COMPARISON OF CHEESE, YOGURT, AND MILK EFFECTS ON GLUCOSE HOMEOSTASIS AND LIVER LIPID ACCUMULATION IN MICE FED HIGH-FAT DIET

by

Salma Moftah Jabir Moftah

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Department of Physiology
University of Alberta

Abstract

Background: Many human observational studies show an inverse or neutral association between dairy consumption and the risk of diabetes. Our lab's previous work suggests that both low and high-fat cheese consumption improves glucose homeostasis in insulin-resistant (IR), prediabetic rats. IR also affects the hepatic lipid metabolism, which can lead to liver injury and non-alcoholic fatty liver disease. Previous studies have shown that low-fat dairy intake can improve liver function and reduce steatosis. This study examines whether regular-fat yogurt and milk have a similar effect on glucose homeostasis as regular-fat cheese and evaluates the impact of regular fat dairy intake on hepatic lipid accumulation in IR mice.

Methods: An 8-week feeding intervention with dairy foods (regular-fat Yogurt, Milk, and Cheese) equivalent to half a serving was performed in a high-fat diet-fed (HFD) insulin-resistant male C57Bl6/J mice (N=48 with n=12/group). A low-fat diet (LFD) control group was included (n=12). Mice were weighed weekly, and body fat mass was measured at week 6. To evaluate IR and hepatic glucose output capacity, insulin tolerance test (ITT) and a pyruvate tolerance test (PTT) with n=6 mice/group were administered one week before euthanasia. Fasting serum at the time of euthanasia was used to measure alanine aminotransferase (ALT), triglyceride (TG), and non-esterified fatty acids (NEFA). Frozen liver tissue was used to measure liver TG. Liver histology was done to quantify the accumulation of fat droplets following dairy consumption.

Results: All mice on HFD had significantly more body fat % than the LFD group independent of dairy consumption. However, there was no significant effect of diet on ITT

and PTT. Milk diet significantly lowered serum TG, while Yogurt elevated both serum and hepatic TG with no significant impact on ALT compared to the LFD group. The dairy foods had no significant effect on serum NEFA; however, liver histology showed improvement in hepatic lipid-related morphological characteristics in the Milk group. This improvement was evidenced by decreased macrovesicle area and increased microvesicle area compared to the HFD group.

Conclusion: The results did not support our hypothesis that regular fat dairy, in particular Milk, Yogurt, and Cheese improve insulin sensitivity. However, milk consumption, even in a small amount (equivalent to half a serving), was beneficial in reducing serum TG and improving hepatic lipid metabolism.

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List of Abbreviations

15:0C: Pentadecanoic acid

ALT: Alanine aminotransferase

ANOVA: Analysis of variance

AST: Aspartate aminotransferase

ATP: Adenosine triphosphate

AUC: Area under the curve

BCAAs: Branched chain aminoacids

BW: Body weight

FBG: Fasting blood glucose

Ca^{2+}: Calcium

CDC: Centers for Disease Control and Prevention

CLA: Cis-9, trans-11 conjugated linoleic acid

ddH2O: Distilled water

DPP-4: Dipeptidyl peptidase-4

ER: Endoplasmic reticulum stress

FAS: Fatty acid synthase

FFA: Free fatty acids

Foxa2: Forkhead box protein A2

G6Pase: Glucose 6-phosphatase

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GIP: Glucose-dependent insulinotropic polypeptide

GLP-1: Glucagon-like peptide 1

GLUT: Glucose transporter

GPR40: Free fatty acid receptor 1

HbA1C: Glycated hemoglobin A

HDL: High density lipoprotein

HFD: high fat diet

HOMA-IR: Homeostasis model assessment of insulin resistance

iAUC: Incremental area under the curve

IDF: International Diabetes Federation

IGT: Impaired glucose tolerance

IR: Insulin resistance

IRS: Insulin receptor substrate

ITT: Insulin tolerance test

K: Potassium

KATP: ATP sensitive potassium channel

LDL: Low density lipoprotein

LFD: Low fat diet

MCD: methionine- and choline-deficient diet

MetS: Metabolic syndrome

MFGE8: milk fat globule-epidermal growth factor 8

MFGM: Milk fat globule membrane

mTOR: Mammalian target of rapamycin

NASH: Non-alcoholic steatohepatitis

NAFLD: Non-alcoholic fatty liver disease

NEFA: non-esterified fatty acids

OAA: Oxaloacetate

OGTT: Oral glucose tolerance test

PC/LPC species: Phosphatidylcholine/Lysophosphatidylcholine

PEPCK: Phosphoenolpyruvate carboxykinase

PEP: phosphoenolpyruvate

PL: Polar lipid

PTT: pyruvate tolerance test

PUFA: polyunsaturated fatty acid

RCTs: Randomized controlled trials

RT: Room temperature

SFAs: Saturated fatty acids

SREBP-1: Sterol element binding protein 1

T2D: Type 2 diabetes

TG: Triglycerides

t-16:1n-7C: Trans-palmitoleate

TNF-α: Tumor necrosis factor-alpha

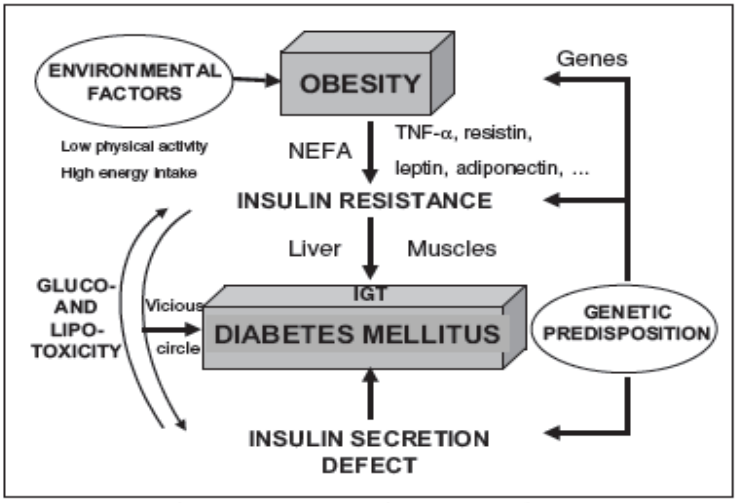
VLDL: very- low-density lipoprotein

Chapter 1 : Introduction

1.1 Diabetes Prevalence and Diagnosis

Diabetes is the fastest-growing chronic disease worldwide. The prevalence of diabetes in the world was 6.4% in 2010 (1) and the estimated number of individuals with diagnosed diabetes will be increased by 54% in 2030 (1). Type 2 Diabetes (T2D) or non-insulin dependent diabetes is the most common type and accounts for about 91% of diabetes cases (2). Insulin resistance (IR) and beta-cell dysfunction are the key factors for T2D development (3). T2D is caused by a combination of genetic and environmental factors that influence beta-cell activity and tissue insulin sensitivity (liver, adipose tissue, and pancreas) (3). People at risk for T2D, such as those with obesity, show an early symptom of IR due to low-grade inflammation originating largely in the expanded white adipose tissue, leading to insulin hypersecretion from pancreatic beta-cells to compensate for the insulin demand. Insulin sensitivity is reduced by 30% in obese with normal blood glucose levels (euglycemic) compared to lean people (4). This compensation process continues until insulin secretion is no longer enough to maintain the physiological range of blood glucose concentration. According to the Centers for Disease Control and Prevention (CDC), prediabetes is a critical health condition where blood glucose levels are higher than normal but not high enough to be identified as diabetes. The fasting blood glucose (FBG) in prediabetes is 6.1-7 mmol/L, while in diabetes, it is ≥7 mmol/L (5). In the USA, 1 in every three adults has prediabetes. This condition could last for up to 15 years before the diagnosis of diabetes (4); therefore, early

intervention can lead to diabetes prevention. Figure 1–1 summarizes the contribution of genetic and environmental factors to the pathophysiology of T2D (3).



Abbreviation: NEFA, Non-esterified fatty acid. TNF- α: Tumor necrosis factor-alpha. IGT, impaired glucose tolerance.

Figure 1-1 Contribution of genetic predisposition and environment factors in the pathogenesis of type 2 diabetes and interplay between defective insulin secretion and insulin resistance leading to a vicious circle explaining the progression from impaired glucose tolerance (IGT) to type 2 diabetes and the progressive aggravation of the disease. Adapted from Scheen 2003. (4)

1.2 Insulin Physiology

Insulin is a peptide hormone that consists of 2 polypeptides chains, A (21 amino acid residues) and B (30 amino acid residues), which are linked together by two disulfide bonds (6). It is produced in the beta-cells of the islets of Langerhans of the pancreas in response to glucose, which is the main secretagogue, and other nutrients. When the plasma glucose concentration is above 3.3 mmol/L (8), glucose is transported into beta-cells mainly via GLUT1 and 3 in humans and GLUT2 in rodents, then phosphorylated by glucokinase (8). An increase in ATP concentration due to metabolization of glucose leads to closure of K_{ATP} channels, leading to membrane depolarization, opening of voltage gated Ca^{2+} channels, Ca^{2+} influx, increased intracellular calcium concentration, and finally

exocytosis of insulin granules. In the resting state, K^+ efflux through an open K^{ATP} channel maintains the beta-cell membrane at a negative potential where the Ca^{2+} voltage channel is closed (6). Insulin is transported via the portal circulation to exert its action and for its clearance by the liver, the half-life of insulin is 3-5 min, and up to 80% of the insulin is cleared through the liver (9).

The "fuel sensing" beta-cells are stimulated to release insulin in response to high blood glucose levels, typically after the body consume carbohydrate rich meal. Blood glucose concentrations are significantly affected by nutrient intake and energy expenditure such as fasting and exercise. To keep blood glucose concentration within the normal range, the pancreas switches the release of insulin and glucagon to regulate glucose uptake and hepatic glucose production (8).

Insulin plays an important role in regulating carbohydrate, lipid, and amino acid metabolism (8). Insulin stimulates utilization and storage of glucose as glycogen in the liver by activating glucokinase and glycogen synthase, thus increasing glycogen storage. In addition, insulin inhibits glucose production and release by suppressing hepatic gluconeogenesis and glycogenolysis (10). Insulin stimulates skeletal muscle's glucose uptake by promoting the expression and translocation of GLUT4, an insulin-regulated glucose transporter, to the cell membrane (11). Moreover, insulin stimulates amino acid uptake in skeletal muscle and promotes protein synthesis and glycogen storage. Adipocytes are also highly insulin responsive, and insulin enhances triglyceride synthesis and storage, stimulates glucose transport, and inhibits lipolysis in adipose tissue (12). Suppose insulin secretion and/or action is disturbed. In that case, insulin-sensitive tissues

are unable to generate a coordinated normal glucose-lowering response that includes suppression of endogenous glucose production, suppression of lipolysis, and net synthesis of glycogen, thus requiring increased insulin secretion to compensate for insulin resistance. Therefore, plasma glucose concentration rises, causing hyperglycemia, and if not treated, the condition can progress to T2D (13). Understanding the pathophysiology of IR is necessary to understand T2D development better.

1.3 Insulin Resistance

Insulin resistance (IR) is a complex metabolic disorder in which the body's cells are not responding properly to secreted insulin (15). This condition is commonly seen in obese and older adults; however, young, and lean individuals are also shown to be affected (15). In insulin-dependent cells, proper insulin signaling is crucial because when insulin sensitivity is impaired, the entry of glucose into adipocytes and skeletal muscle cells is reduced (14). IR is a leading risk factor for diabetes, atherosclerosis, non-alcoholic fatty liver disease (NAFLD), and obesity-related cancers (15). Multiple factors drive the development of IR, such as gluco-lipotoxicity, reactive oxygen species (ROS), and an increase in the level of pro-inflammatory cytokines (16). Obesity is a chronic inflammatory condition that has been linked to T2D and IR (17). Tumor necrosis factor-alpha (TNF-α) is one of the critical pro-inflammatory mediators that causes low-grade inflammation in vital organs and leads to IR (18). It does so by reducing the expression of GLUT4 in adipose tissue and muscle, inducing serine phosphorylation of IRS-1, thus impairing peripheral insulin signaling (16,17). TNF-α inhibits beta-oxidation, increases plasma FFAs, and inhibits lipoprotein lipase (18). TNF-α stimulates lipolysis by reducing the

expression of cell death-inducing DFF45-like effector C which plays an important role in regulating TG accumulation and lipolysis in adipocytes (19). Increased lipolysis in turn increases serum non-esterified fatty acid (NEFA) and contribute to the development of IR (18).

Adiponectin is a fat cell-derived peptide, and its receptors are expressed mainly in adipocytes (20). Adiponectin abundance is positively correlated with insulin sensitivity, and its expression is reduced in obese humans and mice (10). Body weight loss significantly enhances plasma adiponectin and improves IR (21). Studies have shown that adiponectin reduces plasma FFAs and stimulates fatty acid oxidation by increasing the expression of involved genes (10,22). Adiponectin reduces the TG content in the liver and muscle tissue by activating protein phosphorylation activator receptor-alpha, which regulates the genes involved in beta-oxidation; furthermore, adiponectin suppresses gluconeogenesis (20). All these mechanisms are suggested to explain the role of adiponectin as a potential treatment for IR (20). Resistin is also a hormone secreted by adipocytes. Its expression level increases in obese mice (10), and its overexpression reduces insulin function at target tissues (23). A study reported that serum resistin positively correlated with body fat content (24). Treatment with anti-resistin antibody improves blood glucose and insulin action in mice with diet-induced obesity (10). A systemic review and meta-analysis concluded that in T2D and obese people, resistin level is positively correlated with IR (23). Many other possible underlying mechanisms are proposed to be involved in IR, as shown in Table 1–1.

Table 1—1 Molecular mechanism involved in insulin resistance adapted from Peters, Yaribeygi et al. 2019 (14)**.**

Molecular mechanism	Role in insulin resistance
Inflammatory mediators and adipokines	Decreases GLUT-4 expression, reduces IRS-1 expression, induces IRS degradation
Free radical overload	Activates inflammatory responses
Obesity and adipocytes importance	Inflammation and impaired insulin signaling
Accelerated insulin degradation	Autoimmune antibodies against insulin or abnormal insulin structure due to mutation
Mitochondrial dysfunction	Oxidative stress, impaired insulin signaling
Reduced the capacity of receptors to binding to insulin	Decrease in the number of insulin receptors, reduction in functional receptors due to mutation, autoimmune antibodies against insulin receptors.
Mutations of GLUT-4	Inhibits glucose entering into dependent cells and impairs subsequent signaling pathways.
ER stress	Disrupts proper protein folding leading to accumulation of misfolded proteins.

ER stress: endoplasmic reticulum stress; IRS-1: insulin receptor substrates-1

1.4 IR and NAFLD

IR is the most common finding in NAFLD (25). Chronic elevation of plasma insulin in IR promotes hepatic lipogenesis and excessive TG accumulation in patients with NAFLD; therefore, NAFLD is strongly associated with metabolic syndrome and T2D (26). Several factors, such as hyperinsulinemia and hyperglycemia, along with elevated FFAs and pro-inflammatory cytokines levels, may alter insulin signaling in various tissues. These metabolic changes that develop and worsen IR are frequently observed in obese and NAFLD patients and predispose them to T2D development (11). Hepatic steatosis is associated with impaired insulin action in the liver, adipose tissue, and skeletal muscle, therefore, IR predicts NAFLD development (27).

In IR conditions, glucose transport is impaired in adipose tissue. However, sensitivity to the antilipolytic insulin effect is relatively maintained, resulting in preserving or expanding adipose stores (12). Furthermore, in IR, fat accumulation in the liver and

muscle is due to the outflow of FFA as a result of inadequate suppression of lipolysis in adipose tissue (11). In NAFLD, inability of insulin to suppress lipolysis in adipose tissue, leads to an increase in FFA released to the liver and an increase in de novo lipogenesis, which results from increased expression of the lipogenic enzyme through activation of the transcription factor SREBP1-c, both increases TG synthesis and decreases fatty acid beta-oxidation (27). Forkhead transcription factor (Foxa-2) stimulates hepatic fatty acid oxidation. However, it is inactivated by phosphorylation by either insulin receptor substrates 1 and 2 (IRS1 or IRS2) signaling pathways. In IR, Foxa2 remains sensitive to insulin, which suppresses its activity, inhibiting FFAs oxidation and causing lipid accumulation (25). Hepatic lipid overload also causes oxidative stress leading to mitochondrial dysfunction, exacerbating inflammation, and activating inflammatory pathways (11). Early normalization of insulin production and secretion is crucial in preventing and reversing obesity, IR, and T2D. Many possible dietary approaches are known to lower insulin, which may improve obesity and insulin sensitivity.

1.5 Dairy Consumption and Risk of Diabetes

Many studies have shown that dietary intervention programs significantly prevent and delay T2D in high-risk people (28–33). A study mentioned that implementing a prevention program and lifestyle adjustment, including a healthy diet and exercise, can prevent or delay about half of the new cases of diabetes; such programs can also prevent its complications (33).

Dairy, such as milk, yogurt, and cheese, is recommended as part of healthy foods, and 2-3 dairy servings/day is suggested by US dietary guidelines for adults and

adolescents (34). Dairy consumption impacts on human health have been investigated in extensive prospective cohort studies and limited randomized controlled trials (RCTs) (35). Many systematic reviews and meta-analyses of observational trials have been conducted to evaluate the association of dairy intake and risk of T2D. The results suggested overall beneficial effect (36–41).

A systemic review and meta-analyses published in 2018 that included studies from America, Asia, Europe, and Oceania evaluated the relationship between dairy and risk of metabolic syndrome (36). In the dose response meta-analysis, they used 6 studies including 3 cohort studies (2227 cases and 9259 participants) and 3 cross-sectional studies (4775 cases and 19818 participants) and found that 200 g/day of milk was associated with 13% lower risk of metabolic syndrome. Another meta-analyses looked at the association of dairy intake and risk of metabolic syndrome in 20 studies and found that the highest milk consumption was associated with 26% lower risk of MetS in elderly people compared to no milk consumption (42). The systematic review has long been thought to provide the greatest level of research evidence as they combine all available data, nevertheless additional data from randomized controlled trials is necessary to verify the observational studies, which cannot control for all possible confounding variables.The epidemiological data (34,38–40,43,44),and some RCTs (39,41) suggested a neutral or mild inverse association of dairy intake with diabetes risk and improvement of insulin sensitivity, which is mainly consistent for yogurt. Systemic review and meta analysis of cohort study found that yogurt consumption has a beneficial effect on body weight and reduces obesity risk, the leading risk factor for T2D (40). Eleven studies from meta-analysis of observational studies suggested that milk consumption is not associated with

diabetes risk, and cheese intake also has no association with T2D (35). However, another study suggested high-fat total dairy and cheese intake had a dose-dependent inverse association with T2D (34). A summary of evidence was published in a meta-analysis that included 22 cohort studies, 579,832 participants, and 43,118 T2D cases, and the results indicated that total dairy intake was inversely associated with T2D risk (35).

On the other hand, the interventional trials are less consistent, with four out of 10 studies in a systematic review of short-and long-term intervention studies suggesting a positive relationship between dairy intake and insulin sensitivity (45). In this systematic review most of the trials were very small and some trials were of healthy people who were assumed to be insulin sensitive. One trial of healthy people had a negative effect and 2 had no difference, therefore in order for benefits of dairy to be observed, a baseline defect in insulin sensitivity might be needed. Also, some studies were very short duration while the author pointed out that studies with a duration of 12-24 weeks had consistent benefits.

