDairy, low-fat dairy diet; MHC-II, major histocompatibility complex class II; MLN, mesenteric lymph node; NBW, normal birthweight; Th, T helper cell. All values are expressed as means ± SEM. A p value was considered as statistically different when < 0.05. Means sharing the same letter were not significantly different from each other.

Table 5.3 *Ex vivo* cytokine production by mitogen stimulated MLN cells in LBW and NBW swine fed different experimental diets

	NBW-Chow	NBW-CHF	LBW-CHF	LBW-HFDairy	LBW-LFDairy	*P*
PWM (T cell and APC mitogen)						
IL-2, pg/ml	156.4±24.4^{a}	124.4±12.6^{a,b}	77.6±5.8^{b}	95.4±14.8^{a,b}	132.7±15.5^{a}	0.005
TNF-α, pg/ml	696.3±84.8^{a}	472.3±118.3^{a,b}	238.2±26.9^{b}	253.7±45.9^{b}	567.3±99.1^{a}	0.002
IFN-γ, pg/ml	200.0±40.5	224.7±32.1	138.7±19.3	136.2±11.8	176.9±29.8	0.128
IL-10, pg/ml	1932±262.1	2076±191.8	1823±215.7	2438±341.9	2504±277.3	0.289
PMA-I (T cell mitogen)						
IL-2, pg/ml	1241±198.5	1112±50.4	1209±315.6	969.2±120.5	787.8±117.4	0.373
TNF-α, pg/ml	1363±126.0	1843±176.9	1383±154.5	1536±117.3	1728±122.6	0.128
IL-6, pg/ml	142.7±7.0^{a,b}	141.3±29.9^{a}	48.8±14.9^{b}	54.6±17.3^{a,b}	133.7±21.7^{a,b}	0.013
LPS (Bacterial challenge)						
TNF-α, pg/ml	37.3±7.8	37.7±3.2	46.4±3.9	49.1±7.1	43.9±3.8	0.442
IL-10, pg/ml	253.4±47.9	210.5±11.1	212.2±6.8	182.3±27.5	214.1±15.9	0.437

APC, antigen presenting cell; CHF, control high-fat diet; HFDairy, high-fat dairy diet; IFN-γ, interferon-gamma; IL, interleukin; LBW, low birthweight; LFDairy, low-fat dairy diet; LPS, lipopolysaccharide; MLN, mesenteric lymph node; NBW, normal birthweight; PMA-I, phorbol myristate acetate-ionomycin; PWM, pokeweed mitogen; TNF-α, tumor necrosis factor-alpha. All values are expressed as means ± SEM. A p value was considered as statistically different when < 0.05. Means sharing the same letter were not significantly different from each other.

Supplementary Table 5.1 AUC of postprandial plasma inflammatory cytokines in LBW and NBW swine fed different experimental diets

AUC	NBW-Chow	NBW-CHF	LBW-CHF	LBW-HFDairy	LBW-LFDairy	*P*
IL-1α	2575±958.8	3871±1023	8658±4154	7019±2653	6834±2155	0.475
IL-1β	23880±8372	26841±7572	83047±36493	55785±25992	53937±19334	0.429
IL-1Ra	60126±5291	54202±7757	91846±23136	98845±19795	70675±15122	0.237
IL-2	16735±6222	23128±7544	63973±30062	49529±22981	41358±16645	0.504
IL-4	36190±17215	74825±24825	195373±107775	165204±80372	160252±56513	0.491
IL-6	8545±3396	10850±2666	31039±14817	22606±10811	20346±7203	0.491
IL-8	12737±457.4	12745±484.7	14335±1010	12414±777.6	12523±271.9	0.315
IL-10	43424±16264	54668±17551	185380±84331	118156±57896	101023±46242	0.415
IL-12	135447±32357	155475±21928	164081±29989	166640±234747	132028±11430	0.759
IL-18	190340±22375	176039±47451	412417±177514	329340±119094	275846±102244	0.574
TNF-α	16995±1034	21088±2512	25769±5053	22466±7321	12896±3067	0.460
IFN-γ	953877±253247	986877±241818	1048659±283696	769488±80860	664181±53537	0.550

AUC, area under the curve; CHF, control high-fat diet; HFDairy, high-fat dairy diet; IFN-γ, interferon-gamma; IL, interleukin; LBW, low birthweight; LFDairy, low-fat dairy diet; NBW, normal birthweight; TNF-α, tumor necrosis factor-alpha. All values are expressed as means ± SEM. A p value was considered as statistically different when < 0.05. Means sharing the same letter were not significantly different from each other.

Supplementary Table 5.2 AUC of postprandial PBMCs phenotype in LBW swine fed different experimental diets

AUC	LBW-CHF	LBW-HFDairy	LBW-LFDairy	*P*
Total CD3+ (T cell)	240.8±22.1	252.3±6.9	250.9±8.4	0.773
CD3+CD4+ (Th cell)	248.2±19.2	221.5±9.9	230.4±9.0	0.345
CD3+CD8+ (cytotoxic T cell)	147.3±21.3	175.9±9.3	169.2±9.2	0.305
Total CD25+ (IL-2 receptor)	90.5±21.4	114.7±5.4	112.6±11.0	0.378
CD3+CD4+CD25+	127.1±25.6	114.7±19.0	117.4±24.8	0.940
CD3+CD8+CD25+	104.3±29.8	144.5±25.3	131.0±33.7	0.639
Total CD284+ (TLR-4)	3.1±0.7[a]	10.0±1.9[b]	12.8±1.2[b]	0.004
Total SLAII (MHC-II)	152.1±21.6	175.5±13.3	175.6±14.6	0.536

AUC, area under the curve; CHF, control high-fat diet; HFDairy, high-fat dairy diet; IL, interleukin; LBW, low birthweight; LFDairy, low-fat dairy diet; MHC-II, major histocompatibility complex class II; Th, T helper cell; TLR-4, toll-like receptor 4. All values are expressed as means ± SEM. A p value was considered as statistically different when < 0.05. Means sharing the same letter were not significantly different from each other.

6 CHAPTER 6 General discussion and conclusions

6.1 Summary of findings and general discussion

For this thesis, we utilized a pre-established swine model of insulin resistance to study the effect of consuming low- and high-fat dairy products on obesity-related cardiometabolic risk factors and immune function in both the *fasting* and *postprandial* state. We confirmed that LBW piglets fed a CHF diet exhibited early signs of insulin resistance. We also demonstrated that LBW-CHF piglets have impaired immune function in both peripheral and local (intestinal) immune system. This finding is in line with studies using both rodent models and humans with obesity and T2D (Lamas et al., 2002; Richard et al., 2017). Of note, while pigs share tremendous similarity with humans, the fact that our findings are consistent with those results collected from other pre-clinical and clinical data suggests reproducible adaptations across multiple species (Bellinger et al., 2006; Pawar et al., 2015; She, Mangat, et al., 2022; Spurlock & Gabler, 2008). Collectively, our findings in pigs provide additional novel mechanistic evidence underpinning the etiology of immune dysfunction in the context of obesity and insulin resistance.

The outcomes from experiments contribute to an advanced understanding of dairy fat *per se* on cardiometabolic risk factors by using dairy products within a similar food matrix. Consumers are continued to be encouraged to avoid dairy products mainly due to the hypothesis that dairy fats are high in SFA and may cause deleterious effect on cardiometabolic risk factors. As we raised earlier, specifically in Canada, this has been fueled by dietary recommendations from Canada's Food

Guide. Of note, the percentage of Canadian population consuming milk has declined from 70.2% to 56.1% as of 2015 (Islam et al., 2021). More specifically, we also know that in Canada (per capita) regular milk (3.25%) availability dropped from 72.78 litters to 10.08 litters during the period of 1960-2015 based on report from Statistic Canada. Consistent with evidence from previous human RCTs (Drouin-Chartier, Côté, et al., 2016), our own data supports the notion that dairy consumption (irrespective of fat content), also has a neutral effect on the fasting lipid profile in swine. We also found similar observations for postprandial lipid profile. Collectively, we did not find any evidence to suggest that dairy fat consumption exerts deleterious effects on cardiometabolic risk factors. In contrast, the consumption of high-fat dairy improved (lowered) fasting glucose concentrations more than low-fat dairy counterparts in a context of high-fat diet. These observations are also consistent with other studies that have shown that dairy fat is associated with improvements in variables of insulin resistance (Fu et al., 2021; Imamura et al., 2018; Venn-Watson et al., 2020).

One of the focal issues of this thesis is that there is a brevity of data investigating how dairy foods potentially modulate immune cell function *per se*, as opposed to relying solely on circulating biomarkers of the inflammatory response (Pei et al., 2018; Stancliffe et al., 2011; Ulven et al., 2019). Our initial position was that high-fat dairy would likely be more beneficial to immune function. In contrast, we observed that consumption of 3 servings per day of low-fat dairy products improved both peripheral and intestinal immune function (particularly T cell function) to a greater extent than high-fat dairy (despite greater improvement in fasting glucose with the high-fat dairy).

We attribute this finding to the difference in the abundance of MFGM and polar lipids (PC and SM) between low- and high-fat dairy products. Indeed, previous studies from our group have demonstrated that low-fat dairy provides substantially more choline per serving than the high-fat products (Lewis et al., 2014; Richard et al., 2016). Intriguingly, we have also observed that the PC:PE ratio in PBMCs can be modulated by dairy intake and followed a similar pattern as reported for immune function. Although there is very limited data directly relating the PC:PE ratio to immune function, a higher ratio (i.e., > 2) has been linked to numerous metabolic diseases such as obesity and liver steatosis (van der Veen et al., 2017). Hence, our data suggest that the PC:PE ratio may be a potential indicator of immune function and explain mechanistically, at least in part, the improved immune function with low-fat dairy intake. Finally, we would conclude that dairy ought to be highlighted as part of a healthy diet, partly due to the maintaining of robust immune function. The summary of the main findings from the present thesis is illustrated in **Figure 6.1**.

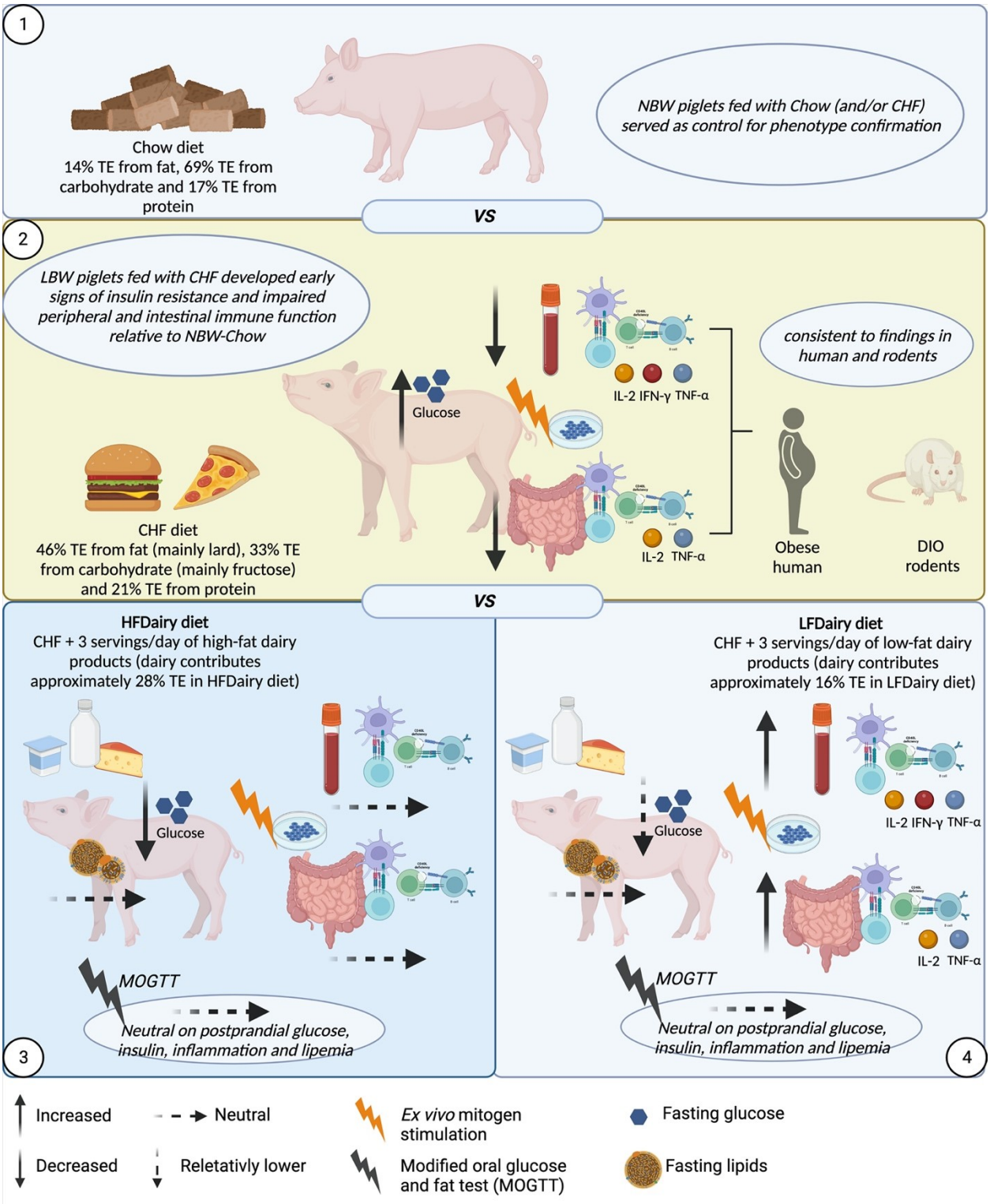

Figure 6.1 Schematic summary of key findings on modulation of cardiometabolic and immune perturbations by dairy products in a swine model of obesity and insulin resistance

APC, antigen presenting cell; CHF, control high-fat diet; DIO, diet induced obese; HFDairy, high-fat dairy diet; IFN-γ, interferon-gamma; IL, interleukin; LBW, low birthweight; LDL-C, low-density lipoprotein cholesterol;

LFDairy, low-fat dairy diet; MOGTT, modified oral glucose and fat tolerance test; NBW, normal birthweight; TE, total energy; TNF-α, tumor necrosis factor-alpha. **(1)** NBW piglets fed with a Chow or CHF diet served as "metabolic control" groups, allowing us to confirm that LBW piglets fed with a high-fat diet (LBW-CHF) developed the same phenotype as seen in obese and insulin resistant humans; **(2)** As expected, LBW-CHF developed elevated fasting glucose concentrations and early signs of insulin resistance. Following *ex vivo* immune cell stimulation, lower production of IL-2, TNF-α and IFN-γ from PBMCs and lower IL-2 and TNF-α from isolated MLN cells were observed, suggesting impaired T and APC cell function compared to NBW-Chow. This is also consistent with findings in individuals with obesity and DIO rodent models; **(3)** Feeding high-fat dairy products (LBW-HFDairy) improved (lowered) fasting glucose while having limited effects on peripheral and intestinal immune function compared to LBW-CHF; **(4)** Feeding low-fat dairy products (LBW-LFDairy) also moderately improved (lowered) fasting glucose while significantly improving the production of IL-2, TNF-α and IFN-γ from PBMCs and IL-2 and TNF-α from isolated MLN cells following *ex vivo* stimulation, suggesting improved T and APC function compared to LBW-CHF. Feeding dairy products, regardless of fat content, had neutral effects on fasting lipid profile. Feeding dairy products, regardless of fat content, also had neutral effects on postprandial lipid profile, glucose tolerance, insulin sensitivity and inflammation following MOGTT in a context of high-fat diet.

