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The effect of milk proteins co-ingested with glucose on blood glucose control in rats

by

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The ingestion of whey protein (WP) leads to the reduction of postprandial blood glucose (BG) response paralleled with an increased insulin response. However, the role of glycomacropeptide (GMP), β-lactoglobulin (β-LG),α-lactalbumin (α-LA), micellar casein (MC), and total milk protein (TMP) remains unclear when these proteins are consumed with a glycemic carbohydrate. The objective of this study was to investigate the effect of co-ingestion of WP, GMP, β-LG, α-LA, MC and TMP with glucose on BG and insulin response in rats. We hypothesized that milk protein fractions co-ingested with glucose will reduce blood glucose response and increase insulin response compared to glucose ingested alone. Methods: A randomized repeated measures study was conducted in rats fitted with jugular vein catheters and vascular access buttons (VAB). Ten male 10-week-old 275-300g Wistar Han rats were gavaged 350mg (allometrically scaled from a human dosage of 10g) of WP, GMP, β-LG, α-LA, MC, TMP co-ingested with glucose or a glucose control dissolved in 3ml of water after being fasted for 6h during daylight. The use of a VAB allowed for the same rat to receive all seven treatments in a random order, with a 48h washout period between treatments. Blood was collected at 0, 15, and 30 min for insulin, and at 0, 15, 30, 60, 90, and 120 min for glucose. Whole blood was analyzed for glucose using a HemoCue 201 Glucose Analyzer, and plasma was analyzed for insulin using a wide range ELISA. The data were tested for normality and analyzed using Two-Way Repeated Measures ANOVA for the effect of time, treatment, and a treatment by time interaction. The data for the area under the curve (AUC) for 2h BG and 30 min insulin were analyzed with One-Way Repeated Measures ANOVA. The differences between the treatments were assessed with Tukey-Kramer post hoc test. Results: There was an effect of time ($P<0.0001$), a treatment by time interaction ($P<0.0001$) but no effect of treatment ($P=0.35$) over 120 min on BG response. There was an effect of time ($P<0.0001$), a treatment by time interaction ($P<0.0001$) and an effect of treatment ($P=0.03$) on BG response over 30 min. WP, GMP, β-LG, α-LA, TMP and MC resulted in a lower BG compared to glucose treatment at 15 and 30 min ($P<0.05$). Blood glucose AUC over 30 min was suppressed after WP treatment compared to glucose alone ($P<0.05$). There was an effect of time ($P<0.0001$) and a treatment by time interaction ($P=0.0002$) but no effect of treatment ($P>0.05$) on insulin response over 30 min. There was no effect of a treatment on insulin AUC over 30 min ($P=0.4$). Results: at the dose of 350mg WP, GMP, β-LG, α-LA, MC and TMP co-ingested with glucose attenuated BG response at 15 and 30 min, and WP co-ingested with glucose attenuated BG response over 30 min compared to glucose alone. The co-ingestion of milk protein fractions with glucose resulted in an elevated insulin response similar to the insulin response when glucose was ingested alone.

Conclusion: co-ingestion of milk protein fractions with glucose result in short-term BG suppression and increased insulin without exacerbated insulin response compared to glucose alone.

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List of Abbreviations

A

α-LA	Alpha-lactalbumin
A1C	Glycated hemoglobin (also HbA1C)

B

BCAA	Branched-chain amino acids
BG	Blood glucose
β-LG	Beta-lactoglobulin
BMI	Body mass index

C

CACF	Carlton Animal Care Facility
CN	Casein
CRL	Charles River Laboratories

D

DPP-4	Dipeptidyl-peptidase-IV (also DPP-IV)

F

FPG	Fasting plasma glucose

G

GDM	Gestational Diabetes
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon-like peptide-1
GLUT	Glucose transporter
GMP	Glycomacropeptide
GTT	Glucose tolerance test

H

HbA1C	Glycated hemoglobin

L

LMIC	Low- and middle-income countries

M

MC	Micellar casein
mTOR	Mechanistic target of rapamycin

O

OGTT	Oral Glucose Tolerance Test

P

PG	Plasma glucose
PNP3M	PinPort injector

T

T1D Type 1 diabetes (also T1DM)
T2D Type 2 diabetes (also T2DM)
TMP Total milk protein

V
VAB Vascular Access Button
VABC Vascular Access Button Cap

W
WP Whey protein
WPC Whey protein concentrate
WPH Whey protein hydrolysate
WPI Whey protein isolate

Chapter 1: Introduction

Diabetes is one of the top chronic diseases in Canada and approximately 10% of Canadians been diagnosed (Diabetes Canada, 2021). Specifically, Type 2 Diabetes (T2D) is a diverse metabolic illness characterized by hyperglycemia caused by inadequate insulin production, insulin resistance, or a combination of the two (Diabetes Canada, 2021). It is currently the world's seventh greatest cause of death (Diabetes Canada, 2021). While incidence has been relatively stable in Canada, prevalence is increasing because of the population growth and higher life expectancy (Diabetes Canada, 2017). While lifestyle changes and medication have been shown to postpone development from pre-diabetes to type 2 diabetes, they have not been shown to successfully reduce long-term incidence and prevalence, and alternate kinds of therapy may be advantageous (Diabetes Canada, 2018). There is strong evidence of anti-hyperglycemic properties of major fractions of milk proteins (casein and whey protein) (Bohdan et.al,2007). However, there is not enough information on the effects of milk protein subunits, including the fractions of casein and whey protein on blood glucose control. Another unexplored area is the effect of milk proteins co-ingested with glucose on blood glucose control. This research gap may be filled in part by this study's objective of examining the short-term blood glucose control effects of diverse milk protein fractions co-ingested with glucose using rat models. Understanding the physiological processes behind blood glucose regulation in response to ingested food components with biological activity may help guide the development of therapeutic food and natural health products aimed at improving blood glucose management.

Chapter 2: Literature Review

2.1. Blood glucose regulation

2.1.1. Blood glucose homeostasis

The endocrine pancreas secretes insulin and glucagon. Both hormones play a crucial function in regulating blood glucose levels (Bano, 2013). If the concentration of either insulin or glucagon rises or declines from its normal range, blood sugar level would possibly spike or drop. The action of both insulin and glucagon to maintain a certain level of blood glucose contributes to glucose homeostasis (Bano, 2013; Wood & Trayhurn, 2003). The plasma glucose concentration is normally maintained within a relatively narrow range, between 3.3 and 6 mmol/L despite wide variations in glucose levels after meals and exercise (Statistics Canada, 2017; Bano,2013; Diabetes Canada, 2018; Hidayat et.al., 2019). There are five predominant types of cells found in pancreatic islets of Langerhans that contribute to the production of hormones involved in glycemic regulation, including β-cells that secret insulin, C-peptide and amylin and represent 65-80% of pancreas, α-cells (15-20% of endocrine cells) that secrete glucagon, γ-cells (3-5% of endocrine cells) that secrete pancreatic polypeptide (PP), δ-cells (3-10% of endocrine cells) that secrete somatostatin, and ghrelin-producing ε-cells (<1% of endocrine cells). Thus, glucagon is released in response to a low blood glucose concentration stimulating the breakdown of glycogen in the liver and resupplying glucose into circulation, while insulin is released in response to increased blood glucose concentration after ingestion of glycemic carbohydrates (e.g., starch and sugars) and facilitating the uptake of glucose by peripheral tissues, including liver, muscles, and adipose tissue, and resulting in the reduction of blood glucose concentration. Among the nutrients other than glycemic carbohydrates, food proteins possess a specific action on hormones regulating blood glucose homeostasis, and their actions are mainly determined by their amino acid composition (Luhovyy & Kathirvel, 2022)

While the peripheral glucose control is coordinated by balanced action of insulin and glucagon, the central nervous system (CNS) can sense insulin, glucagon-like peptide-1 (GLP-1) and some nutrients, including glucose, and regulate blood glucose control via vagal efferent mechanisms leading to a reduced hepatic glucose production (Lam et al., 2009). Plasma glucose is a crucial metabolic fuel utilized by the central nervous system (CNS) that is incapable of producing glucose and therefore relying on glucose supply from the circulation (Lam et al., 2009). Brief hypoglycemia can lead to the transient dysfunction of the CNS causing a stroke-like picture and symptoms associated with behavioural changes, confusion, loss of consciousness, seizures and even as cerebellar stroke (Agrawal et al., 2014). In 1953, Jean Mayer proposed the glucostatic theory of food intake regulation (Mayer, 1953). This theory suggested that the glucose level is sensed by glucose receptors and relayed to the CNS, and when the glucose concentration is low, the feeling of hunger is increased, and when the glucose concentration is high, the feeling of hunger is decreased (Chaput & Tremblay, 2009). Although insulin does not stimulate glucose uptake in the brain, it can cross through blood-brain barrier and regulate many metabolic functions, including hepatic glucose production and the sympathoadrenal response to hypoglycemia (Agrawal et al., 2021). While glucose is a sole energy source in the brain, the sensing of glucose level in the brain helps to regulate peripheral blood glucose level, specifically, the high level of central glucose led to reduced level of blood glucose and insulin, and suppression of blood glucose production in the liver (Lam et al., 2009).

Glucose in plasma is derived from three sources: intestinal absorption from the diet, glycogenolysis, the breakdown of glycogen in the liver, and gluconeogenesis the formation of glucose in the liver and kidney from non-carbohydrate precursors (Bano, 2013; Gasbjerg et al., 2018; Han et al., 2016). Glucose disposal from blood usually occurs after glucose absorption by target tissues and further glycolysis or storing in the form of glycogen (Hers, 1990). While insulin

and glucagon play the major role in blood glucose control, other hormones such as incretins (GLP-1 and GIP), CCK, somatostatin, leptin, catecholamines and neuropeptides such as Neuropeptide Y or vasoactive intestinal peptide, may modulate either pancreatic synthesis or secretion of insulin and glucagon, or hepatic glucose production; however, these central and peripheral mechanisms might be disabled in individuals with obesity or diabetes (Röder et al., 2016) .

2.1.2. Hepatic glucose production

After gastrointestinal absorption of glucose, around 33% of glucose goes into liver, another 33% is utilised by muscle and adipose tissue, and the remaining amount gets used by the brain, kidney and red blood cells (Moore et al., 2012). Following glucose absorption, the liver plays an important role in maintaining glucose homeostasis by storing glucose in form of glycogen and is responsible for the endogenous glucose production. The following metabolic events take place in the liver, including gluconeogenesis, glycogenolysis, glycolysis, and glycogenesis (Han et al., 2016). When the plasma glucose concentration rises, high plasma glucose promotes glucose absorption from the blood stream to be stored as glycogen in the liver (Sharabi et al., 2015). Secondly, pancreatic β-cells start the secretion of insulin to supress hepatic glucose production, and maintain glycogenesis (Sharabi et al., 2015). When glucose levels drop in the blood stream more than the normal range, pancreatic α-cells releases glucagon, which stimulate hepatic glucose production in the processes of gluconeogenesis and glycogenolysis to maintain the normal blood glucose levels (Sharabi et al., 2015). The studies with rodents, dogs and humans demonstrate the direct and indirect effect of insulin in the brain on the suppression of hepatic glucose production; however, the increased level of insulin in the brain of individuals with obesity associated with insulin resistance, or with diabetes failed to suppress hepatic glucose production (Agrawal et al., 2021). Some proteins may affect hepatic glucose production. For example, the proteins from the latex isolated from medicinal

plant *Calotropis procera* and administered to rats (5mg/kg b.w.) has shown the reduction of blood glucose two hours after glucose administration without changes in insulin secretion (de Oliveira et al., 2019). This study has found that latex proteins improved insulin sensitivity, and glucose tolerance at 30 min, and decreased hepatic glucose production at 15 and 30 min as it was confirmed in the experiment with glucagon-induced hepatic glucose release (de Oliveira et al., 2019). It is unknown whether food proteins possess similar effect and can influence hepatic glucose production. Most of the amino acids, except BCAAs, stimulate glucagon secretion and it was hypothesized that plant-based proteins stimulate glucagon release (Chambers et al., 1968; Luhovyy & Kathirvel, 2022; McCarty, 1999; Rocha et al., 1972).