T2D and its complications are increasing, and there is rising evidence of clinically significant sex disparities. T2D is more commonly diagnosed in men when they are younger and have a lower BMI (46); nevertheless, the most significant risk factor, obesity, is more prevalent in women (46). Yet, the epidemiological evidence about sex-specific effects on dairy consumption and diabetes risk are very limited. A systemic review and meta-analysis showed that in adult men, high milk consumption was inversely associated with hypertriglyceridemia, while elderly women showed lower HDL cholesterol with high milk intake (42). Another study included 37,038 women concluded that higher dairy product intake during adolescence is associated with lower risk of T2D (47). While obese

postmenopausal women showed reduced risk of diabetes in response to higher intake of low-fat dairy products (48). Other studies suggested significant association between dairy intake and lower risk of diabetes in women than men (49–51).

The relationship between dairy fat and diabetes risk is uncertain. Some studies linked each specific fatty acid with the risk of diabetes. For instance, even-chain saturated fatty acids (SFAs) like myristic acid, palmitic acid, and stearic acid are associated with higher T2D risk. Markers of dairy fat intake like pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0) are odd-chain SFAs, and are inversely correlated with risk of metabolic disease (52). A systemic review and meta-analysis of observational studies suggested a protective effect of odd chain fatty acids against incident of T2D and showed that C15:0 was inversely associated with the pro-inflammatory mediator TNF-α (53). The same paper reported that higher odd-chain SFAs inversely associated with lower total cholesterol and TG (53). Even though the evidence is reasonably consistent about the benefits of dairy consumption on human health, the mechanisms of beneficial effects are not known precisely. However, animal studies tend to support the overall observations in humans. A study evaluated the effect of low dose supplementation of C15:0 in C57Bl/6J mice for 90 days. The result showed reduced pro-inflammatory mediators such as Interleukin 6, monocyte chemoattractant protein 1(MCP-1), and cytokine compared to non-supplemented control (54). The same study also found lower glucose, cholesterol, and body weight gain in response to C15:0 intake while only a high dose of C17:0 reduced the serum MCP-1 (54). Odd-chain fatty acids also inversely associates with NAFLD and supplementation with odd-chain SFAs is shown to improve hepatic steatosis (39,55). Another study suggested that dairy fat in cheese form significantly reduces total

cholesterol compared to the same amount of fat in other matrices (56). Even though the evidence is reasonably consistent about the benefits of dairy consumption on human health, the mechanisms of beneficial effects are not known precisely.

1.6 Previous Animal Trials for Dairy and Risk of T2D

Since human trials of dairy interventions are limited, animal trials have been conducted to study the impact of dairy consumption on glucose homeostasis to detect whether dairy intake can lower the risk of diabetes and the potential mechanisms for those effects. Calcium, protein, and fat have been examined as potential mediators of dairy's metabolic effects.

In animal model research studies, dairy calcium was suggested to prevent weight and fat regain (57). Also, calcium might suppress hepatic and adipose lipogenesis by its effect on leptin and Glucagon-like peptide 1 (GLP-1) signaling, decreased calcitriol level, and change in gut microbiota composition (58). Another study observed that a high calcium diet increased weight gain and hyperphagia compared to dairy calcium intake, which showed a preventive effect, suggesting that it is not the calcium responsible for these effects but other dairy components (59). An additional proposed hypothesis for calcium-related weight improvement is binding fat to calcium, then excreting the fat as calcium salt in the feces (60).

Dairy protein could be another explanation for dairy's effect on glucose homeostasis. A study showed that total dairy protein intake could prevent fat mass build-up in rats more than casein or whey alone during ad libitum high fat, high sugar feeding

(60). The potential mechanism of dairy protein for metabolic improvement might be through enhancement of weight loss and appetite control in addition to stimulation of protein synthesis, skeletal muscle growth, and function (61). Many studies investigated the effect of dairy fat on metabolic outcomes in obese animals. Supplementing sphingomyelin, which is part of the polar lipid of milk in mice fed HFD causes improvement in hepatic steatosis by reducing hepatic cholesterol and TG(55). Furthermore, adding odd chain fatty acids 15:0C to mice fed methionine-choline deficient for 4 weeks showed a protective effect against liver injury with decrease in AST and normalizing liver weight, suggesting improvement in the liver function due to odd chain fatty acid consumption (55).

Previous work from our lab suggested that trans-11 vaccenic acid, which is found in dairy, can improve glucose homeostasis by enhancing insulin secretion and the growth of islets in diabetic rats. This effect was associated with increased GPR40, a fat signaling protein found on beta-cells and the key protein mediating free fatty acid potentiation of insulin (62,63). Many studies suggested that milk consumption was associated with lower adiposity and increased insulin sensitivity(64–69). Furthermore, fermented milk enhances glucose and lipid metabolism by decreasing body weight and circulating glucose, besides eliciting dyslipidemia and insulin sensitivity (70).

The effect of yogurt intake on glucose homeostasis was evident in many studies (71–74). All studies suggested that yogurt modulates gut microbiota and alters hepatic lipid metabolism to regulate glucose homeostasis. A study evaluating the effect of ripening duration observed that 35 days of ripening duration improved glucose tolerance

and showed a significant reduction in adipose tissue along with a decrease in hepatic lipid content (75).

A study conducted in our lab in 2017 investigated the effect of low fat and high fat (LF/HF) cheese on glucose homeostasis in prediabetic and diabetic rat models. The results showed increased hepatic insulin sensitivity during the insulin tolerance test for HF and LF cheese in prediabetic groups. In contrast, only LF cheese improved glucose tolerance among the diabetic group. The study suggested that cheese consumption (LF/HF) may enhance glucose homeostasis in the prediabetic model, while only LF cheese improved glucose tolerance in the diabetic model (76). Table 1–2 shows a summary of experiments studying the effect of dairy on glucose homeostasis in animals.

Table 1—2 Rodent studies feeding milk, yogurt, or cheese and their effects on glucose homeostasis

Authors/country, year.	Aim	Type of dairy	Treatment details	Finding
Song et al. Korea 2016	To investigate the antidiabetic effect of fermented milk containing CLA on type 2 diabetes mice.	Fermented milk (FM+CLA)	32 male, diabetic C57BL/KsJ-db/db mice and 8 normalC57mice. Feeding for 6 weeks	FM+CLA ↓body weight and FBG in diabetic mice. Improved OGTT, ITT ↑HDL, ↓LDL&TG ↓Hepatic enzymes Improved hepatic ballooning
Matsumoto et al. Japan 2009	To determine whether the beneficial effects of cow milk on reduced insulin sensitivity are experimentally reproducible using normal dose of cow milk in rats.	Dextrine based diet vs Sucrose based diet. Cowmilk CM vs artificial milk AM.	24 male F344 rats. Feeding for 7weeks	CM Improved OGTT ↓ insulin,↓FBG, and ↓fructosamine

Yoshimura et al. Brazil 2018	To evaluate the supplementation with milk naturally enriched with PUFA and polyphenols in rats with diabetes.	Whole milk (COM-M) vs milk enriched with PUFA (PUFA+M) vs milk enriched with PUFA+ polyphenols (PUFA/P+M).	40 male, streptozotocin-treated diabetic, Wistar rats. Feeding for 35 days	COM-M Improved OGTT ↓Fructosamine PUFA+M ↓LDL, ↓FBG PUFA/P+M ↓LDL, ↑GCM mass
Eller et al. Canada 2010	To determine the effect of different ca-enriched dairy protein sources on weight gain in obese Sprague-Dawley rats.	Skim milk powder (SMP) vs casein vs whey protein	64 high-fat, high-fructose diet obese Sprague-Dawley rats. Feeding for 8weeks.	SMP ↓body fat% ↓body weight ↓FBG Improved HOMA-IR ↓SREBP1c
Trinchese et al. Italy 2018	To evaluate whether skeletal muscle mitochondrial function, efficiency and dynamics is differently affected by cow, human, and donkey milk, supplementation.	Cow milk vs human milk vs donkey milk.	28 male Wistar rats. Feeding for 4 weeks.	Cow milk ↑body weight ↑leptin ↓ adiponectin
Kergoat et al. France 1992	To examine whether chronic milk feeding leads to an impairment of insulin-mediated glucose metabolism.	Raw cow milk (RCM)	Female Wistar rats. Feeding for 18 days.	RCM ↓hepatic glucose production.
Yamin et al. Israel 2013	To assess whether long term supplementation with low or whole fat milk would differentially affect body weight, hormone secretion, and key metabolic pathway in liver and white adipose tissue in newly weaned mice.	3% milk vs 1% milk	Male newly weaned C57BL/6 mice. Feeding for 17weeks.	3% milk ↑body weight and food intake ↓FBG ↓ hepatic FAS ↓WAT FAS
Qu et al. China 2018	To explore the mechanism of bioactivity of Lac-Q14 as starter culture in fermented yogurt in diabetic rats.	Yogurt/Lac-Q14 (Y/L-Q14) separated from yak yogurt	40 male Wistar rats. FBG>11.1mmol/L treatment for 13 weeks	Y/L-Q14 Improved OGTT ↓G6P and PEPCK expression(liver). ↑GLP-1 ↓TG, LDL.
Lasker et al. Bangladesh 2019	To determine the effects of yogurt supplementation on fat deposition, oxidative stress,	HFD+ yogurt	24 male, diet-induced obese, Wistar rats. Feeding for 8weeks.	Yogurt ↓body weight Improved OGTT ↓FBG Improved hepatic

	inflammation and fibrosis in the liver of obese rats.			steatosis ↓AST,↓TG,↓LDL Normalize oxidative stress markers.
Miao et al. China 2012	To assess if Synbiotic can improve the survival of bacteria crossing the upper part of the gastrointestinal tract. Thereby enhancing their effects in the large bowel. In addition, their effects might be additive or even synergistic.	Commercial yogurt (CY) vs synbiotic supplemented fermented milk (SSFM).	100 Wistar rats (50 males and 50 females) divided into 5 groups of diet. Feeding for 30 days.	SSFM ↓glucose level ↓TG
Johnson et al. USA 2007	To examined whether yogurt supplementation attenuated the weight gain and insulin resistance in mice.	Dried yogurt powder (DYP)	113 F1 generation male mice fed moderate-fat diet. Feeding for 4 weeks.	DYP ↓body weight ↑basal glucose uptake in adipose tissue ↑ energy content of feces.
Hanning et al. Canada 2018	To determine effects on glucose and insulin tolerance of feeding LF or HF to rats fed a HFD.	HFD+cheese(LF/HF).	64 male prediabetic Sprague-Dawley rats fed HFD. Treatment for 8 weeks	LF/HF cheese ↑ body weight. Improved ITT ↓ G6Pase (liver). Normalized certain PC/LPC species in serum.
Geurts et al. France 2012	To determine the influence of administration of dairy (cheese-based) products on glucose tolerance, hepatic lipid content, and profiles.	0,15, and 35 days of ripening cheese.	30 db/db male C57BL/6. Feeding for 4 weeks	35 days ripened cheese Improved glucose tolerance ↓oxidative stress markers ↓hepatic lipid content ↓mRNA expression of FAS

Abbreviations: CLA-conjugated linoleic acid, FBG-fasting blood glucose, OGTT-oral glucose tolerance test, ITT-insulin tolerance test, HDL-high density lipoprotein cholesterol, LDL-low density lipoprotein cholesterol, TG-triglyceride, TC-total cholesterol, LF-low fat, HFD-high fat diet, ALT-alanine transaminase, G6P-glucose 6 phosphatase, PC-phosphatidylcholine, LPC-lysophosphatidylcholine, GLUT2-glucose transporter 2, Lac-Q14-Lactobacillus casie, PEPCK-phosphoenolpyruvate carboxykinase, GCM-gastrocnemius muscle, GLP-1-glucagon like peptide-1, PUFA-polyunsaturated fatty acid, ALT/ALP ratio-alanine transaminase/alkaline phosphatase ratio, DIO-diet induced obesity, HOMA-IR-homeostatic model assessment of insulin resistance, SREBP1c-sterol regulatory element-binding transcription factor1, PPAR gamma-peroxisome proliferator-activated receptor gamma, FAS-fatty acyl synthase, WAT-white adipose tissue, L casei-lactobacillus casei, FFAs- free fatty acids, F1 generation-cross between BTBR males and C57Bl6 females.

These studies show that milk, yogurt, and cheese intake can improve metabolic risk markers besides their beneficial effect as a rich source of valuable micronutrients. Also, they can potentially use various physiological activities, including reduced body weight and enhanced glucose tolerance. Moreover, dairy consumption showed improvement in lipid metabolism, which is independent of weight change. Epidemiological evidence seems to support the inverse association between dairy consumption and its potential impact on counteracting chronic diseases such as type 2 diabetes. However, significant variability in the studies' focus, duration of treatment, and type of animals with different physiological backgrounds contributed to the result's differences. Lately, experts have suggested that considering the food as a whole instead of looking at specific nutrients, as the combination of food elements and their structure could change their physiological effects (77).

1.7 Dairy Matrix

Foods contain many different nutrients that are connected in a complex structure called the food matrix (78). The structure of the food and the nutrients within will determine the nutrient digestion and absorption and affect the food’s whole nutritional properties. The food matrix may affect health differently compared to single nutrients. Traditionally, the relationship between health and diet was assessed based on individual food components such as protein, fat, carbohydrate, and micronutrients separately. While most current dietary guidelines include dairy products as part of a healthy diet, they recommend low-fat or fat-free versions to reduce saturated fat intake (78) this strategy does not consider any potential benefits of a food matrix that includes fats.

Dairy components including fat, protein (whey and casein), calcium, magnesium, phosphate, potassium, and milk fat globule membrane (MFGM) are probably the main reasons for the beneficial effects of dairy on human health (39). MFGM is the biological membrane that encases milk's lipid droplets, and all high-fat dairy is rich in MFGM (except butter) (79), However, low fat dairy, in particular buttermilk, is the greatest source of MFGM (80). Dairy's form and matrix influence its effect on wellbeing (81). The physical state of the dairy food could impact appetite and gastric emptying. For instance, a study found that intake of solid dairies such as cheese and yogurt inhibited hunger more than liquid dairy like milk (82). Traditional dairy foods are believed to be "functional" foods that possibly prevent disease (81). The functional properties depend on the bioaccessibility and bioavailability of dairy food (81). The bioaccessibility of a nutritional component is represented by its release from its food matrix into the gastrointestinal system, where the bioavailability is represented by the fraction that is absorbed and reaches the bloodstream (81).

Cow's milk has 32 g of protein per liter, making it an excellent supply of essential amino acids, with a wide range of biological activities, including antimicrobial, enzymes, and growth factors. Dairy protein contains casein and whey protein in an 80:20 ratio, both are absorbed at various times after digestion, implying a natural time-dependent mechanism for amino acid and peptide distribution (83). Dairy protein has been shown to improve satiety as a direct result of particular peptides, in addition to the satiety effects of intact casein and whey protein. According to the same study, specific peptides known as glycol-macropeptides are produced during the cheese production process. These

peptides have been found to activate pancreatic and stomach satiety hormones compared to whey protein alone (81).

Potassium is an intracellular cation and plays important roles in cellular function, membrane polarization, energy metabolism and fluid balance (84). Abnormal potassium homeostasis can lead to heart, muscle, and nerve dysfunction. Dairy is rich in potassium and makes a significant contribution to dietary potassium intake. Eight ounces of yogurt contains 352 mg and a cup of whole milk provides 349 mg of potassium (84). WHO recommends a potassium intake of at least 3510 mg/day for adults (84). Dairy consumption in the US population is below the recommended intake, which in part contributes to inadequate dietary potassium intake (84).

Choline is a vital nutrient and is present in all cells as a precursor for phospholipid production, such as phosphatidylcholine (PC) and sphingomyelin (SM). Both are found in the cell membrane and play crucial roles in cell signaling (80). Choline impacts liver function and is required for very low-density lipoprotein VLDL production to transporter fat from the liver. Low choline is found to be associated with fatty liver and liver damage (85). Dairy products are a good source of choline, and milk has about 32.9 mg total choline, 2.2 mg PC, and 1.1 mg SM per serving (86). It is clear that the dairy matrix impacts its physiological effect, but how much dairy should we take to get its benefits?

1.8 Comparison of Recommended and Actual Doses of Dairy Used in Research

Dairy amount recommendations vary worldwide, ranging from 1-5 servings/day (87). In Canada, the 2019 update of Canada's Food Guide considers dairy part of the protein group and does not specify a recommended amount, while the US guideline

recommends 3 servings/day as part of a healthy dietary pattern. The recommended serving size is 250 ml of milk, 175 g of yogurt, and 50 g of cheese (87). Several human research studies have evaluated the effect of dairy dose and type on health outcomes. A meta-analysis of observational studies found a different amount of dairy intake was associated with a lower risk of MetS (36); a summary of these findings is presented in Table 1–3.

Table 1—3 Summary of the association of dairy dose and type with the risk of T2D adapted from Lee et al 2018 (36)

Type of dairy	No. of studies	Amount of dairy	Finding
Total dairy	9	200g/day	associated with 9% lower MetS risk.
Milk	6	200g/day	associated with 13% lower MetS risk
Yogurt	3	100g/day	associated with 18% lower MetS risk

In a meta-analysis of 22 prospective cohort studies on dairy intake, small protective linear associations of T2D risk for total and low-fat milk intake were observed that were dose-dependent (3% and 4% risk reduction, per 200 g/day) (35). A non-linear association for yogurt intake with 80 g/day having the lowest risk (14% risk reduction), with no additional benefit seen above this dose (35). This result aligned with the result from Framingham Heart Study Offspring cohort. The study investigated the relationships between dairy product intake and long-term risk of prediabetes in healthy participants and risk of T2D among people with prediabetes in the middle-aged adult cohort (34). In this study, total, low-fat, and high-fat dairy consumption was associated with 39%, 32%, and 25% lower risk of incident prediabetes, respectively. Prediabetes Incidence was

nonlinearly associated with total, low-fat, skim milk, whole milk, and yogurt intakes. Only high-fat dairy and cheese showed evidence of dose-responsive, inverse associations with incident T2D, with 70% and 63% lower risk, respectively.

Another meta-analysis published 2019 showed that total dairy product consumption was inversely associated with the risk of MetS (9 studies). Low-fat dairy and total yogurt consumption were inversely associated with the risk of MetS (2 studies and 4 studies respectively). Low-fat yogurt and whole-fat yogurt were inversely associated with the risk of MetS (2 studies each). Total milk consumption was inversely associated with the risk of MetS (6 studies). No association between whole-fat dairy consumption with MetS risk. The findings indicate that the consumption of total and low-fat dairy products, milk, and yogurt is inversely associated with the risk of MetS (37).

A comprehensive study of dairy product intake in the multicentric European Prospective Investigation into Cancer and Nutrition (EPIC) cohort of European populations found that; Europeans consume high amounts of dairy compared to American and Asian populations (88). The study showed that intake of 286 g/day in women and 250 g/day in men was not associated with T2D risk for total dairy or milk intake; however, yogurt and fermented milk consumption were associated with decreased risk of T2D. The same study suggested that cheese intake was inversely associated with the risk of T2D and persisted even with increased amounts of cheese intake (88).