6.2 Limitations

In the present thesis, although LBW-CHF piglets did not develop impaired postprandial glucose tolerance at 11 weeks of age, we have demonstrated that LBW-CHF piglets did show early signs of insulin resistance by 12 weeks of age. Indeed, the age of the piglets and/or the duration of the intervention may have played a role in preventing the development of frank IR. On the other hand, we also noticed that the birthweight of LBW-CHF piglets in the present study were much higher overall and caught up to NBW groups much earlier (at 5 weeks of age) than for previous studies from our group (Fontaine et al., 2019). This is likely due to the fact that our piglets are from a generation after multiple cross of the strain, which yield stronger offspring and is often referred by the term as "hybrid vigor".

We also wish to acknowledge that we did not explore sex differences in the current series of

experiments as we elected to only use male piglets. Indeed, observational studies reported that men are generally at higher risk of developing impaired fasting glucose and have higher prevalence of T2D (Tramunt et al., 2020). Women are usually thought to be protected pre-menopause and this is partly due to the sex hormone estrogen, which have direct effect on body composition, glucose, and insulin homeostasis (Geer & Shen, 2009). But this may not always be the case, in a study of 13 European cohorts, postprandial glucose intolerance was more prevalent in women than in men (Qiao, 2003). McKnight et al showed that glucose intolerance was affected by sex and visceral adiposity in Yucatan miniature pigs, while female pigs had significant higher glucose AUC and glucose AUC/ insulin AUC ratio compared to male littermates (McKnight et al., 2012). Recent work from our group has also shown that sex-differences modulate immune function in high-fat fed rats, where female Wistar rats developed a milder obesity phenotype and maintained enhanced *ex vivo* cytokine production compared to male rats. (Tibaes et al., 2022). Collectively, these findings suggest a potential need to include both female and male in future research and explore the sex differences on obesity-related cardiometabolic and immune perturbations.

A final note to raise was that we took the approach to match overall energy intake among groups by pair-feeding the pigs and substituting portions of energy from the CHF diet with dairy products. We recognize that the high-fat dairy products contain more fat and slightly less protein compared to the low-fat dairy products (leading to an overall higher fat content in HFDairy diet). As discussed in Chapters 4 and 5, this may have (plausibility) contributed to an elevation of certain fasting inflammatory markers (but not in the *postprandial* state) in LBW-HFDairy piglets.

Although the diets were not matched for all macronutrients; however, this is more reflecting a real-life scenario in human conditions. As when incorporating dairy in people's habitual diet, the macronutrient composition also varies.

From a mechanistic perspective, future studies could consider using more specific anti-CD3/CD28 antibodies as an alternative approach to assess T cell function. Mitogens such as PWM, PHA, and PMA-i are plant lectins that bind to extracellular membrane glycoproteins (Kay, 1991; Lis & Sharon, 1998), which do not necessarily mimic the cell activation in a physiological relevant manner. PWM is also both T and APC mitogen. Amplification factor produced by simulated APCs can also somewhat increase the production of cytokines including IL-2 from T cells. Additionally, we cannot attribute the cytokine produced (i.e., TNF-α and IFN-γ) by a specific cell type population since we only used mitogen. Hence, intracellular staining technique coupled with flow cytometry can allow us to identify the cytokines produced by a specific immune cell subset following *ex vivo* mitogen stimulation (McKinnon, 2018).

6.3 Future areas of research

We have discussed the concept that swine are a useful intermediate between rodents and humans and findings derived from the present thesis are potentially applicable to the human condition. However, we need to appreciate that "RCT" is the gold standard in nutritional research and it would be advantageous to be able to verify our ‘food matrix’ approach using dairy products in a clinical

trial in humans. Despite a number of well-designed RCTs have studied the effect of different types (and/or fat content) of dairy food on immune-related factors (i.e., inflammation, insulin resistance), none of them have reported or investigated the actual immune function. It is also worth noting that we could test the hypothesis to determine if polar lipids in dairy are the main component responsible for the improved immune function. Since low-fat dairy appears to have greater benefits based on findings from the present thesis, we could also compare the effect of low-fat dairy alone to a fortified dairy counterpart (i.e., with added MFGM and polar lipids). We also know that gut microbiota plays a crucial role in shaping early immune development and modulating immune function (Wu & Wu, 2012; Yoo et al., 2020) and therefore, it would be more interesting to know if fermented low-fat dairy products exert greater impacts on immune function, compared to non-fermented counterparts.

From an analytical perspective, other integrative approaches such as genomic or metabolomic analyses could also be considered to explore potential mechanisms related to immunomodulatory effect of dairy consumption. For instance, blood metabolites including asparagine, alanine, glutamate, glutamine, oxoglutaramate, fumarate, and pyruvate have all been found to be associated with *ex vivo* cytokine production from stimulated immune cells in humans (Chu et al., 2021). For further advancement, analyses that includes measurements of body composition and/or gut microbiota should also be considered, since body composition (particularly visceral adipose) relates to insulin sensitivity and gut microbiota has been linked to immune system development and function. Moreover, invasive analyses that require biopsies such as immunohistochemistry and

gut-permeability (histological approach, i.e., tight junction expression) (Bischoff et al., 2014; Pawar et al., 2015) can offer an additional opportunity to identify underlying molecular mechanisms underpinning the etiology.

6.4 Policy making on Canada's Food Guide

In the development of the new Canada's Food Guide, all industry-funded studies were excluded from the review of the scientific evidence of dairy and human health, as commented by the Director General of the Office of Nutrition Policy and Promotion of Health Canada (Sharon Kirkey, 2019). This strategy may indeed, at least in part, reduced perceived bias during the process of policy making. However, it also resulted in unnecessary exclusions of large amount of well-designed, high-quality scientific evidence. In fact, the vast majority of scientific research on dairy foods and its impact on health conducted in Canada have been receiving funds, partially or solely, from Dairy Farmers of Canada.

In addition, the current Canada's Food Guide clearly encouraged a dietary pattern switch from animal-based to plant-based foods. Indeed, pulses and/or grain-based foods provide good amount of fiber, polyphenols, and plant-derived protein, which deliver profound health benefits to humans (Bouchard et al., 2022). However, from a consumer's perspective, they may tend to just choose commercial plant-based foods and/or dairy substitutes. In fact, many of the commercial plant-based foods can be classified as ultra-processed food in retail stores. Findings from a large cohort

(n = 21212) have reported that avoidance of dairy/animal-based foods was associated with a higher consumption of ultra-processed food which do not improve the nutritional quality of diet (Gehring et al., 2021). In contrast, milk and dairy products are well-known for their high nutritional values as they provide significant amount of most vitamins and minerals in the diet with the exception of iron and vitamin C.

The jury is still out there whether the demoting of dairy in the new Canada's Food Guide support a healthy dietary pattern. On the other hand, dairy consumption is still recommended and has remained as one of the crucial dietary categories in the 2022 Chinese Food Guide Pagoda and 2020-2025 Dietary Guidelines for Americans. In Canada, dairy products are now part of the protein category and emphasize on low-fat dairy consumption for protein intake. In the way forward, dairy should also be considered as a crucial dietary category for supporting growth and providing many other health benefits, including immune development and function.

6.5 Final conclusion

Overall, the research presented in this thesis has provided new mechanistic insight to support the role of dairy products, specifically milk, yogurt, and cheese, in counteracting some of the obesity-related cardiometabolic and immune perturbations. We have demonstrated that high-fat dairy can improve fasting concentration of glucose more than low-fat dairy, whereas low-fat dairy provided greater benefits to peripheral and intestinal immune function. Regardless of the fat content, dairy

consumption has a neutral effect on blood lipid profile, in both *fasting* and *postprandial* states. Hence, we believe that findings from the present thesis continue to support a positive image for dairy consumption for the general public, and could be utilized to translate in human conditions and direct the future work of clinical trials.

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APPENDIX A The interplay of obesity, dyslipidemia and immune dysfunction: a brief overview on pathophysiology, animal models, and nutritional modulation

APPENDIX A is a review paper that has been published in Frontiers in Nutrition

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Introduction

Obesity is one of the most common non-communicable diseases globally and is often accompanied by several complications such as insulin resistance, diabetes, atherosclerosis, hypertension and cancer (de Heredia et al., 2012). According to the Obesity Canada, over 30% of Canadian adults have obesity and may need medical intervention. Elevated fasting triglycerides (TG) and low-density lipoprotein cholesterol (LDL) levels are often seen in individuals with obesity (Klop et al., 2013), which underpins a concomitant increase in cardiovascular disease (CVD) risk. On the other hand, obesity is also widely recognized as being associated with a state of chronic low-grade systemic inflammation with elevated levels of circulating pro-inflammatory cytokines (de Heredia et al., 2012). More recent discoveries suggested that obesity and diabetes are associated with marked changes in the immune system systemically and in metabolically relevant tissues (Luck et al., 2015), to an extent that impairs normal immune function (Richard et al., 2017). In that regard, patients with obesity-related metabolic complications (i.e. type-2 diabetes, hypertension) were also found to have an impaired immune response to infection, influenza and COVID-19 (Green & Beck, 2017; Kang et al., 2020). These findings suggest that obesity plays a key role in modulating immune function, development of metabolic complications, and related cardiometabolic diseases. In addition, obesity-related dyslipidemia may also directly modulate immune function where TG-rich lipoproteins are able to induce leukocyte activation, particularly in the postprandial state (Alipour et al., 2008). The scope of the present review article is to provide a brief overview on the current understanding of immune changes in the context of obesity, applicable research models that are advancing this field of research, and potential nutritional strategies with a particular focus

on dietary intake of choline and foods high in choline to improve the immune dysfunction in the context of obesity and insulin resistance.

Obesity is associated with systemic inflammation

Studies have demonstrated that subjects with obesity have elevated circulating pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and C-reactive protein (CRP) (Festa et al., 2001; Halle et al., 2004; Park et al., 2005). There is the notion that adipose tissue is one main contributor through producing substantial amounts of TNF-α and IL-6 (Coppack, 2001). It is known that these pro-inflammatory cytokines impair normal insulin signaling pathways and therefore can lead to insulin resistance as well as dyslipidemia. However, the role of IL-6 per se may depend on the target organ. For example, IL-6 has been shown to promote glucose uptake by skeletal muscles towards improving whole body glucose homeostasis (Glund et al., 2007). Nevertheless, how inflammation is initiated in the context of obesity and further leads to a state of chronic low-grade systemic inflammation still remains to be fully elucidated.

Bornstein et al. were the first to report the involvement of macrophages in adipose tissue leading to inflammation (Bornstein et al., 2000). This discovery has led to an emergent hypothesis that defines the relationship between obesity-related inflammation and impaired immunity. Latter studies found that macrophage infiltration in adipose tissue is increased in obesity (Weisberg et al., 2003; Xu et al., 2003). Infiltrating macrophages appear as aggregated crown-like structures and tend to shift from M2 (anti-inflammatory) to M1 (pro-inflammatory) phenotypes (de Heredia et

al., 2012). However, recent findings have challenged the initial simplified notion of M1/M2 phenotypes in adipose tissue. The populations of adipose tissue macrophages appear to have more diverse phenotypes (i.e. metabolic activated or oxidized), with distinct surface markers activated by a wide range of stimuli such as free fatty acids or glucose (Russo & Lumeng, 2018) Mechanisms of macrophage recruitment into adipose tissue are still not fully understood, but appear to be partially due to adipose tissue expansion, leptin resistance and adipocyte death (Surmi & Hasty, 2008). M1 macrophages are pro-inflammatory and secrete substantial amounts of TNF-α and IL-6, while also secreting other pro-inflammatory mediators such as monocyte chemoattractant protein-1 (MCP-1) that can recruit additional circulating monocytes (Makki et al., 2013; Surmi & Hasty, 2008). The understanding on macrophage infiltration is still evolving while other pro-inflammatory immune cells including CD8+ cytotoxic T cells, CD4+ T helper (Th) 1 cells, neutrophils as well as B cells (particularly the B2 subtype that promote inflammation) have also been observed during the progression of obesity in animal models (Makki et al., 2013; Srikakulapu & Mcnamara, 2020). Similarly, in mice, an accumulation of CD4+ Th cells and CD8+ cytotoxic T cells and reduction of FoxP3+ T regulatory (Treg) cells have also been observed in the small and large intestine in a diet-induced obesity (DIO) model (Luck et al., 2015). Notably, intestinal barrier integrity has also been proposed to be compromised during obesity, leading to gut-derived lipopolysaccharide (LPS) translocation that may further contribute to systemic inflammation (Luck et al., 2015).