2.1.3. Glucose transporters

Glucose transporters (GLUT) family of facilitative glucose transporters includes 14 members (GLUT 1-12) encoded by the *SLC2A1–SLC2A14* genes that are identified with the most of them being integral membrane proteins engaged in facilitated diffusion of hexoses, including glucose and fructose, and other sugar-related molecules such as inositol, glucosamine, or metabolites such as uric acid (Szablewski, 2021). Based on their composition and characteristics, glucose transporters are divided into three subfamilies that were previously classified as glucose transporters, GLUT1-4 (class I); fructose transporters, GLUT5, GLUT7, GLUT9 and GLUT11 (class II); and class III included GLUT6, GLUT8, GLUT10, GLUT12, and the myo-inositol transporter HMIT1 (Joost & Thorens, 2001). Glucose transporters are not only involved in glucose uptake, but may have other physiological roles such as glucose sensing (Chadt & Al-Hasani, 2020). For example, GLUT2 is required for the function of hepatoportal glucose sensor (Burcelin et al., 2000). The histological distribution of glucose transporters and their biological roles are different. Thus, GLUT1 is the main glucose transporter in non-parenchymal cells while GLUT2 is the most

abundant GLUT isoform in hepatocytes (Chadt & Al-Hasani, 2020). GLUT1 is ubiquitously present in all tissues facilitating the basal glucose uptake (Pragallapati & Manyam, 2019). The glucose transporters GLUT1 and GLUT4 facilitate glucose transport into insulin-sensitive cells (Kumagai et al.,1994). The functional difference between these transporters is that GLUT1 is insulin-independent while GLUT4 is insulin-dependent which spawned the hypothesis that the insulin resistance is determined by the imbalance of GLUT1 and GLUT4 function (Ebeling et al., 1998). This hypothesis stated that glucose uptake by muscle tissue via GLUT4 transporters is utilized primarily for glycogenesis or for glycolysis; however, when glucose uptake is facilitated mainly via GLUT1 transporter in muscle cells and metabolized via multiple pathways, hexosamines may be formed and negatively impact GLUT4 that in turn may negatively impact insulin-mediated glucose uptake resulting in insulin resistance (Ebeling et al., 1998). Although this hypothesis was not further elaborated upon a study with rats found that GLUT4 transporter showed a concentration-dependent competition between glucose and glucosamine uptake, and has an effect on the expression of Farnesoid X receptor in liver of fructose-fed rats exacerbating insulin resistance (Chien et al., 2009).

A recent review has summarized the present knowledge on the role of food components on glucose transporters (Abioye et al., 2022). Among those compounds that can affect the expression or activity of glucose transporters are black pea protein hydrolysates and peptides, plant extracts including those from onion, potato, strawberry, coffee, tea, and other plant ingredients (Abioye et al., 2022). A study with weaning pigs fed with isoleucine-supplemented diet found that the expression of GLUT1 in red muscles, GLUT4 in red muscle, white muscle and intermediate muscle, and Na+/glucose co-transporter 1 and GLUT2 in small intestine was enhanced (Salehi et al., 2012). This observation resonates with the fact that isoleucine content is high in whey protein and known to reduce postprandial blood glucose (Akhavan et al., 2010). Isoleucine along with

other BCAAs have strong insulinotropic effect and the content of isoleucine in whey protein and casein is one of the highest among other food proteins (Luhovyy & Kathirvel, 2022). A new study conducted with cell lines demonstrated that BCAAs significantly increase glucose uptake and revert decreased glucose uptake induced by endoplasmic reticulum stress or glycolysis inhibition (Iwai et al., 2022). This study also revealed that BCAA enhance the translocation of GLUTs proteins to the plasma membrane over time (Iwai et al., 2022). The effect of BCAAs on glucose transporters suggest that the reduction of blood glucose after the ingestion of whey protein might be due to multiple mechanisms, including the effect of amino acids on glucose transporters.

2.1.4. Incretins and blood glucose control

Incretins are the group of hormones which are usually secreted from enteroendocrine cells (Nauck & Meier, 2018). The main role of incretins is to regulate the plasma insulin concentration after food intake (Kim & Egan, 2008). At normal physiological conditions, incretins works as insulinotropic hormones (Holst et al., 2021). The phenomenon known as an "incretin effect" provides around 50% of insulin secretion after glucose ingestion (Holst et al., 2021). Two main incretins include glucagon like peptide-1 (GLP-1), and glucose-dependant insulinotropic polypeptide (GIP) (Campbell & Drucker, 2013). While they are both considered to be insulinogenic substances, GIP, independently of the dose, does not stimulate insulin secretion in patients with T2DM, while GLP-1 does (Holst, 2019). The other difference between GIP and GLP-1 is that GIP regulates blood glucose absorption and glycemic excursion only following enteral glucose challenge (Sherwood & Adams, 2004). GIP is a peptide hormone containing 42 amino acids and mainly secreted from K-cells in the proximal duodenum (Fridolf et al., 1992). GIP is produced from a 153-amino acid precursor pre-pro-GIP (Yavropoulou & Yovos, 2010). The secretion of GIP increases after the ingestion of food leading to the inhibition of gastric acid

secretion and motility (Vella, A., & drucker, D. J., 2011). The other sites of GIP secretion include brain, submandibular salivary glands and the stomach, while GIP receptors are expressed in various organs and tissues (Park, 2016). GIP binds with their receptor, which is a class B G-protein cognate receptor (McIntosh et al., 2009). The receptor activation induces the insulin secretion from pancreatic β-cells (McIntosh et al., 2009). The mechanism of GIP action is mediated via multiple GIP receptors present on β-cells that are activated after binding with GIP that leads to increased concentration of intracellular cAMP and downstream increase of [Ca^{2+}] leading to the release of insulin (Gupta & Raja, 2021). The other function of GIP is the inhibition of glucagon secretion and stimulation of adipogenesis (Park, M. K., 2016). Both GIP and GLP-1 are found to be potent therapy options for T2DM patients (Gasbjerg et al., 2018).

GLP-1 is produced in L-cells of the small intestine in response to food ingestion (Nadkarni et al., 2014). The key physiological reactions to GLP-1 include glucose-dependent insulin secretion, glucagon secretion inhibition, and gastric acid secretion and gastric emptying inhibition (Aziz et al., 2005). The activation of the GLP-1 receptor is responsible for all of GLP-1's functions (Ahmed & Khalique, 2019). This peptide's possible utility in the treatment of diabetes has been studied (Antony & Vijayan, 2021); however, the GLP-1 receptor agonists are recognized as the second line of antihyperglycemic agent for use in T2DM according to the Diabetes Canada Clinical Practice Guidelines Expert Committee (Lipscombe et al., 2018). Glucagon like peptide 1(GLP-1) demonstrated antidiabetogenic activity in type 2 diabetes individuals by boosting insulin production, decreasing glucagon secretion, prolonging stomach emptying time, and promoting satiety; however, DPP IV can quickly deteriorate it (Aziz et al., 2005). Glucagon like peptide 1(GLP-1) maintains the glucose levels by increasing gastric emptying time and small intestine motility in non-fasting state (Mojsov, 2000). Glucagon like peptide 1(GLP-1) stimulate the glucose dependent insulin secretion and inhibit glucagon secretion from islets cell and reduce the

hyperglycemic condition with the help of development of GLP-1 receptor agonists (Nadkarni et al., 2014). It also regulates glucose level by supressing the glucagon release from α cells. In β-cells it promotes insulin biosynthesis, and insulin secretion (Drucker, 2018).

Food proteins affect glycaemic control via multiple mechanisms, including their effect on incretins. Thus, both whey protein and soy protein provided as pre-meal protein drinks containing 9g of protein resulted in increased level of incretins after ingestion (Gunnerud et al., 2012). In another study with 39 participants, the GLP-1 response was lower after ingestion of 15g of pea protein hydrolysate compared to 15g of milk protein control (Diepvens et al., 2008). This suggests that the responses of incretins may be determined by both dose and origin, while the other factors may include the presence of other nutrients such as glucose that would reproduce the effects of mixed meals.

2.2. Hyperglycemia

2.2.1. Pathophysiology of impaired blood glucose control

Insulin is an endocrine peptide hormone that interacts with plasma membrane-bound receptors in target cells to induce an anabolic response to nutrients (Ralston, 2002). Type 1 diabetes mellitus is characterized by a total lack of insulin whereas, T2DM involves resistance of the body's peripheral tissues to the effects of insulin (Moini, 2019). Type 2 diabetes mellitus is caused by a decrease of β-cell function, as well as chronic insulin resistance (Srinivasan & Poduri, 2007). Numerous research involving animals and humans reveal that obesity is the primary pathogenic factor behind the development of insulin resistance (Galicia-Garcia et al., 2020). In T1DM, the liver can produce glucose, but there is only a limited amount of storage for glycogen. When insulin is absent, gluconeogenesis is uncontrolled, and blood glucose levels are higher (Moini, 2019).

T2DM develops when β-cells are unable to secrete enough insulin to compensate for reduced insulin sensitivity, which is caused by β-cell malfunction, resulting in an elevated blood glucose level (Chiu et al., 2006). In T2DM, insulin resistance causes the body to react as there is an absence of insulin, even though it is elevated in blood but could not get attached to the receptors to produce the effect resulting in hyperglycemia (Moini, 2019). In the early stages of T2DM, insulin levels are often very high and fluctuate that further leads to the development of the disease. T2DM usually develops in adults and its more common with aging. Plasma glucose levels are higher after food intake in older people than in youngsters. The higher levels need more time to get back to normal, partly due to increased accumulation of visceral and abdominal fat, with decreased muscle mass (Moini, 2019). Clinically, T2DM is much more prevalent than T1DM (Gagliardi & Wittert, 2007). In Canada, 90% of diabetes cases are T2DM 9% T1DM, and less than 1% of a different type (Health Canada, 2017).

2.2.2. Insulin sensitivity and resistance

2.2.2.1. Sensitivity to insulin

Insulin sensitivity refers to the degree to which the body's cells respond to insulin (Czech, 2017). Increased insulin sensitivity enables the body's cells to use blood glucose more efficiently, hence lowering blood sugar (Borghouts & Keizer, 2000). Insulin sensitivity differs across individuals. Individuals with low insulin sensitivity, also known as insulin resistance, will require more insulin, either from their own pancreas or through injections, to maintain stable blood glucose levels (Freeman & Pennings, 2022). Insulin resistance is a symptom of the body's glucose metabolic abnormalities and may result in other health concerns such as high blood pressure and cholesterol levels (Freeman & Pennings, 2022). However, having increased insulin sensitivity might pose complications for patients with T1DM, particularly in childeren (Atkinson et al., 2014). For

individuals with T1DM having a high insulin sensitivity may occasionally increase the risk of hypoglycemia if the individual is very insulin sensitive (Sesti, 2006). Insulin resistance impairs glucose disposal, resulting in a high insulin production and hyperinsulinemia (Freeman & Pennings, 2022). Insufficiency of insulin can result in a range of health concerns. Typically, the body attempts to compensate for decreased insulin sensitivity by generating more insulin (Atkinson et al., 2014). However, an elevated insulin level in the circulatory system has been linked to blood vessel damage, hypertension, heart disease and heart failure, obesity, osteoporosis, and possibly cancer (Giacco et.al., 2010). Stress and sickness can have a short-term effect on insulin sensitivity. Insulin resistance, or low insulin sensitivity, is more commonly linked with type 2 diabetes, although it can also develop in type 1 diabetics. “Double diabetes” is a term that refers to individuals who have type 1 diabetes and insulin resistance (Kietsiriroje et al., 2019). The causes of insulin resistance are not completely understood; however, the role of excessive body weight in triggering insulin resistance has been discussed. Thus, a substantial correlations between an accumulation of fat surrounding the organs and impaired insulin sensitivity has been shown (Haythorne & Ashcroft, 2021). Lifestyle interventions may increase and improve insulin sensitivity. Physical exercise is vital and has a significant influence on insulin sensitivity (Giacco et.al., 2010). Foods that are high in fibre and have a low glycemic index and glycemic load can also help increase insulin sensitivity (Wilding, 2014). Insulin resistance plays a significant role in the pathophysiology of the metabolic syndrome and type 2 diabetes, although the mechanisms behind it remain unknown. The fundamental metabolic anomaly in insulin resistant type 2 diabetes is a deficiency in insulin-stimulated glucose transport in skeletal muscle (Wondmkun, 2020). Insulin resistance, defined as a decreased response to normal circulating insulin levels, plays a significant role in the aetiology of type 2 diabetes (Båvenholm et al., 2003; Goldstein, 2002). The body begins producing insulin when glucose enters the circulatory system, mostly via dietary

carbohydrate breakdown and absorption (Boden et al., 2015). Under normal conditions, insulin reactivity causes glucose to be absorbed into body cells for use as energy, resulting in a drop in the concentration of glucose in the blood stream, resulting in a normal range of glucose in the blood stream (Wang et al., 2019). Type 2 diabetes mellitus is defined by four primary metabolic abnormalities: decreased insulin action, obesity, increased endogenous glucose output, and insulin secretory dysfunction (Chatterjee et al., 2017). Insulin maintains glucose homeostasis by inhibiting hepatic glucose synthesis and boosting glucose absorption, predominantly into skeletal muscle and adipose tissue (Sesti, 2006). The bulk of insulin-stimulated glucose absorption occurs in skeletal muscle, and over 80% of this glucose is ultimately stored as glycogen (Taylor, 2012).