Animal models have been used in some studies to evaluate the effects of dairy consumption on diabetes-related outcomes. Based on my literature review, varying amounts of dairy were used in animal experiments. Rat models have been widely used

to evaluate the relationship between dairy intake and its effect on health outcomes. The amount of milk provided ranged from 22-25ml or 5% of the body weight; these amounts resulted in a significant beneficial effect on glucose and lipid metabolism by decreasing FBG, improving hepatic steatosis, and reducing inflammation (64,65,68,72,89). In the mouse studies, the dose was inconsistent. Some studies did not mention how much dairy was given, providing ad libitum dairy consumption (90,91). Other studies specified the amount of dairy such as 100g/kg of the diet for cheese, which is about 300 mg (75), 10.75g/100g of diet for yogurt intervention which is about 323 mg (90), while the milk intake in the mouse studies ranged from 0.5 to 2 ml per day (92,93).

In summary, dairy products have beneficial effects on human health, particularly metabolism, which are well established and confirmed by a significant number of previous human studies and animal trials. However, no study compared different products of dairy and their effects on glucose and lipid metabolism in one experiment; also, the mechanism of these effects is unclear, and it is unknown if all dairy has the same mechanism of action.

This project aims to narrow this gap by comparing the effects of cheese, milk, and yogurt products with higher fat content on glucose homeostasis in prediabetic mice and exploring possible mechanisms. We hypothesize that dairy products including regular fat milk and yogurt have a similar effect to regular fat cheese on prediabetic mice by improving insulin sensitivity. However, the mechanism by which regular fat cheese, milk, and yogurt improve glucose and lipid metabolism could be different because milk is not a fermented dairy product like cheese and yogurt. Therefore, the objectives of this study are:

1. To define and compare the metabolic phenotype of prediabetic mice with respect to circulating glucose, insulin, total cholesterol, triglycerides, and in vivo insulin sensitivity after including regular fat milk, yogurt, or cheese in the diet.

2. Based on previous findings that cheese alters liver metabolic indices, compare glucose and lipid metabolism markers in the liver in response to regular fat milk, yogurt, and cheese intake in the prediabetic model.

Chapter 2 : Methods

2.1 In Vivo Protocols

2.1.1 Animal Treatment and Protocol

All animal protocols were approved by the Animal Care and Use Committees at the University of Alberta (animal use protocol AUP00003066). The guidelines of the Canadian Council on Animal Care were followed for all procedures. 6-week-old C57BL/6 mice were purchased from Charles River Canada (St. Constant, QC, Canada) and shipped to the University of Alberta. The mice were acclimatized to the facility for one week and housed as they arrived, with 4 sex-matched animals per cage under a reverse 12:12 light: dark cycle. A high-fat diet (HFD) was provided ad libitum to 4 groups of each sex to induce insulin resistance, while one group of each sex was provided a low-fat diet (LFD). After one week, the dairy product (Milk, Yogurt, Cheese) was provided 2 hours each day to individual mice from the same cage after 4 hours of fasting. HFD and LFD control groups were provided with half a Cheerio to control the handling and novel food supplied to the dairy groups. Dairy feeding was done 5 days/week for 8 weeks. The first week was considered a training period after which the mice readily consumed all dairy products. Body weight was monitored weekly, and the baseline was when I introduced HFD/LFD (one week before dairy started). Food intake was measured over 24 hours 3 times during the last week of the study. At week 7, the mice were randomized to undergo either an insulin tolerance test (ITT) or a pyruvate tolerance test (PTT).

We conducted 3 trials. In the first and second trials, we used 40 mice in each, 20 males and 20 females, but because the HFD failed to induce IR in female mice, we used only

male mice in the last trial. Thus, the total number of mice was 60 males (12 per group) and 40 females (8 per group) Figure 2–1.

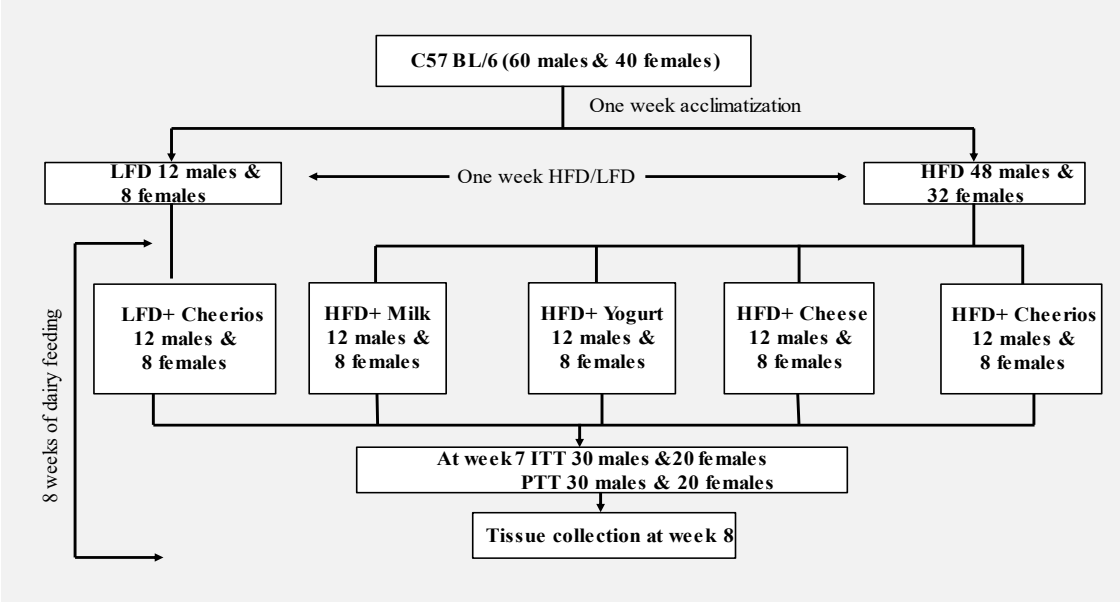


Figure2-1Schematic diagram of experiment design

2.1.2 Diet

The mice were randomly distributed into 5 diet groups as follow:

1. High-fat control (HFD) with 45 kcal% fat.
2. Low-fat control (LFD) with 10 kcal% fat.
3. HFD+Milk (Lucerne 3.25 g% fat).
4. HFD+Yogurt (ACTIVIA with active probiotics, 2.9 g% fat).
5. HFD+Cheese (Armstrong with 31 g% fat), it is similar to high fat cheese used in the cheese project (76). Table 2—1.
6. For the control groups, we provided half of a plain Cheerio (General Mills) to control for handling, which might affect stress and, therefore, blood glucose and hormones Table 2–2

Table 2—1 Dairy product classification according to milk fat %

Dairy used in this project	Milk fat %
Regular-fat Milk	3.25%
Regular-fat Yogurt	2.9%
Regular-fat Cheese OR High-fat Cheese	31%
Low-fat Cheese	20%

7. The dairy products were chosen because they are regularly consumed by Canadians and are palatable to mice. The ingredient composition of the HFD and LFD are summarized in Table 2–1. The macronutrient composition of each diet is summarized in Table 2–2.

Table 2—2 Composition of the experimental diets (g/kg)

Ingredients	HFD(D12451I)	LFD(D12450B)
Casein, 80 Mesh	233.1 g	189.6 g
Cystine, L	3.5 g	2.8 g
Starch, Corn	84.8 g	298.6 g
Maltodextrin 10	116.5 g	33.2 g
Sucrose	201.4 g	331.7 g
Cellulose, BW200	58.3 g	47.4 g
Soybean Oil	29.1 g	23.7 g
Lard	206.8 g	19 g
Mineral Mix S10026	11.7 g	9.5 g
Dicalcium phosphate	15.1 g	12.3 g
Calcium Carbonate	6.4 g	5.2 g
Potassium Citrate, 1 H2O	19.2 g	15.6g
Vitamin Mix V10001	11.7 g	9.5 g
Choline Bitartrate	2.3 g	1.9 g
FD&C	Red Dye#40 0.06 g	Yellow Dye#5 0.05 g
Total	1000 g	1000 g
Kcal/g	4.73	3.85

Note: all values are adjusted to reflect weight in 1000 g of diet. HFD, High fat diet. LFD, Low fat diet.

2.1.3 Dairy dose calculation

The amount of dairy was calculated based on the body weight. The amount was supposed to be equivalent of two human adult servings of regular dairy product. The recommended serving size according to American guideline is 250 ml for milk, 175 gm for yogurt and 50 gm for cheese (87). Adult humans consume 2000 calories per day and one serving of milk has 150 calories, one serving of yogurt has 175 calories, and one serving of cheese has 200 calories. One serving of milk is equals to 7.5% of the total calories per day for adult human, while one serving of yogurt is equals to 8.75%, and one serving cheese is equals to 10% of total daily calories for human adult. Mice consume about 4 g of food (~16 Kcal/day) (94). The calculation and final amount of dairy foods are presented in Table 2—2. In the previous study the amount of cheese (31% fat cheddar cheese) was 1.85 g/ day. This was calculated based on the total rat food intake of 20.3 g/day and the diet containing 9.12 g of cheese/100 g of food (76).

Table 2—3 Amount of dairy for mouse diets calculated according to serving sizes for human diets

	Calories/serving	Serving size	Total calories intake in human adult	Total calories intake in mice	Energy from one dairy serving for mice	1 serving of dairy for mice	2 serving equivalents of dairy for mice	What we gave in our trial
			2000	16				
3.25% milk	150	250 ml			1.2 Kcal	2 ml	4 ml	0.425 ml
2.9% yogurt	175	175 ml			1.4 Kcal	1.4 ml	2.8 ml	0.3 ml
31% cheddar cheese	200	50 g			1.6 Kcal	0.4 g	0.8 g	0.05 g

Table 2—4 Nutritional composition of experimental food, Milk, Yogurt, Cheese, (per half serving).

	Milk	Milk (given to mice)	Yogurt	Yogurt(given to mice)	Cheese	Cheese (given to mice)
Measure (half serving)	125 ml	0.425 ml	87.5 ml	0.300 ml	25 g	0.05 g
Weight (g)	129	0.439	92.5	0.317142857	25	0.05
Energy (Kcal)	77.5	0.26	91.5	0.31	101	0.20
Energy (KJ)	323.5	1.10	383.5	1.31	424	0.85
Protein (mg)	4000	13.60	3500	12.00	6000	12.00
Carbohydrate (mg)	6000	20.40	15000	51.43	500	1.00
Total sugar (mg)	7000	23.80	12500	42.86	trace	trace
Total fat (mg)	4000	13.60	2000	6.86	8500	17.00
Saturated fat (mg)	2700	9.18	1150	3.94	5250	10.50
Cholesterol (mg)	13	0.04	8.5	0.03	26.5	0.05
Calcium (mg)	145.5	0.49	113.5	0.39	180.5	0.36
Average Choline* (mg)	16.45	0.06	12.8	0.04	7.1	0.01
Iron (mg)	0.05	0.00	0.05	0.00	0.15	0.00
Sodium (mg)	51.5	0.18	49	0.17	155.5	0.31
Potassium (mg)	184.5	0.63	167.5	0.57	24.5	0.05
Magnesium (mg)	13	0.04	10	0.03	7	0.01
Phosphorous (mg)	117.5	0.40	83.5	0.29	128	0.26
Vitamine A (RAE)	36	0.12	_	_	66.5	0.13
Vitamine D (mcg)	1.35	0.00	_	_	0.05	0.00
Folate (DFE)	6.5	0.02	_	_	4.5	0.01
Vitamine B12 (mcg)	0.565	0.00	0.155	0.00	0.21	0.00
Riboflavin (mg)	0.235	0.00	0.075	0.00	0.095	0.00

Abbreviation,mcg-microgram, RAE-rational activity equivalent, DFE- dietary folate equivalent. Value adjusted to half serving and to the equivalent amount given to the mice. Average choline* adopted from C. Richard et al. / Journal of Food Composition and Analysis 45 (2016) 1–8 (86)

Table 2—5 Diet Macronutrient Composition expressed as % of Kcal for HFD, LFD, HFD+Milk, HFD+Yogurt, and HFD+Cheese.

	HFD	LFD	HFD+Milk	HFD+Yogurt	HFD+Cheese
Protein	20%	20%	19.6%	19.5%	19.8%
Fat	45%	10%	44.2%	43.9%	44.5%
Carbohydrate	35%	70%	34.4%	34%	34.6%
Dairy protein	-	-	0.5%	0.4%	0.4%
Dairy fat	-	-	0.5%	0.3%	0.5%
Dairy carbohydrate	-	-	0.8%	1.8%	0.06%

Abbreviation, HFD:high fat diet. LFD:low fat diet

2.1.4 Insulin Tolerance Test (ITT)

The purpose of the ITT is to assess changes in insulin sensitivity (95) in response to Milk, Yogurt, and Cheese intake. ITT was performed on half of the animals in each diet group following 4 hours of fasting (n=30 males and 20 females). This fasting period was chosen to ensure that while insulin would have been cleared from circulation, glycogen stores would not have been depleted by prolonged fasting (95). Blood glucose concentration was measured from a tail vein sample using a glucometer (Contour Next, Bayer, Leverkusen, Germany) at time zero. Human insulin (Sigma Aldrich, St. Louis, MO, United States) was diluted using saline solution, and a dose of 26 µg/kg body weight was given intraperitoneally to the mice. Blood glucose was measured at 15, 30, 60, 90, and 120 minutes.

2.1.5 Pyruvate Tolerance Test (PTT)

The purpose of PTT is to measure hepatic gluconeogenesis upon provision of the substrate pyruvate (96). PTT was conducted on the other half of the animals (n=30 males and 20 females) following overnight fasting. Blood glucose was measured at time point 0 by glucometer (Contour Next, Bayer, Leverkusen, Germany), then sodium pyruvate solution was injected intraperitoneally at a dose of 2g/kg. Glucose was measured at 10, 20, 40, 60, 90, and 120 minutes.

2.2 Tissue Collection

Animals were fasted overnight prior to euthanasia at the end of the 8th week. Half of the animals (n=30) were injected with 13 µg/kg body weight of insulin in saline 10

minutes before euthanasia using CO_2, and tissues were collected. A 500-1000 µl blood sample was collected by cardiac puncture, then centrifuged for 10 minutes at 4000 RPM and 4C to separate serum. Liver, epididymal fat, brown adipose tissue, skeletal muscle, ileum, and colon were removed, washed in saline, and weighed. Samples of liver, colon, and fat were prepared for histology by fixing in phosphate-buffered formalin. The remaining tissues were frozen in liquid nitrogen and stored at -80 ^{o}C until further study.

2.3 In Vitro Protocol

Serum obtained at the time of tissue collection from animals not injected with exogenous insulin was used to quantify endogenous fasting insulin secretion using ALPCO mouse insulin Elisa kit (Alpco, Salem, NH, USA). Serum was also used to measure plasma triglycerides (TG) and non-esterified fatty acids (NEFA) (Wako Pure Chemical Industries Ltd., Chuo-ku, Osaka, Japan). Direct colorimetric enzymatic reactions were conducted as per the manufacturer's instructions. ALT activity was measured in serum samples using a commercially available kit (Abcam, Cambridge, MA, USA) following the manufacturer's instructions.

2.3.1 Liver Lipid Content

Frozen liver samples of approximately 100 mg were washed with cold PBS, then resuspended and homogenized in 1 mL of 5% NP-40/ddH2O solution using a sonicator for 1 minute. Samples were then heated to 80 – 100°C in a water bath for 5 minutes until the solution became cloudy, then cooled down to room temperature. Samples were centrifuged for 2 minutes to remove any insoluble material. Finally, samples were diluted

to 10-fold with ddH2O before proceeding according to the kit manufacturer's instructions (Wako Pure Chemical Industries Ltd., Chuo-ku, Osaka, Japan).

2.3.2 Immunoblotting

Approximately 100 mg of liver samples were weighed out and added to labeled 1.5 mL screw cap microtubes (DiaTEC, Kitchener, ON, Canada) with ~250 µL of 1mm glass beads (BioSpec Products, United States) and 300 µL of LIPA buffer. Samples were homogenized for 40 seconds two times with 5 minutes in between, with the samples kept on ice. Then all supernatant was aspirated into new microcentrifuge tubes. Tubes were centrifuged at 12,000 RPM for 20 minutes at 4°C. The protein concentration of each sample was determined using a colorimetric assay (in duplicate) using the modified LOWRY protein assay method. A microplate layout was created, and 40 µL of standards, or 1 µL of liver + 39µL of ddH2O was added, in duplicate, to each well. Bovine serum albumin was used as a protein standard. With a multichannel pipette, 200µL of Lowry Reagent (Sigma, St. Louis, MO, United States) was added to each well and incubated at room temperature for 10 minutes. Folin & Ciocalteu Phenol Reagent 2.0N (Thermo Scientific, Waltham, MA, United States) was diluted 1 in 2 with ddH2O, and 20µL of the dilute mixture was added to each well and incubated for 30 minutes. Absorbance was measured at 750 nm using a spectrophotometer. Concentrations were interpolated in GraphPad Prism (GraphPad Software Inc., CA, United States) using bovine serum albumin standards to calibrate the readings. For immunoblotting, protein samples were diluted into a final concentration of 2µg/µl with LIPA buffer and SDS loading buffer, boiled at 100°C for 5 minutes, and cooled on ice. About 40µg of protein was loaded for each sample. Proteins were separated on 10% SDS-PAGE gels and transferred to the

nitrocellulose membrane. Membranes were blocked for 1 hour with 5% skim milk in Tris-buffered saline (TBS), then incubated with primary antibodies overnight in a cold room. The following day the membranes were washed 3 times for 5 minutes each and then incubated with 2.5% skim milk in TBS and appropriate secondary antibodies for 1 hour. Blots were developed using ECL (ThermoFisher Scientific) and imaged with a Bio-Rad ChemiDoc MP imaging system. A summary of antibodies used in the western blot is in Table 2–3.

Table 2—6 Antibodies and dilutions used in western blot

Antibody	Source	Company/Catalog Number	Dilution	Incubation Condition
Anti-glucose-6 phosphatase α (G6Pase- α)	Rabbit polyclonal antibody	Santa Cruz #Sc-25840	1:200	4°C, overnight
Anti-phosphoenolpyruvate carboxykinase (PEPCK)	Rabbit polyclonal antibody	Cayman Chemical #10004943	1:200	4°C, overnight
Anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	Mouse Monoclonal IgG1	Invitrogen #MA5-15738	1:1000	4°C, overnight
Anti-Mouse IgG	Goat	Sigma-Aldrich #A4416	1:2000	RT 1 hour
Anti-Rabbit IgG	Goat	Sigma-Aldrich #A9169	1:4000	R.T, 1 Hour

Abbreviation: R.T., room temperature.

2.3.3 Liver Histology

At the time of tissue collection, we observed some differences between mice's liver color as some were pink and others were yellowish-brown. Liver tissue prepared in paraffin blocks was cut into 5 μm sections and affixed onto glass slides. One slide for

each sample was stained using hematoxylin & eosin. 7 photo microscopic images with 20X magnification of each slide were taken using a Zeiss AxioCam HR microscope attached to a Canon Power Shot camera. Liver samples were used to measure macrovesicles area, microvesicles area, the total number of vesicles, microvesicular steatosis, and macrovesicular steatosis. Vesicle size greater than 15μ was considered as macrovesicles, whereas vesicle size less than 15μ was considered as microvesicles (97). ImageJ software “freehand selections” tool was used to measure fat droplets.

2.3.4 Echo MRI

Echo-MRI Whole Body Composition Analyzer was used to measure the mouse body composition including fat tissue, lean tissue, free water, and total water.40 males and 20 females were used, and mice were placed in appropriate-diameter tubes to make the mice remain still, then put into the machine slot and scanned. Data for fat mass and lean mass were collected and presented as a percentage of body weight.