Obesity is a heterogenous chronic disease that is influenced by multiple factors such as the

environment and genetics. Obesity is also associated with a number of metabolic perturbations that can vary on an individual basis. The notion of metabolically healthy obesity (MHO) which can be defined as individuals with obesity that do not have other comorbidities such as hyperglycemia, dyslipidemia or hypertension, has drawn substantial attention in the research field (Magkos, 2019). MHO individuals can even exhibit improved insulin sensitivity, better adipose tissue function, a favorable adipokine secretion pattern, as well as less immune cell infiltrations into adipose tissue (Blüher, 2020). Yet, there is still a debate as to whether MHO individuals are truly healthy or if they will eventually develop metabolic perturbations associated with their obesity. In that regard, it has been shown that MHO subjects display a remarkably higher risk of developing type 2 diabetes and CVD compared to metabolically healthy lean subjects (Magkos, 2019). Findings from a meta-analysis of 59 studies found that nearly 50% of MHO subjects developed one or more metabolic abnormalities within a 10-year window (Lin et al., 2017). We have also recently demonstrated that MHO subjects appear to have elevated plasma CRP levels (3.6 ± 3.1 mg/L), which can be categorized as low-grade systemic inflammation (Richard et al., 2017). These findings suggest that MHO individuals might be on the right trajectory for developing metabolic complications including systemic inflammation. Nevertheless, the extent to which MHO individuals have “normal” immune function when compared to healthy lean individuals remains to be investigated.

Understanding of immune dysfunction in the context of obesity

In the clinical setting, a number of studies have confirmed the strong association between obesity,

body mass index and post-surgical infections (Dowsey & Choong, 2008; Lillenfeld et al., 1988; Zhang & Li, 2018). More recently, scientists have identified that obesity is an independent risk factor for COVID-19 mortality (Peters et al., 2021). A meta-analysis of 20 cohort studies involving 28355 patients showed that patients with obesity displayed nearly twice the risk for unfavorable outcomes related to COVID-19, along with a 50% increased risk of death (Hoong et al., 2021). These emerging clinical observations support the notion that obesity may lead to immune dysfunction.

The two major arms of the immune system, namely innate and adaptive immunity, work closely together and are regulated by key messengers such as cytokines. There is an appreciable shift from anti- to pro-inflammatory immune cells in adipose tissue in the context of obesity (SantaCruz-Calvo et al., 2022), which leads to a constant exposure to higher circulating levels of pro-inflammatory cytokines. How these physiological changes will in turn impair the magnitude of their response to immune challenges remains unclear. IL-2 is a crucial cytokine produced by T cells that regulates both cell proliferation and differentiation. In 2002, Lamas et al. assessed immune responses to foreign stimuli by ex vivo mitogen stimulation. Cafeteria DIO Wistar rats had a significantly lower production of IL-2 from splenocytes after mitogen stimulation compared to lean controls, suggesting impaired T cell function (Lamas et al., 2002). Consistent with this, Richard et al. compared immune function between subjects with obesity (stage 0; i.e. MHO) and subjects with obesity and type-2 diabetes (stage 2; i.e. with type 2 diabetes). Stage 2 patients produced significantly lower levels of IL-2, IL-6 and TNF-α from peripheral blood mononuclear

cells (PBMC) after T cell mitogen stimulation, suggesting impaired T cell function compared to stage 0 subjects. In the same study, stage 2 patients also produced lower levels of IL-6 after LPS stimulation, suggesting impaired antigen presenting cell function compared to stage 0 subjects (Richard et al., 2017).

More recently, Li et al. observed a striking loss of Treg cells in visceral adipose tissue of obese mice, driven by IFN-γ producing dendritic cells (Li et al., 2021). The loss of circulating Treg cells has also been previously observed in humans in the context of obesity (Wagner et al., 2013). Conversely, van der Weerd et al. found that morbidly obese subjects (BMI > 40) had higher absolute counts of Treg as well as naïve and memory T cells in PBMC compared to lean subjects (van der Weerd et al., 2012) Higher proportions of CD4+ Th cells in PBMC were also observed in obese subjects (BMI > 35) compared to lean controls (O'Rourke et al., 2005). In addition to obesity, factors such as hyperglycemia, dyslipidemia and insulin resistance have been shown to further modulate circulating T cell subtypes by increasing the ratio of Th1 to Th2 cells (Matia-Garcia et al., 2021). Recent findings from our group also demonstrated that hyperglycemia in obesity and type 2 diabetes leads to an increased proportion of activated Th cells (expressing CD278), cytotoxic T cells as well as inflammatory monocytes (CD14+CRTh2+) in PBMC compared to individuals with obesity that are metabolically healthy (Richard et al., 2017). Hence, obesity and/or hyperglycemia not only affect immune cells response to stimuli, but also the proportions of circulating T cell subsets.

Luck et al. recently found that obesity also significantly impairs intestinal B cell homeostasis and functions in a manner that reduces immunoglobulin (Ig) A+ B cells and IgA producing plasma cells in mesenteric lymph nodes, and secretory IgA antibody concentrations in colon in DIO mice (Luck et al., 2019). IgA is known to defend mucosal surfaces against pathogen breaching (de Sousa-Pereira & Woof, 2019). High-fat diet fed IgA deficient mice were also found to have increased intestinal permeability and levels of serum endotoxins compared to wild type controls (Luck et al., 2019). Therefore, we can speculate that the dysregulation of intestinal B cell homeostasis and IgA production in the context of obesity further contributes to local and systemic inflammation. In another study, splenic B cells from DIO mice poorly responded to CpG+anti IgM stimulation ex vivo compared to lean controls, suggesting that obesity also impairs peripheral B cell functions, particularly the ability to produce IgM and IgG antibodies. In the same study, B cells from PBMC of subjects with obesity produced less IL-6 upon ex vivo stimulation despite having elevated proportions of total B cells compared to lean subjects (Kosaraju et al., 2017). DIO mice were also shown to have impaired antibody production upon influenza virus infection (Milner et al., 2013). Moreover, vaccinated individuals with obesity still displayed a remarkably higher risk of developing influenza compared to vaccinated lean subjects during the past influenza seasons (Neidich et al., 2017). Thus, obesity seems to affect the proportion and functions of B cells not only at the local levels (i.e. adipose tissue and intestine) but also in circulation.

The interaction between obesity-induced dyslipidemia and immune dysfunction

Obesity induced chronic systemic inflammation impairs insulin signaling pathways via

mechanisms such as serine phosphorylation of the insulin receptor and insulin receptor substrate-1 via the activation of IkB kinase, c-Jun N-terminal kinase and protein kinase C (Lauterbach & Wunderlich, 2017). At the same time, hypertriglyceridemia is thought to be a result of overproduction of TG-rich lipoproteins from the liver, and accelerated free fatty acids efflux from adipose tissue. On the other hand, overproduction of TG -rich chylomicrons from the intestine has also been observed in the context of obesity and insulin resistance (Adeli & Lewis, 2008). Obesity and insulin resistance are also thought to induce postprandial hypertriglyceridemia, which is a result of overproduction of chylomicrons from the intestine, overproduction of very-low density lipoprotein (VLDL) from the liver as well as limited endothelium lipoprotein lipase activity (Klop et al., 2012). Qin et al. demonstrated that TNF-α has a direct role in the overproduction of postprandial apoB48-containing lipoproteins (chylomicron) while upregulating II1-α, II6, and TNF-α gene expression and downregulating the gene expression of proteins related to insulin signaling in hamster enterocytes (Qin et al., 2010). Consequently, there appears to be evidence that inflammation contributes to dyslipidemia both in the fasting and postprandial states, and may in turn enhance systemic inflammation and impair the corresponding immune response.

Consistent with this finding, an in vitro study demonstrated that TG-rich lipoproteins activate human aortic endothelium cells to express vascular cell adhesion molecules (de Vries et al., 2014). These adhesion molecules are in turn able to bind to circulating monocytes and facilitate their migration into the subendothelial space. Remnants of TG-rich lipoproteins are also small enough to penetrate the subendothelial space and offer additional sources of plaque-derived cholesterol

(Duran & Pradhan, 2021). Internalized lipoprotein-cholesterol can subsequently activate numerous inflammatory signaling pathways and initiate the production of pro-inflammatory cytokines and chemokines (de Vries et al., 2014). Also of note are studies that reported postprandial activation of neutrophils and monocytes while also increasing the total leukocyte count (reflected by expression of CD11b and CD66b) in human subjects after consumption of high fat meals (Alipour et al., 2008; A. J. H. H. M. V. Oostrom et al., 2004; A. J. H. H. M. van Oostrom, Sijmonsma, Rabelink, et al., 2003; A. J. H. H. M. van Oostrom, Sijmonsma, Verseyden, et al., 2003). Similar to postprandial TG dynamics, postprandial neutrophil counts are dramatically increased to a peak level in the first two hours and reach a plateau after four hours following a standard oral fat challenge (A. J. H. H. M. van Oostrom, Sijmonsma, Verseyden, et al., 2003). Stable isotope tracers, such as ^{13}C-labelled palmitic acid and deuterium have been applied to modern nutritional studies in understanding endogenous lipid metabolism (Jones et al., 1985; Leitch & Jones, 1993). Taking advantage of this groundbreaking innovation of isotopic tracers in metabolic research, a study in which an oral fat load was conducted in eleven subjects using stable isotope tracers (^{13}C-palmitate) demonstrated that postprandial leukocytes became enriched with meal-derived ^{13}C-palmitate (Alipour et al., 2008). This finding revealed a putative direct interaction between postprandial lipids and circulating leukocytes in that postprandial TG-rich lipoproteins may be directly phagocytosed or postprandial free fatty acid up-taken by leukocytes.

These important findings suggest that hypertriglyceridemia can not only induce inflammation at a cellular level, but also serves as an activator of circulating leukocytes. The triglyceride-mediated

immune cell activation may ultimately contribute to the progression of atherosclerosis and dysregulated immune function in the context of obesity. A comprehensive summary of the links between obesity, dyslipidemia and immune dysfunction is illustrated in **Figure 1**.

Applicable research models of obesity, dyslipidemia and immunology

Rodents

Rodent models including rats and mice have long been applied to the field of research since the first development of a rat strain in 1920. In contrast to humans, high-density lipoprotein particles (HDL) were found to be the primary carrier of lipids in rats and mice. However, rats have been a good model for recapitulating the human findings, at least in certain types of immunological studies. For instance, gut-associated lymphoid tissue (GALT) is one of the most important secondary lymphoid organs that is linked to numerous metabolic complications. Rats and humans share substantial similarities in the GALT anatomy and they both have lymphocyte-filled villi (Pérez-Cano et al., 2012). Given the development of modern genetic engineering, numerous rat and mouse models have been introduced to meet specific research needs. Some of the first phenotypic models used in this field include the fatty Zucker rat (Zucker & Zucker, 1961). The fa/fa homozygous Zucker rat strain spontaneously develops obesity, insulin resistance and hypertriglyceridemia (Russell & Proctor, 2006). Later, another strain of Zucker rat, the Zucker diabetic fatty rat was developed (Richard G. Peterson, 2001). Other phenotypic rats of this kind have been developed such as the JCR:LA-cp rats. Homozygous JCR:LA-cp rats have impaired chylomicron and VLDL metabolism in the context of insulin resistance (Russell & Proctor, 2006;

Vine et al., 2007). Ruth et al. have utilized the JCR:LA-cp rat model and showed that splenocytes of obese rats produced less IL-2 than lean controls after T cell mitogen stimulation, suggesting an impaired T cell function (Ruth et al., 2010). However, spontaneous phenotypic rat models may be less relevant to understand the development of obesity in human and its nutritional modulation since they do not require diet-induced approaches. Lamas et al. was the first to demonstrate that cafeteria DIO Wistar rats had impaired T cell function compared to lean controls characterized by a lower IL-2 production from splenocytes after T cell mitogen stimulation (Lamas et al., 2002). Sprague Dawley rats are another well-known wild-type model and often serve as the stock background for numerous genetic-engineered models (Rex et al., 2007). Recently, our lab demonstrated the benefits of supplementing rodent diets with different forms of choline, an essential nutrient, in Sprague Dawley rats, on the immune system (Azarcoya-Barrera et al., 2020, 2021; Dellschaft et al., 2015; Lewis et al., 2016).

Some spontaneous mutation models such as ob/ob and db/db mice have also been successful models to feature leptin deficiency and/or defective leptin binding and leading to obesity, dyslipidemia and other related commodities (Russell & Proctor, 2006). ob/ob model is certainly one of the most used models in unravelling molecular basis of obesity-induced perturbations. Hsu et al. also found that ob/ob mice have an impaired pulmonary bacteria clearance after Streptococcus pneumoniae challenge, and increased death (Hsu et al., 2007). Leptin plays a crucial role in modulating immune function, such as increasing T cell proliferation, and modulating expression of activation markers on both helper and cytotoxic T cells (Milner & Beck, 2012).

Patients with obesity often display hyperleptinemia and leptin resistance, whereas this condition is certainly absent in ob/ob mice. These models are also monogenetic which is a rare condition in humans. Therefore, the DIO mouse model has emerged as a useful alternative. Luck et al. showed that DIO C57BL/6 mice have a shift to pro-inflammatory immune cells in intestinal lamina (Luck et al., 2015). This polarization of intestinal immune cells is characterized by an increased proportion of Th1 cells and cytotoxic T cells and a reduction in Treg cells (Luck et al., 2015). Amar et al. have also found that DIO mice respond poorly to P.gingivalis infection compared to the wild type lean control (Amar et al., 2007). However, careful consideration must also be taken when employing DIO mice models, as phytoestrogens are often absent in high fat diets but present in chow diets and known to impact rodent physiology (Milner & Beck, 2012). Therefore, when assessing the effect of diet/nutrients on the immune system using rodent models, special attention should be considered in carefully balancing the composition of chow/low-fat and high-fat diets. Moreover, IL-8 is a crucial chemokine for neutrophil recruitment during inflammation, however IL8 gene is also absent in mice (Hein & Griebel, 2013). Adaptive immunity such as T and B cell development in mice and Fc receptor expression were also found to be quite different from humans (Mestas & Hughes, 2004).

It is worth noting a variety of polygenic and even transgenic rodent models, such as GLUT4 glucose transporters mice, are also dominating the research and unravelling the molecular basis of obesity-induced cardiometabolic complications (Suleiman et al., 2020). More recently, CRISPR-Cas9 mediated leptin and leptin receptor knockout mouse models were also introduced (Roh et al.,

2018). Despite a large body of evidence demonstrating the usefulness of employing rodent models in obesity-related research and using modern genetic engineering to introduce specific, attractive and sophisticated gene-knockout models, we must admit that these conditions are rare in humans. Additionally, differences in anatomy, physiology, lipid metabolism and immune systems between rodents and humans also needs to be taken into account when interpreting the findings from these studies.