2.3. Diabetes

2.3.1. Classification of diabetes

Majority of cases of diabetes can be broadly classified into 2 categories: type 1 diabetes and type 2 diabetes, but there are also other types of diabetes such as gestational diabetes and monogenic diabetes (Atkinson et al., 2014). Type 1 diabetes or juvenile occurs because of primary β-cell destruction with insulin deficiency, which is prone to ketoacidosis (Atkinson et al., 2014). Type 2 diabetes occurs mainly due to insulin resistance with relative insulin deficiency to a secretory defect with insulin resistance (Chatterjee et al., 2017). Gestational diabetes mainly occurs during the pregnancy due to prominent glucose intolerance. It can be resolved after pregnancy or can convert into T2DM (Postic et al., 1994). Monogenic diabetes is a rare type caused by genetic defect of β-cell function which usually occurs in a young individual(American Diabetes Association, 2014). Differentiating between type 1, type 2 and monogenic diabetes is important but can be difficult at the time of diagnosis in certain situations (American Diabetes Association, 2014). One

monogenic form to highlight is neonatal diabetes, which typically presents by six months of age and is indistinguishable from type 1 diabetes in its clinical features (Atkinson et al., 2014).

2.3.1.1. T1DM

Type 1 diabetes (T1DM)is the primary result of pancreatic β-cell destruction that can be due to an autoimmune process or unknown etiology with consequent insulin deficiency, which is prone to ketoacidosis (Atkinson et al., 2014). Although type 1 diabetes can be diagnosed at any age; however, it is one of the most chronic diseases of childhood (Health Canada, 2011). Peaks in presentation occurs between 5-7 years of age and at or near puberty. According to Statistic Canada, in 2017, about 7.3% of Canadians aged 12 and older (roughly 2.3 million people) reported being diagnosed with type 1 diabetes (Statistics Canada, 2018). Type 1 diabetes is usually present in individuals without a family history. Only 10-15% of the patients have a first- or second degree relative with the disease. The main genes predisposing someone to T1DM are within the major histocompatibility complex region, often called HLA (human leucocyte antigen) and located on chromosome 6 (Paschou et al., 2017). HLA complex polymorphic alleles are responsible for 40-50% of the genetic risk of T1DM development (Paschou et al., 2017). Environmental factors also play an important role in the pathogenesis of T1DM Much evidence of this derives from the study of monozygotic twins, where occurrence of the disease in both siblings varies around 50% and never reaches 100%. The environmental factors include viruses, toxins, and nutrients. The precise effect of these factors remains unclear, but it is important to be identified. Immunological factors could be one of the causes too (Raman, 2016).

2.3.1.2. T2DM

Type 2 diabetes the most common metabolic disorders in the world. The development of T2DM is primarily caused by the defect in β-cell functions and inability of insulin sensitive tissues to

respond to insulin. Insulin synthesis and release must precisely meet the metabolic demand, if not then the imbalance in insulin release and its action which leads to the pathogenesis of T2D (Health Canada, 2012).

Epidemiological data shows that diabetes caused 4.2 million deaths in 2019; and about 463 million adults aged between 20 to 79 were living with diabetes, and the numbers are more likely to go up around 700 million by 2045 (Khan et al., 2020). In 2021, approximately 537 million adults (age 20-79) are living with diabetes, and it is projected to rise to 643 million by 2030 (Statistics Canada, 2022). Patients with diabetes have a 15% higher risk of developing chronic health conditions and mortality than people without diabetes with cardiovascular disease. According to Diabetes Canada's recent data, there are more than 5.7 million Canadians who have T2DM, however, there are 11.7 million Canadians living with diabetes or prediabetes (Diabetes Canada, 2022). Diabetes risk factors include a complex combination of genetic, metabolic, and environmental factors that interact with one another contributing to its prevalence. Predisposition to T2DM due to non-modifiable risk factors has a strong genetic basis (CDC, 2022). Globally, the prevalence and incidence of T2DM are found to vary widely depending on ethnicity and geographical region with Japanese, Hispanics and native Americans having the highest risks (CDC, 2022). Obesity is the strongest risk factor for T2DM and is associated with metabolic abnormalities resulting in insulin resistance. There is an inverse relationship between body mass index(BMI)and the age at diagnosis of T2DM. The exact mechanisms by which obesity induces T2DM and insulin resistance is still unclear. A sedentary lifestyle is another risk factor for T2DM as shown by the women's health study, which showed a reduction of 34% in developing T2DM in participants walking 2-3 h a week or at least 40 min a week (Diabetes Canada,2018). There are three benefits of physical activity in the prevention and management of diabetes. First, the contraction of skeletal muscle cells induces an increased blood flow into muscle, enhancing glucose uptake from plasma. Second,

physical activity reduces the intra-abdominal fat, which is known as the risk factor that promotes IR. Finally, moderate intensity exercise has been shown to improve glucose uptake by 40% (Kirwan et al., 2017). Physical activity improves glucose uptake and insulin sensitivity, but it can also improve or even reverse inflammation and oxidative stress, which are T2DM predisposing factors (Wilding, 2014).

2.3.1.3. Gestational diabetes

Gestational diabetes is a serious pregnancy complication, in which women without previously diagnosed diabetes develop chronic hyperglycemia during gestation(National Diabetes Data Group, 1979). In most cases, this hyperglycemia is a result of impaired glucose tolerance due to pancreatic β-cell dysfunction on a background of chronic insulin resistance (National Diabetes Data Group, 1979). The American Diabetes Association formally classifies GDM as a diabetes first diagnosed in the second or third trimester of pregnancy that is not clearly pre-existing T1DM or T2DM. Epidemiological studies of risk factors for GDM are limited and are typically afflicted by confounding factors (Kampmann et al., 2015). There are severe risk factors associated with GDM such as overweight/obesity, excessive gestational weight gain, westernized diet, ethnicity, genetic polymorphism, advanced maternal age, intrauterine environment, family, and personal history of GDM, and other diseases of insulin resistance, such as polycystic ovarian syndrome (PCOS) (Lee et al., 2018). Each risk factor is associated directly or indirectly with the β-cell dysfunction and /or insulin sensitivity (Lee et al., 2018). GDM is associated with antenatal depression and can also increase the risk of additional pregnancy complications, including preterm birth and preeclampsia, and in many cases surgical delivery of the baby is required (Craig et al., 2020). In 2010/11, the rate of gestational diabetes was 54.5 per 1,000 deliveries (Health Canada, 2016).

2.3.1.4. Diagnostic markers for T2DM

There are several recognized biomarkers to detect T2D, such as fasting plasma glucose and glycated hemoglobin A_{1c} (HbA_{1c}) (Sattar et al., 2019). Blood glucose test and HbA_{1c} are important biomarkers to confirm prediabetes, type 1, type 2, or gestational diabetes. Glucose tolerance test (OGTT) measures the blood glucose before and after drinking the liquid containing sugar (Diabetes Canada, 2022). Overnight fasting is required for this test and before the test fasting blood glucose gets measured and then the patient is required to drink the liquid containing glucose and the blood sugar level is measured at hr, 2hr and possibly 3hrs afterward (Cox & Edelman, 2009). HbA_{1c} measures average blood sugar level over the past 2 or 3 months, and it is the most used biomarker to diagnose prediabetes and diabetes (Diabetes Canada, 2022). HbA_{1c} forms when glucose attaches to the amino-terminal group of the beta subunit of hemoglobin (Cox & Edelman, 2009). It reflects chronic glycemia rather than glucose level at a single time point. An A_{1c} below 5.7% is considered as normal, the level between 5.7 to 6.4% indicates prediabetes and 6.5% of higher indicated the diabetes (Diabetes Canada,2022). Increased HbA_{1c} levels are associated with increased risk of morbidity and mortality (National Diabetes Data Group, 1979). Some research studies state that higher HbA_{1c} levels also increases the risk of cardiovascular diseases (CVD), cancer andcause mortality. This diagnostic marker hasseveral advantages over fasting plasma glucose (FPS), and oral glucose tolerance test (OGTT) including greater convenience as fasting is not required and hasgreater pre-analytical stability (Chiu et al., 2006).

2.4.The role of foods in blood glucose control

2.4.1. Role of macronutrients

2.4.1.1. Carbohydrates

Carbohydrates are the nutrients with the highest influence on postprandial blood glucose, and it is well known that carbohydrate intake monitoring and matching mealtime insulin dosages is an essential technique in glycemic control (de Munter et al., 2007). The major dietary carbohydrates are classified into different classes based on the degree of polymerisation (de Munter et al., 2007). Carbohydrates simply can be defined as sugars, starches, and fibre. Carbohydrates are often referred to as simple and complex.

The physiological classification of CHO proposed by Englyst and Hudson divide all carbohydrates to available (can produce blood glucose) and resistant (i.e., cannot be broken down and absorbed in the small intestine) (Englyst & Hudson, 1996). According to Englyst's classification, starches are divided into categories, including RDS, SDS and RS. Rapidly digestible starch (RDS) is hydrolysed and absorbed in the small intestine and have a high glycemic response. Slowly digestible starch (SDS) also gets hydrolysed and absorbed in small intestine over longer period, whereas resistant starch (RS) does not get hydrolysed in the small intestine and is passed undigested into the large intestine where it is fermented by intestinal microbiota. CHO also contains non-starch polysaccharides and oligosaccharides that are classified as dietary fibre resistant to hydrolysis in the small intestine, and they do not have any glycemic response (Englyst & Hudson, 1996).

2.4.1.2.Role of amino acids, and peptides in blood glucose control

Amino acids can be physiologically divided into two groups: essential and non-essential amino acids. Essential amino acids are not produced in the human body. They are usually acquired through diet (Lopez & Mohiuddin, 2022). Whey protein and casein contains both essential and non essential amino acids (Rafiq et al., 2016). Amino acid consumption may have an important role in glucose homeostasis, causing glucose lowering effects, indicating that amino acid intake with a meal may commence the glucose lowering effects (Hall et al., 2003). Amino acids have been shown to have an important function in managing blood glucose levels. Many investigations have been conducted to determine the role of amino acids in glucose metabolism. Gunnerud et al. (2012) conducted a trial with 14 individuals who ate the conventional meal (ham sandwich) with pre-meal protein drinks (Gunnerud et al., 2012). The protein beverages were made from whey or soy protein isolates, with or without the inclusion of five amino acids (isoleucine, leucine, lysine, threonine, and valine) or the five referenced amino acids plus arginine (Gunnerud et al., 2012). When compared to the normal meal, all the protein drinks reduced blood glucose levels (Gunnerud et al., 2012). Furthermore, research on Sprague-Dawley rats conducted by Bernand et al. (2011) revealed that amino acids have a glucose-lowering impact (Bernard et al., 2011). The rats were given either glucose, glucose plus an amino acid combination, glucose+ amino acid mixture + increased leucine concentration, or water (Bernard et al., 2011). When compared to glucose, blood glucose levels in amino acid supplement groups were shown to be lower (Bernard et al., 2011).

2.4.1.3.Role of peptides in blood glucose control

Bioactive peptides are peptide fragments formed during the proteolytic cleavage or maturation of functional proteins. These peptides control blood glucose levels by inhibiting key enzymes such as amylase, DPP IV, α-glucosidase, and serving as an agonist of glucagon-like peptide 1 (GLP-1) (Aziz et al., 2005).Bioactive peptides are abundant in dairy products such as milk, cheese, yoghurt,

and other cultured dairy foods (Greenwood et al., 2011). Milk proteins, in particular, are a rich source of bioactive peptides with several health advantages (Madureira et al., 2007). Animal milk is high in nutrients and contains two key proteins: whey protein and casein. Both have been recognised as having a high concentration of bioactive peptides (Antony & Vijayan, 2021). Long terms exposure to cow's milk improved the insulin sensitivity in rats when fed with high sucrose diet and reduced the prevalence of insulin resistance (Matsumoto et al., 2009). One of the mechanisms involved in glycaemic effect of milk proteins and peptides is the inhibition of DPP IV that can result in reduced blood glucose levels. DPP IV inhibitors are found naturally in milk protein (Tulipano et al., 2011).

Whey protein are found to be an potent DPP IV inhibitor in preclinical and clinical trials (Tulipano et al., 2011). Several peptides with anti-diabetic potential have been discovered, with the ability to lower blood glucose levels, increase insulin absorption, and inhibit critical enzymes involved in the development and progression of diabetes (Aziz et al., 2005). Dietary proteins are a diverse group of foods that are high in these peptides.