2.4 Statistical analysis

Male and female mice were analyzed separately. Data were analyzed by GraphPad PRISM software for all experiments and expressed as the mean ± SEM. They were first checked for normal distribution using the Shapiro-Wilk test and analyzed by one-way or two-way ANOVA (or equivalent nonparametric tests as appropriate). Post-hoc tests were adjusted for multiple comparisons. A p-value < 0.05 was considered statistically significant. The total decrement in blood glucose from baseline for ITT was measured using the Prism software and considered peaks that went below the baseline only, thus it was called “the area of the curve” (AOC). While for PTT, we measured the

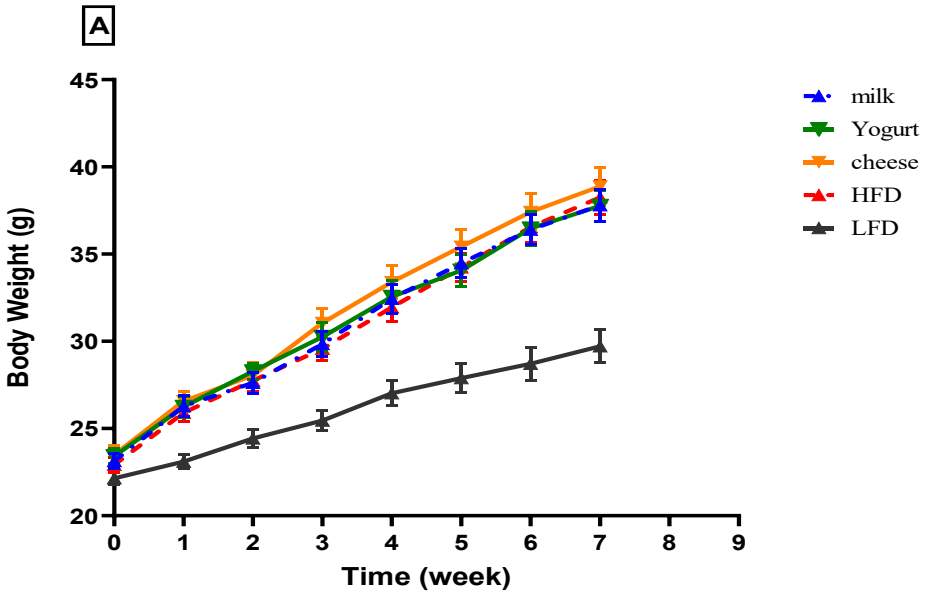
total and incremental area under the curve (AUC, iAUC) by measuring the peaks above zero and above the baseline, respectively.

Chapter 3 : Results

3.1 Effect of Dairy Consumption on Metabolic Phenotype in Insulin Resistant Male Mice

3.1.1 Weight Changes and Caloric Intake

There was a significant effect of diet (P<0.0001), time (P<0.0001), subject (P<0.0001), and interaction of diet × time (P<0.0001) on body weight (Figure 3-1(A)). The body weight increased in the Yogurt group at the first week of feeding relative to LFD (P=0.0248). By the second week of dairy feeding, all the HFD groups were significantly higher in body weight than the LFD group until the end of the study. Body weight changes showed that all HFD groups gained similar amount of weight compared to LFD group (Figure 3-1(B)). Energy intake was normalized for body weight (kcal/kg of body weight) for each cage and expressed as the mean intake per mouse. There was no significant effect of diet on energy intake (Table 3—1).



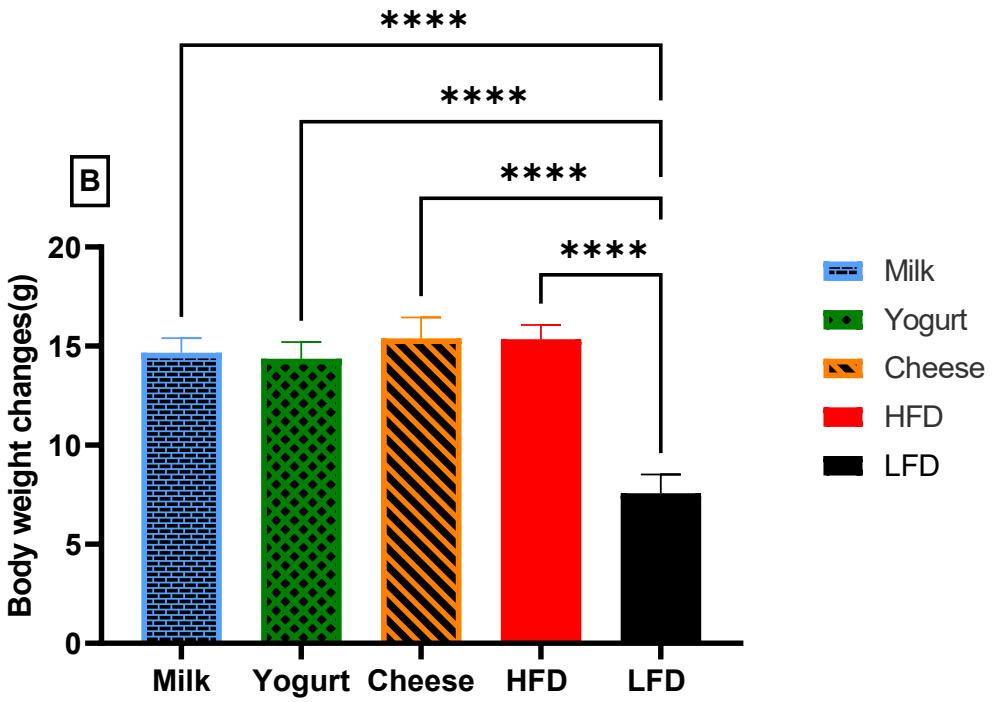


Figure 3-1 Weekly body weight for male mice fed HFD+Milk, HFD+Yogurt, HFD+Cheese, HFD, or LFD diets(A). Body weight changes for male mice fed HFD+Milk, HFD+Yogurt, HFD+Cheese, HFD, or LFD diets(B). Data are presented as mean ± SEM. One-way ANOVA followed by Tukey's multiple comparison test was performed. N per group=12 (three trials combined).

3.1.2 Fasting Blood Glucose and Insulin Concentrations:

Fasting blood glucose was obtained in mice following 4 hours of fasting prior to ITT and overnight fasting prior to PTT (Figure 3-2). There was a significant difference in blood glucose concentrations in the Milk group after 4 hours of fasting (P=0.001) and in all HFD groups after overnight fasting (P=0.005) compared to the LFD group. There was no significant difference in insulin concentrations between groups. A summary of the metabolic phenotype is presented in (Table 3—1). Due to insufficient samples, we could not calculate HOMA-IR to represent the degree of insulin resistance.

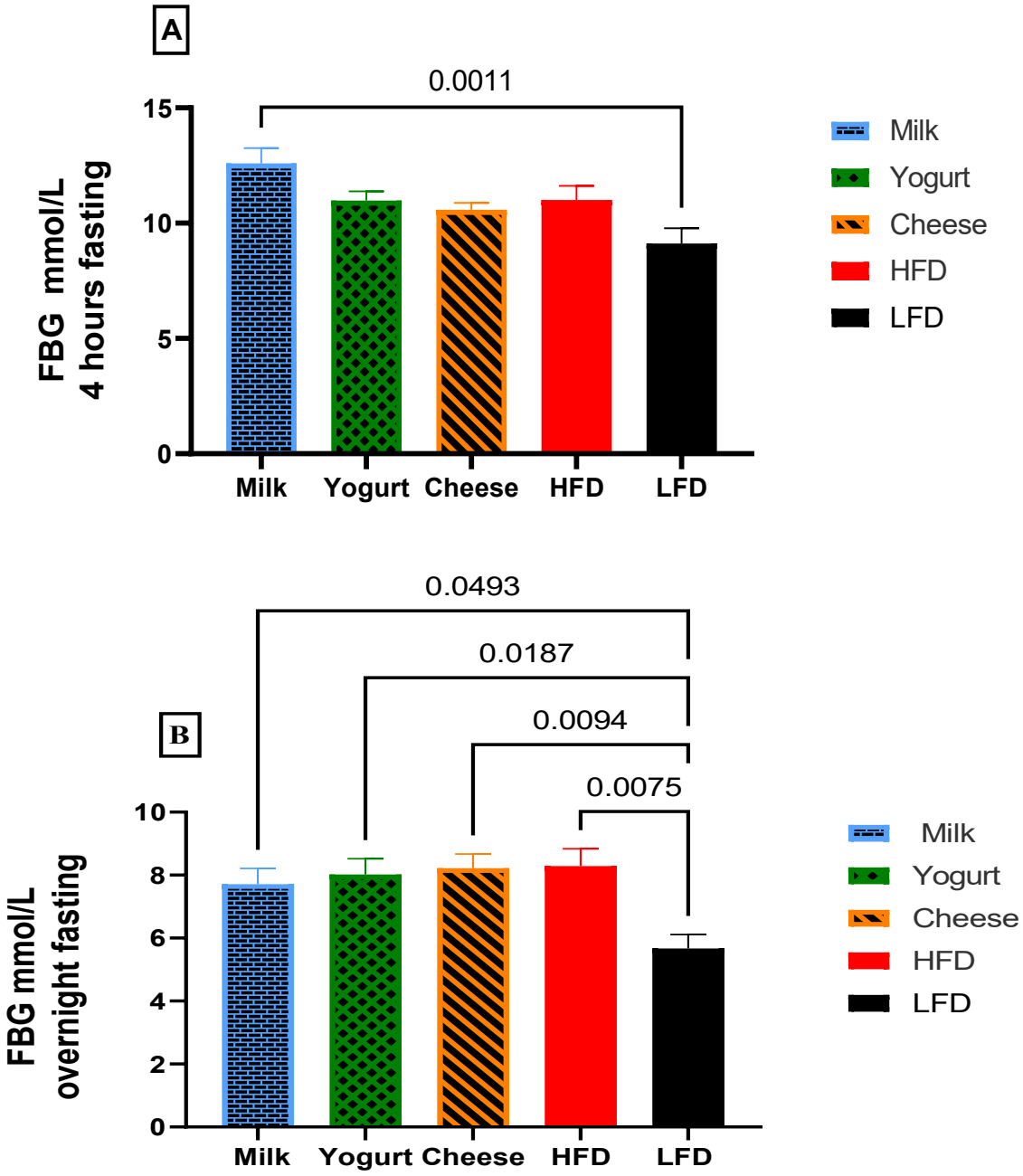


Figure 3-2 Blood glucose concentration after 4 hours of fasting (A) and overnight fasting (B) for mice fed HFD+Milk, HFD+Yogurt, HFD+Cheese, HFD, or LFD diets. Data are presented as mean ± SEM. One-way ANOVA test was performed followed by Tukey's test for multiple comparisons. n per group=6.

Table 3—1 Metabolic Profile of Male Mice

	Diet group					
	Milk	**Yogurt**	**Cheese**	**HFD**	**LFD**	**P-value**
Initial BW (g)	23.1±0.5 n=12	23.4±0.4 n=12	23.5±0.6 n=12	22.9±0.4 n=12	22.1±0.3 n=12	0.2212
Final BW (g)	37.8±0.9⁑ n=12	37.8±0.9⁑ n=12	38.9±1.1⁑ n=12	38.3±1⁑ n=12	29.7±0.9 n=12	<0.0001
BW changes (g)	14.7±0.7⁑ n=12	14.4±0.9⁑ n=12	15.4±1.1⁑ n=12	15.3±0.7⁑ n=12	7.6±1 n=12	0.0005
FBG(mmol/L) overnight fast	7.7±0.5* n=6	8.0±0.5* n=6	8.2±0.5⁑ n=6	8.3±0.6⁑ n=6	5.7±0.4 n=6	0.005
FBG(mmol/L) 4hrs fast	12.6±0.7⁑ n=6	10.98±0.4 n=6	10.58±0.3 n=6	11.0±0.6 n=6	9.1±0.7 n=6	0.004
Fasting Insulin (ng/ml)	2±0.4 n=3	1.1±0.3 n=4	2.9±1 n=3	0.8±0.3 n=4	1.4±0.1 n=2	0.7079
Food intake Kcal/mouse/day	12.5± 1.4 n=12	12.9± 1.6 n=12	13.4± 1.1 n=12	12.7±0.4 n=12	10.8± 1.3 n=12	0.66

* p<0.05, ** p<0.01 compared with LFD by one-way Anova. Abbreviation: HFD: High fat diet, LFD: Low fat diet, BW:Body weight, FBG: Fasting blood glucose.

3.1.3 Insulin Tolerance Test (ITT)

The ITT represents the efficiency of whole-body insulin action. ITT was performed on half of the mice to determine the degree to which blood glucose concentrations fell following intraperitoneal insulin injection. (Figure 3-3(A)) compared the the effect of insulin bolus between HFD and LFD group. HFD group showed higher fasting blood glucose and impaired blood glucose disappearance while LFD group showed faster drop of blood glucose concentration indicating higher response to insulin injection. The diet effect was statistically significant (Time, P<0.0001, Diet, P=0.03, Time×Diet, P=0.02), however when we expressed the data as % of baseline, the differences between HFD and LFD group remain significant Figure 7(B), (Time<0.0001, Diet, P=0.052, Time×Diet=0.01), suggesting mice in HFD group successfully induced IR.

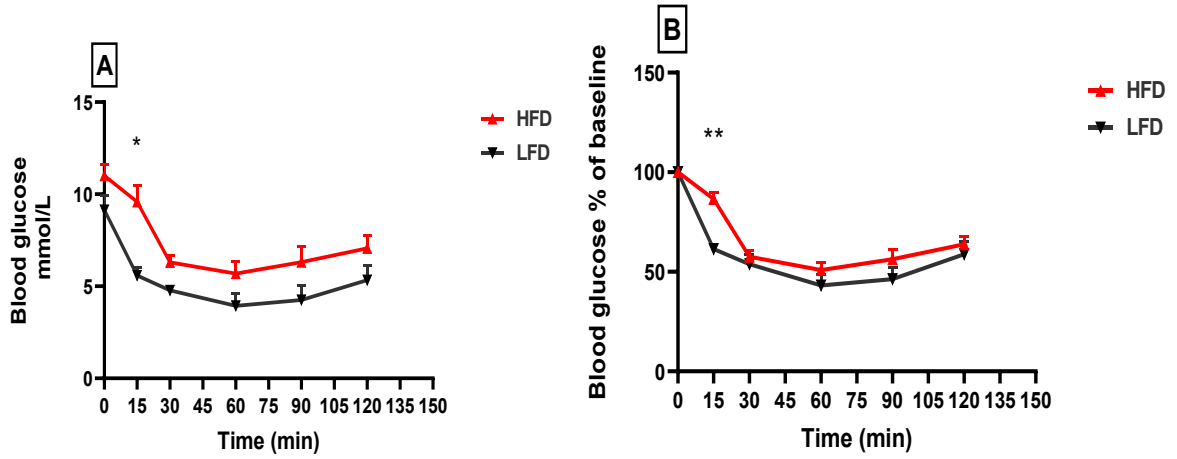


Figure 3-3 Effects of 7 weeks of diet feeding on ITT in HFD and LFD control group. The value of blood glucose is shown as absolute mean±SEM, n=6(A). P-value, Time<0.0001, Diet=0.03, and Time×Diet=0.02. *= at 15 min P= 0.02. And as mean % of basal glucose±SEM, n=6 (B). P-value, Time<0.0001, Diet=0.052, and Time×Diet=0.01. ** P=0.002. Two-way ANOVA followed by Tukey's multiple comparison test was performed.

At 15 minutes of ITT, the milk, yogurt, and HFD group's blood glucose were higher than the LFD group with P= 0.04, 0.01, and 0.02, respectively. When data were expressed as % of baseline, the effect of exogenous insulin was similar between groups except for the HFD group, which was significantly different compared to LFD (P=0.002) Figure 3-3 (B). The effect of exogenous insulin in the milk group appeared to be prolonged until 90 minutes, whereas blood glucose concentrations began to rebound by 30-60 minutes for all other groups. When examining the AUC, we subtracted the baseline area and measured the negative peak only. Here, the milk group showed about 50% higher response (indicating greater insulin sensitivity) than other groups Figure 3–5.

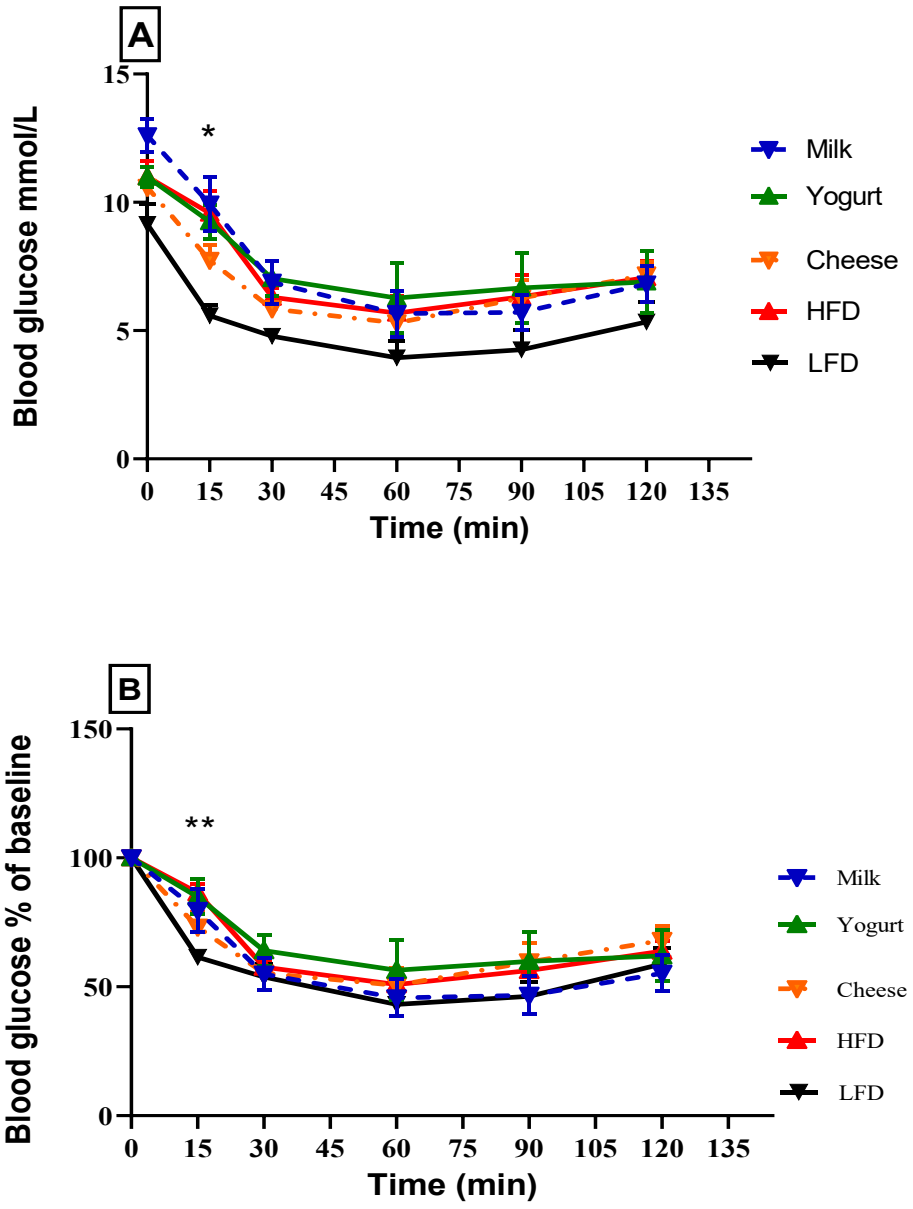


Figure 3-4 Effects of 7 weeks of diet feeding on insulin tolerance in HFD+Milk, HFD+Yogurt, HFD+Cheese, HFD, and LFD groups. ITT was performed on mice following a 4-hours fast. The values of blood glucose are shown as absolute mean ± SEM, n=6. (A) and as mean % of basal glucose ± SEM, n=6. (B). Two-way ANOVA followed by Tukey's test was performed.