Large animal model-swine

In contrast to rodent models, large animals such as pigs share tremendous similarities with humans in terms of anatomy, genetics and physiology. To highlight the similarities, pig-to-primate organ transplantation has been successfully achieved (Meurens et al., 2012). Intriguingly, studies have demonstrated that intrauterine growth restriction pigs are more susceptible to insulin resistance and related metabolic complications later in life, making this a potentially useful model to study obesity-related complications in humans (McKnight et al., 2012). The immune system composition of the pig also closely resembles that of humans, whereas mice only share about 10% similarity (Meurens et al., 2012). For instance, 50 to 70% peripheral leukocytes are neutrophils in pigs which is quite similar to humans, and IL-8 has a direct ortholog in pigs for neutrophil recruitment (Meurens et al., 2012). Circulating levels of IL-6 in pigs appear to be high and primarily originate from adipocytes, and dietary fatty acids were found to modulate endotoxin (LPS) levels and toll-like receptor 4 (TLR4) expression in swine adipose tissue (Spurlock & Gabler, 2008). These findings highlight that pigs share similarities in immune response with humans and

could be an alternative approach to study obesity-related immune dysfunction.

Currently, a number of swine models have been established to study obesity-related metabolic complications and pathologies, including the Yucutan miniature pig model and Ossabaw pigs (Myrie et al., 2011, 2017; Spurlock & Gabler, 2008). Yucutan miniature pigs have been shown to spontaneously develop impaired lipid and glucose metabolism (56). The Ossabaw pig model has also been described to develop criteria of metabolic syndrome including early insulin resistance, hypertriglyceridemia, hypertension, and visceral adiposity when fed a high fat diet (Spurlock & Gabler, 2008). Recently, our own group has also established a swine model of insulin resistance using Duroc x Large White-Landrace low birth weight (LBW) piglets fed with high fat diet. The LBW high fat diet fed piglets developed signs of early insulin resistance, impaired postprandial TG metabolism and high hepatic bridging fibrosis after six weeks of intervention (Fontaine et al., 2019; Singh et al., 2021).

Consistent with the findings that were found in rodent models and humans, recent work from our group has confirmed that high-fat diet induced insulin resistance in LBW swine exhibit impaired IL-2 production from PBMC after ex vivo mitogen stimulation, suggesting impaired immune function, particular to T cell function (She et al., 2021). Pawar et al. has also found increased expression of TNF-α in abdominal subcutaneous adipose tissue of obese pigs fed with high-fat/high-fructose diet, along with macrophage infiltration in both abdominal and pericardial adipose tissues, which paralleled the observations made in humans with obesity (Pawar et al.,

2015). The fact that these findings are consistent across different animal models as well as in humans further advances the concept that immune function is significantly impaired in the context of obesity and insulin resistance. It also highlights that swine may serve as a great intermediate between rodents and humans in studying obesity-related perturbations.

However, the swine model has major drawbacks such as being difficult to manage and maintain, and requiring specialized facilities. Routine and proper socializations with humans are also required to minimize pigs' stress and behavioral problems. Additionally, they can be very expensive to purchase. For instance, a 6 month-age miniature pig is approximately 1000 US dollars (Russell & Proctor, 2006). Similar to rodent models, a number of genetically modified diabetic pigs (such as CRISPR-Cas9 mediated) are also being introduced to meet specific research needs (Perleberg et al., 2018; Zou et al., 2019); however, these strains are highly dedicated and can be even more expensive to purchase. Hence, swine strains that are originally maintained for food production may be the more economical and practical choice for large animal research in nutritional studies.

Other animal models

In addition to the commonly used rodent models and swine models, a number of other species are also advancing the field of research. For instance, ferrets have been recognized as a useful intermediate model between rodent and non-human primate models in biomedical research, particularly for their use in researching infectious respiratory diseases (Johnson & Ghedin, 2020;

Stittelaar et al., 2016) Human pathogens are able to naturally infect ferrets and reproduce human diseases, highlighting the importance of this model in immunological research in order to recapitulate human findings. DIO (National Institute of Health, USA ongoing project) and CRISPR-Cas9 genetic modified ferret models are also gradually introduced (Albrecht et al., 2018). However, this model is currently very rare in nutritional studies. Limitations such as lack of commercial antibodies is one of the main drawbacks. Rabbit is a species that can develop all components of the metabolic syndrome similarly to humans and are easy to handle and cost-effective (Lozano et al., 2019). DIO rabbit models have also been widely used in understanding obesity-related cardiometabolic complications (Alarcon et al., 2018; Carroll et al., 1996). They are also extremely sensitive to cholesterol and therefore, more often used as a pre-clinical model for atherosclerosis research (Russell & Proctor, 2006). Rabbits have also been used in a series of immunological studies and became major sources for the production of mono- and polyclonal antibodies (Esteves et al., 2018). Finally, non-human primates such as monkeys and rhesus are no doubt the most ideal model since they are highly relevant to human anatomy and physiology, are omnivorous and able to develop metabolic complications with aging (Russell & Proctor, 2006; Suleiman et al., 2020). Several DIO non-human primate models such as a high-fat high-sugar diet fed baboon model, a high-fat diet fed marmoset model as well as a Japanese macaque model were also developed to mimic the phenotype of obesity-related complications in humans (Kleinert et al., 2018). However, major drawbacks of non-human primates are lack of approved facilities, long life span and risk of transmitting infectious viruses to humans.

Nutritional strategies to improve immune dysfunction in the context of obesity

One of the first lines of intervention to improve cardiometabolic health and immune function is certainly weight loss and physical activity. Physically active subjects were found to have lower circulating CRP, CD14+CD16+ monocytes and LPS-induced TNF-α production from whole blood compared to physically inactive individuals (Timmerman et al., 2008). Exercise was also found to modulate adipose tissue macrophage infiltration, in a manner that downregulated MCP-1 following a moderate-intensity excise protocol (Soltani et al., 2020). In addition to physical exercise, a number of pharmacological therapies have also been developed with promising potential to improve obesity and type-2 diabetes associated immune dysfunction, such as etanercept (TNF-α blockade) (Dominguez et al., 2005).

Besides genetic and environmental factors, obesity is often caused by excessive energy intake combined with reduced energy expenditure, reinforcing the fact that adequate and appropriate nutrition is required. Accumulating evidence has indicated that consumption of a high-fat meal has a direct role in promoting postprandial inflammation and endotoxemia in humans (Erridge et al., 2007). However, nutrients have diverse roles and often act differently at modulating immune function. Saturated fatty acids (SFA) were found to be pro-inflammatory and share similar structural components of gut bacteria endotoxins (LPS). Among all SFA, lauric acid is thought to exert the most pro-inflammatory properties, whereas myristic and stearic acids tend to exert the least (Fritsche, 2015). Polyunsaturated fatty acids (PUFA) of the omega-6 family (linoleic acid, LA; arachidonic acid, ARA) are generally considered pro-inflammatory whereas omega-3

(eicosapentaenoic acid, EPA; docosahexaenoic acid, DHA) are considered anti-inflammatory. This is due to the fact that their derivate metabolites (i.e. eicosanoids and resolvins) primarily exert pro/anti-inflammatory activities, respectively. However, this is an oversimplification since some of ARA derived eicosanoids, such as lipoxin 4, can also be anti-inflammatory and not all EPA derived metabolites are anti-inflammatory (Calder, 2010). ARA derived lipoxins and epi-lipoxins were also found to promote resolution of inflammation by regulating the life cycle of immune cells including neutrophils and macrophages (Chandrasekharan & Sharma-Wali, 2015). The recent work conducted at Texas Tech University by Davis and colleagues demonstrated that EPA ameliorated gene expression of inflammatory markers in DIO B6 mice, along with reducing macrophage infiltration into epididymal white adipose tissue, in a dose-dependent manner (Davis et al., 2021). Additionally, other bioactive fatty acids such as ruminant-derived trans fats have been shown to be beneficial to immune function including actions on IL-2 and TNF-α production after mitogen stimulation (Blewett et al., 2009; Ruth et al., 2010).

Micronutrients and immune function

Selenium, a trace element, has critical functional roles in a range of physiological responses. Selenium deficiency has also been found to be associated with a higher risk of cancer and CVD (Childs et al., 2019). Selenium supplementation was found to increase T cell proliferation and IFN-γ production in adults with marginal selenium status (Broome et al., 2004). The key roles of a number of other micronutrients including Vitamin A, B, C, D and E, and trace minerals in supporting immune function are also widely appreciated and reviewed elsewhere (Calder, 2020).

In recent years, a better understanding of the role of dietary choline at modulating immune function have also emerged. Choline serves as the precursor for functional and structural components of cell membranes and critical to a number of molecules including neurotransmitters and lipoproteins. Dietary forms of choline can be divided into water-soluble forms (i.e. free choline (FC), phosphocholine (PCho) and glycerophosphocholine (GPC)) and lipid-soluble forms (phosphatidylcholine (PC) and sphingomyelin (SM)) (Wiedeman et al., 2018). We have previously demonstrated that feeding choline in the form of PC to Sprague-Dawley dams during lactation improves IL-2, IL-6 and IFN-γ production from splenocytes after ex vivo mitogen stimulation in their offspring pups, highlighting the critical role of choline in offspring's immune system development (Lewis et al., 2016). Consistent with this, recent work from our own group has also found that feeding buttermilk-derived choline forms (37% PC, 34% SM, 17% GPC) to Sprague Dawley dams during pregnancy and lactation led to a higher IL-2, TNF-α and IFN-γ production from splenocytes after mitogen stimulation compared to the control diet in lactating dams (Azarcoya-Barrera et al., 2020), suggesting an improved immune function during lactation. Later, we have also demonstrated that the lipid soluble form of choline (especially PC and SM), in the suckling and weaning diet, improved immune function early in life in Sprague Dawley dams' offspring (Azarcoya-Barrera et al., 2021). These pre-clinical findings remain to be confirmed in humans. Nevertheless, emerging evidence is highlighting the promising role of choline and its different forms, along with other important micronutrients, in supporting the function of the immune system in different life stages in the context of obesity.

Dairy intake, insulin resistance and immune function

Biomarkers for dairy fat intake have been found to inversely associate with variables of insulin resistance and incidence of type-2 diabetes in a pooled analysis of 12 cohort studies (Imamura et al., 2018). However, high SFA found in dairy fat has traditionally been a concern to many health organizations, including Health Canada, who revised the Canada's food guide in 2019 which no longer recommends 2-3 servings of dairy products/day. Accumulating clinical evidence has shown that dairy consumption has no impact on inflammation particularly in subjects with obesity or are overweight (Labonté et al., 2013; Labontè et al., 2014). Another recent systematic review has concluded that consumption of various forms of dairy products may have either favorable or neutral effects on several cardiometabolic risk factors, including the lipid profile and systemic inflammation (Drouin-Chartier et al., 2016). Interestingly, low-fat yogurt consumption was also found to attenuate postprandial inflammation by decreasing the plasma LPS binding protein to CD14 ratio and IL-6 concentrations (Pei et al., 2018). The recent preliminary findings from our group found that consumption of 3 servings/day of low-fat dairy products normalized IL-2 production from PBMC in a swine model of insulin resistance, suggesting improved T cell function as compared to controls (She et al., 2021). The immune-protective benefits associated with dairy intake is also likely due to the presence of dietary choline. Our group has assessed the amount of choline in a variety of commercial dairy products in Canada and reported that there is a negative correlation between the fat content in dairy and choline content suggesting that low-fat dairy foods generally contain higher amount of choline (Richard et al., 2016). Moreover, a cohort study has reported that the main sources of dietary choline consumed by the population come from dairy,

meat and egg (Lewis et al., 2014). Therefore, dairy is an important source of choline and especially lipid soluble forms of choline that could potentially beneficially affect immune function in the context of obesity.

Conclusion

The rising concern in the global prevalence of obesity and its associated health consequences has drawn substantial attention from scientists in the areas of physiology, immunology and nutrition. Although numerous underlying mechanisms that link obesity and immunity are still poorly understood, we have highlighted a number of obesity-related cardiometabolic and immune dysfunctions that underpin the obesogenic etiology. Research models that are driving this field of research range from small animal models of rodents to emerging large swine animal models. In the past few years, numerous pharmacological and nutritional strategies to improve immune dysfunction in the context of obesity have been developed to meet specific needs. Despite this, ongoing research is required in order to translate these findings into tenable advances in the treatment or risk management of obesity in humans.