2.4.1.4. Fats and free fatty acids

The American Diabetes Association recommends that individuals consume more monounsaturated and polyunsaturated fats in their diets than saturated and trans fats. It is widely established that high-calorie diets promote weight gain, insulin resistance, and blood sugar dysregulation, all of which can raise the risk of developing diabetes. Saturated fatty acids (SFA) are abundant in dairy fat; approximately 70% of milk fat is composed of SFA (Imamura et al., 2016). Pereira et al. discovered that dairy intake was inversely linked to the prevalence of metabolic variables associated with insulin resistance in young individuals who were overweight at baseline (Pereira et al., 2002). There is no long-term research on the relationship between the quality of dietary fat

and the risk of diabetes. However, several short-term research on the effects of dietary fats on insulin sensitivity, insulin secretion, and glycemic control have been conducted. In a randomized controlled crossover research, isocaloric meals were taken for four weeks by 25 healthy adults, with all menus cooked in a metabolic kitchen. There was no difference in insulin sensitivity between diets high in saturated fatty acids and monounsaturated fatty acids (von Frankenberg et al., 2017). In average, milk contains about 33 g of total fats. More than half of the milk fatty acids are saturated, accounting to about 19 g/L whole milk and it has been found to be increasing the cholesterol levels in the body (Haug et al., 2007). Oleic acid is the unsaturated fatty acid with the highest concentration in milk in around 8 g/L whole milk and found to be beneficial for health (Haug et al., 2007). The other unsaturated fatty acids found in milk include conjugated linoleic acid (CLA), including *trans* CLA, trans-palmitoleic acid, t16:1n-7, and others. The pooled analysis of 16 prospective cohort studies encompassing63,682 participants concluded that the higher intake of milk as judged by the level of 15:0, 17:0, and t16:1n-7 were associated with a lower risk of T2D (Imamura et al., 2018). On the other hand, a recent intervention study with 72 adults with MetS who were assigned to three groups consuming either low-fat dairy, high-fat dairy and no dairy found no changes in glycaemia or HbA1c; however, they found the reduction of insulin sensitivity after dairy consumption (Schmidt et al., 2021). In his editorial comments, Dr. Lamarche discussed plausible explanation of these results, including the authors' speculation of specific insulinotropic effect of dairy or the possible effect of dairy on gut microbiota; however, the interpretation of these results as well as the finding of causality require more mechanistic studies (Lamarche, 2021). One of the potential explanations for the observed effect could be an effect of dairy proteins on insulin sensitivity; however, whether the changes in insulin sensitivity without other changes in glycemic control are meaningful is still to be explored.

2.5.Role of Dairy Products in Blood glucose control

Dairy proteins are present in dairy foods such as milk, yogurt, and cheese, or they can be purchased in isolated or concentrated forms as supplements. Milk products with a low glycemic index and high calcium, vitamin D, fatty acids, and protein content may play an important role in diabetes management (Dairy Nutrition,2020). Dairy foods include low-fat milk, full-fat milk, yogurt, and cheese. According to Struijk et al. (2012), cheese and fermented dairy products contain glucose-regulating properties (Struijk et al., 2012). According to Dairy Nutrition (2020), there are various molecular pathways through which milk products contribute significantly to the management of impaired blood glucose by promoting weight loss and lowering the risk of hypertension and metabolic syndrome (Dairy Nutrition,2020). Tremblay & Gilbert (2013) state that milk and whey may have insulinotropic effects, that medium chain fatty acids found in milk products enhance insulin sensitivity, and that peptides and minerals found in milk products may help lower blood pressure and body weight (Tremblay & Gilbert, 2013).

When ingested in sufficient quantities, casein or whey proteins have a variety of health benefits. When consumed with CHO, milk proteins tend to reduce the glycemic response compared to the CHO diet alone (Frid et al., 2005, Nuttall et al., 1984). This shows the potential insulinotropic efficiency of milk proteins (Petersen et al., 2009). Whey protein, specifically, has a stronger effect on glycemic control compared to the other milk proteins as it is attributed to rapid digestion and release of amino acids and bioactive peptides during digestion (Petersen et al., 2009). The effects of whey protein on short term appetite, food intake, and blood glucose control were reviewed earlier (Luhovyy et al., 2007). It has been shown that at least 10g of whey protein is required to reduce blood glucose response, and 20g of whey protein is needed to reduce a short-term food intake (Akhavan et al., 2010). The same study showed that the effect of whey protein observed for the dose of 10g was not reproduced with the same dose of whey protein hydrolysate suggesting

that some unique peptides with specific effect on blood glucose control might be forming from the digestion of intact whey protein (Akhavan et al., 2010). When the same dose of 10g was compared for preload of whey protein and glucose, the preload with glucose resulted in a higher level of PYY and GLP-1, and lower level of insulin after the subsequent meal at 30 min between 50 and 230 min after preload (Akhavan et al., 2014).

Milk proteins can modulate the stomach emptying rate, stimulate insulin release from pancreatic β-cells, and lowers postprandial glucose levels, especially in type 2 diabetes with mild to moderate hemoglobin A1c (Hidayat, 2019). Another possibility is that bioactive peptides from milk proteins block the DPP-4 enzyme in the proximal gut, so inhibiting the breakdown of the GLP-1 and GIP, which are important for insulin release (Jakubowicz, 2012). Numerous investigations demonstrate that milk proteins may help to lower postprandial glucose levels and increase postprandial insulin responses. (Jakubowicz et al., 2017; Akhavan et al., 2014; Nilsson, Holst, and Bjorck, 2007; Gunnerud et al., 2012; and Veldhorst et al., 2009). Manders et al. (2005) recruited 10 T2DM patients to investigate the effect of co-ingested protein and amino acid mixture with carbohydrates on blood glucose level (Manders et al., 2005). The result of the study shows that co-ingestion of carbohydrate with a mixture containing casein hydrolysate, leucine and phenylalanine increased the insulin secretion when compared to the carbohydrate treatment alone (Manders et al., 2025). This study indicates that nutritional interventions that improves insulin secretion can be an effective tool in the treatment of T2DM(Manders et al., 2005). Another research done by Mortensen etal. (2012) investigated four isocaloric test meals in randomized order in 12 diabetic subjects (Mortenson et al., 2012). The test meals contained 45g of carbohydrate in combination with 3 different whey protein treatments (whey protein isolate, hydrolysate, and ALA) (Mortenson et al., 2012). There was a significant difference in terms of insulin secretion for all the treatments

(Mortenson et al., 2012). Whey protein hydrolysate and isolate form produced a higher insulin response compared to carbohydrate treatments alone (Mortensen et al., 2012).

2.5.1. Casein

Casein proteins (CN), which account for about 80% of bovine milk proteins, combine with calcium phosphate to create casein micelles (Brader et al., 2010). Casein micelles include four distinct types of caseins: α_{S1}-CN, α_{S2}-CN, β-CN, and k-CN (Hristov et al., 2016). These proteins have distinct primary amino acid sequences, occupy distinct locations within the micelle, and perform distinct roles (Hristov et al., 2016). Certain proteins are involved in the transport of calcium phosphate, whereas others are involved in the stability of other caseins and micelles (Hall et al., 2003). Milk's primary role is to supply critical amino acids and minerals necessary for the formation and operation of muscle and other tissues. Milk proteins coagulate fast in a newborn's stomach because they are physically designed to form big complexes with calcium phosphate. Casein fluctuates in composition and concentration throughout lactation (Pezeshki et.al, 2015). Milk proteins have been classified into two groups and are no longer considered to be a homogenous protein (Newmark, L., 2018). However, each of these two groups (CN and WP) is represented by various fractions and some of them are now commercially produced and available on the market. Casein accounts for about 75-80% of total milk proteins and precipitates at a pH of 4.6 at 30°C. Under identical circumstances, the remaining portion, serum or whey protein, is soluble (Farrell et al., 2004). The remaining proteins in milk are glycoproteins in trace amounts. From huge colloidal particles termed casein micelles, casein proteins and calcium phosphate are synthesized (Hristov et al., 2016). The primary function of casein micelles is to provide casein molecules fluidity and to solubilize phosphate and calcium. The α_{S1}-CN accounts for up to 40% of the CN fraction in bovine milk and is represented by eight genetic variants A,B,C,D,E,F,G and H with α_{S1}-CN B-8P as the reference protein containing 199 amino acids, including eight

phosphoseryl residues (Farrell et al., 2004). α_{S2}-CN accounts for up to 10% of the CN fraction in bovine milk and is recognized as the most hydrophilic of all caseins (Farrell et al., 2004). α_{S2}-CN is represented by four genetic variants, A, B, C and D with α_{S2}-CN A-11P as the reference protein containing 207 amino acids, including 11 phosphoryl residues, and intramolecular disulfide bond (Farrell et al., 2004).

β-CN known as the most hydrophobic casein accounts for 45 percent of bovine milk casein and is represented by 12 genetic variants, A^1, A^2, A^3, B, C, D, E, F, G, H^1, H^2 and I, with β-CN A2-5P as the reference protein containing 209 amino acids, including five phosphoseryl residues and no Cys (Farrell et al., 2004). β-CN is susceptible to plasmin which breaks down a part of β-CN releasing the fragments previously considered as separate protein fractions, $\gamma_{1,2,3}$-CNs, now known as β-$CN_{29\text{-}209}$, β-$CN_{106\text{-}209}$ and β-$CN_{108\text{-}209}$ (Farrell et al., 2004). Additionally, the components previously known as proteose peptone components 5, 8-fast, and 8-slow are now recognized as β-$CN_{1\text{-}105}$ or β-$CN_{1\text{-}107}$, β-$CN_{1\text{-}28}$ and β-$CN_{29\text{-}105}$ (Farrell et al., 2004). Two genetic variants of β-CN, A^1 and A^2, differ for one amino acid at position 67. β-CN A^1 contains His while β-CN A^2 contains Pro. This difference results in the production of different oligopeptides β-casomorphin-7 or β-casomorphin-9, respectively. A systematic review of 15 randomized controlled trials, 2 case-control studies, and 8 ecological studies concluded that based on the studies published by 2017, there is moderate evidence suggesting a link between the consumption of β-CN A^1 and adverse digestive health effects (Küllenberg de Gaudry et al., 2019) and a potential link between β-CN A^1 and T1D (Chia et al., 2017). On the other hand, the positive health effects were reported for β-casomorphin-7 (ul Haq et al., 2014). While most of commercial milk contains both A^1 and A^2 β-CN produced from the cows secreting either A^1 or A^2, the new products containing only A^2 β-CN and claiming improved digestive health have been commercialized such as "Gut-friendly" A2 milk in Canada (*Gut-Friendly Milk That's Easier to Digest | A2 Milk™ Canada*, n.d.).k-CN is represented by 11

genetic variants, A, B, C, D, E, F^1, F^2, G^1, G^2, H, I and J, with κ-CN A-1P as the reference protein containing 169 amino acids, including one phosphoseryl residue and no carbohydrate groups (Farrell et al., 2004). The cleavage of k-CN by chymosin during the cheesemaking leads to the hydrolysis of peptide bond between Phe_{105} and Met_{106} resulting in disintegration of casein micelle and formation of two k-CN products: para-κ-CN (k-CN_{1-105}) that remains with the curd, and caseinmacropeptide (CMP, k-$CN_{106-169}$) that becomes a part of sweet whey and then concentrated into whey protein (Farrell et al., 2004). Post-translational modification of k-CN results in the variety of the products, including 14 glycosylated forms, a glycosylated and non-phosphorylated form, and multiphosphorylated forms (Farrell et al., 2004).

The effect of caseins on the reduction of postprandial glycaemia is retained under diabetic conditions. Thus, Manders et al. (2005) demonstrated that co-ingesting casein with carbohydrate significantly enhanced insulin response and blood glucose clearance in ten individuals with a long-term diagnosis of T2DM by lowering postprandial blood glucose levels associated with carbohydrate consumption (Manders et al., 2005). Additionally, they discovered that supplementing casein with the branched-chain amino acid leucine enhances insulin responsiveness and glucose metabolism more than casein alone (Manders et al., 2005). The presence of leucine in whey protein may contribute significantly to its enhanced insulin secretagogue properties when compared to casein and other proteins with lower leucine levels. In 11 participants with well-controlled T2DM, Brader et al. (2010) studied the acute effect of the meals containing 80g of fat (control), 80g fat and 45g of casein (PRO-meal), 80g fat and 45g of carbohydrate (CHO-meal), and 80g fat, 45g casein and 45g carbohydrate (PRO-CHO-meal) (Brader et al., 2010). The level of both insulin and glucagon was increased after PRO- and PRO-CHO-meals (Brader et al., 2010). PRO-CHO-meal also resulted in an increased level of GIP while the level of GLP-1 was not

different (Brader et al., 2010). This observation is very interesting as it is known that GIP is not capable to stimulate insulin secretion in patients with T2DM (Holst, 2019).

Milk proteins, including caseins and whey proteins are precursors of bioactive peptides that have various activities, including α-glucosidase and DPP IV inhibition, and the enhancement of insulin sensitivity that may contribute to the low glycaemic effects observed for dairy products and milk proteins (Tulipano, 2020).

2.5.2. Whey protein

Whey proteins (WP) are defined as the group of milk proteins that remain soluble in milk serum or whey after precipitation of CN at pH 4.6 and 20°C (Farrell et al., 2004). WP are globular molecules with a high-proportion of α-helix motifs and a balanced distribution of acidic/basic and hydrophobic/hydrophilic amino acids throughout their polypeptide chains (Hall et al., 2003; Madureira et al., 2007). WP consist of β-lactoglobulin (β-LG), α-lactalbumin (α-LA), immunoglobulins (Ig), bovine serum albumin (SA), bovine lactoferrin (LF), and lactoperoxidase (LPO) (Ali, 2019; Ali, 2014; Anema, 2014). WP has the highest content of insulinotropic amino acids, including Iso, Leu, Lys, Thr, and Val, among the other food proteins (Luhovyy & Kathirvel, 2022).