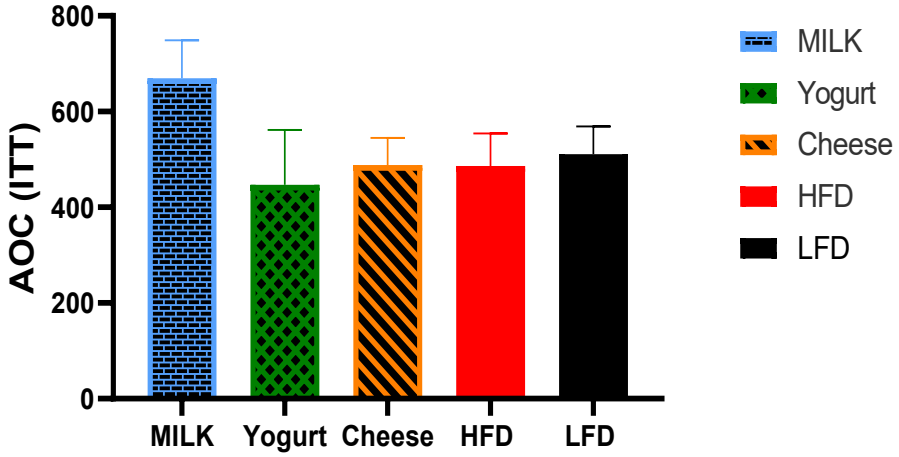


Figure 3-5. Graph showed the area of the curve AOC data for the ITT as mean ± SEM, n=6, One-way ANOVA was used to evaluate statistical differences, but none were found (P=0.3). (AOC calculated based on data presented in (Figure 3-4 (A)).

3.1.4 Pyruvate Tolerance Test

Gluconeogenesis capacity is assessed by the pyruvate tolerance test (PTT) by monitoring glycemic excursions after administering a pyruvate bolus after overnight fasting. The differences between HFD and LFD groups were significant with P= 0.02 for Diet, 0.0002 for the time in absolute values. When we adjusted for the blood glucose baseline, only time was significant (P<0.0001) (Figure 3–6).

For the dairy group, the absolute value of blood glucose showed a significant effect of Time, P<0.0001, Diet, P=0.003, Time × Diet= 0.9, while when we express the data as % of the baseline only time was significantly different with P<0.0001 Figure 3-7. AUC was significantly higher in all HFD groups compared to the LFD group, with milk P=0.03, yogurt P=0.009, cheese P=0.007, and HFD P=0.01; however, this effect disappeared when we normalized the blood glucose concentration to the baseline P=0.8. This high response is

mainly due to somewhat higher fasting glucose because when normalized to fasting glucose, differences after pyruvate administration were attenuated (Figure 3–8).

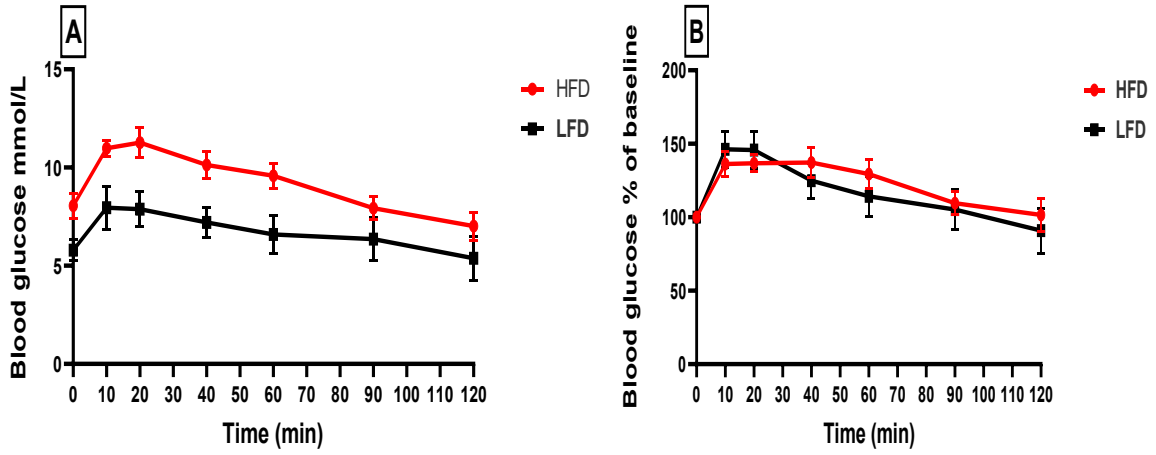
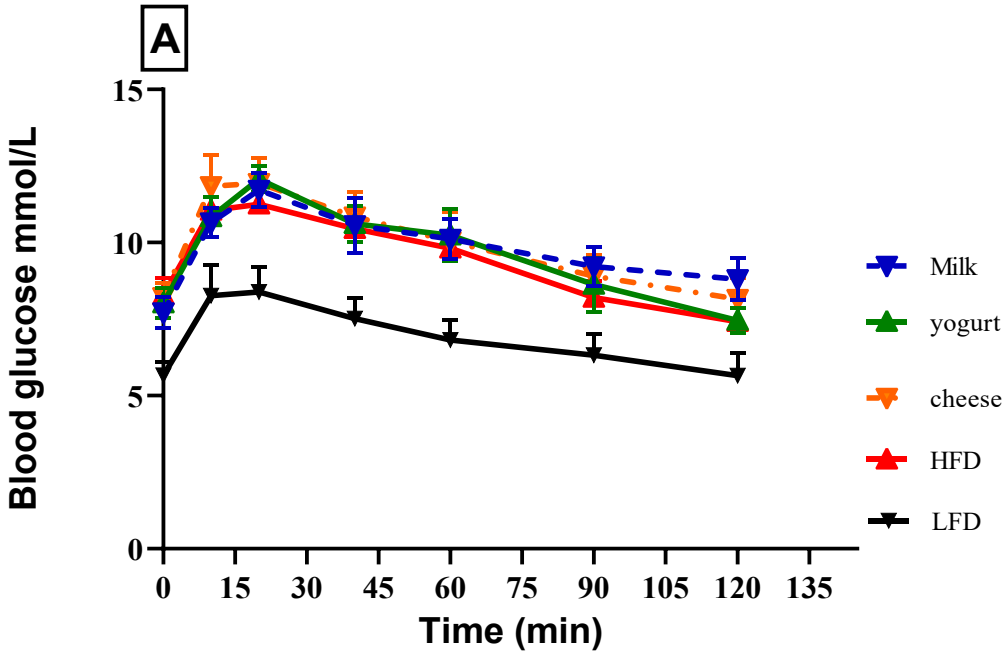


Figure 3-6 Effects of 7 weeks of diet feeding on pyruvate tolerance in HFD and LFD groups. PTT were performed on mice following an overnight fast. The blood glucose values are shown as absolute mean ± SEM, n=6 (A) and as mean % of basal glucose ± SEM, n=6 (B). Using Tukey multiple comparison tests following one-way ANOVA, no significant differences were found.



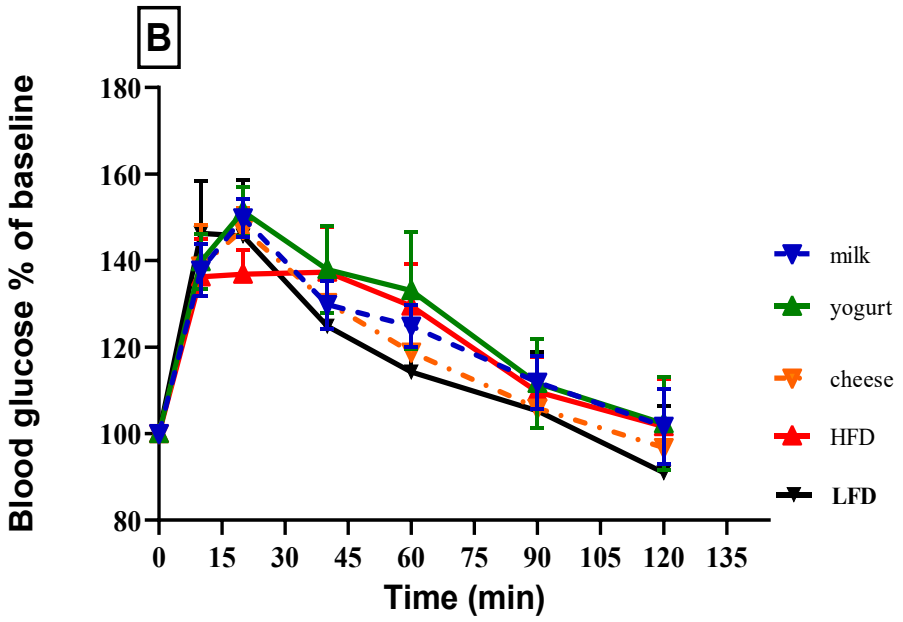


Figure 3-7 Effects of 7 weeks of diet feeding on pyruvate tolerance in HFD and LFD groups. PTT was performed on mice following an overnight fast. The blood glucose values are shown as absolute mean ± SEM, n=6 (A), and as mean % of basal glucose ± SEM, n=6 (B).

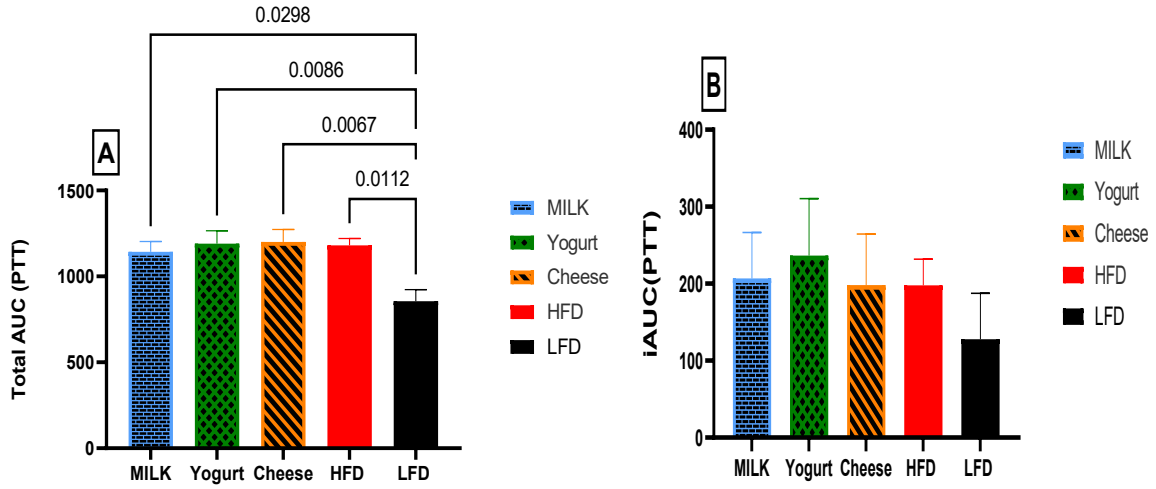


Figure 3-8 Graph (A)showed the total area under the curve data for the PTT as mean ± SEM, n=6. One-way ANOVA (P=0.004) followed by Tukey's test was used to evaluate statistical differences, while (B) represents Incremental changes in glucose (iAUC) (P=0.8).

3.1.5 Epididymal Fat Weight

There was a significant effect of diet on the epididymal fat weight relative to body weight. All HFD groups showed a higher percentage of epididymal fat weight compared to LFD with ($p<0.0001$) (Figure 3–9).

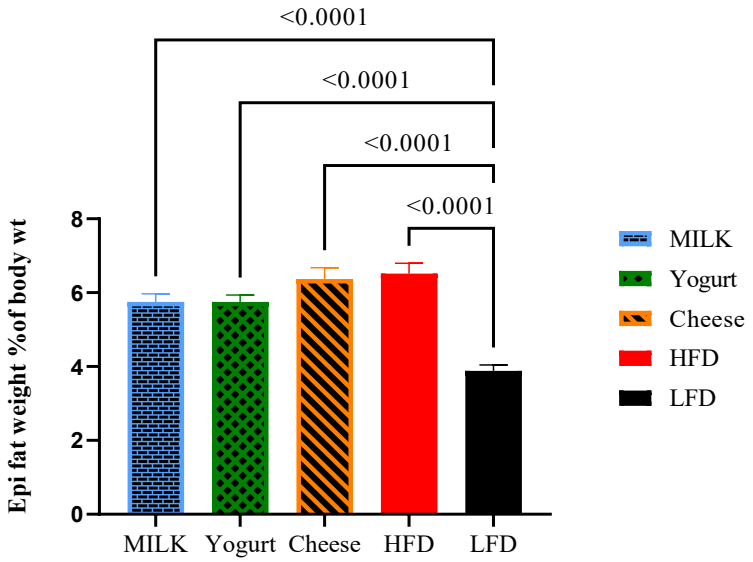


Figure 3-9 Epididymal fat weight as % of total body weight for mice fed HFD+Milk, HFD+Yogurt, HFD+Cheese, HFD, or LFD diets. Data are presented as mean ± SEM. One-way ANOVA followed by Tukey's multiple comparison test was performed. n per group HFD+Milk=11, HFD+Yogurt=10, HFD+Cheese=10, HFD=12, and LFD=8.

3.1.6 Body Composition

ECHO MRI was used to measure the whole-body fat, lean, and free water in the mice. Fat mass was significantly high among all HFD groups compared to LFD ($P<0.0001$), while the lean mass was significantly higher in the LFD group compared to all HFD groups ($P<0.0001$). No significant differences were found between HFD groups with or without dairy (Figure 3–10).

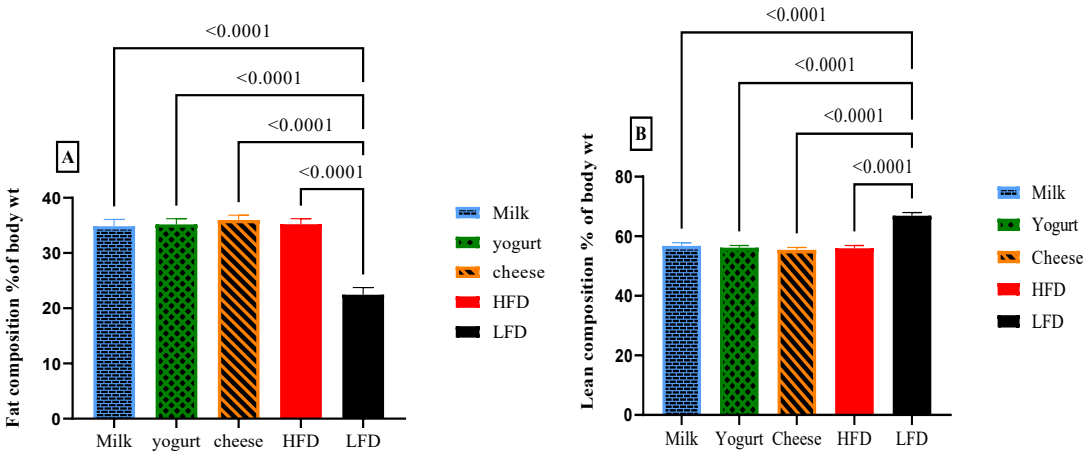


Figure 3-10 MRI-measured body composition for mice fed HFD+Milk, HFD+Yogurt, HFD+Cheese, HFD, or LFD diets. % fat mass (A) and % lean mass (B). Data are presented as mean ± SEM. One-way ANOVA followed by Tukey's multiple comparison test was performed. n per group=8.

3.2 Effect of Dairy Consumption on Serum and Hepatic Lipid Phenotype in IR Mice

3.2.1 Liver Weight

Liver weight was presented as a percentage of total body weight. There was no significant effect of diet on the liver weight (P=0.1) (Figure 3–11).

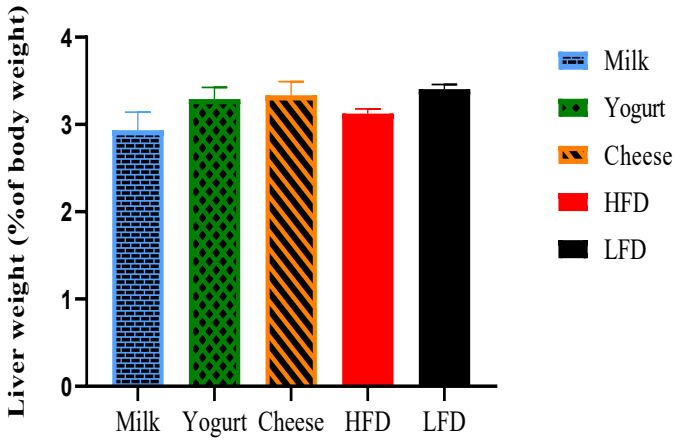


Figure 3-11 Liver weight as % of total body weight for mice fed HFD+Milk, HFD+Yogurt, HFD+Cheese, HFD, or LFD diets. Data are presented as mean ± SEM. One-way ANOVA was performed.n per group HFD+Milk=11, HFD+Yogurt=12, HFD+Cheese=10, HFD=11, and LFD=8.

3.2.2 Serum TG and NEFA

Serum at the time of euthanasia was used (overnight fasting) to evaluate the concentration of TG. The yogurt group had the highest mean TG and was significantly higher than both Milk (P=0.0493) and LFD (P=0.0365). No other statistically significant differences were found in Figure 3–12. Serum NEFA analysis showed no significant differences between groups Figure 3–13.

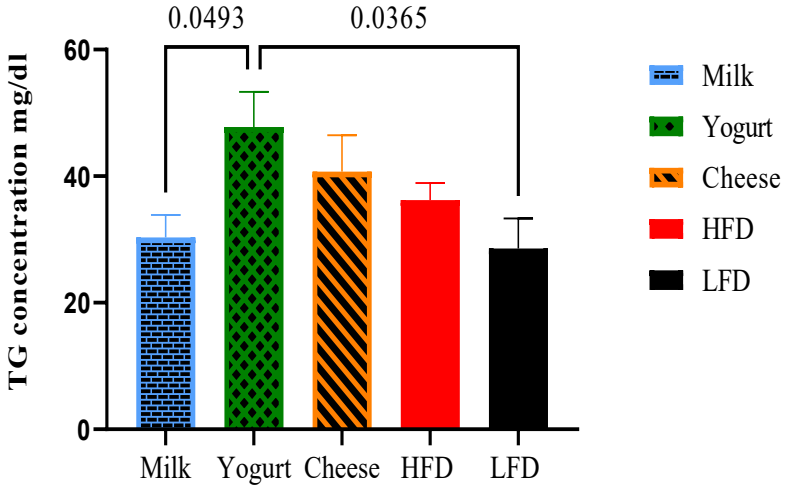


Figure 3-12 Serum TG for mice fed HFD+Milk, HFD+Yogurt, HFD+Cheese, HFD, or LFD diets. Data are presented as mean ± SEM. One-way ANOVA followed by Tukey's multiple comparison test was performed. n per group HFD+Milk=11, HFD+Yogurt=12, HFD+Cheese=9, HFD=12, and LFD=9.