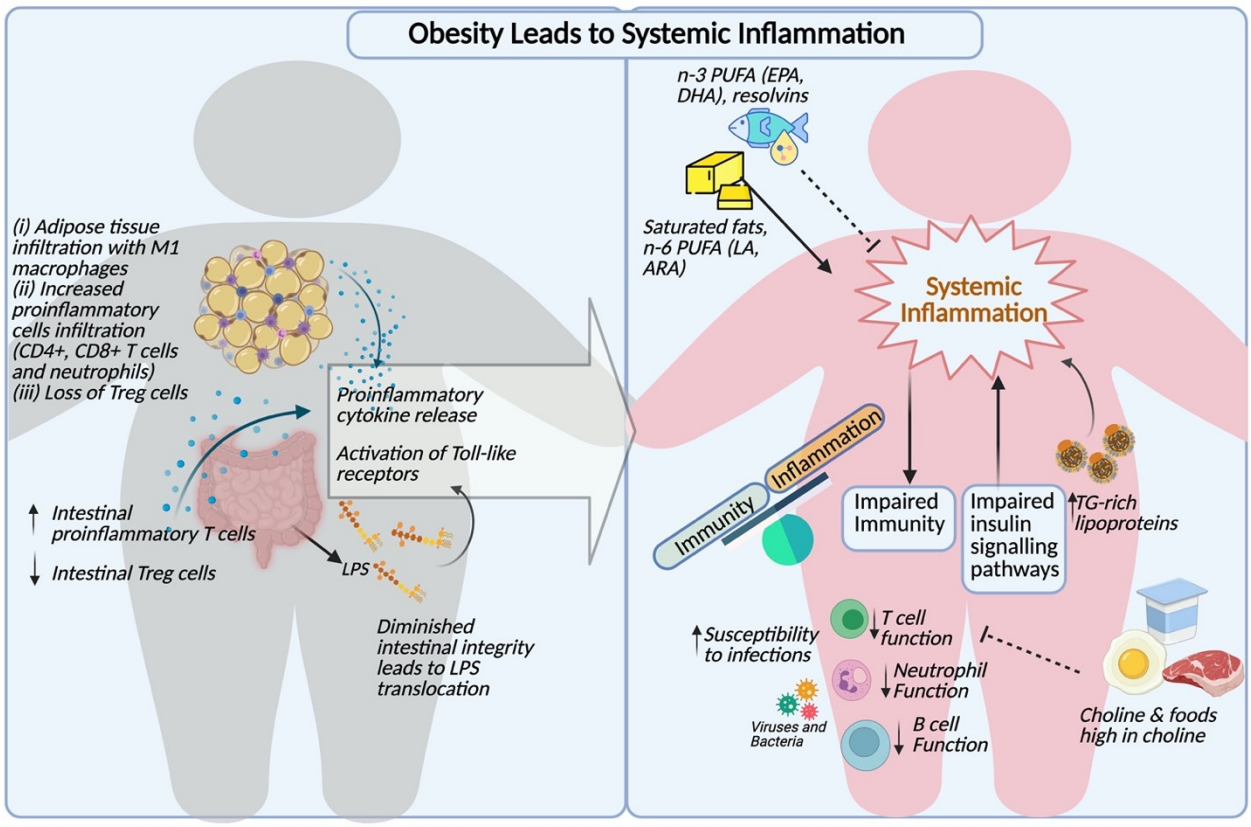

Figure 1 Comprehensive summary of obesity induced inflammation, dyslipidemia and impaired immunity (Created with BioRender.com)

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APPENDIX B Preferential deposition of dairy derived fatty acids in muscle tissue is partially due to the upregulation of CD36 in a low-birth-weight swine model

APPENDIX B is a research paper that investigate the modulation of dairy products on lipid transporter (i.e., CD36) and pork meat quality in LBW swine model of insulin resistance. The manuscript is written by Kun Wang and has been submitted to Frontiers in Veterinary Science for publication (revision in progress as of October 2022)

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INTRODUCTION

Metabolic syndrome (MetS) is a worldwide health issue with the prevalence rising dramatically in the past decade (Saklayen, 2018). Large animal models (such as swine) are now being developed to routinely study metabolic diseases in order to better translate to the human condition (Zhang & Lerman, 2016). Our previous research has established an innovative approach to study MetS and insulin resistance (IR) in a low birth weight (LBW) swine model. Results revealed that LBW swine fed a high fat (HF) diet were susceptible to IR and developed a preferential intestinal lipid absorption, hypertriglyceridemia and muscle steatosis (Fontaine et al., 2019). It is well known that in many animal models, a HF diet can lead to increased lipid absorption and over-production of chylomicrons, which further contributes to hypertriglyceridemia. Hypertriglyceridemia has been associated with ectopic lipid accumulation in peripheral tissues such as the liver and muscle (Zou et al., 2020). Yet, the molecular understanding of the mechanisms associated with intestinal lipid absorption and muscle lipid deposition during IR remain unclear. Long-standing evidence suggests that fatty acid transporters such as cluster of differentiation 36 (CD36), fatty acid transporter 4 (FATP4) and fatty acid binding protein 2 (FABP2) are expressed on the surface membrane of enterocytes and/or myocytes and can facilitate the uptake of long chain fatty acids into tissues (Dash et al., 2015; Glatz & Luiken, 2017; Said, 2018). Vesicle associated membrane protein 7 (VAMP7) was also found to play a functional role on the regulation of intestinal pre-chylomicron transport vesicle (PCTV) (Siddiqi et al., 2006). In this study, we hypothesized that protein expressions of these candidate transporters in enterocytes and/or myocytes in LBW swine could potentially explain the development of hypertriglyceridemia and ectopic lipotoxicity, which

implicate post-translational regulation.

Low birth weight swine are often not valued in the pork production industry due to reduced growth rate and limited economic gain. However, we also have identified that preferential lipid deposition in muscle tissue may yield a human health benefit by increasing the proportion of beneficial fatty acids. Dairy-derived fatty acids such as myristic acid (C14:0), pentadecanoic acid (C15:0) and margaric acid (C17:0) have been shown to be valid biomarkers to assess dairy intake, which can be utilized to investigate the specific muscle lipid incorporation (Sofie Biong et al., 2006). Additionally, there has been significant debate over the impact of dairy consumption on both cardiometabolic health and glycemic control in humans. Interestingly, odd chain fatty acids (C15:0 and C17:0) from dairy have been associated with lower risk of type II diabetes and total mortality (Imamura et al., 2018; Zhuang et al., 2019). As a result, we have proposed that increasing dairy product intake in LBW swine may be a suitable approach to alleviate metabolic consequences of IR specifically in LBW swine.

The overall aim of this study was to delineate the molecular transport candidates responsible for intestinal lipid absorption and muscle lipid deposition in LBW swine; and secondly to determine dietary fatty acids accumulation in pork with increased dairy intake. We hypothesized that the consumption of 3 servings/day of full-fat dairy products would improve insulin sensitivity. We also proposed that increasing dietary intake of dairy-derived fatty acids would result in higher proportion of these fatty acids being incorporated into the muscle, likely facilitated by candidate fatty acid transporters.

MATERIALS AND METHODS

Ethics approval

All procedures involving animals were in accordance with the Canada Council on Animal Care (CCAC) guidelines and approved by the University of Alberta's Animal Ethics Committee under the protocol number AUP00001184.

Animal and housing

Details regarding animal care were previously described (She et al., 2022). Briefly, pigs were obtained from the bio-secure Swine Research and Technology Center (SRTC), Department of Agriculture, Food and Nutritional Science, University of Alberta, Canada. Piglets were the product of a cross between a Duroc boar and Large White/Landrace sows. All living conditions (water, food, temperature, hygiene, etc.) were provided properly by trained staff under the guidelines of animal ethics. Piglets were co-housed (2~3 animals per pen) from 5 weeks of age to 9 weeks of age, then were single-housed from 9 weeks of age to 12 weeks of age due to their larger size. Regardless of housing condition, pigs were socialized by facility staff or researchers on a daily basis.

Study design and diets

Details regarding study design and experimental diets were previously described (She et al., 2022). At birth, full-term newborn male piglets were weighed and selected based on normal birth weight (NBW) and low birth weight (LBW). A 95% confidence interval (CI) was used to categorize piglets as LBW (below 95% CI) or NBW (within or above 95% CI). All piglets were weaned at 3-weeks of age and fed a standard grower diet until 5-weeks of age. At 5-weeks of age, NBW and LBW piglets were fed a 1-week transition diet, then were randomly assigned to three experimental

diets: 1-chow diet, 2-HF diet or 3-HF diet supplemented with 3 servings per 2000kcal/day of full fat dairy products. The groups are as follow: NBW-Chow (n=5), NBW-HF (n=6), LBW-HF (n=8) and LBW-HF+Dairy (n=5). Chow and HF diet were provided ad libitum. Both HF and HF+Dairy diets were isocaloric, and piglets on HF+Dairy diet were pair-fed to piglets on HF diet to ensure similar energy intake. The nutritional composition of chow and HF diets has been previously described (Fontaine et al., 2019). Briefly, HF diet consisted of 46%, 33% and 21% of total energies from fat (mainly lard), carbohydrates (mainly fructose) and protein respectively. 1% w/w cholesterol was added. On the contrary, chow diet consisted of 14%, 69% and 17% of total energies from fat, carbohydrates and protein respectively. Dairy extract (high-lactose whey) was added to support the growth of piglets. Regarding HF+Dairy diet, servings of dairy products (per 2000 kcal/day) included: whole milk powder (3.25% fat, 33g; Bulk Barn, Canada), plain yogurt (10% fat, 175ml; Liberté, Canada) and mozzarella cheese (28% fat, 50g; No Name®, Canada). Food intake and body weight were measured on a daily and weekly basis respectively. At 12-weeks of age, piglets were fasted overnight, and fasting blood was collected using capillary tubes. Piglets were then euthanized, exsanguinated and eviscerated by trained staff at SRTC, followed by tissue collection (Figure 1).

Sample collection and processing

Fasting blood samples along with muscle and mucosal scrapings (jejunum) were collected and flushed with ice-cold sterile PBS (phosphate buffered saline, pH 7.4). Tissue samples were snap frozen in liquid nitrogen before being stored in a -80°C freezer.

Biochemical analysis

Fasting plasma samples were assessed for glucose concentrations and lipid profiles using commercially available colorimetric kits for glucose, triglycerides (TG), total cholesterol (TC), low-density lipoprotein (LDL) and high-density lipoprotein (HDL). All kits were purchased from Wako Pure Chemical Industries Ltd. (Tokyo, Japan). Fasting insulin levels were measured via porcine-specific enzyme-linked immunosorbent assay (ELISA) kits (Mercodia, USA). Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) was used to evaluate IR (Matthews et al., 1985).

Triglyceride in jejunum mucosal scrapings and muscle samples (biceps femoris) was determined by the colorimetric test (Wako Pure Chemical Industries Ltd, Tokyo, Japan). Samples were pulverized using liquid nitrogen in radio-immuno-precipitation assay (RIPA) buffer and centrifuged at 4800 rpm for 10 mins, and supernatant was collected. Triglyceride concentration of the supernatant was measured using the same colorimetric kit as mentioned above.

Fatty acids composition

Plasma and ham muscle (biceps femoris) were used to assess fatty acid compositions via gas chromatography equipped with a flame ionization detector (Agilent 8890) as described previously (She et al., 2022). Briefly, muscle samples were homogenized and only homogenates were collected. Total lipids were extracted using the method of Folch et al. (Folch et al., 1957) in which they were saponified and then methylated with hexane and BF3 (boron trifluoride) as described previously (Cruz-Hernandez et al., 2004; Folch et al., 1957).

Fatty acid methyl esters were identified by chromatography with GLC standards (Sigma-Aldrich) and from the NIST 05 MS Library Database. Quantification of the fatty acid methyl ester profiles

was made by considering the relative peak areas, expressed as the relative percentage of the individual area of each one as related to the total area of compounds in the chromatogram.

Protein analysis

Western blot was used to determine candidate fatty acid transporters expression in mucosal scrapings of the jejunum and biceps femoris, using an adapted method as previously described (Krysa et al., 2021).

Briefly, after homogenization, proteins were separated by SDS-PAGE on a 4~12% Bis-tris NuPAGE Gel (ThermoFisher Scientific, Waltham, MA, USA) using the BioRad Criterion System and electro-blotted to polyvinylidene difluoride (PVDF) membranes. Membranes were incubated overnight with primary antibodies (Anti-CD36, 1;1000 dilution, #ab133625, Abcam; Anti-FABP2, 1;1000 dilution, #ab231070, Abcam; Anti-FATP4, 1:1000 dilution, #ab200353, Abcam; Anti-VAMP7, 1:1000 dilution, #NBP2-41183, Novus Biologicals; Anti-beta actin, 1:5000 dilution, #ab8226, Abcam), followed by the incubation of corresponding secondary antibodies. Protein bands were visualized by ECL luminescent solution. ImageJ (https://imagej.nih.gov/ij/, NIH, USA) was used for the quantification of selected proteins.

Carcass traits

Swine carcasses were transported to Agri-Food Discovery Place (University of Alberta, Edmonton, Alberta, Canada) immediately and weighed (recorded as hot carcass weight) before being stored in a 4°C cooler for 24 hours.

Subcutaneous fat depth, loin muscle depth and subjective marbling score were all measured by using a steak (2.54cm) between the last 3rd and 4th rib of loin eye muscle. The maximum depth

of the muscle perpendicular to the maximum of the width was measured to determine loin muscle depth, and subcutaneous fat depth was measured in the same line of the muscle depth (Teixeira et al., 2021). Subjective marbling score was assessed by trained personnel based on National Pork Board Pork Quality Standards on a scale from 0 to 10 where 0=devoid and 10=abundant presence of marbling. Measurements of subcutaneous fat depth and muscle depth were also used to calculate the predicted leanness, namely Canadian Lean Yield %, using the formula previously described (Pomar and M. Marcoux, 2003).

The proximate analysis to determine the crude fat content (intramuscular fat) has been described previously (Roy et al., 2018, 2021). Briefly, after muscle and fat depth measurements, epimysium and external fat in the steak were trimmed off, cut into small cubes, frozen, lyophilized and grounded, followed by crude fat extraction.

Statistical analysis

Data are reported as mean±SE. IBM SPSS (version 26.0) was used for statistical analysis. Fasting data were analyzed by one-way ANOVA with diet as the main effect and in cases where a p-value was significant, post-hoc comparison tests was performed with the Tukey's HSD adjustment for multiple comparisons among groups. For fatty acids profile, since dairy extract was added into the chow diet to support the piglets' growth, the LBW-HF control group was used for comparison with the LBW-HF+Dairy and was compared directly by student's t-test. The use of superscript letters (a,b) only refers to statistical difference when the letters differ. Different letters do not assume numerically higher or lower when comparing statistical difference. All tests with p-value <0.05 were considered statistically significant.

Results

Growth performance and food intake

As expected, all LBW piglets had significantly lower birth weight than NBW piglets ($p<0.05$, Supplementary Table 1). Chow-fed NBW piglets had significantly lower average daily energy intake and energy to gain ratio, compared with HF-fed piglets ($p<0.05$, Supplementary Table 1). No other significant differences were found in terms of average daily gain and final body weight among all groups.

Fasting plasma biomedical variables in response to HF diet and/or dairy product

High fat-fed LBW swine exhibited significantly higher fasting plasma glucose level ($p<0.05$) and tended to have higher fasting plasma insulin ($p=0.091$) and HOMA-IR score ($p=0.086$) than Chow-fed NBW swine (Table 1). Feeding dairy products as a part of a HF diet in LBW swine improved plasma glucose concentration to values no longer significantly different from NBW-Chow (Table 1). High fat diet significantly elevated fasting TC and LDL levels compared with the chow diet, regardless of dairy intake (Table 1).

TG contents in muscle and mucosal scrapings of jejunum

Despite no difference in circulating concentrations of TG, muscle samples from HF-fed LBW swine contained significantly higher TG content compared to Chow-fed NBW swine ($p<0.05$, Figure 2B). The introduction of a dairy source for dietary fat did not change muscle TG content in LBW-HF+Dairy group (Figure 2B). No significant difference was observed in intestinal TG content among groups (Figure 2A).