β-LG is the major bovine whey protein is represented by 11 genetic variants, A, B, C, D, E, F, G, H, I, J and W, with β-LG B as the reference protein containing 162 amino acids (Farrell et al., 2004). One of the unique characteristics of β-LG is the ability to bind hydrophobic and amphiphilic molecules ranging from hexane to palmitic acid to vitamin D (Farrell et al., 2004). α-LA is the second major protein in bovine whey and represented by three genetic variants, A, B and C, with A and B being predominant variants, although B variant is more prevalent (Farrell et al., 2004). α-LA contains 123 amino acids and is defined as a calcium metalloprotein for its ability to bind

calcium, zinc, and other metals (Farrell et al., 2004). Whey proteins account for about 17% of the protein in milk. They include a high concentration of branched chain amino acids, such as leucine and isoleucine. α-LA and β-LG account for approximately 25% and 65% of total whey protein in bovine milk, respectively (Madureira et al., 2007). When heated to 70°C, these globular proteins alter their structure, reveal reactive groups, and combine with one another and with caseins in milk (Madureira et al., 2007). Blood proteins such as SA and Ig are absorbed into milk from blood and are recovered as whey proteins. GMP, the glycosylated form of CMP, is found in sweet whey, which is generated when chymosin (rennin) cleaves κ-casein as it was described above for κ-CN. Apart from caseins and whey proteins, milk includes a protein-like material known as a non-protein nitrogen (NPN) fraction (Fox et al.,1998). The effects of various WP products were investigated in the studies using a preload paradigm and various doses. Thus, Mortensen et al. (2012) investigated the effects of mixed isocaloric meals containing 100g of butter, 45g of carbohydrate, and another 45g of either WP isolate, or WP hydrolysate, or WP enriched with α-LA, or WP enriched with GMP on postprandial metabolic responses in 12 individuals with T2D (Mortensen et al., 2012). The data was obtained over an 8-hour postprandial period and revealed a significant increase in insulin response in the first 30 minutes following treatments with WP isolate, or WP hydrolysate compared to the treatments with α-LA and GMP (Mortensen et al., 2012). Frid et al. (2005) evaluated the effect of high GI breakfast supplemented either with 18g of whey protein or lean ham and lactose and high GI lunch meals on 14 participants aged 29-69 y with T2D (Frid et al., 2005). The insulin response was significantly higher after the breakfast and lunch served with WP, while blood glucose after the lunch was reduced (Frid et al., 2005). The incretins response was different: the treatments with WP resulted in significantly greater GIP than the treatment with lactose and ham, while there was no difference in GLP-1 response (Frid et al., 2005).

2.5.3. Comparative effects of casein, whey protein and their fractions

While WP is extensively used as the natural health product, most of the dairy products have a higher content of CN than WP. While both CN and WP have all essential amino acids and are high in BCAAs and other insulinogenic amino acids (Luhovyy & Kathirvel, 2022), they have distinct kinetics after ingestion. Thus, CN is referred as a slow protein based on the elevated concentration of leucine up to seven hours after ingestion, while WP is known as a fast protein digested within a much shorter period of time (Boirie et al., 1997). It is known that milk has a strong insulin response that cannot be predicted by its low glycemic index (Ostman et al., 2001). However, cow's milk protein contains approximately 80% of CN and 20% of WP. In the elegant experiment using cow's milk and a product with reversed protein content (i.e., higher WP than CN), the product with reversed protein composition led to a higher insulin and C-peptide iAUC compared to cow's milk; however, both products resulted in a similar mild glycemic response (Toffolon et al., 2021). The study conducted with nine lean healthy adults using 48g of WP or CN as an ingredient in an isocaloric liquid preload formulated with double cream, maltodextrin and water, found the increased amino acid concentration, CCK, GLP-1 and GIP over three hours after the preload with WP compared to CN (Hall et al., 2003). However, this study did not report in details blood glucose responses to CN and WP treatments, only indicating that there was no difference in plasma blood glucose, and therefore it is difficult to link the changes in incretins in relation to postprandial glycemia.

Both CN and WP are represented by multiple individual proteins that may have distinct physiological effects compared to their original composite fraction. While some of these proteins are commercialized and available as food ingredients on the market, more information becomes available about their unique physiological properties. The recent study has demonstrated a distinct

effect of β-LG from WP on insulin and glucagon secretion in individuals with T2D after OGTT (Smedegaard et al., 2021). β-LG led to the higher levels of insulin, glucagon and, paradoxically, blood glucose compared to WP (Smedegaard et al., 2021). The two major proteins in WPI, α-LA, which accounts for approximately 19%, and β-LG, which accounts for approximately 65% of WP, respectively, exhibit distinct physical and functional features, including temperature and pH-dependent aggregation and loss of solubility (Farrell et al., 2004). Additionally, serum albumin, immunoglobulins, lactoferrin, transferrin, and a variety of other proteins and enzymes are constituents of WP and their effects need to be further investigated. While β-LG is the major component of bovine WP, α-LA is a major fraction of human WP (Goulding et al., 2020). Thus, β-LG is found in cow's milk while there is α-LA in human milk (Goulding et al., 2020). Such interspecies differences in milk composition led to the development of infant formulas, children's foods, and senior foods enhanced with α-LA, while the addition of β-LG can be utilized to boost the gel strength of meals or nutritious beverages (Farrell et al., 2004).

Another milk protein fraction that originates from κ-CN, however, is considered as WP constituent is GMP that has 64 amino acids and may have a various degree of glycosylation (Farrell et al., 2004). One of the most prominent characteristics of GMP is the absence of aromatic amino acids, including phenylalanine, tryptophan, tyrosine, and histidine (Etzel, 2004). The absence of phenylalanine in GMP makes this fraction useful for the food formulation aimed for the individuals with phenylketonuria (PKU) (Lim et al., 2007). GMP also has anti-inflammatory properties and may reduce the symptoms of intestinal colitis as it has been shown in the study with rats (López-Posadas et al., 2010). On the other hand, GMP has a high content of isoleucine (Etzel, 2004) which is the only amino acid known to reduce the secretion of glucagon (Luhovyy & Kathirvel, 2022b). The total content of BCAA in GMP is 22.5% which is higher than in α-LA (21%) but lower than in β-LG (25.1%), although the content of isoleucine in GMP is the highest compared to α-LA and

β-LG (Etzel, 2004). The distinct physiochemical properties of milk proteins and the difference in their amino acid composition suggests that their effects on blood glucose control may differ as well.

2.6. Investigating the effects of milk proteins on blood glucose using laboratory animals

There are multiple animal models of human diabetes in experimental animals. They include spontaneous autoimmune models such as streptozotocin-induced damage of β-cells, genetically-induced models such as Goto-Kakizaki model resulting in the development of adult onset T2DM earlier in life, genetically engineered model such as KK mouse strain that exhibit T2D associated with hyperglycemia, glucose intolerance and microalbuminuria, surgical model using pancreatectomy with the reduced mass of β-cells, or virus-induced models using viruses that stimulate autoreactive T-cells, or destruct β-cells (Kottaisamy et al., 2021). The wide spectrum of animal models with the experimental diabetes and its etiologies poses the question which animal model would be the most adequate to investigate the effect of milk proteins on blood glucose control. As stipulated in Health Canada's guidance document on postprandial blood glucose reduction health claims made on food, the effect of food should be established first with healthy individuals in order to see the normal physiological response to ingested food (Health Canada, 2017) Similarly, the normal physiological responses needs to be assessed with laboratory animals first.

Certain diabetic animals contain robust beta cells that maintain a strong, life-long insulin-secreting potential throughout their lives, including Zucker fatty rats (ZFR), ob/ob (obesity), K.K. mouse, and (corpulent) cp rat group (Srinivasan, 2017). ZFR and ZDF rats, as well as ob/ob, DB/DB, and K.K. mice, all have a similar constitution of obesity and insulin resistance (Srinivasan, 2017).

Gregerson et al. (2013) investigated the effects of dietary supplementation with various milk protein fractions in Zucker Diabetic Fatty rats (ZDF) and normal Wistar rats (Gregersen et al., 2013) Therefore, they discovered that ZDF rats fed a diet with WP, WPH, CN, and α-LA had a lower increase in HbA1c and a lower glucagon response over 13 weeks, and a lower glycemic response after OGTT at week 18 compared to ZDF rats fed a standard chow diet (Gregersen et al., 2013). The changes in Wistar rat group were not significant possibly due to the insufficient sample size required to detect the observed effect size that was lower compared to ZDF rats that have developed a high level of HbA1C over the intervention period. However, the other study using Wistar rats that were put on a dietary pattern that mimic the unhealthy and healthy dietary patterns in humans did find the significant changes after the intervention with WP (Tong et al., 2014). In that study, Wistar rats were fed a high-fat diet for 16 weeks to establish a non-obese insulin resistance and then switched back to the regular AIN-93 diet containing various amount of WP (0-15%) or 1.6% Leu which was like the content of Leu in 15% WP. After 8 weeks of AIN-93 diet with or without WP/Leu supplementation, the fasting insulin and homeostasis model assessment-insulin resistance and BG after OGTT was significantly decreased in the 15% WP and 1.6% Leu groups compared to the diet with 0% WP/Leu (Tong et al., 2014). This study suggests that the supplementation with WP at 15% might reverse the risk of insulin resistance developed after a high-fat dietary intervention, and the mechanisms of this effect are determined by the content of Leu in WP (Tong et al., 2014). Although in this study, the 1.6% dose of Leu reproduced the effect seen for 15% WP, this was not the case in the study conducted by Aziz and colleagues who demonstrated that the amino acid mixture could not result in a similar effect demonstrated by WP suggesting the role of peptides produced during WP digestion in blood glucose control mediated via the action of GLP-1 and GLP-1 receptors (Aziz et al., 2005).

In conclusion, impaired BG control presents a serious challenge for public health and increase the risk of diabetes and other comorbidities. Milk proteins present the safe and effective approach to aid in blood glucose control by reducing postprandial glycaemia via multiple mechanisms, including insulinogenic effect of milk protein amino acids, their effect on the release of incretins, and other metabolic effects realized via neurohumoral mechanisms. While the effects of main milk proteins, CN and WP has been well established, the effect of WP fractions is not well investigated. Many studies with humans and animals have utilized GTT to assess the effect of mainly high doses of milk proteins on blood glucose excursion; however, GTT rather represents a supraphysiological glucose load (Viswanath et al., 2006). This study will investigate how the co-ingestion of WP and glucose provided in the same moderate dose will affect short-term glycaemia and insulin response in Wistar HAN rats.

Chapter 3

Rationale, objective, and hypothesis

3.1. Rationale

Diabetes is a major worldwide health problem due to the comorbidities associated with it. Numerous therapy alternatives are available for diabetes care; however, they have been shown to be ineffective at reducing diabetes prevalence, necessitating the development of novel treatment options. Adhering to a healthy diet and making lifestyle changes may be possible therapy choices. Dairy proteins, which contain all essential and branch chained amino acids and are precursors of bioactive peptides, may have a considerable influence on blood glucose regulation. Numerous investigations provided strong evidence that the ingestion of milk proteins, particularly whey protein, results in a lower glycemic and higher insulin responses. However, the significance of other milk protein fractions remains unknown. Additionally, the effects of milk protein fractions on blood glucose levels, when consumed concurrently with glucose, remain unknown. The purpose of this study is to determine how milk protein fractions impact blood glucose response and insulin secretion when consumed in conjunction with glucose solution. Examining various milk protein fractions in conjunction with sugar may aid in understanding the efficiency of milk protein fractions in regulating glycemic response for the purpose of developing beneficial food products.

3.2. Objective:

To investigate the effect of milk protein fractions co-ingested with glucose on glycemia and insulin response in Wistar Han rats.

3.2.1. Specific objective:

To investigate the effect of whey protein isolate (WPI), micellar casein (CN), total milk protein (TMP), alpha-lactalbumin (α-LA), beta-lactoglobulin (β-LG), and glycomacropeptide (GMP) on blood glucose and insulin responses over 120 and 30 min, respectively, in Wistar Han rats.

3.3. Hypothesis:

Milk protein fractions co-ingested with glucose will reduce blood glucose response and increase insulin response compared to glucose ingested alone.

Chapter 4: Methodology

4.1. Study Design

The impact of commercial WPI, CN, GMP, α-LA, β-LG, and TMP co-ingested with glucose and glucose control was determined using a randomized repeated-measures design in sixteen Wistar-Han rats. Each rat has received all treatments in a random order. The random allocation to the treatments was done using random.org software (Appendix C). Ethical approval was obtained from Dalhousie University Committee on Laboratory Animals (Protocol #19-022).

4.2. Protocol for the Study

Sixteen Wistar Han rats with jugular vein catheters were purchased and transported separately from Charles River Laboratories in Massachusetts, USA. They were fitted with a button cap for the vascular access button upon arrival (VAB). Following arrival, we flushed the catheters with saline and locked them with heparinized glycerol. The rats were given identification, placed in pairs in cages, and acclimated over a seven-day period. Rats were randomly randomized to treatments following the acclimatization phase. All rats were sorted into four groups of four. After a six-hour daylight fast, group 1 begun at 1:30 pm on day 1, group 2, group 3, and group 4 had begun at the same time as group 1 on subsequent days (Appendix C). There was a 72-hour (3-day) washout period between sessions. Each rat was given seven different treatments in a random sequence. During the washout phase, the rats' catheters were flushed every three days.