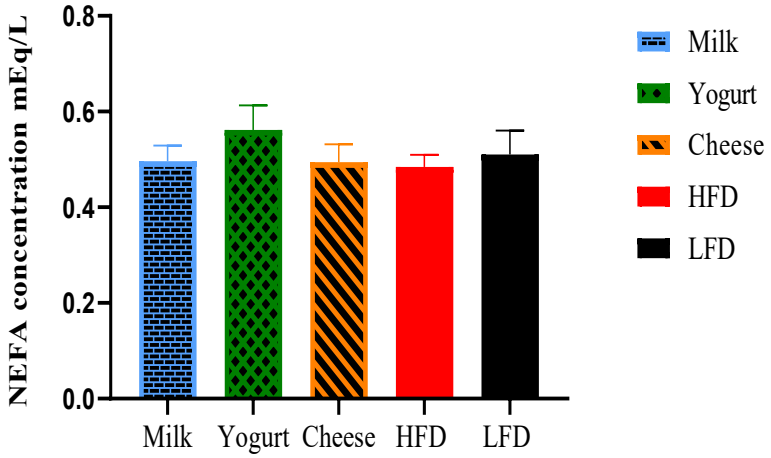


Figure 3-13 Serum NEFA for mice fed HFD+Milk, HFD+Yogurt, HFD+Cheese, HFD, or LFD diets. Data are presented as mean ± SEM. One-way ANOVA was performed. n per group: HFD+Milk=11, HFD+Yogurt=12, HFD+Cheese=9, HFD=12, and LFD=9

3.2.3 Liver Triglyceride

There was a significant effect of Yogurt on liver TG content (P=0.0073) compared to LFD. No other statistically significant differences were found in Figure 3–14.

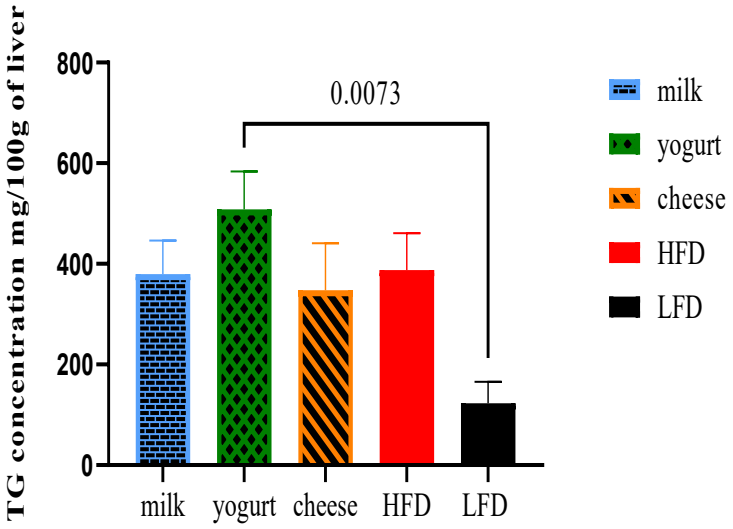


Figure 3-14 Liver TG for mice fed HFD+Milk, HFD+Yogurt, HFD+Cheese, HFD, or LFD diets. Data are presented as mean ± SEM. One-way ANOVA followed by Tukey's multiple comparison test was performed. n per group HFD+Milk=6, HFD+Yogurt=6, HFD+Cheese=6, HFD=6, and LFD=6.

3.2.4 Alanine Aminotransferase (ALT)

Serum ALT was measured to rule out liver inflammation due to HFD consumption using serum collected at the time of euthanasia. No significant differences between groups were detected in Figure 3–15.

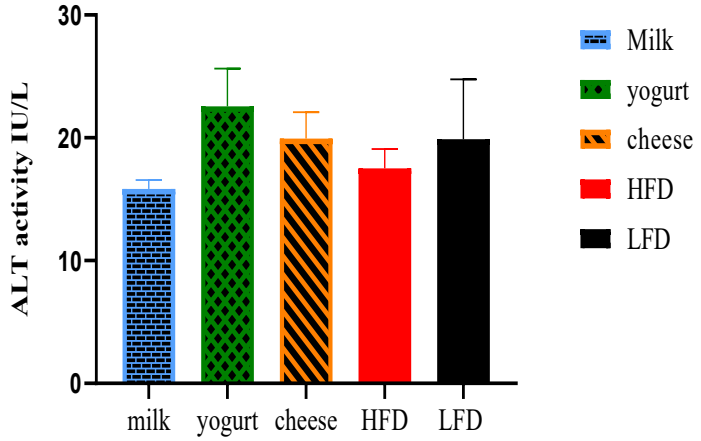
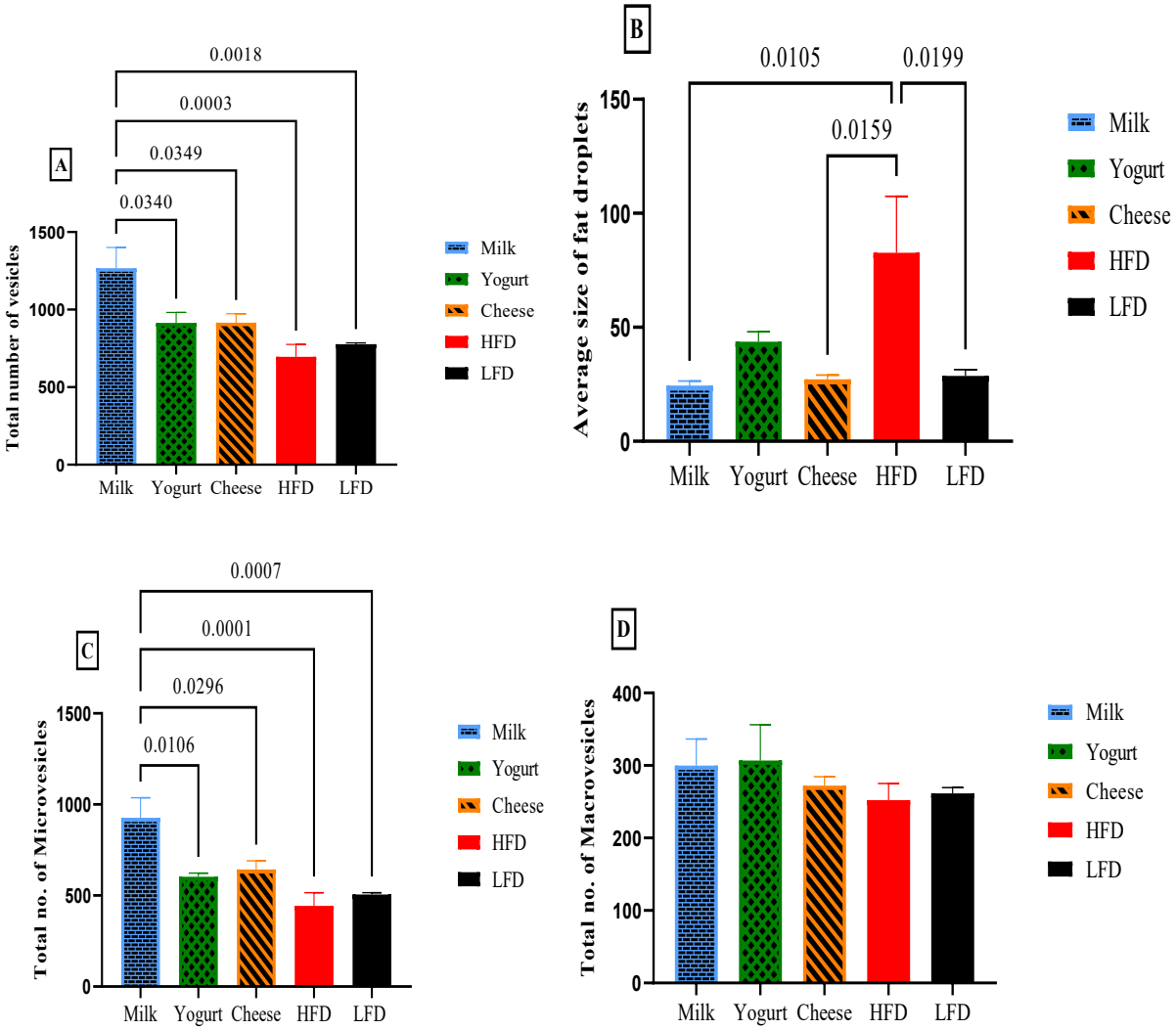


Figure 3-15 ALT activity for mice fed HFD+Milk, HFD+Yogurt, HFD+Cheese, HFD, or LFD diets. Data are presented as mean ± SEM. One-way ANOVA was performed. n per group HFD+Milk=6, HFD+Yogurt=6, HFD+Cheese=6, HFD=6, and LFD=6.

3.2.5 Liver Histology

The total number of fat droplets was significantly higher in the Milk group compared with any group (Figure 3–16(A)). The size of fat droplets was smaller in the LFD, Milk, and Cheese groups than the HFD group with P=0.02, 0.01, and 0.02, respectively (Figure 3–16 (B)). Microsteatosis is assessed using the total number of microvesicles ($\leq 15\mu m$) (97), which was significantly higher in the Milk group compared to all other groups (Figure 3–16(C)). Conversely, the number of macrosteatotic vesicles (>15 μm) was not statistically different between groups (Figure 3–16(D)).

The steatosis area % is the percentage of macro or microvesicles relative to the total vesicle number. Microsteatosis % was significantly higher in the Milk group compared to the HFD group (P = 0.0379) (Figure 3–16 (F)), while macrosteatosis % was higher in the HFD group compared to the Milk group (P = 0.0385) (Figure 3–16 (F)).



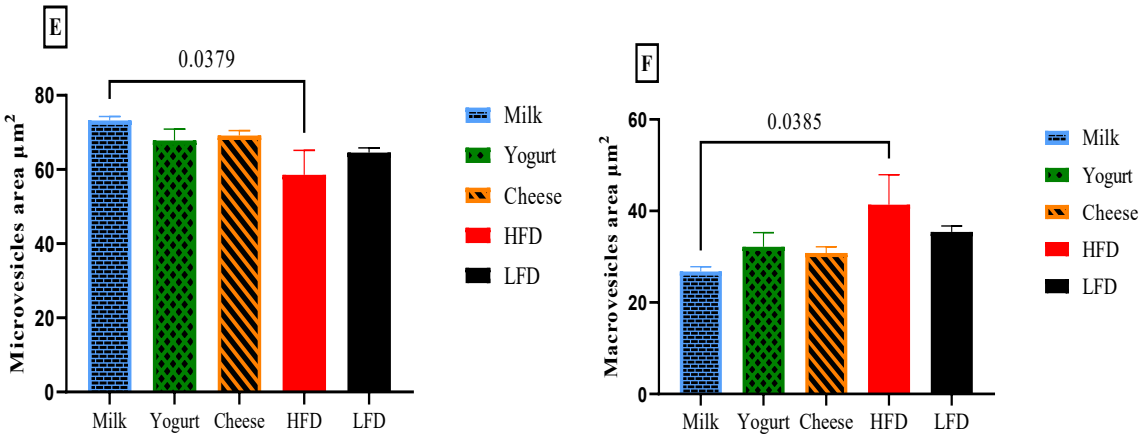


Figure 3-16 Effect of diet on hepatic lipid accumulation. N = 6 per diet group. The total number of vesicles(A). The average size of vesicles (B), microsteatosis (C). Macrosteatosis (D). Microvesicle area %(E). Macrovesicle area% (F). One-way ANOVA followed by Tukey's multiple comparison test was performed.

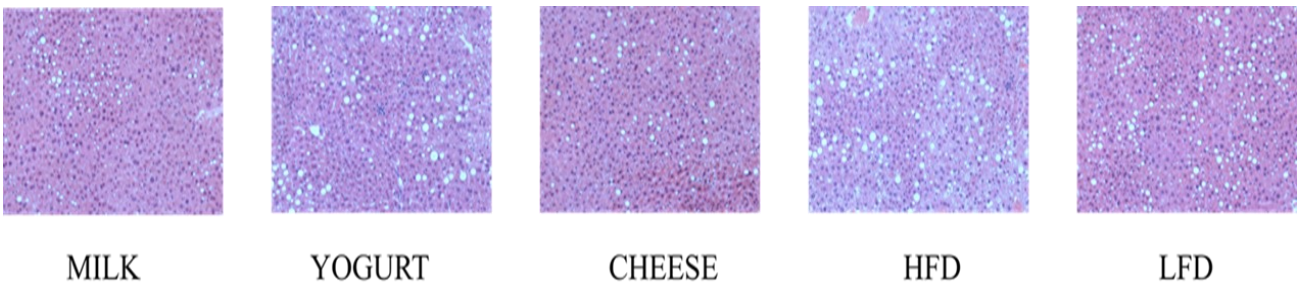
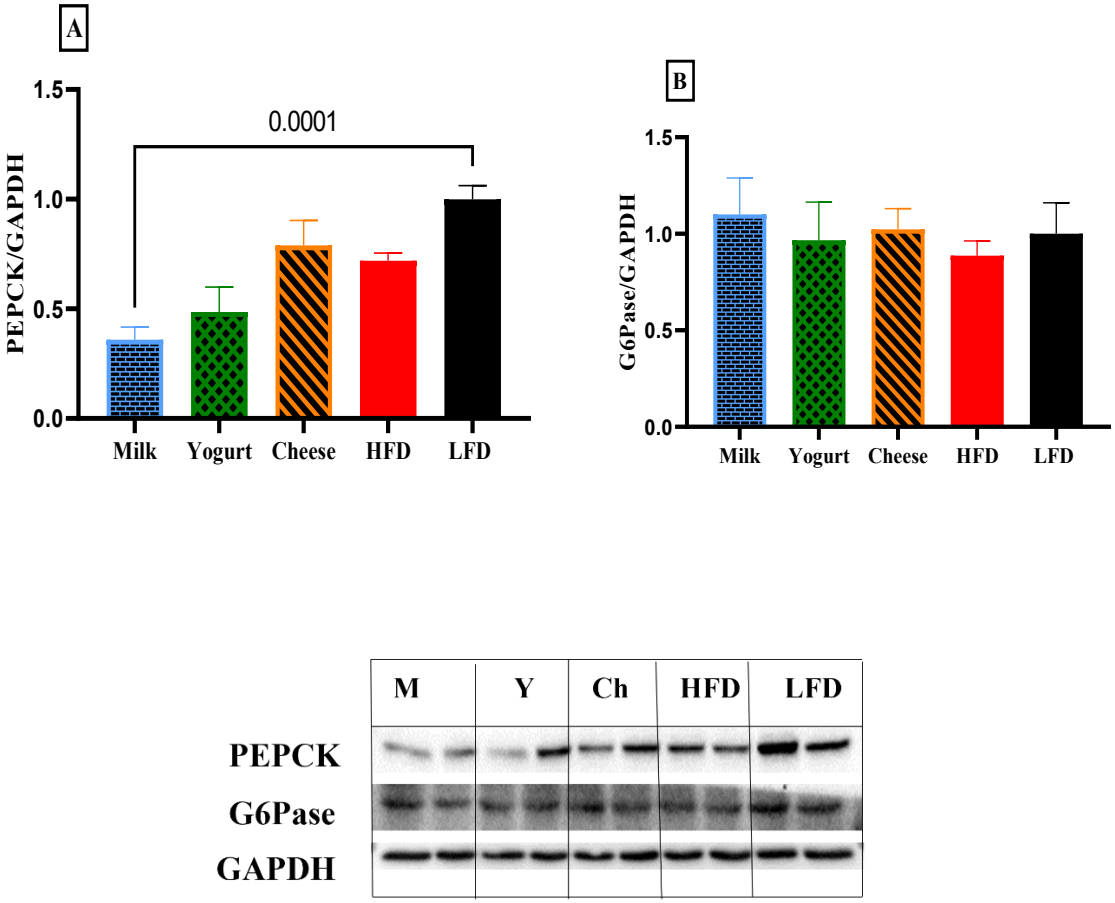


Figure 3-17 Effect of diet on hepatic lipid accumulation. N=30 (N = 6 Per diet group). Mouse liver samples were stained with hematoxylin & eosin. Photo microscopic images of liver tissue using 20X objective lens.

3.2.6 Immunoblotting

The abundance of rate-limiting enzymes of hepatic gluconeogenesis was evaluated. A significant effect was detected in the Milk group compared to the LFD group for PEPCK abundance (P = 0.0318) (Figure 3–18 (A)), while G6Pase was not different between groups (Figure 3–18 (B)).



M, milk. Y, yogurt. Ch, cheese. HFD, high-fat diet. LFD, low-fat diet

Figure 3-18 Effects of Dairy diets on relative protein abundance of (A) PEPCK (n=6) and (B) G6Pase (n=6). PEPCK and G6Pase values were normalized to GAPDH. One-way ANOVA followed by Tukey, a multiple comparison test, was performed. There was a significant effect of Milk on PEPCK protein abundance (P=0.03) compared with LFD. No differences were observed for G6Pase abundance.

Chapter 4 : Effect of Dairy Consumption on Metabolic Phenotype in Female Mice

We used 40 female mice in the first and second trials in order to compare sex differences in the effect of dairy foods on insulin resistance. Body weight was significantly increased among HFD and dairy groups compared to the LFD group, with a significant effect of both time ($P<0.0001$) and diet ($P<0.0001$) (Figure 4—1). The change in body weight was greatest in the milk group, consistent with having the highest epididymal fat weight, and a trend to highest total body fat (Table 4—1).

Data for the ITT (Figure 4—2) were not complete because most of the mice could not finish the test due to hypoglycemia, even those in the HFD group and even after the insulin dose was reduced by half in trial 2. Blood glucose dropped to <3mmol/L, necessitating the administration of glucose solution to 10 out of 20 mice. This effect indicates that all the mice were highly insulin sensitive.

PTT showed a significant effect of diet ($P=0.0084$), time ($P=<0.0001$), and subject ($P<0.0001$). The Yogurt group showed increased glucose concentration only at 20 minutes ($P=0.01$) compared to the LFD group. The Cheese group showed increased glucose level at 20 min ($P=0.006$), at 40 minutes ($P=0.04$), at 60 min ($P= 0.05$), and at 90 minutes ($P=0.01$) compared to LFD. In contrast, the Milk group showed increased glucose concentration at 20 min ($P=0.002$), at 60 min ($P=0.04$), and at 90 min ($P=0.02$) compared to the LFD group (Figure 4–3). The majority of these differences were likely dependent on slightly elevated fasting blood glucose in the Milk and Yogurt groups. Metabolic data are summarized in Table 4–1.

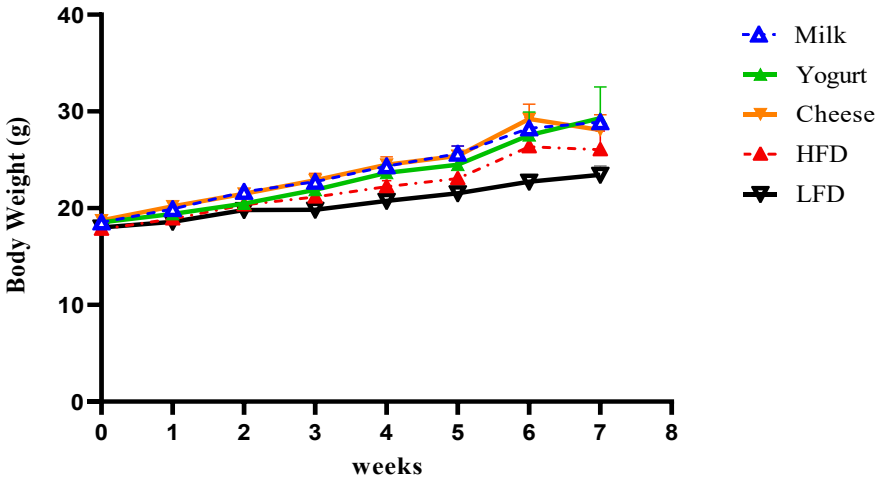


Figure 4-1 Weekly Body weight for female mice fed HFD+Milk, HFD+Yogurt, HFD+Cheese, HFD, or LFD diets. Data are presented as mean ± SEM. One-way ANOVA was performed. N per group=8.