Fatty acid profile in plasma

High fat feeding significantly increased plasma levels of eicosapentaenoic acid (EPA, C20:5n3) in NBW and LBW swine compared with Chow-fed NBW swine ($p<0.05$, Table 2). Further, feeding dairy products significantly increased margaric acid (C17:0) possibly at the expense of EPA (C20:5n3) when compared to LBW swine fed a HF diet ($p<0.05$, Table 2).

Fatty acid profile in muscle

High fat feeding significantly reduced the n-6/n-3 ratio in biceps femoris from both NBW and LBW swine compared with Chow-fed NBW swine ($p<0.05$, Table 3). Feeding dairy products increased myristic acid (C14:0, $p<0.05$), docosapentaenoic acid (DPA, C22:5n3, $p<0.05$) and docosahexaenoic acid (DHA, C22:6n3, $p=0.092$) relative to high fat feeding alone (Table 3).

Expression of candidate fatty acid transporters

All high fat-fed LBW swine (regardless of dairy intake) exhibited an upregulation of CD36 expression compared with NBW littermates in both small intestine (Figure 3A, 3B) and muscle (biceps femoris) (Figure 3D, 3E) ($p<0.05$). No significant difference was found regarding the expression of FABP2 among groups (Figure 3A, 3C). No significant differences were found regarding the expression of other candidate transporters or related proteins such as FATP4 and VAMP7 (data not shown).

Carcass traits and meat quality

High fat feeding did not significantly change carcass traits or meat quality in NBW and LBW swine compared with Chow-fed NBW swine (Table 4). Feeding dairy products significantly decreased the subcutaneous fat depth, which further increased Canadian Lean Yield % in LBW-HF+Dairy group compared to LBW-HF group ($p<0.05$, Table 4). No significant difference was

observed in other indices of pork quality such as the subjective pork marbling score.

DISCUSSION

In the present study, we utilized a LBW swine model of IR to investigate the molecular transport pathway on intestinal and muscle lipid metabolism. The increased intestinal lipid absorption and muscle steatosis in LBW swine are likely explained by the upregulation of CD36. We also assessed the impact of dairy intake on metabolic consequences and confirmed that LBW piglets consuming 3 servings/day of full-fat dairy products (compared to HF-diet alone) exhibited improved insulin sensitivity, enhanced incorporation of dietary long-chain polyunsaturated fatty acids into pork, as well as increased predicted lean yield of the carcass.

Early insulin resistance induced by HF diet can be ameliorated by dairy intake

Low birth weight infants have been shown to have an increased risk of developing MetS later in life (de Mendonça et al., 2020; Ferrie et al., 2006). Animal studies also suggest that LBW rodents or piglets are more susceptible to hypertension, increased pro-inflammatory cytokines, and/or IR during adulthood (Barnett et al., 2017; Fontaine et al., 2019). Our study is consistent with these findings further highlighting that LBW piglets can be susceptible to develop fasting hyperglycemia and hyperinsulinemia (early IR) when consuming a HF diet. Additionally, our findings also support potential inverse association between dairy intake and IR in LBW swine. Ruminant-derived dairy products contain a diverse fatty acid profile, which mainly consists of short-chain (C4:0) and medium-chain fatty acids (C6:0 to C12:0), myristic acid (C14:0), palmitic acid (C16:0), monounsaturated fatty acids (MUFAS) (i.e. oleic acid, C18:1n9), polyunsaturated fatty acids (PUFAs) and unique odd-chain fatty acids (i.e. C15:0 and C17:0) (Abdullah et al., 2015). Butyric

acid (C4:0) and myristic acid (C14:0) have been shown to improve insulin sensitivity in mice (Amine et al., 2021; Gao et al., 2009; Takato et al., 2017). Odd-chain fatty acids such as pentadecanoic acid (C15:0) and margaric acid (C17:0) have also been found to lower the risk of type II diabetes and total mortality in humans (Imamura et al., 2018; Zhuang et al., 2019). Furthermore, Vitamin K2 (which can be uniquely synthesized by bacteria from cheese production), may also ameliorate IR by potentially modulating pro-inflammatory cytokine levels (Shea et al., 2008; Struijk et al., 2013). The dietary intake of menaquinones (Vitamin K2) has also been inversely associated with the risk of type II diabetes (Beulens et al., 2010).

Incorporation of dietary-derived fatty acids in the muscle tissue (pork)

Triglyceride is the major storage form of lipid in muscle. The HF diet used in this study was found to elevate muscle TG content suggesting muscle steatosis in LBW swine, which is consistent with the previous studies using the same approach (Fontaine et al., 2019). However, other methods such as magnetic resonance imaging and computing tomography should also be used in future studies to confirm muscle steatosis. To further identify fatty acids incorporation, we analyzed the fatty acids composition in both the plasma and muscle via gas chromatography. We know that increasing dietary fat intake can alter fatty acid composition in meat. We also highlight that despite fishmeal being used as a source of long-chain PUFA, it did not significantly elevate EPA, DPA or DHA level in muscle in either NBW or LBW swine fed a HF diet compared with NBW-Chow group. However, we did find that dairy products raised DPA and DHA in muscle of LBW swine, suggesting a preferential incorporation of long-chain fatty acids into the muscle. Of relevance to this, an earlier study has indicated that feeding dairy may potentially enhance long-chain omega-

3 fatty acids incorporation in tissues, provided that alpha-linoleic acid (ALA, C18:3n3) supply from the diet is sufficient (Drouin et al., 2018). For instance, myristic acid (C14:0) has been found to activate delta-6 desaturase in cultured rat liver cells, and delta-6 desaturase is one of the rate-limiting enzymes involved in the synthesis of long-chain PUFA (Jan et al., 2004). Additionally, fatty acids shunted to mitochondrial beta-oxidation are based on the number of carbon and double bonds with the rate being reported as follows: short-chain fatty acids > medium-chain fatty acids > ALA (DeLany et al., 2000; Leyton et al., 1987). Despite ALA exhibiting a high affinity for the beta-oxidation, short- and medium- chain fatty acids have even higher priority for the oxidation pathway and can spare ALA from being oxidized. It is also thought the spared ALA can be re-directed towards the conversion pathway of long-chain PUFA (Drouin et al., 2018). Consequently, we speculate here that long-chain omega 3 fatty acid synthesis pathway could be indirectly influenced by the addition of dairy products.

Fatty acid transporter candidates

We hypothesized that preferential increased lipid deposition in muscle tissue as well as the corresponding elevated intestinal lipid absorption in LBW swine could be due to the upregulation of fatty acid transporters or relevant proteins such as: CD36 (expressed in skeletal muscle and small intestine, regulating the uptake of long-chain fatty acids); FATP4 (expressed on the brush border membrane of the small intestine to transport fatty acids); FABP2 (expressed in the epithelial cells of the mucosal layer in small intestine, involved in fatty acid uptake); or VAMP7 (involved in the PCTV trafficking together with CD36 in the small intestine) (Dash et al., 2015; Glatz & Luiken, 2017; Said, 2018; Siddiqi et al., 2006). We found a significant elevation of both intestinal

and muscle CD36 (but not FABP2) in LBW swine under a HF diet. Our data support the notion of post-translational modification, as well as an underlying role of CD36 in intestinal lipid absorption (Fontaine et al., 2019). Studies in mice indicate that the phenotype of the FABP2 knockout displayed similar fecal lipid content compared with wild-type when fed a HF diet, implying that FABP2 may not be essential for fatty acid uptake in the small intestine (Gajda et al., 2013; Lackey et al., 2020; Lagakos et al., 2011). In contrast, CD36 knockout mice have impaired fat absorption and decreased chylomicron production in the proximal segment of small intestine with no significant compensatory pathways found, suggesting a predominant role in intestinal lipid absorption (Nassir et al., 2007). CD36 has also been shown to be the fundamental membrane protein involved in the long-chain fatty acid uptake into skeletal myocytes, which results from the activation of peroxisome proliferator-activated receptors (PPARs) induced by specific fatty acids (Glatz & Luiken, 2017). Overexpression of muscle-specific PPARα in a transgenic mouse model led to a five-fold increase in CD36 gene expression (Finck et al., 2005). CD36 is transferred from endosomes to sarcolemma to facilitate fatty acid uptake in muscle cells, induced by muscle contraction or insulin (Luiken et al., 2002, 2003). Hyperglycemia may also contribute to muscle steatosis by increasing de novo lipogenesis activity in muscle, which further triggers abnormal CD36 translocation (Savage et al., 2007). Collectively, our data supports consistent findings of the important role of CD36 in intestinal and muscle lipid metabolism.

The impact of dairy products on carcass traits

We speculate that the preferential muscle lipid uptake in LBW-HF+Dairy swine may potentially be at the expense of decreasing fat content in adipose tissue (as we have seen a decreased

subcutaneous fat thickness). This in turn may further indirectly elevate the Canadian Lean Yield %. Lean yield content is one of the factors determining pork carcass value in the Canadian grading system, which can be predicted via backfat and loin muscle depth measurements (Pomar and M. Marcoux, 2003). Low birth weight swine potentially exhibit severe growth retardation (Gondret et al., 2005; Rehfeldt & Kuhn, 2006; Yan et al., 2017). Interestingly, it was notable that dairy products in this study compensated the shortcoming in growth performance of LBW swine by increasing the predicted lean yield. Leucine has been found to enhance myogenesis in skeletal muscle in piglets (Wan et al., 2016). Dairy products are one of the good sources of essential amino acids and contained more leucine than traditional plant-based diet (soybean or wheat) (Gorissen et al., 2018). Increased leanness may improve the carcass grading index, as well as increasing the economic value (Pomar et al., 2009). This finding may suggest the importance of bioactive nutrients (such as those from the dairy) to improve the LBW carcass value in swine.

CONCLUSION

In summary, findings from this study provide evidence on the mechanistic pathway in intestinal and muscle lipid metabolism in an innovative LBW swine model. We have learned that increased intestinal lipid absorption and preferential muscle lipid deposition in LBW swine can be explained by the upregulation of CD36. We have also demonstrated that increasing dairy intake can enhance the incorporation of dietary long-chain PUFA into pork, as well as increasing the predicted lean yield of the carcass.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial

relationships that could be construed as a potential conflict of interest.

Author Contributions

SDP and CR designed and obtained funding for this study. HLB and MKD provided expertise on meat science and animal model. KW, YS, RM, AM and BCR conducted research experiments. KW performed the statistical analysis and wrote the manuscript under the supervision of SDP and CR. CR and SDP have the primary responsibility for the final content. All authors have read and approved the final manuscript.

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Table 1 Fasting plasma biochemistry in NBW and LBW swine fed experimental diets at 12-weeks of age.

Fasting plasma levels	NBW-Chow	NBW-HF	LBW-HF	LBW-HF+Dairy	*P*-value
Glucose, mmol/L	4.44±0.31[a]	5.99±0.29[b]	6.27±0.36[b]	5.18±0.28[ab]	0.004
Insulin, mU/L	3.66±0.36	5.63±0.88	9.91±3.27	3.59±0.58	0.091
HOMA-IR	0.72±0.07	1.52±0.27	3.04±1.19	0.83±0.15	0.086
TC, mg/dL	92.3±4.4[a]	300.2±25.4[b]	252.6±17.4[b]	241.1±24.0[b]	<0.001
LDL, mg/dL	54.1±2.0[a]	177.4±14.5[b]	158.3±15.4[b]	145.2±13.7[b]	<0.001
HDL, mg/dL	24.4±3.1	26.1±3.5	24.1±2.8	19.5±1.0	0.469
TG, mg/dL	24.3±2.6	25.2±4.9	29.4±2.1	38.7±10.5	0.308

Values are presented as mean±SE. Groups that do not share similar letters are statistically significant according to the Tukey's HSD test for multiple comparisons. NBW: normal birth weight; LBW: low birth weight; HF: high fat diet; HF+Dairy: HF diet supplemented with dairy products; HOMA-IR: Homeostatic Model Assessment for Insulin Resistance; TC: total cholesterol; LDL: low density lipoprotein; HDL: high density lipoprotein; TG: triglyceride.

Table 2 Fatty acids compositions in plasma (%) in NBW and LBW swine fed experimental diets at 12-weeks of age.

					P-value[1]	
Fatty acids	**NBW-Chow**	**NBW-HF**	**LBW-HF**	**LBW-HF+Dairy**	**1**	**2**
C14:0	1.5±0.09^{b}	1.23±0.06^{ab}	1.04±0.07^{a}	1.02±0.13^{a}	0.003	0.861
C15:0	0.7±0.05^{b}	0.53±0.04^{a}	0.44±0.03^{a}	0.43±0.03^{a}	<0.001	0.918
C16:0	22.11±1.27^{ab}	20.12±0.24^{a}	21.55±0.69^{ab}	23.99±0.92^{b}	0.032	0.055
C17:0	0.98±0.11^{b}	0.47±0.02^{a}	0.5±0.03^{a}	0.68±0.09^{a}	<0.001	0.043
C18:0	18.99±1.06	18.23±0.49	19.49±0.76	21.34±1.15	0.132	0.188
C18:1	30.12±1.30^{b}	30.86±0.49^{b}	29.44±0.63^{b}	26.31±0.69^{a}	0.005	0.008
C18:2n6	17.86±0.24	20.21±0.60	19.36±0.69	18.34±0.42	0.058	0.301
C18:3n6	0.14±0.03	0.16±0.01	0.16±0.03	0.2±0.03	0.640	0.469
C18:3n3	0.24±0.03	0.16±0.02	0.2±0.05	0.17±0.02	0.469	0.680
C20:0	1.15±0.16^{b}	0.71±0.03^{a}	0.65±0.04^{a}	0.57±0.03^{a}	<0.001	0.233
C20:1n9	0.21±0.02	0.19±0.01	0.19±0.01	0.16±0.01	0.270	0.203
C20:2n6	0.32±0.01^{ab}	0.32±0.01^{b}	0.29±0.01^{ab}	0.26±0.03^{a}	0.029	0.272
C20:3n3	0.32±0.04^{a}	0.65±0.02^{b}	0.65±0.05^{b}	0.76±0.10^{b}	<0.001	0.318
C20:4n6	3.47±0.58	2.86±0.17	2.8±0.20	2.87±0.28	0.442	0.830
C20:5n3 (EPA)	0.34±0.06^{a}	0.96±0.05^{b}	0.96±0.10^{b}	0.65±0.05^{a}	<0.001	0.04
C22:5n3 (DPA)	0.37±0.08	0.34±0.03	0.31±0.03	0.29±0.04	0.691	0.681
C22:6n3 (DHA)	0.25±0.06^{a}	0.91±0.07^{b}	0.85±0.10^{b}	0.83±0.13^{b}	<0.001	0.932
Total SFA	45.43±1.94^{ab}	41.28±0.63^{a}	43.67±1.11^{ab}	48.04±0.89^{b}	0.008	0.019
Total MUFA	31.26±1.41^{b}	32.15±0.52^{b}	30.74±0.68^{ab}	27.58±0.75^{a}	0.010	0.012
Total PUFA	23.31±0.97	26.57±0.60	25.59±0.84	24.38±0.60	0.059	0.327
Total n-6	21.79±0.81	23.56±0.55	22.61±0.69	21.67±0.42	0.216	0.343
Total n-3	1.52±0.16^{a}	3.01±0.12^{b}	2.97±0.27^{b}	2.71±0.28^{b}	0.001	0.526
n-6/n-3 ratio	14.79±1.06^{b}	7.87±0.30^{a}	8.03±0.70^{a}	8.35±0.86^{a}	<0.001	0.780

Values are presented as means±SE. Groups that do not share similar letters are statistically significant according to the Tukey's HSD test for multiple comparisons. NBW: normal birth weight; LBW: low birth weight; HF: high fat diet; HF+Dairy: HF diet supplemented with dairy products; EPA: eicosapentaenoic acid; DPA: docosapentaenoic acid; DHA: docosahexaenoic acid; SFA: saturated fatty acids;

MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids. n-6: omega 6 fatty acids: n-3: omega 3 fatty acids.
[1]Contrast: 1=one way ANOVA; 2=LBW-HF vs LBW-HF+Dairy.