Blood samples were taken from animals throughout each day session utilizing Pinports (PNP3M, Instech Laboratories) attached to 2 mL syringes via a Luer Slip Tip. The catheter was flushed with two times the dead space volume (90 ul) soon after the blood sample was obtained and then locked with the dead space volume (45 ul). The dead space volume was 45 ul. 70ul of whole blood was

drawn at 0, 15, and 30 minutes, and 10ul of blood was obtained at 60, 90, and 120 minutes (Fig 4.1). This results in a total collection of 240 ul of blood, or around 1% to 2% of total blood volume. Three days of washing out was sufficient to refill the blood volume. A six-hour daylight fast was chosen based on past study and discussion with Dr. Sue Pearce, the CACF(Carlton animal care facility, Dalhousie University) veterinarian and well-reviewed literature.

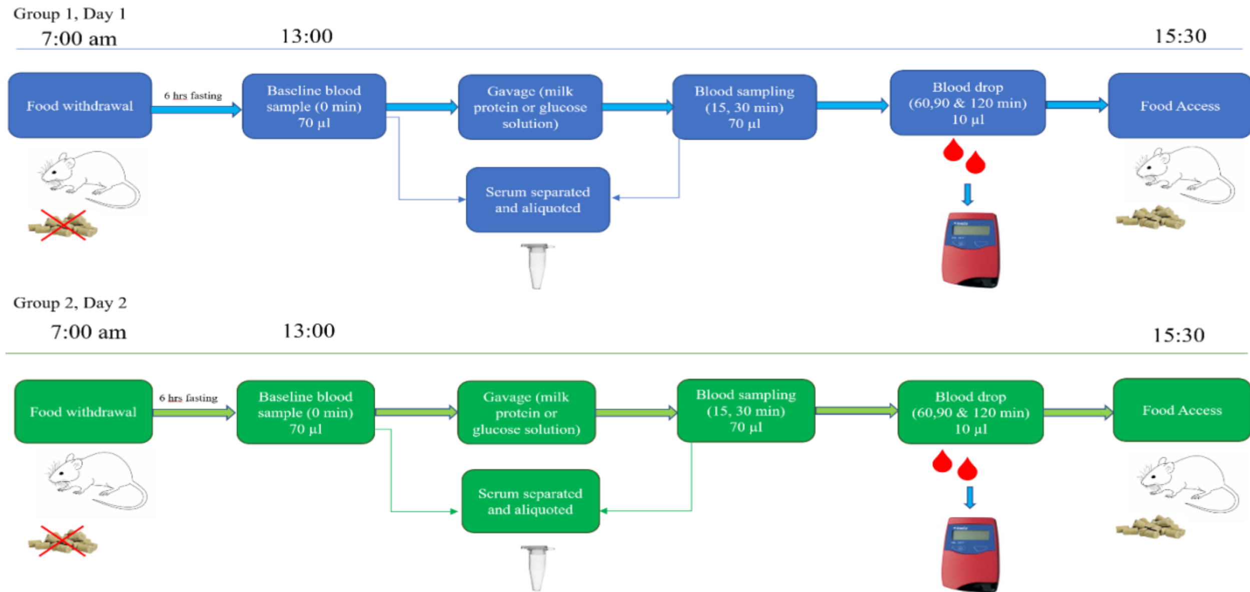


Figure 4.1

4.3. Treatments

4.3.1. Treatment Dosage

While a variety of various milk protein dosages have been investigated, it has been shown that a 10g human dose is efficient at dramatically lowering blood glucose following a single consumption (Akhavan et al., 2010, 2014; Drummond et al., 2018). This dose is consistent with Health Canada's "Draft Guidance Document on Food Health Claims Related to Post-Prandial Glycaemic Response Reduction," which mandates that the serving size be proportionate to the intended mode of intake (i.e., snack or meal) (Health Canada, 2013). To guarantee that solutions containing 10% wt/vol

was suitable for gavaging, they were tested. Allometric scaling was used to convert the 10 g dosage to an animal equivalent dose (AED). This was accomplished with a correction factor (K_m), which is an estimation of a species' average body weight (kg) divided by its average body surface area (m2) (Nair & Jacob, 2016). The typical human body weight is 60 kg, the K_m value is 7, and the rats' body weight is 0.3 kg (Nair & Jacob, 2016). As a result, the AED for the rats utilized in this investigation is as follows: AED = Human dose (mg / kg) × K_m ratio

AED = ((10 g * 1,000 mg/g) / 60 kg) x 7

AED = 1,166.67 mg/kg

AED = 1,166.67 mg/kg *0.3 kg

AED = 350 mg

All the treatments were prepared in bulk solutions to make up enough for 16 rats. The appropriate temperature was maintained during the preparation. There was no issue in terms of solubility for any proteins except for the Micellar casein. It took more time to prepare the treatment of Micellar casein compared to the rest of the treatments as casein was slowly dissolving but did not cause any issue with the formation.

4.3.2. Treatment Information

Table 4.1

	Manufacturer	Product Information	Dosage Calculation
Whey protein isolate	Agropur Dairy Cooperative (Saint-Hubert, QC, Canada)	BiPRO 9500 Whey protein isolate	87% As is Protein 350/0.87 = 402.3 mg

Glycomacropeptide	Agropur Dairy Cooperative (Saint-Hubert, QC, Canada)	BiPRO GMP 9000	93.6 As Is Protein 350/0.936 = 373.93
α-lactalbumin	Agropur Dairy Cooperative (Saint-Hubert, QC, Canada)	BiPRO Alpha 9000 α-lactalbumin	92.5% As Is Protein 350/0.925 = 378.4 mg
β-lactoglobulin	MilliporeSigma (St. Louis, MO, USA)	β-lactoglobulin from bovine milk >99%	99% As Is Protein 350/0.99 = 353.5 mg
Glucose	MilliporeSigma (St. Louis, MO, USA)	Dextrose	100% As Is Glucose 350/1 = 350 mg
Total milk protein	Milk Specialties Global	Non-commercial isolated milk protein concentrate (90%) (MPI 90)	87% As Is Protein 350/0.87 = 402.3 mg
Casein	Milk Specialties Global	Caseinate-Micellar casein	86% As Is Protein 350/0.86 = 406.9 mg

4.4.Animals

4.4.1. General Characteristics

Wistar-HAN rats are described by manufacturer (Charles River Laboratories) as albino rats suitable as a general multipurpose model that can be used for safety and efficacy testing, aging, oncology, and surgical model. A sample size of 7 (n=10) 300 g 8-week-old Wistar HAN rats was considered sufficient to detect 15% difference in blood glucose ($\alpha = 0.05$ and $\beta = 0.08$). A similar sample size was used in previous studies investigating the effect of proteins on blood glucose using laboratory animals (Aziz et al., 2005; Baena et al., 2016; Drummond et al., 2018). To account for possible loss of animals during the experiments due to patency of catheter, a sample of 16 had been used. Animals had jugular vein catheters placed and fitted with Instech vascular access button by Charles River which makes repeated blood collection process easier and do not put animal under much stress and discomfort. Intravenous catheterization allows sufficient and easy blood collection from catheter in the volume sufficient to measure blood glucose in whole blood, and insulin in blood plasma at repeated intervals at each day. This method allows using the same animal for multiple sessions and therefore reduce the number of animals required for the study. Using repeated measures design allows to reduce biological variability as the results from each session are compared within the same animal.

The health of rats was regularly monitored from receiving and over all experimental sessions and wash-out period.

4.4.2. Gavage

Treatments were given through oral gavage immediately following the collection of fasting blood sample (time point 0 min). While other oral feeding techniques have been devised, their protocols need the use of a sweetener, which has a negative effect on blood glucose, and are inaccurate in

their dose administration (Atcha et al., 2010; Wheeler et al., 2007). While gavaging does pose risks, several steps were taken to minimize this. Not only does the use of a disposable plastic flexible gavaging needle (FTP-15-78, Instech Laboratories, Inc., PA, USA) lower the danger of esophageal perforation, but it also reduces the stress levels associated with recurrent gavaging (Arantes-Rodrigues et al., 2012; Jones et al., 2016; Murphy et al., 2001). The use of a 3mL volume has been determined to be both suitable and safe for testing (Brudzynski & Ociepa, 1992). Repeated gavaging using metal gavage needles has been shown to produce weight loss in animals when the animals were weighed at each session. Normally, while gavaging a rat, the animal is held by the scruff of the neck and the gavage needle is inserted vertically; however, the VAB inhibits this, so to overcome these hindrances, the animal was wrapped in a towel to restrict motion and gavaged horizontally as reported earlier (Boughter, 2020), and this study followed the same gavaging technique.

4.4.3. Blood Collection

Blood was collected at intervals of 0, 15, 30, 60, 90, and 120 minutes, totaling 240 uL. Blood was obtained at time points 0, 15, and 30 minutes to test both blood glucose and insulin, and then at 60, 90, and 120 minutes to assess solely blood glucose. After blood collection at each given time point of 0, 15, 30, the blood was centrifuged at 2,500×*g* at 4°C for 10 minutes using a refrigerated centrifuge (VWR Symphony, Germany). Blood plasma was collected and aliquoted in volume of 20ul in two 0.5ml microtubes and was stored in -80°C ultrafreezer for insulin detection.

4.4.4. Animal training and ethics clearance

This study has been reviewed and approved by the University Committee on Laboratory Animals at Dalhousie University (Protocol 19-022). All study personnel who were working with animals obtained animal training in the care and management of laboratory animals (Appendix A) as well as rat-specific technical procedures (Appendix B). Because animals lack the capacity to agree, the principle of 3R (Replacement, Reduction, and Refinement) is required to guarantee that the danger of damage is reduced. This was accomplished using a small number of animals to assure accurate results that account for attrition, a shorter fasting period to facilitate recovery, and the use of innovative catheter technology to decrease discomfort in the animals. Daily health checks were conducted on the animals. At the conclusion of the trial, animals were sedated with isoflurane and euthanized with carbon dioxide.

4.5. Data Analysis

4.5.1. Body weight

Body weight of all the animals was measured upon arrival and then at the day of each session. Generally, it is considered that during the study the animals should be gaining the weight, and the weight gain in all the rats was observed. Please refer to the table 5.2. with body weights.

4.5.2. Blood glucose

Blood glucose was measured from baseline to 120 min timepoint using Hemocue 201+ (HemoCue AB, Ängelholm, Sweden) blood glucose spectrophotometer. One drop(5uL) of whole blood was placed on the disposable cuvette and measured. This method is fast, accurate, and needs a very small volume of whole blood to measure glucose.

4.5.3. Insulin

Blood was immediately transferred to a Microvette 100 K3E tube (Sarstedt AG & Co. KG, Nümbrecht, Germany), inverted ten times, and centrifuged for 10 minutes at 2,500 × *g* at 4°C using a refrigerated centrifuge (Symphony, VWR International, Germany). The plasma from the blood was then extracted using a pipettor and aliquoted into two 0.5 ml microtubes (Sarstedt AG & Co. KG, Nümbrecht, Germany). Following aliquoting, the samples were placed in a microtube box and frozen at -80°C for analysis later. Insulin analysis was done using ELISA (Enzyme linked immunosorbent assay) (Appendix D)

Table 4.3 Data Collection Timeline (Animals shown as R1-R16)

Day	Group #	Session #	Treatment						
			Whey Protein	α-LA	β-LG	GMP	MC	TMP	Glucose
Day 1-7 (Acclimatization)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
8	1	1	R1	R2		R4			
9	2	1	R5	R6	R7				
10	3	1	R10	R11	R12				
11	4	1	R14		R16				
12	1	2		R1	R2		R4		
13	2	2			R6	R7			
14	3	2			R10	R11	R12		
15	4	2			R14		R16		
16	1	3			R1	R2		R4	
17	2	3				R6	R7		
18	3	3				R10	R11	R12	
19	4	3				R14		R16	
20	1	4				R1	R2		R4
21	2	4					R6	R7	
22	3	4					R10	R11	R12
23	4	4					R14		R16
24	1	5					R1	R2	R4

25	2	5						R6	R7
26	3	5	R12					R10	R11
27	4	5						R14	R16
28	1	6		R4				R1	R2
29	2	6		R7					R6
30	3	6		R11	R12				R10
31	4	6		R16					R14
32	1	7		R2		R4			R1
33	2	7		R6	R7				
34	3	7		R10	R11	R12			
35	4	7		R14		R16			

4.5.4. Statistical analysis

GraphPad Prism version 8.0 was used to analyze the data (GraphPad Software, CA, USA). The mean and standard deviation was used to express the results. $P<0.05$ values were deemed statistically significant. A two-way repeated measures ANOVA was used to determine the effects of treatment, time, and treatment by time interaction. A Tukey-Kramer post hoc test was be used to determine pairwise differences. Insulin and glucose area under the curves were plotted using the trapezoid technique and the effect of treatment on BG or Insulin AUC and the differences between the treatments were determined using a one-way ANOVA with Tukey's-Kramer post-hoc test.