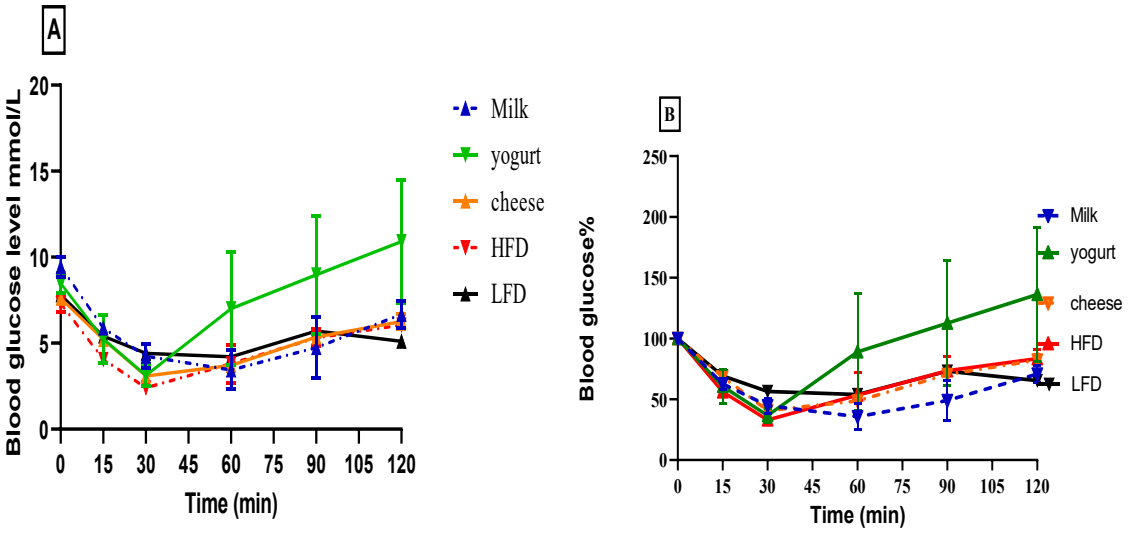


Figure 4—2 Figure 3-4 Effects of 7 weeks of diet feeding on insulin tolerance in HFD+Milk, HFD+Yogurt, HFD+Cheese, HFD, and LFD groups. ITT was performed on mice following a 4-hours fast. The values of blood glucose are shown as absolute mean ± SEM, n=6. (A) and as mean % of basal glucose ± SEM, n=6. (B). Two-way ANOVA followed by Tukey's test was performed

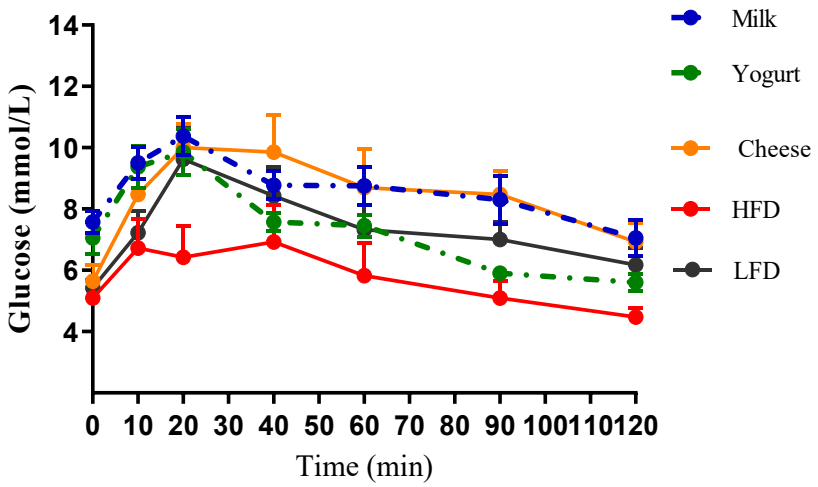


Figure 4—3 Effects of 7 weeks of diet feeding on pyruvate tolerance in HFD+Milk, HFD+Yogurt, HFD+Cheese, HFD, and LFD female mice. PTT was performed on mice following an overnight fast. The values of blood glucose are shown as absolute mean ± SEM, n=4. Tukey, multiple comparison tests following one-way ANOVA, was used.

Table 4—1 Metabolic profile of female mice

Diet group	Milk	Yogurt	Cheese	HFD	LFD	P-value
BW change(g)	7.1±0.7 * N=8	6.0±1.0 N=8	6.7±1.0 N=8	5.3±0.6 N=8	3.5±0.6 N=8	0.02
FBG (mmol/L) 4Hrs fast	9.3±0.4 N=3	8.3±0.4 N=3	8.4±0.5 N=2	7.9±0.5 N=2	7.6±0.4 N=1	0.2
FBG (mmol/L) overnight fast	7.6±0.3# N=4	7.1±0.5 N=4	5.7±0.5 N=4	5.1±0.6 N=4	5.4±0.7 N=4	0.02
Epi fat (% BW)	4.8±0.3* N=8	3.3±0.3 N=8	3.5±0.3 N=8	3±0.3 N=8	3.0±0.4 N=8	0.004
Liver WT (% BW)	3.2±0.2 N=8	3.3±0.2 N=8	3.2±0.2 * N=8	3.6±0.1 N=8	3.9±0.1 N=8	0.03
Fat mass (% BW)	31.0±2.6 N=4	26.2±5.3 N=4	26.6±3.3 N=4	21.3±3.0 N=4	16.8±1.8 N=4	0.08
Lean mass (% BW)	63.0±1.2 N=4	64.0±4.4 N=4	63.5±3.0 N=4	68.6±2.8 N=4	72.4±1.8 N=4	0.012
Food intake (Kcal/day)	13.9±0.4 N=8	14.51±0.3 N=8	14.45±0.1 N=8	13.17±0.1 N=8	11.99±1.0 N=8	0.07

** p<0.05 compared with LFD. # P< 0.05 compared to HFD. BW: body weight, FBG: fasting blood glucose, Epi fat: epididymal fat, WT: weight.*

A literature review reported that female mice are less susceptible to diet induced obesity and its complications (98). Female mice are protected against metabolic changes and insulin resistance when fed high fat diet (99,100); in particular, female C57BL/6 mice are known to respond differently to HFD feeding due to the protective effect of estrogen (99). Female mice fed HFD develop mild IR and hyperinsulinemia (101) and hypercholesteremia (99) compared to male mice. Female mice take longer time to gain weight in response to HFD feeding with 129 days in female compared to 46 days in male (101). Female mice also show constant blood glucose level up to 14 weeks of HFD feeding while male mice showed significant hyperglycemia (99). These data are consistent with our findings that, although female mice fed HFD gained weight, it was about half that of male mice (7 g vs 15 g). The female mice fed HFD also had similar insulin sensitivity compared with the LFD controls based on the need to stabilize blood glucose with exogenous glucose in all groups. Therefore, it was not possible to investigate whether dairy interventions improved insulin sensitivity.

Human epidemiological data have also shown sex variations in obesity, cardiometabolic risk factors, response to treatments, and diabetes complication development (102). There is no doubt that investigations aimed to prevent or reverse obesity and treat its complication like in our project should taking sex differences in account with the intention of enhance their translational value to human as sex affect the metabolic parameters and its applicability to humans. To overcome the insulin sensitivity in female mice and increase their ability to induce IR in response to HFD feeding we could increase the duration of trial in the future studies.

Chapter 5 : Discussion and Conclusion

5.1 Summary of Hypotheses and Main Findings

The purpose of the current research was to evaluate and compare the effect of Milk, Yogurt, and Cheese consumption on glucose homeostasis in HFD induced prediabetic mice and explore the possible mechanisms contributing to differences in insulin sensitivity and liver lipid metabolism. Previous work by our group showed a beneficial effect of low fat and high fat (LF/HF) cheese on insulin sensitivity in prediabetic rat models. In vivo experiments demonstrated increased hepatic insulin sensitivity during the insulin tolerance test for HF and LF cheese among prediabetic groups without affecting body weight. The study suggested that cheese consumption (LF/HF) may improve glucose homeostasis in the prediabetes model (76). In the current research, we hypothesized that regular fat milk and yogurt would have a similar effect to regular fat cheese in prediabetic mice by improving insulin sensitivity. Overall, the data do not support the hypothesis and are not similar to our previous lab work using Cheese. This is probably because of the very low dairy dose used in this research, which was underestimated due to an error in the calculated value. Nevertheless, the results did show beneficial effects of a low amount of milk on liver morphology related to lipid storage. The data indicate that the food matrix or nutritional changes that occur during processing affect the ability of individual dairy products to alter metabolism.

5.2 HFD as a Model for Prediabetes and Non-Alcoholic Fatty Liver Disease

Generally, a research animal model must ideally yield repeatable, reliable results, be commercially available, and be inexpensive for development (103). Rodents such as

rats and mice are the most used species for experimental studies of glucose homeostasis (104). Compared with mice, rats have a big body, making it easy for catheterization and surgical procedures and larger blood volume that allows for multiple samples. The small body of the mouse limits the number of blood samples and makes the surgical procedures more challenging to perform. However, mouse models well established as a genetically modified, have genes removed (knocked out), and be transgenic to evaluate specific genes and pathways than rats (104).

Our model is C57BL/6J on HFD to induce IR, as our primary hypothesis was to assess the effect of dairy on insulin sensitivity. Many studies have shown that male C57BL/6J mice develop obesity, IR, diabetes, and hypertension when fed HFD, mimicking the human metabolic syndrome (105). The high-fat diet C57BL/6J mouse model was shown to be accompanied by IR, as determined by glucose tolerance tests, and insufficient insulin secretion compensation by the insulin-resistant islets. Therefore, the model is often used to study the pathophysiology of impaired glucose tolerance (IGT) and type 2 diabetes (106). However, there are some limitations for using this model such as the remarkable variation in the degree of IR between female and male mice, despite weight gain, due to the effect of estrogen (99). Furthermore, male mice develope significant hyperglycemia and develop obesity sooner and more severely than female mice in response to HFD feeding (98). Many researchers use male mice and ignore the sex differences, therefore, affecting the translational value of the research outcome as obesity and IR are common in female humans (98). These sex-dependent data are consistent with our findings that, precluded us determining whether dairy interventions improved insulin sensitivity in female mice.

Another limitation of rodents as an experimental model is the heterogeneity in response to HFD with only 60% developing obesity (98). There are other physiological differences, such as the primary site for glucose disposal in human being skeletal muscle, whereas liver is the primary site in mice (98). Thus, hepatic adaptations to dairy in humans might not be as pronounced as those observed in mice.

C57BL/6J mice fed HFD may also be a suitable model for NAFLD. As we developed our secondary hypothesis that the low dose of dairy could have a beneficial effect on hepatic lipid accumulation, our animal model was still useful for assessing NAFLD. Mice fed a 60% HFD exhibit strong steatosis, hyperlipidemia, and hyperinsulinemia after 10-12 weeks, while rats on a similar diet needed only 7 weeks to develop the same features (103). The literature shows that mice fed HFD for 50 weeks developed metabolic syndrome and steatohepatitis with mild fibrosis (107). C57BL/6 mice fed a diet of fast food that included high cholesterol, high saturated fatty acid, and high fructose for 6 months, had the features of metabolic syndrome, NAFLD, and NASH with progressive fibrosis (107). Another study suggested that a high fat/calorie diet with high fructose/glucose in drinking water is ideal for developing significant steatosis, necroptosis, inflammatory reaction, IR, and fibrotic progression. Therefore, it represents a great model for treatment intervention or pathophysiologic study of NAFLD and NASH (108). The methionine- and choline-deficient (MCD) diet caused fatty liver in the mice; however, the mechanism by which this occurs is pathologically different from human NAFLD, which is one of the most important criticisms of this dietary model (103). In the current research, our model showed increased body weight in response to 8 weeks feeding of 45% HFD compared to the LFD group. The body fat composition was significantly higher in the HFD

group as well as the epidydimal fat weight. Histology of the liver showed increased accumulation of fat droplets in the HFD groups compared to the LFD group even after a relatively short period of HFD feeding. Thus, the HFD-fed C57BL/6J mouse may be considered a model of the early progression of NAFLD.

5.3 Effects of Dairy and Its Components on Hepatic Lipid Storage

Improvement of hepatic lipid storage in the Milk group was the most significant finding in this research. Milk consumption had a positive effect on the development of liver steatosis, which is supported by reduced macrovesicles area and increased microvesicle area compared to the HFD group. Liver steatosis is the accumulation of lipid in hepatocytes and is the mild form of NAFLD, the most common chronic liver disease in Western countries (109). There are two forms of hepatic steatosis depending on the size of the lipid droplet: macro-vesicular, a single giant lipid droplet defined in the hepatocyte with nucleus displacement, and micro-vesicular steatosis, where the hepatocytes are packed with small lipid droplets without nucleus displacement (109). Microsteatosis is suggested to be the primary form of hepatic steatosis. The small lipid droplets may merge to form large droplets with the progression of the disease (110). Our data, particularly for Milk, is consistent with other research findings, suggesting that microsteatosis is a transitional condition between non-steatotosis and macrosteatosis (110). Milk may have slowed or prevented that progression. Furthermore, published studies have shown that microsteatosis was found to be associated with less sign of fibrosis, lobular inflammation, IR, and hepatic damage than macrosteatosis (111,112).

Our findings raise the question as to what attribute of milk, as compared with cheese or yogurt, is responsible for the reduction in hepatic lipid. Several components of dairy are proposed to exhibit bioactivity that could contribute, including certain fatty acids, the milk fat globule membrane, which has many components including phospholipids and choline, calcium and vitamin D, and dairy protein. The effect of dairy fat on health has often been debated in the literature because of the potentially harmful outcomes caused by saturated fatty acid (SFA) on metabolic health (55). Metabolic dysfunction including IR, glucose intolerance, dyslipidemia, hypertension, and central obesity, contribute to cardiometabolic disease, which is one of the global leading causes of mortality and morbidity (55). Excessive energy consumption can be a factor leading to obesity and IR (113). Saturated fat mainly refers to the dietary long-chain fatty acids, which are linked to the risk of cardiovascular disease. At the same time, dairy products contain some medium and short-chain saturated fatty acids, which have beneficial effects on health (113). However, dairy fatty acids such as trans-16:1n-7, phytanic acid, C:15, and C:17 were also proposed as an active component to stimulate hepatic beta-oxidation and/or inhibit de novo lipogenesis (52,114,115). In our project, it is unlikely that dairy fat had an effect because the amount of fat contributed by cheese and milk to overall diet of mice was equal but we did not see similar improvement in the hepatic lipid accumulation. Figure 5–1 provides a summary of proposed mechanisms that demonstrates the beneficial effect of dairy fat on liver fat, which could improve insulin sensitivity and reduce FBG (113).

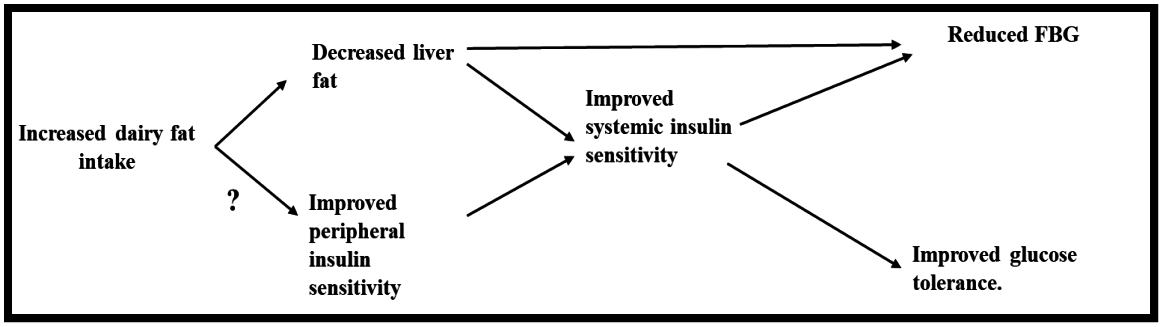


Figure 5-1 Proposed mechanism through which higher intakes of dairy fat in general, or trans-palmitoleic acid in particular, may affect fasting glucose concentrations and glucose tolerance include a reduced liver fat content leading to improved hepatic and systemic insulin sensitivity. Adapted from Mario et al.2021 (113)**.**

Choline is one of the nutrients increased by milk that may contribute to improved hepatic lipid storage. Milk has approximately 50% and 100% higher choline than yogurt and cheese, respectively (Table 2—4), and is involved in many physiological functions. The choline metabolite PC represents 40-50% of cell membranes and 70-95% of phospholipids in lipoproteins and bile (116). Low choline intake is reported to be associated with NAFLD. This may be because choline is required for the synthesis of very low-density lipoprotein (VLDL), which is responsible for TG transport from the liver. And any defect in VLDL production will cause TG accumulation in the liver (116). A study has shown that 3 weeks supplementation with a choline-rich diet for postmenopausal women resolved NAFLD (117).

Polar lipids (PL), one of the MFGM components along with protein and cholesterol, account for 1% of total milk lipid and certain sub-classes (sphingolipids) may play an important role in reducing hepatic lipid accumulation (118). PL includes glycerophospholipids, such as PC, and sphingolipids, such as SM. Both are responsible for emulsifying TG in the aqueous phase of milk. Many studies have investigated the

effect of the consumption of extract of PL on lipid metabolism. The results show that PL-rich dairy milk extract (PLRDME) significantly reduces liver weight, total cholesterol, TG, and serum lipid in mice fed HFD (79). The same study showed that PLRDME decreases expression of enzymes involved in fatty acid synthesis and inhibits 7α-hydroxylase mRNA expression (the rate-limiting enzyme in bile acid synthesis), (79) consistent with the finding that milk SM inhibits intestinal lipid absorption and increases TG/cholesterol excretion with feces in obese/diabetic mice (119). SM is relatively resistant to solubilization in bile salt micelles, leading to uncomplete hydrolysis in the upper intestinal part where the most lipid hydrolysis occurs. SM might interact with other lipids and reduce their rate of hydrolysis, solubilization, and transfer to enterocytes (119). Overall, sphingolipids might therefore decrease liver lipid both directly and indirectly by its effects on gut lipid metabolism.

Phospholipid is the most important compound in the cell membrane and plays a crucial role in gastrointestinal barrier integrity and improving systemic inflammation and lipid metabolism (120). HFD consumption results in intestinal barrier dysfunction, triggering metabolic endotoxemia and causing metabolic disorders (120,121), which play in important role in immune response involved in the development of systemic inflammation, IR, obesity, and NAFLD (122,123). PC supplementation reduces oxidative stress and improves gut barrier function by improving the expression of tight junction (TJ) protein (124). The same study showed that PC supplementation substantially inhibited the intestinal mucosal damage caused by LPS by improving villi length (124). In our research we could hypothesize that milk PC and SM contributed to improved hepatic steatosis by improving the intestinal permeability and repairing the damage caused by

LPS due to HFD consumption. To test this hypothesis, we could confirm the amount of MFGM in our dairy products and assess gut permeability.