Table 3 Fatty acids compositions in *biceps femoris* (%) in NBW and LBW swine fed experimental diets at 12-weeks of age.

					***P*-value[1]**	
Fatty acids	**NBW-Chow**	**NBW-HF**	**LBW-HF**	**LBW-HF+Dairy**	**1**	**2**
C14:0	1.16±0.05[a]	1.27±0.03[a]	1.25±0.05[a]	2.28±0.20[b]	<0.001	<0.001
C15:0	0.38±0.26	0.17±0.04	0.26±0.09	0.28±0.03	0.765	0.319
C16:0	23.79±1.27	23.15±0.33	23.23±0.50	25.03±0.29	0.301	0.027
C17:0	0.44±0.18	0.22±0.05	0.29±0.02	0.38±0.04	0.520	0.12
C18:0	12.23±0.71	13.18±1.38	13.73±1.44	13.45±0.51	0.839	0.884
C18:1	38.63±3.04	40.93±1.37	39.52±1.15	37.03±0.83	0.528	0.153
C18:2n6	16.04±0.95	13.85±0.68	14.68±0.88	13.65±0.99	0.260	0.468
C18:3n6	0.05±0.02	0.02±0.006	0.03±0.01	0.02±0.01	0.474	0.583
C18:3n3	0.14±0.02	0.16±0.01	0.14±0.02	0.1±0.02	0.247	0.204
C20:0	1.55±0.25[b]	0.95±0.06[ab]	0.99±0.14[ab]	0.73±0.06[a]	0.014	0.208
C20:1n9	0.53±0.10	0.59±0.03	0.55±0.04	0.46±0.03	0.420	0.121
C20:2n6	0.45±0.07	0.52±0.02	0.5±0.03	0.44±0.02	0.505	0.191
C20:3n3	0.22±0.04[a]	0.27±0.04[ab]	0.3±0.04[ab]	0.39±0.04[b]	0.082	0.209
C20:4n6	1.43±0.29	1.11±0.26	1.28±0.21	1.79±0.34	0.406	0.207
C20:5n3 (EPA)	0.26±0.11	0.38±0.06	0.38±0.06	0.48±0.08	0.357	0.392
C22:5n3 (DPA)	0.28±0.10	0.32±0.04	0.3±0.02	0.43±0.05	0.366	0.038
C22:6n3 (DHA)	0.24±0.16[a]	0.44±0.03[ab]	0.4±0.08[ab]	0.54±0.05[b]	0.110	0.092
Total SFA	39.55±2.12	38.95±1.31	39.79±1.49	42.15±0.61	0.565	0.255
Total MUFA	41.34±3.29	43.96±1.59	42.19±1.33	40.0±1.09	0.593	0.278
Total PUFA	19.10±1.30	17.07±1.09	18.02±1.10	17.85±1.52	0.698	0.929
Total n-6	17.97±1.19	15.50±0.94	16.49±1.07	15.91±1.32	0.448	0.739
Total n-3	1.14±0.40[a]	1.56±0.15[ab]	1.52±0.17[ab]	1.94±0.21[b]	0.02	0.076
n-6/n-3 ratio	21.12±4.13[b]	10.08±0.42[a]	11.78±2.01[a]	8.27±0.28[a]	0.006	0.100

Values are presented as means±SE. Groups that do not share similar letters are statistically significant according to the Tukey's HSD test for multiple comparisons. NBW: normal birth weight; LBW: low birth weight; HF: high fat diet; HF+Dairy: HF diet supplemented with dairy products; EPA: eicosapentaenoic acid; DPA: docosapentaenoic acid; DHA: docosahexaenoic acid; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids. n-6: omega 6 fatty acids: n-3: omega 3 fatty acids.
[1]Contrast: 1=one way ANOVA; 2=LBW-HF vs LBW-HF+Dairy

Table 4 Carcass traits of NBW and LBW swine fed experimental diets at 12-weeks of age.

Carcass characteristics	NBW-Chow	NBW-HF	LBW-HF	LBW-HF+Dairy	*P*-value
Hot carcass weight, kg	37.08±1.66[ab]	40.77±1.28[b]	36.26±2.04[ab]	33.44±0.88[a]	0.061
Subcutaneous fat depth, cm	1.21±0.14[ab]	1.45±0.09[b]	1.40±0.14[b]	0.90±0.04[a]	0.023
Loin muscle depth, cm	3.76±0.38	4.62±0.34	4.56±0.25	4.62±0.19	0.193
Canadian lean yield[1], %	62.51±0.58[ab]	61.69±0.34[a]	62.00±0.72[a]	64.79±0.34[b]	0.008
Intramuscular fat, %	1.59±0.10	2.40±0.28	2.37±0.26	2.51±0.33	0.121
Subjective pork marbling score	1.40±0.24	2.17±0.4	2.00±0.27	1.80±0.37	0.280

Values are shown as means ±SE. Groups that do not share similar letters are statistically significant according to the Tukey's HSD test for multiple comparisons. NBW: normal birth weight; LBW: low birth weight; HF: high fat diet; HF+Dairy: HF diet supplemented with dairy products.

Canadian lean yield (%) = 68.1863-(0.7833 × fat depth)+(0.0689 × muscle depth)+(0.0080 × fat depth^2)-(0.0002 × muscle depth^2)+(0.0006 × fat depth × muscle depth)

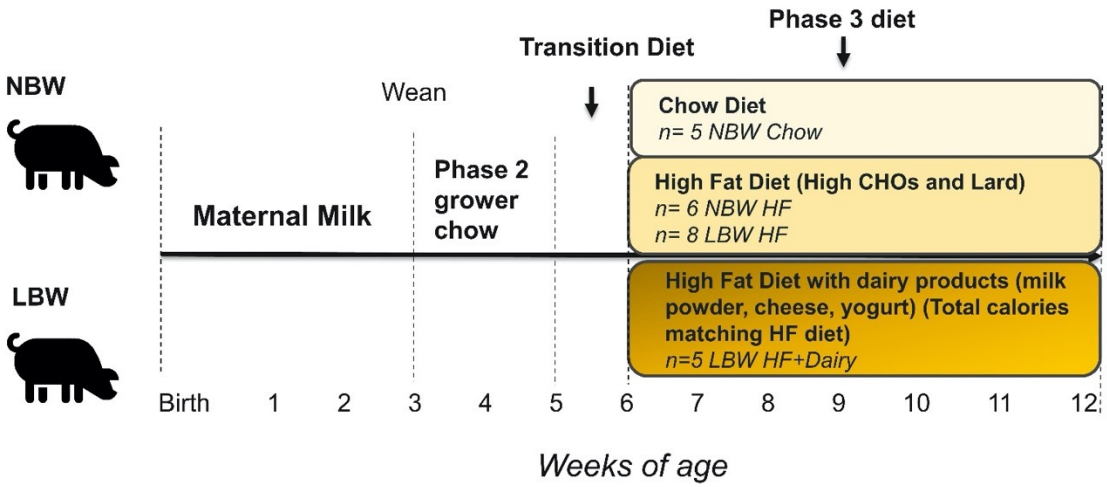

Figure 1 Study design to investigate intestinal and muscle lipid metabolism under the impact of dairy products in LBW swine model of insulin resistance.

Abbreviations: CHO: carbohydrate; NBW: normal birth weight; LBW: low birth weight; HF: high fat diet; HF+Dairy: HF diet supplemented with dairy products.

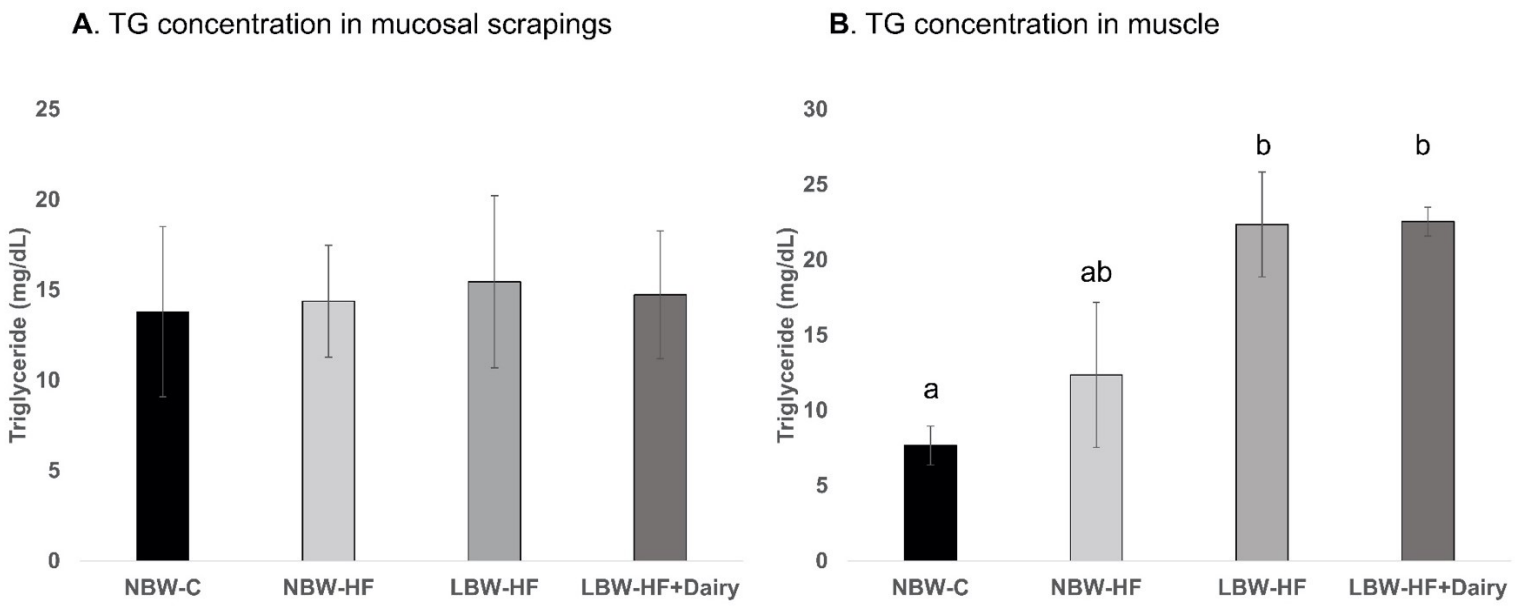

Figure 2 Comparison of TG levels (mg/dL) in mucosal scrapings (**A**) and biceps femoris (**B**) in NBW and LBW swine fed experimental diets at 12-weeks of age.

Abbreviations: TG: triglyceride; NBW: normal birth weight; LBW: low birth weight; HF: high fat diet; HF+Dairy: HF diet supplemented with dairy products.