Chapter 5: Results

5.1 Weight of Rats

Ten 10-week-old male Wistar Han Rats with an average weight of 327 ± 16 g (Table 5.1) were able to complete all the 7 treatments. There was no significant weight loss during the duration of the study (Table 5.2).

Table 5.1 Mean weight (g) ± SD of rats throughout the duration of the study.

Animal Number	Average weight (g) ± SD
Rat 1	339±9
Rat 2	327±15
Rat 4	326±15
Rat 6	340±18
Rat 7	320±18
Rat 10	324±15
Rat 11	319±17
Rat 12	312±12
Rat 14	317±18
Rat 16	332±16

Table 5.2 Weight (g) of rats throughout the duration of the study

Animal Number	Session (#) / Weight (g)						
	1	2	3	4	5	6	7
Rat 1	324	332	337	342	342	346	350
Rat 2	302	316	322	329	334	339	345
Rat 4	305	310	318	326	336	343	346
Rat 6	311	325	337	343	350	355	361
Rat 7	292	304	315	321	330	336	341
Rat 10	302	311	319	324	330	338	344
Rat 11	293	305	313	310	312	325	330
Rat 12	295	308	316	323	327	335	343
Rat 14	290	302	310	317	325	332	341
Rat 16	297	314	323	337	343	351	357

5.2. Blood Glucose

0-120 min

There was an effect of time ($P<0.0001$) and a treatment by time interaction($P<0.0001$) but no effect of treatment ($P=0.35$) on blood glucose over 120 min.

0-60 min

There was an effect of time ($P<0.0001$) and a treatment by time interaction($P<0.0001$) but no effect of treatment ($P=0.15$) on blood glucose over 60 min.

0-30 min

There was an effect of time ($P<0.0001$), treatment ($P<0.0001$), and a treatment by time interaction ($P<0.0001$) on blood glucose over 30 min. The treatments with WP, β-LG, α-LA, , and MC resulted in a lower blood glucose compared to the glucose treatment at 15 min ($P<0.05$). There was no difference between the treatments with TMP and glucose at 15 min ($P>0.05$).

The treatments with β-LG and WP resulted in a lower blood glucose compared to the glucose treatment at 30 min ($P<0.05$). There was no difference between the treatments with WP, α-LA, GMP, MC and glucose at 30 min ($P>0.05$).

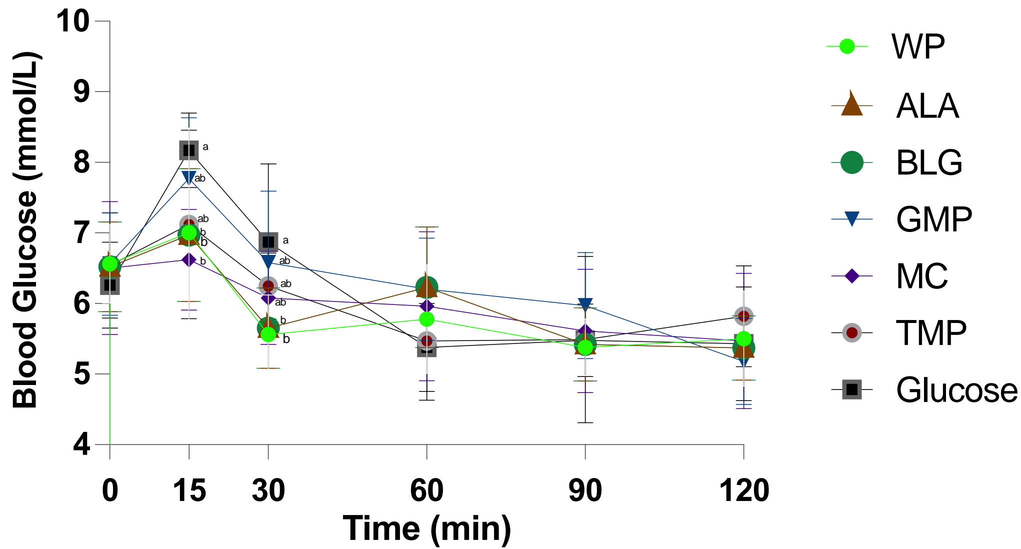
Table 5.3 Mean Blood Glucose by Treatment over 120 min

Treatment	Mean Blood Glucose					
	0 min	15 min	30 min	60 min	90 min	120 min
WP	6.6 ± 0.5	7.0 ± 1.5^{b}	5.6 ± 0.6^{b}	5.8 ± 0.8	5.4 ± 0.4	5.5 ± 0.7
ALA	6.8 ± 0.7	6.7 ± 1.3^{b}	5.9 ± 0.6^{ab}	6.2 ± 0.9	5.9 ± 1.0	5.2 ± 0.4
BLG	6.5 ± 0.6	7.0 ± 0.9^{b}	5.7 ± 0.6^{b}	6.2 ± 0.9	5.4 ± 0.5	5.4 ± 0.5
GMP	6.5 ± 0.6	7.8 ± 0.6^{ab}	6.6 ± 0.6^{ab}	6.2 ± 0.8	6.0 ± 0.5	5.2 ± 0.5
MC	6.5 ± 0.9	6.6 ± 0.7^{b}	6.1 ± 0.6^{ab}	6.0 ± 1.1	5.6 ± 0.9	5.5 ± 0.9
TMP	6.5 ± 0.8	7.1 ± 1.3^{ab}	6.2 ± 0.5^{ab}	5.5 ± 0.8	5.5 ± 1.2	5.8 ± 0.7
Glucose	6.3 ± 0.6	8.1 ± 0.5^{a}	6.9 ± 1.1^{a}	5.4 ± 0.6	5.5 ± 0.5	5.4 ± 0.8

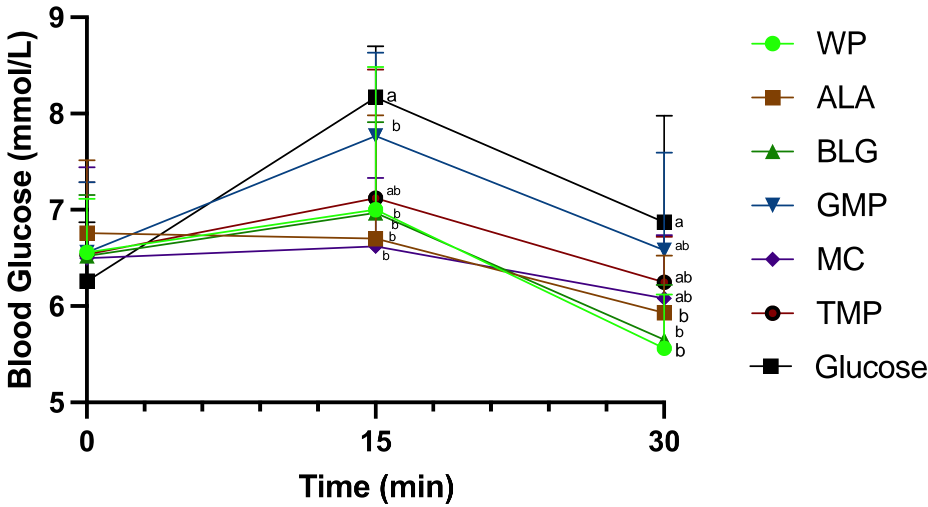
Means ± SD, Abbreviations: WP=whey protein, β-LG *=beta-lactoglobulin,* α-LA *=alpha-lactalbumin, GMP= glycomacropeptide, MC=micellar casein, TMP= Total milk protein. n = 10. Effect of time ($P<0.0001$), and a time by treatment interaction ($P<0.0001$). Values with different letters are significantly different within the same time point ($P<0.05$).*

Figure 5.2. Mean Blood Glucose by Treatment over 120 min (A) and over 30 min (B)

A



B



Mean±SD. Abbreviations: WP=whey protein, BLG=beta-lactoglobulin, ALA=alpha-lactalbumin, GMP= glycomacropeptide,TMP= Total milk protein, MC= Micellar casein. Values with different letters are significantly different (P<0.05).

5.3. Blood glucose AUC

0-120 min

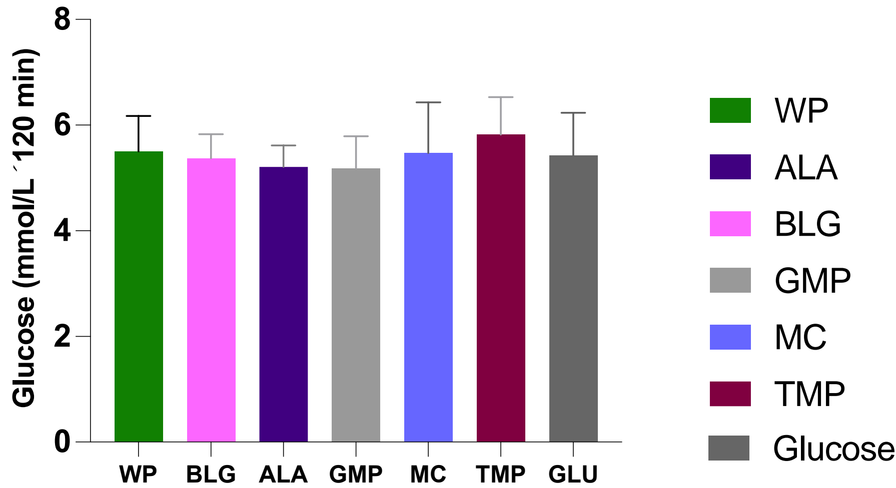
There was no effect of treatment on blood glucose AUC over 120 min (P=0.4).

0-30 mins

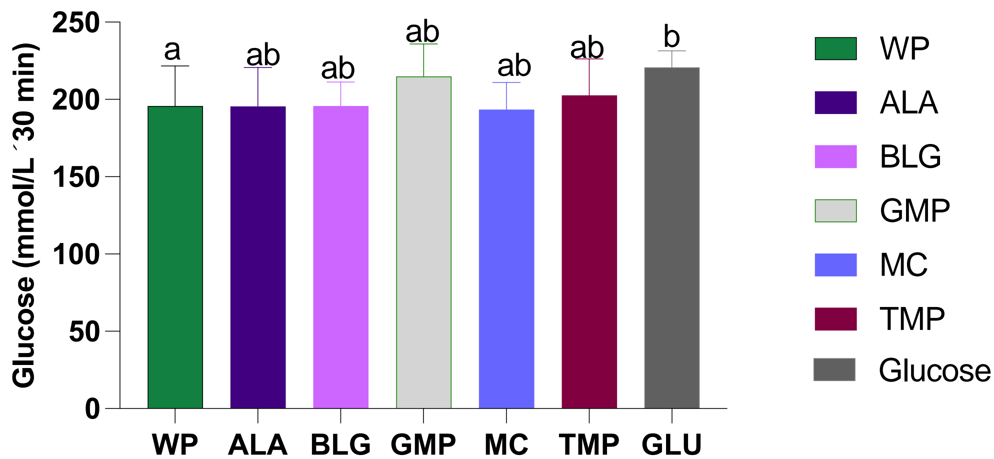
There was an effect of treatment on blood glucose AUC over 30 min (P=0.016). The treatment with WP resulted in a lower BG AUC compared to the treatments with glucose (P<0.05). The treatments with ALA, GMP, MC, TMP and glucose resulted in a similar BG AUC over 30 min (P>0.05).

Figure 5.3. Blood glucose AUC over 120 min (A) and 30 min (B)

(A)



(B)



Mean ± SD. Abbreviations: WP=whey protein, BLG=beta-lactoglobulin, ALA=alpha-lactalbumin, GMP= glycomacropeptide, MC=micellar casein, TMP= Total milk protein. One-way ANOVA with Tukey's-Kramer post-hoc test. Values with different letters are significantly different (P<0.05).

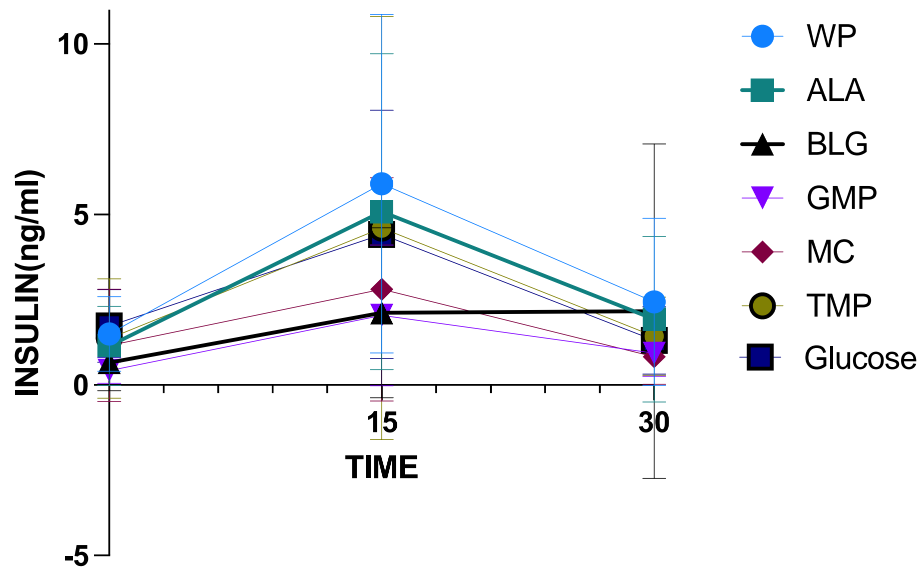
5.4 Plasma Insulin

There was an effect of time ($P<0.0001$), and time by treatment interaction ($P<0.0001$) but no effect of treatment ($P=0.3$) on insulin response over 30 min (Figure 5.5).