Another possibility is milk fat globule-epidermal growth factor 8 (MFGE8). This protein is a membrane-associated glycoprotein found in milk and mammary epithelial cells and is known to play a role in the physiological and pathophysiological processes of immune and inflammatory response and liver homeostasis (125). MFGE8 originated from milk fat globules, and its structure is similar to the epithelial growth factor. During inflammation, its expression increases in the mammary gland during lactation and decreases in the liver, spleen, lung, kidney, and intestine. The most commonly known function for MFGE8 is to promote the clearance of apoptotic cells and maintain cellular homeostasis (126). Whey protein is known to include MFGE8. Calcium and Vit K, which are also present in milk, serve as cofactors of MFGE8 (126). In NAFLD, a study has shown that MFGE8 is reduced in hepatocytes, and its deletion leads to increased hepatic lipid accumulation and inflammatory response under metabolic challenges (125). Many studies suggested that maintaining the level of MFGE8 in the liver could be used to treat NAFLD (125,127).

Another component of milk that might be relevant to improved hepatic lipid storage is calcium. Dairy calcium was suggested to prevent weight and fat regain (128). Also, calcium might suppress hepatic and adipose lipogenesis by improving leptin and GLP-1 signaling, decreasing calcitriol levels, and changing gut microbiota composition (58). An additional proposed hypothesis for calcium-related weight reduction is binding fat to calcium, leading to excretion of the fat as calcium salt with feces (60). However, other

studies investigated the effect of a high calcium diet compared to dairy calcium. These studies concluded that a high calcium diet increased weight gain and hyperphagia compared to dairy calcium intake, which showed a preventive effect, suggesting that it is not the calcium responsible for these effects, but other components of the dairy might be related which support the idea of the whole dairy matrix instead of single components (59,129). Increasing dietary Ca^{2+} was proposed as a mechanism by which dairy might be responsible for weight loss by regulation of the circulating calcitriol (1,25-dihydroxy vitamin D) (1,25(OH)2 D) levels (130). High calcium diets may suppress calcitriol activity, therefore reducing adipocyte's number due to inhibition of the fatty acid synthesis and activation of lipolysis. The same study concluded that increasing the amount of dairy intake did not enhance weight loss compared to caloric restriction (130). According to our results, the dairy calcium is unlikely to play a role in the improvement of hepatic steatosis as cheese has more calcium than milk (Table 2—4).

According to Canadian guidelines for dairy intake, one serving of milk has the highest amount of vitamin D compared to one serving of yogurt while cheese does not have vitamin D. However, the yogurt we used was fortified with vit D and according to the dairy product's label, 100 g of yogurt has 15% of daily required of vit D while 250 ml of milk has 13%. My result favors milk over cheese for the improvement of hepatic steatosis, which is consistent with relative vitamin D content but does not explain the lack of effect of yogurt. Vitamin D influences calcium homeostasis in addition to its role in modulating the inflammatory response (123). Many human studies suggested that vitamin D intake is inversely associated with obesity and NAFLD (123,131,132). Interestingly, a study showed that glycemic control was worse in the winter season when the vitamin D

deficiency is higher (123). The study highlighted the effect of vitamin D on the NAFLD pathophysiology mechanism, for instance, vitamin D suppresses the activity of adipocyte renin-angiotensin system (RAS), thus increasing adiponectin secretion (123). Therefore, deficiency of vitamin D leads to decreased adiponectin secretion, which is linked to IR and NAFLD (123). Vitamin D deficiency negatively impacts gut innate immunity by causing gut dysbiosis and low-grade systemic inflammation, which is a major driving factor behind insulin resistance and metabolic dysfunction (131). Another study suggested that vitamin D supplementation might improve NAFLD by inflammation reduction, however the precise mechanism of action remained unclear (132). In our project, its unlikely that vitamin D played a role in the improvement of hepatic lipid steatosis since yogurt has about the same vitamin D as milk.

Dairy protein, including whey and casein, comprising 2.8% and 0.7% of total weight of milk respectively, was the focus of many studies due to its beneficial effect in metabolic syndrome and diabetes (133). Whey and casein contain a relatively high concentration of branched amino acids (BCAAs). Whey protein is rich in the BCAA leucine, which activates protein synthesis and stimulates insulin and GIP secretion and expression (133,134). Also, whey protein intake causes significant inhibition of DPP-4 activity, which was found to be increased in NAFLD patients, leading to slower degradation of incretin hormones (135). High activity of DPP-4 limits the activity of GLP-1, which may contribute to steatosis (135). Another study suggested that whey protein supplementation may improves hepatic steatosis and reduces oxidative stress (136). Casein increases the intestinal transit time and increases the feeling of fullness, while whey protein increases satiety signals and diminishes food intake (137). Compared to whey or casein alone, total dairy protein

consumption can protect against increased adiposity in rats fed HFD (60). Dairy protein has an antioxidant activity, which might be beneficial because increased oxidative stress is associated with most diet-related chronic diseases (138). Casein has the most antioxidant activity; therefore, cheese has the highest amount of antioxidants as cheese has more protein due to the fermentation process (138). Conversely, milk has more whey protein than cheese and yogurt because during processing, whey is the liquid left over after the caseins in milk have been coagulated and filtered off (139). Figure 5–2 summarizes the potential effects of whey protein. In our model, there does not seem to be a role of dairy protein in improving the liver steatosis because we did not see any effect of milk intake on body weight, food intake or insulin secretion, which would be expected by increasing whey intake.

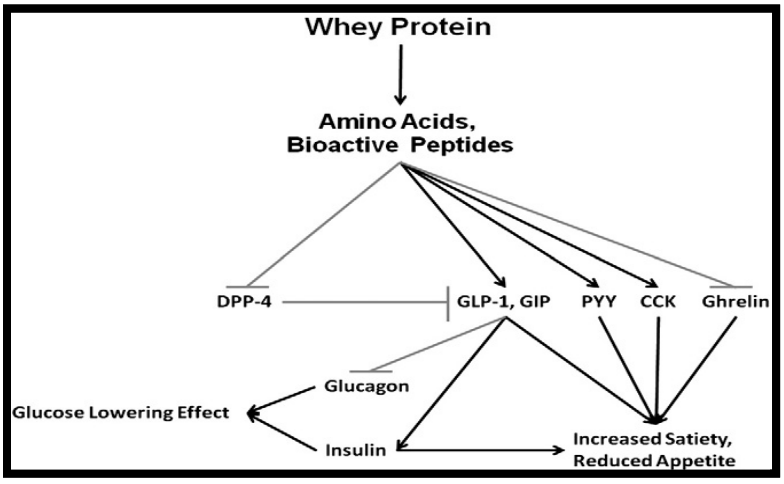


Figure 5-2 Metabolic effects of whey protein. Adapted from Jakubowicz D, Froy O, 2012 (134)**.**

According to the discussion above, we can summarize the possible mechanism of action by which milk improved the hepatic steatosis. The role of fatty acids, calcium, vitamin D, and protein are excluded based on the reasons mentioned in the discussion

and thus the focus in the discussion of future research will be on the role of phospholipids, choline, and MFGE8 (section 5.8).

Changes in fat metabolism seemed relatively specific to liver because we observed a significant increase in epididymal fat mass as well as total body fat among all HFD groups compared with the LFD. Unexpectedly in our experiment, the Yogurt group showed a higher concentration of serum and liver TG compared to LFD. The beneficial effect of yogurt intake on circulating lipids accumulation was suggested in many studies. Rosquist et al. concluded that consumption of yogurt enhanced with Lactobacillus acidophilus La5 and Bifidobacterium lactis Bb12 caused improvement in total cholesterol compared with conventional yogurt. At the same time, conventional yogurt reduced the total and LDL cholesterol but did not alter HDL cholesterol or TG (140). In 2010 a study concluded that both probiotic and conventional Yogurt positively affected the subjects' lipid profile, suggesting that consumption of fermented dairy can improve lipid profile independent of its probiotic content (141). We used a sweetened yogurt, which might explain our result for high TG. Intake of sugar-sweetened beverages causes dyslipidemia through ectopic fat accumulation, de novo lipogenesis, visceral adiposity, and hypertriglyceridemia (142). A recent review on dairy fat effects on human health has shown benefits of cheese consumption on hepatic lipid content; moreover, longer ripening duration of the cheese affects the cheese matrix and improves the health response (55). We contacted the Armstrong company regarding our Cheese ripening duration, but we did not get a firm answer.

IR and obesity are the hallmarks of NAFLD (143) Hepatic IR is thought to be involved in the etiology of NAFLD as a result of inflammation and deposition of lipid metabolites such as ceramide and diacylglycerol in the liver. Insulin stimulates de novo lipogenesis and inhibits hepatic gluconeogenesis; however, hepatic IR is associated with inability of insulin to suppress the gluconeogenesis, but the de novo lipogenesis is maintained. Reductions in ß-oxidation occur in association with increases in hepatic de novo lipogenesis. Insulin induces lipogenesis, increases the malonyl-CoA synthesis, which inhibits carnitine palmitoyl transferase-1 and lowers fatty acid oxidation. As a result, this abnormal lipid handling prompts the advancement of liver damage and NAFLD.

Hepatic IR is associated with increased VLDL-triglyceride production and impairment in LDL clearance from circulation, which contributes to dyslipidemia (55). Our results did not show any significant changes in the insulin sensitivity among dairy groups compared to the HFD group with ITT. Previous work showed improvement in the insulin sensitivity demonstrated by ITT and reduce G6Pase abundance after 8 weeks of high and low-fat cheese intake, However the liver histology of the prediabetic rats did not show any improvement in hepatic lipid accumulation (76). Interestingly, we observed improvement in the hepatic lipid storage in the Milk group independent of any effect on insulin sensitivity. The small dose of dairy might negate any effect of dairy on insulin sensitivity, and also, the duration of treatment might be too short.

5.4 Effect of Dairy on Markers of Liver Damage

Liver enzymes such as ALT, aspartate aminotransferase (AST), and gamma-glutamyl transferase (GGT), have been recognized as markers of liver damage and

identified as markers of NAFLD (144). ALT is an enzyme that catalyzes the transfer of the amino group from alanine to 2-oxoglutarate. High serum activity indicates liver cellular destruction (145) Many studies have shown that serum ALT levels significantly increased in patients with hepatic steatosis. In contrast, serum AST levels were associated with NAFLD patients, but serum GGT levels were correlated with the level of liver TG and used as a biomarker for NAFLD. However, serum ALT level is more specific as a marker of liver inflammation as ALT activity is positively correlated with intrahepatic triglyceride (146) therefore, the elevation of ALT is associated with IR and hepatic steatosis. A study showed that whey protein improved the bodyweight of rats and reduced hepatic TG, serum glucose, and liver enzymes (136). In contrast, another study observed that consuming three servings of low-fat dairy improved liver function and reduced systemic inflammation among subjects with MetS (147). However, in our experiment, ALT wasn't increased in HFD groups compared with the LFD group. Even though HFD did cause fatty liver, it wasn't severe enough to induce liver damage.

5.5 Effect of Dairy Intake on In Vivo and Hepatic Glucose Metabolism

Although we did not observe any effect of a low dose of dairy foods on insulin sensitivity, we were interested in whether gluconeogenic enzymes were altered in abundance in line with previous studies (76). Fasting causes a drop in blood glucose due to a lack of meal intake and, as a result, there is a decrease in glucose absorbed from the intestines (148). Therefore, hepatic gluconeogenesis maintains the systemic blood glucose by compensating for food supply interruptions (149). As the animal transitions between fed and fasted phases, the liver is the primary tissue that maintains metabolic balance. Fasting increases hepatic glucose and ketone body production, triacylglycerol

buildup, and glycogen depletion (150). Insulin normally suppresses PEPCK gene transcription and gluconeogenesis in the liver. However, in many animal species, obesity, and type 2 diabetes models, gluconeogenesis and PEPCK mRNA levels are 2–3 times higher in diabetic than in non-diabetic animals. These findings indicate that hyperglycemia in these animal models may result from insulin signaling problems, leading to PEPCK gene transcription not being suppressed (151). Previous work in IR rats suggested that cheese might modulate rate-limiting enzymes of gluconeogenesis (76). We assessed the impact of dairy consumption on the rate-limiting enzyme for gluconeogenesis (PEPCK and G6Pase) and found suppression of PEPCK abundance by Milk compared with LFD; however, the G6Pase was similar among groups. PEPCK catalyzes the conversion of oxaloacetate (OAA) to phosphoenolpyruvate (PEP) as the first step to produce glucose (149). In the previous cheese study, there was an effect of high and low-fat cheese only on G6Pase compared to the control group (76). The same study suggested that reduced G6Pase and PEPCK in the liver contributed to reduced gluconeogenesis in the second phase of the insulin tolerance test. In our research, it is likely that the dose of dairy was too low to test this hypothesis, therefore we would either increase the amount of dairy or extend the duration of the intervention since it might take longer to see an effect at the low dose.

We observed a significant increase in body weight among HFD groups compared to LFD as expected. Our lab's previous work showed a significant weight gain in prediabetic rats after HF/LF cheese feeding (76). Weight gain in response to dairy intake was consistent with a meta-analysis including 29 RCTs and 2101 participants to evaluate the effect of dairy intake on body weight. No evidence found from their collected data

supports the beneficial effect of dairy intake on body weight in long-term studies without calorie restriction (152).

To evaluate the effect of milk, yogurt, and cheese on insulin sensitivity, we performed ITT to measure the response of prediabetic mice to an exogenous insulin bolus. Ideally, in ITT, the plasma glucose concentration drops due to the inhibitory effect of insulin on endogenous glucose production and stimulatory effect on glucose uptake by muscle, whereby a faster drop indicates insulin sensitivity. The effect of exogenous insulin lasts for 3-15 minutes only due to counterregulatory hormones (153). In our experiment, we did not observe any significant changes between groups. To assess the impact of dairy consumption on hepatic gluconeogenesis animals were fasted overnight to deplete the glycogen storage and ensure that glucose production comes from available precursors to compensate for the high glucose disposal (pyruvate) instead of glycogen breakdown (154). Herein, the result indicates a significant difference in the basal blood glucose concentration between groups, which affects PTT outcome. These differences disappeared when we normalized the blood glucose to the baseline, suggesting that the diet did not affect the PTT. A study showed that insulin sensitivity is related to decreased body weight due to the beneficial effect of dairy protein (60), but in our model, we did not observe improvement in the body weight of the mice, which might explain why we did not see any effect on insulin sensitivity as an effect of dairy intake.

The method we used to convert the amount of dairy from a human serving to an equivalent amount for mice is based on the caloric intake. A limitation of this approach is that it did not fully consider the biochemical and functional system differences between

mice and humans which in turn affect pharmacokinetics of drugs (155). Allometric scaling (body surface area (BSA) normalization) is the method used to convert drug doses from animal to human (and vice versa), and it is widely used in the pharmacology field (156). BSA is based on oxygen utilization, caloric expenditure, basal metabolism, blood volume, and circulating plasma protein (156). Allometric scaling is used to calculate the safe initial dose for the animal to test a new drug effect. Because mice have a higher surface:volume ratio and higher metabolic rate than human, this results in a higher dose per body weight in mice than humans. By basing our calculated dose on caloric intake we did take into account the higher metabolic rate of the mice; moreover, by basing the dose on the recommended intake of dairy for humans, it means that it is achievable by humans without displacing too many calories and nutrients from other foods. Human studies showed that a relatively small amount of dairy have a benefit (34,36,38). Therefore, the smaller dose may be sufficient at least for some effects.

5.6 Conclusions

Our research aimed to evaluate the effect of dairy foods differing in their matrix as well as a nutrient profile on glucose homeostasis. Our intention was to provide dairy in an amount equivalent to the recommended dose for adult humans (2-3 servings/day); however, we ended up using less than one serving a day of dairy. Consumption of milk, yogurt, and cheese had no effect on insulin sensitivity, glucose homeostasis, and body weight with a half serving a day. That is likely due to the low dose or possibly insufficient duration of treatment. On the other hand, the Milk group showed reduced hepatic lipid accumulation in IR mice compared to the HFD group, suggesting a beneficial effect of regular milk intake even with a half serving a day.

However, these beneficial effects of milk are independent of body weight and insulin sensitivity. This study contributes to understanding dairy consumption's effect as a healthy choice to improve human health, particularly IR and NAFLD. Considerably more work will need to be done to confirm the result before applying it in real life.

5.7 Limitations

The first limitation of the current study is that the actual dairy dose used in this research was relatively small compared to that used in our previous work (76), and upon which we developed our current hypothesis. The dairy dose used in this study was only 0.5 serving instead of 2 servings. This happened due to an error that occurred in the calculation of the required dairy dose for this research. Unfortunately, we realized this error after we had completed the whole trial. Therefore, the results and the research outcomes have been negatively influenced by such error. As a result, the focus of the research shifted to discuss the impact of a small dairy dose on hepatic lipid instead of the effect of dairy on insulin sensitivity. Another limitation is that female mice failed to induce IR, as noted by others (99), so we could not examine any sex-related differences regarding the impact of dairy.

The epidemic of COVID-19 and related interruptions had a significant influence on this project's progress. During the Covid restriction period, there were multiple closures for the lab and no guarantee that it would not be shut off again, which affected the trial's duration. We were obliged to end the trial early. And restrictions have limited our ability to perform some planned tests (for example, metabolomics analysis) and access to some equipment. Furthermore, the re-entry plan for the lab has affected our work as social

distancing rules made shadowing for learning more difficult. The idea that learning from others in the lab by observing a procedure that appears wrong, discussing some data, and detecting a problem with lab members has been drastically decreased due to the covid situation. This is a challenge, especially for those unfamiliar with the lab equipment and need to be taught how to use them and perform an experiment with a distant mentor. Personal factors such as requirements for children to stay home from school also had a negative impact. Nevertheless, most of the original objectives of the research were met.

5.8 Future Recommendations

The work presented in this thesis demonstrates that half a serving of milk primarily affects hepatic lipid accumulation, leading to improved liver steatosis. However, the mechanism by which milk consumption led to this improvement is unclear and requires further investigation. Considering the possible pathways leading to hepatic lipid accumulation, it would be worth investigating the impact of milk intake on both fatty acid synthesis (de novo lipogenesis) and fatty acid oxidation by measuring the abundance and activity of key rate-limiting enzymes in each pathway. Furthermore, the effect of milk consumption on VLDL concentration could be studied as high choline concentration in milk may increase VLDL synthesis.

Considering the recommended dairy amount and given the beneficial effect of dairy in this study with a lower dosage, conducting a dose-response study with a higher amount of dairy might allow us to investigate further a possible positive effect of dairy on insulin sensitivity and hepatic lipid accumulation. In addition, an interesting point is to increase the feeding period to evaluate if the beneficial effects of dairy are transient or sustained over time.

An intriguing area of research is comparing whole food matrix intake to individual food components such as bioactive peptides, fatty acids, and probiotics. In this study, we used whole milk, but several individual components from milk such as Ca^{+2}, protein, phospholipid, choline, and other milk nutrient components have been shown to promote beneficial effects on body weight and insulin sensitivity. Therefore, it would be interesting to compare the effect of whole milk ingestion with MFGE8 on insulin sensitivity and hepatic steatosis to identify the role the food matrix plays in the observed effect.

Moreover, in our work, we hypothesized that milk PC and SM improved hepatic steatosis by improving the intestinal permeability and repairing the damage caused by LPS due to HFD consumption; however, we did not investigate changes in the intestine in future work. It would be interesting to assess gut permeability, for instance, by conducting a lactulose/mannitol (Lac/Man) test (157) to assess the status of epithelial barrier integrity after milk consumption.

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