Values are presented as mean±SE. Groups that do not share similar letters are statistically significant according to the Tukey's HSD test for multiple comparisons

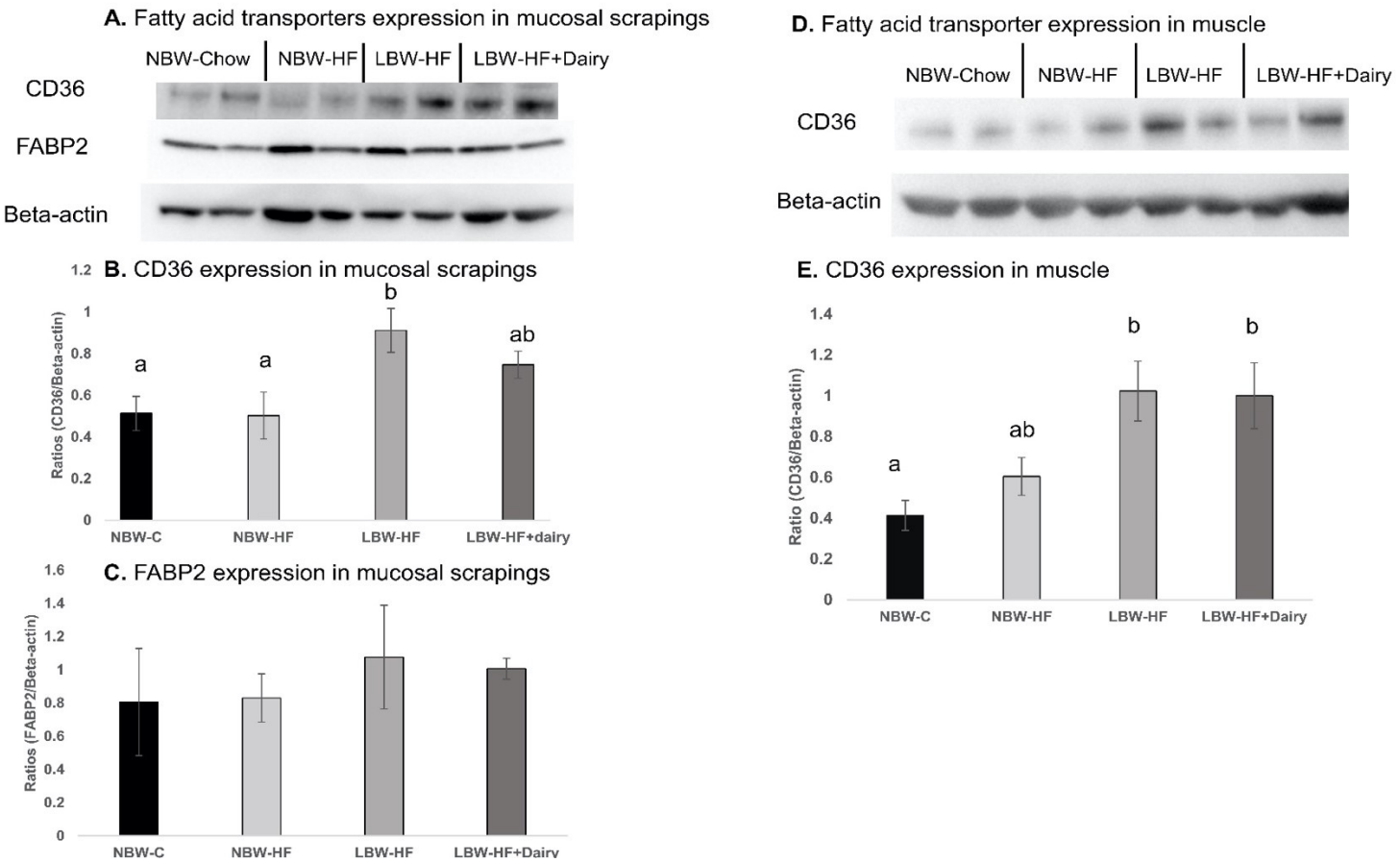

Figure 3 Expressions of CD36 and FABP2 relative to beta-actin in mucosal scrapings (**A, B, C**) and/or *biceps femoris* (**D, E**) in NBW and LBW swine fed experimental diets at 12-weeks of age.

Abbreviations: CD36: cluster of differentiation 36; FABP2: fatty acid binding protein 2; NBW: normal birth weight; LBW: low birth weight; HF: high fat diet; HF+Dairy: HF diet supplemented with dairy products.

Values are presented as mean±SE. Groups that do not share similar letters are statistically significant according to the Tukey's HSD test for multiple comparisons

Supplementary Table 1 Growth performance and feed consumption of NBW and LBW swine fed experimental diets.

Growth and feed intake	NBW-Chow	NBW-HF	LBW-HF	LBW-HF+Dairy	*P-value*
Birth weight, kg	1.620 ± 0.066[a]	1.733 ± 0.095[a]	1.228 ± 0.081[b]	1.225 ± 0.065[b]	<0.001
Final weight, kg	48.44 ± 3.164	53.62 ± 1.970	48.10 ± 2.771	50.53 ± 2.806	0.618
Average daily gain, g/d	779.2 ± 47.34	854.0 ± 15.80	794.7 ± 41.00	824.1 ± 40.47	0.723
Average daily energy intake, kcal/d	4076.9 ± 290[a]	5716.7 ± 340[b]	5313.5 ± 238[b]	5677.9 ± 172[b]	<0.01
Energy to gain ratio	5.23 ± 0.14[a]	6.67 ± 0.29[b]	6.72 ± 0.15[b]	6.97 ± 0.10[b]	<0.01

Values are shown as means ±SEM. Groups that do not share similar letters are statistically significant according to the Tukey's HSD test for multiple comparisons. NBW: normal birth weight; LBW: low birth weight; HF: high fat diet; HF+Dairy: HF diet supplemented with dairy products. Details were described previously (She et al., 2022).

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APPENDIX C Standard operating procedures

Blood

- On all samples we will perform immunofluorescence and complete blood counts on whole blood and cytokine assays on cells isolated from gradient.
- **RUN CBC AND IF FROM WHOLE BLOOD SAMPLES**
- To isolate cells, spin sample (using red buckets) for 10 min @ 3000 rpm
- Remove plasma and store in ~500uL aliquots at -80°C , make sure there is a 1mL aliquot for lipids analysis
- Top up blood tube with 1%BSA in PBS (add same amount as what is in the tube ~3 mLs) to dilute and re-suspend buffy coat.
- Gently layer cell suspension over 5 mLs Histopaque (should have 1 histopaque tube per blood tube).
- Spin samples at 1800 rpm for 30 min at room temperature with no brake (large centrifuge).
- Remove the lymphocyte band at the gradient interface and transfer to a 50 mL tube.
- For RBC's save the RBC's from gradient for lipid extraction (see protocol below)
- Top off to 25 mL with 1%BSA in PBS and spin at 1500 rpm for 10 min at 4°C to wash off Histopaque.
- Wash second time with 10mL 1%BSA in PBS
- Discard supernatant and add 5 mLs 5% CCM and count.
- Add 20 µL of 1:1 trypan blue to 20 µL sample and load onto hemocytometer.
- Count cells and determine concentration of cells/mL for use in mitogen stimulation
- Pellet the remaining cells, spin samples at 1500 rpm @ 4°C for 5 min
- Discard supernatant and resuspend in 3 mL 20% CCM
- Divide remaining cells between 2 cryovials with 10% DMSO in 20% CCM for immune assays and one pre pellet tube

Mesenteric Lymph nodes

- Sterilize all instruments and screens (soak overnight in 70% ETOH).
- In a sterile petri dish, transfer tissue into dish, and discard storage buffer into liquid waste
- Wet tissue and screen with buffer (0.5% BSA in KRH)
- Trim excess fat, connective tissue, blood vessels etc., from around the node
- Cut node into little pieces and push cells through the nylon mesh using the barrel of sterile syringe and buffer, use buffer to keep tissue wet and to rinse screen after
- Collect buffer with cells through a 40um sterile filter into a 50mL conical tube, rinse with buffer to remove debris

- Spin samples at 1500 rpm @ 4°C for 5 min to pellet cells. (Program 8), discard supernatant.
- Wash pellet with 10 mL 1% BSA in PBS using program 8, discard supernatant
- Add 5 mLs 5% CCM and resuspend pellet.
- Take a 1mL aliquot for lipids, freeze at -80°C
- Add 20 µL of 1:1 trypan blue to 20 µL sample and load onto hemocytometer.
- Count cells and determine concentration of cells/mL for use in mitogen stimulation and immunofluorescence
- Pellet remaining cells, spin samples at 1500 rpm @ 4°C for 5 min, and remove supernatant
- Resuspend in 3mL 20% CCM and divide between two cryovials and one pre pellet tube
- Freeze leftover cells in 10% DMSO in 20% CCM for immune assays

Peyer's Patches

- Remove excess mesenteric and fat tissue from the intestine
- Rinse the intestine 2 times with Hank's + DTT/EDTA buffer. DTT/EDTA removes the mucous and must be made fresh everyday.
- Cut open intestine down the mesenteric line and dissect out the Peyer's patch.
- Chop up the entire patch into small pieces and incubate 20-30 min at 37°C in a waterbath with 40 mLs with Hank's + DTT buffer. Seal tubes with parafilm to keep water out.
- Vortex vigorously, and discard supernatant without disturbing tissue
- Repeat incubation for the second time mixing as before
- Discard supernatant and push intestinal tissue through sterile screens.
- Keep tissue wet with HBSS+DTT buffer while squishing
- Collect buffer with cells through a 40um sterile filter into a 50mL conical tube, rinse with buffer to remove debris
- Pellet sample using program 13, discard supernatant
- Add 6 mLs 40% Percoll and resuspend pellet.
- Layer cell suspension over 6 mls 70% percoll and centrifuge 30 min no brake at 1800 rpm (large centrifuge).
- Remove cells from interface and wash with 20mL 1% BSA in PBS using program 13, discard supernatant
- Wash pellet with 10 mL 1% BSA in PBS using program 8/13, discard supernatant
- Add 5 mLs 5% CCM to resuspend pellet and count.
- Add 20 µL of 1:1 trypan blue to 20 µL sample and load onto hemocytometer.
- Count cells and determine concentration of cells/mL for use in mitogen stimulation and immunofluorescence
- Pellet the remaining cells, spin samples at 1500 rpm @ 4°C for 5 min
- Discard supernatant and resuspend in 2 mL 20% CCM
- Divide remaining cells between 2 cryovials with 10% DMSO in 20% CCM for immune assays

Immunoflorescence

- Pre-condition V well plate with 5% FCS in PBS for ~30 minutes at room temperature.
- Add 100 µL whole blood to appropriate wells.
- Add 200 µL lysis buffer (mix with pipet –go up and down numerous times so that you are properly mixing the lysis buffer and the blood) incubate 15 min @ RT°C.
- Centrifuge (program 22), discard liquid and vortex cell pellet.
- Repeat lysis step.
- Add 50-100 µL of MES, and PP samples (5% CCM suspension) to appropriate wells (~100 000 cells).
- Wash cells one time by adding 200 µL 5% FCS in PBS and spin (program 22). Dump supernatant in sink. Vortex plate to break up pellet.
- Add 20 µL of diluted CD25 Add 20ul of IF buffer to all other wells
- Incubate 30 minutes @ 4°C.
- Wash plate 1X
- Add Star PE to CD25 well ++ add all other antibodies (according to Template)
- Wash 2 times by adding 200 µL buffer and spin (1000 rpm for 3 min). Dump supernatant in sink. Vortex plate to break up pellet.
- Transfer FOX wells to another plate/IF tubes before carrying on with next step
- Add 200ul of cell fix to remaining wells
- Cover in foil

FOXP3 Intracellular Staining Procedures:

1. Perform cell surface staining as described above
2. Add 100ul of 1X BioLegend's FOXP3 Fix/Perm solution to each well/tube and incubate at room temperature in the dark for 30 minutes.
3. Spin down the cells and remove the supernatant.
4. Wash once with 200ul 1X BioLegend's FOXP3 Perm buffer.
5. Re-suspend cells in 100ul 1X BioLegend's FOXP3 Perm buffer, incubate at room temperature in the dark for 5 minutes
6. Spin down cells and discard the supernatant, Vortex
7. Add 20ul of diluted FOX antibody and incubate at room temperature in the dark for 30 minutes. 20ul perm buffer if you want unstained
8. Wash twice with cell staining buffer, and resuspend in 300ul Cell Fix

RBC extraction from Gradient (does not have to be sterile) for lipids

Take 1ml of RBC pellet from bottom of gradient
Wash 3X with 2mls saline (1000 rpm for 5 min)

After final wash remove supernatant and add 1ml water.
Vortex and remove 1 ml of cell suspension and freeze in 1.5mL microcentrifuge tube.

Peyer Patch buffer HBBS –Hank's- sterile filter
1L -dd water
0.4g--KCL
0.1g-- KH_2PO_4
8g—NaCl—
0.35g—NaH_2CO_2
1g—glucose
0.584g EDTA
pH=7.2
**For isolation add1mM of DTT—0.154g
Add the day of kills and sterile filter

5%CCM—1L
1 pack of RPM1
2.0g of NaHCO3
50mL of FCS
25mL of Hepes
1mL of 2-mercaptoethanol (stock solution 2.5umol/L)
10mL of antibiotic/antimycotic-sterile filter

KRH- Krebs Ringer Bicarbonate buffer—We have stock bottles of the individual buffers and then we add 25ml of each and make up to 1L. We then add 5 g of BSA. Let it sit a couple of hours at room temp so that it dissolves and then we pH to 7.4 and sterile filter.
**we always add the $CaCl_2$ last to avoid precipitation. We also add 1% antibiotic/antimycotic to this buffer. (10mls/L)

CHEMICAL	[BUFFER]	[STOCK]	g/L for stock
NaCl	130 mM	5.20 M	303.6
HEPES	10 mM	0.40 M	104.0
KCl	5.2 mM	0.21 M	15.6
** $CaCl_2$	1.4 mM	0.060 M	6.2
NaH_2PO_4	1.0 mM	0.040 M	5.68
$MgSO_4$	1.4 mM	0.056 M	6.74

Cytokine Stimulation

- Cell concentration. Total 3.75 X 10^6 cells per 3mL culture tube
- Add required amount of 5% FCS complete culture media (CCM) in each culture tube before starting (amount in brackets at end of mitogens)
- Individually calculate the required volume of cell suspension to add in order to get 3.75 X 10^6 cells per culture tube (3mL X 1.25 X 10^6 cells/ mL). Remove this volume of media before adding the cell suspensions (volume should not change)

- Prepare ConA and PHA mitogens following below dilutions, add required amount of mitogen to culture tubes and place in cell culture incubator for 48 hours.

Mitogens:

1. UNS (unstimulated) cells only.—300ul CCM (2.7mls CCM)
2. LPS –2ul per mL 6ul per tube (3mls CCM)
3. PMAI – 2ul per ml 6ul per tube (3mls CCM)
4. ConA –50mg/ml stock (PBS)-aliquot 10ul and freeze. Add 990ul CCM. Take 22oul and dilute to 2mls. Add 300ul (per tube) for a final concentration of 5ug/ml. (2.7mls CCM)
5. PWM(dilute 5mg vial in 5mL)-(1mg/mL) (PBS) stock –add 165ul per tube (2.8ml CCM)
6. PHA – 5mg/ml Add 1mL sterile PBS (pH=7.4) to 5mg vial. Aliquot 50ul and freeze. To aliquot add 950uL CCM. Add 100ul per million cells for a final concentration of 25ug/mL. Add 300ul per tube (2.7ml CCM)

- After ~48hrs incubation, spin samples at 1500rpm for 10 min to pellet cells.
- Carefully remove ~500-600uL aliquots of supernatant, taking care to not disturb the pellet, and place in 1.5ml eppendorf tubes (5 for each mitogen) freeze at –80 ^{o}C.
- Wash pellet with 1mL sterile PBS and pellet again.
- Remove all liquid and freeze at –80^{o}C