Table 5.4 Plasma insulin over 30 min

Treatment	Insulin ng/ml ± SD		
	0 min	15 min	30 min
WP	1.5 ± 1.1	5.9 ± 4.9	2.4 ± 2.4
α-LA	1.2 ± 1.2	5.1 ± 4.6	1.9 ± 2.4
β-LG	0.7 ± 0.8	2.1 ± 2.5	2.2 ±2.4
GMP	0.4 ± 0.4	2.0 ± 2.0	0.9 ± 0.6
MC	1.5 ± 0.3	4.5 ± 0.1	1.4 ± 0.0
TMP	1.4 ± 1.7	4.6 ± 6.2	1.4 ± 1.1
Glucose	1.7 ± 1	4.4 ± 3.6	1.3 ± 0.9

Figure 5.5. Insulin response over 30 min

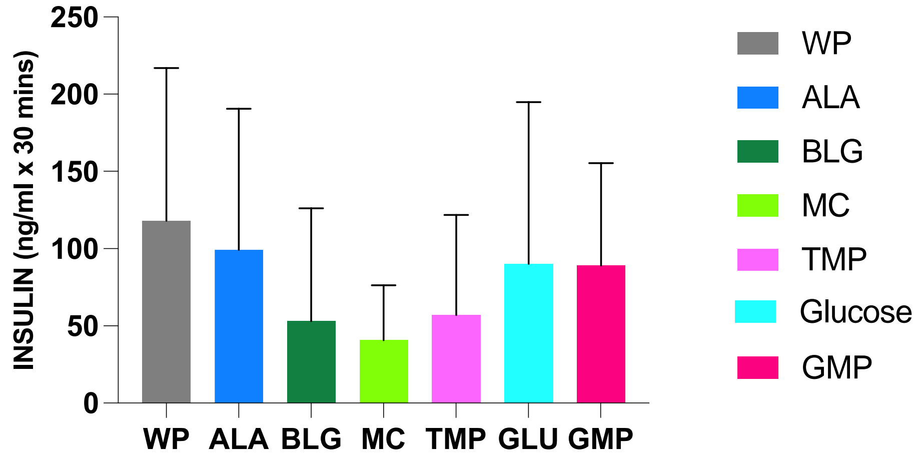


Mean ± SD. Mean ± SD. Abbreviations: WP=whey protein, BLG=beta-lactoglobulin, ALA=alpha-lactalbumin, GMP= glycomacropeptide, MC=micellar casein, TMP= Total milk protein. One way ANOVA (treatment and time) with Turkey's-Karmer post-hoc test.

5.6. Plasma Insulin AUC

There was no effect of treatment on insulin AUC over 30 min (P=0.3).

Figure 5.6. Plasma Insulin AUC over 30 min



Mean ± SD, Abbreviations: WP=whey protein, BLG=beta-lactoglobulin, ALA=alpha-lactalbumin, GMP= glycomacropeptide. MC – micellar casein, TMP – total milk protein, GLU - glucose. One-way ANOVA with Tukey's-Kramer post-hoc test.

Chapter 6: Discussion

To the author's knowledge, this is the first study which investigated the effect of milk proteins co-ingested with glucose on blood glucose control in Wistar Han rats. The finding of this study supports the first part of our hypothesis that the co-ingestion of milk protein fractions and glucose will result in distinct blood glucose response. Thus, the effect of milk protein fractions on blood glucose sustained for 30 min and was not observed for 60 min or 120 min. The suppression of glycaemic response at 15 and 30 min, and over 30 min was not the same for milk protein fractions (Figure 5.2.1., Table 5.3). Furthermore, only the treatment with WP resulted in a lower BG response over 30 min.

The second part of our hypothesis assumed that milk protein fractions co-ingested with glucose will result in a higher insulin response compared to glucose alone due to a cumulative insulin stimulation effect known for glucose and for milk proteins. However, all treatments with milk protein fractions and glucose resulted in a similar insulin response as the treatment with glucose alone (Figure Table 5.4). This finding suggests that the insulin response after the ingestion of the solution containing both insulinogenic nutrients – glucose and milk proteins, does not exacerbate insulin response.

Findings from this study support previous evidence showing milk proteins ability to reduce blood glucose response (Eussen et al., 2016; Hidayat et al., 2019; Law et al., 2017; Luhovyy et al., 2007b). At 15-minute, blood glucose response was lower in WP, α-LA, β-LG, and MC co-ingested with glucose compared to only glucose treatment (Figure 5.2). However, there was no significant difference seen for GMP, and TMP treatments compared to the treatment with glucose alone. The lack of the effect at 15 min can be potentially explained by a longer digestion of these proteins in stomach that could be confirmed by kinetics of amino acid release or paracetamol gastric emptying or similar tests in future studies (Willems & Quartero, 2001) In the same pattern, at 30 minutes,

the treatments with WP and β-LG resulted in a lower BG response compared to glucose. This observation is also confirmed by BG AUC over 30 min that was significantly lower after only WP treatment compared to the treatment with glucose. This observation is in line with the previous studies showing that WP may exert a better glycaemic control compared to WPH or amino acid mixtures (Luhovyy et al., 2007). The lack of the effect of milk protein fractions co-ingested with glucose on BG response over 60 min or over 120 min may be also explained by a fluid nature of the treatments and relatively short time required for their digestion and absorption than it would be expected for a solid food. However, in the previous study conducted with milk proteins only, the treatment effect on BG was observed over 120 min (Vandenboer et.al, 2020).

Many studies have suggested that milk proteins are responsible for reducing the absorption of glucose by various insulin independent mechanisms such as the delay in gastric emptying (Brun et al., 2012). In addition, milk proteins and glucose stimulate insulin secretion and therefore may result in stronger elevation in insulin secretion and more rapid glucose uptake within 30 min.

For insulin response, there was no significant difference found between the treatments. WP has an ability to increase the insulin secretion and insulin sensitivity. WP has the highest content of insulinotropic amino acids, including Iso, Leu, Lys, Thr, and Val, among the other food proteins (Luhovyy & Kathirvel, 2022).

α-Lactalbumin is one of the fractions of whey protein which accounts for around 25% of WP. Both whey protein and ALA attenuated the glucose in blood stream by elevating the insulin secretion and reducing gastric emptying time (Nauck & Meier, 2018). Interestingly, while WP did attenuate blood glucose response at 15- and 30-minutes (Figure 5.1), no significant difference between the treatments with WP and glucose was found on insulin response (Figure 5.5) and insulin AUC (Figure 5.6. The reason for not finding the significant difference could be the power of the study. After the loss of six animals due to the loss in the patency of catheters, the sample

size was not adequate to detect the differences in insulin response between the treatments. Additionally, as β-LG accounts for around 75% in WP, it was surprising not to find the significant difference on BG AUC compared with the treatment with glucose as it was seen with WP. As discussed above, WP is rapidly digested and releases the amino acids in blood stream (Akhavan et al., 2010); however, the effect of WP on blood glucose response is mediated not only by amino acids but potentially by bioactive peptides formed during digestion (Luhovyy et al., 2007a). That could justify that whey protein and its fractions may attenuate glycemic response by insulin dependent and insulin independent mechanisms. A study conducted by Salehi and colleagues with mice suggests that WP increases postprandial insulin due to increased serum levels of certain amino acids (Salehi et al., 2012); however, amino acids alone might not explain the observed differences between milk protein fractions that are known to have a high content of insulinogenic amino acids. In previous study conducted by Vandenboer, the treatment with GMP alone resulted in suppressed insulin response compared to the same dose of glucose (Vandenboer, 2020). However, this effect has not been observed in the present study when GMP was co-ingested with glucose. The total content of BCAA in GMP is 22.5% and it has no phenylalanine and other aromatic amino acids including tryptophan that is also considered as insulinogenic amino acid (Luhovyy & Kathirvel, 2022b). However, the fact that GMP alone results in a lower insulin response suggests that GMP can be used as a protein of choice to prevent high postprandial insulin response; however, based on the results of this study, such intervention or specially designed food should not have glucose or other rapidly digestible carbohydrate. Especially, the individuals with phenylketonuria and hyperinsulinemia may benefit from the food formulated with GMP and low content of glycaemic carbohydrate. Most of milk proteins contains a certain (and in case of WP abundant) amount of essential amino acids and branched chain amino acids (Landi et al., 2021). Branched chain amino acids have been found to stimulate incretin, which increases the insulin

secretion and maintain the level of insulin in blood stream. (McGregor & Poppitt, 2013). WP and BCAAs have been found to stimulate incretin secretion, which aid in blood glucose regulation (Chen & Reimer, 2009; Pezeshki et al., 2015).

In summary, all the protein treatments attenuated blood glucose response at 15-minute after ingestion that may suggest about their potential use for those applications in which acute postprandial glycaemic response should be reduced. However, only WP demonstrated a sustained effect on BG over 30 min. None of the protein fractions led to reduced glycaemia over 120 min that could be explained by the presence of glucose in the treatment, high insulin response and a fast glucose uptake by peripheral tissues over 30 min. It can be concluded that all protein treatments stimulate insulin secretion; however, they do not exacerbate insulin response compared to glucose alone that makes them safe in terms of expected hyperinsulinemia that was not observed in this study.

There have been many techniques such as anesthesia, movement restriction, catheterization that are well established in similar research; however, the uniqueness of this study design allowed a pain-free and stress-free blood withdrawal during multiple times in conscious animals. Doing a repeated measure experiment with multiple collection time points is quite rare and challenging in rats due to the technical complexities. However, this experimental model not only facilitates repeated blood collection due to catheterization but also reduced the number of animals required for the data collection and reduce the data variability as all results are compared within the same animal.

6.2. Limitations and challenges

There are several limitations of this study. One of the most significant limitations is a lower volume of blood that could be collected from animals. Therefore, it was impossible to analyze blood

samples for the other analytes to see the changes, for example, in C-peptide, incretins and DPP-IV inhibition. The use of 10 g dose which is equivalent to the amount of protein shown to be effective in BG reduction in human studies might not be the ideal dose for animals; however, these results provide further direction for the future studies to evaluate the effect of even smaller doses of milk proteins ingested either alone or in combination with glucose.

In terms of technical limitations, the patency of the catheters was the technical challenge: based on our observations, the jugular vein catheter is only ideal to use for collecting the blood for a short intervention up to 15 days, whereas this study protocol was for 40 days. Six rats were lost due to the catheter failure. Additionally, the stress level associated with the gavage is also a factor that could affect blood glucose response. This challenge could be solved with the study using the milk protein fractions mixed into the solid diets; however, some of the fractions (β-LG) were not available only in preparative quantities.

Chapter 7: Future Directions

The finding of this study shows the potential use of whey protein fractions in development of functional foods aiming at reduction in blood glucose after food ingestion. Finding the similar dose which increases the efficiency and efficacy of the treatment could be the next step. As there are many findings about the role of amino acids in blood glucose control, it would be important to see the concentrations of individual amino acids in blood over 30 min after the ingestion of milk protein fractions. Methodology should be refined to withdraw more blood to enable the analysis of stress hormones, incretins, and amino acids, which may give the clear picture on effect of proteins with co-ingestion and how stress impacts the blood glucose levels. It would be important to see the acute effect of various doses of milk proteins, especially GMP, on blood glucose levels when ingested alone or with glucose. Such future investigation will provide benefits for the people with PKU accompanied with metabolic disorders.

8. References

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Appendices

Appendix A Animal care certificate

TO: **Devanshi Desai**

CERTIFICATION #: **2020-019**

The above is your certification number for successfully completing the exam **"Introduction to the Care and Use of Laboratory Animals"** based on the following material:

- On-line review of CCAC's ***Core Stream*** AND ***Animals Housed in Vivaria Stream*** modules

Completion of the exam fulfills the initial Canadian Council on Animal Care (CCAC) requirement for the National Institutional Animal User Training Program (NIAUT).

Please retain this number and provide it with any protocols submitted to the University Committee on Laboratory Animals for review.

Date: February 6, 2020

Jennifer Devitt
Training Coordinator
jennifer.devitt@dal.ca
494-8507

Appendix B Training Certificate

PRACTICAL TRAINING CERTIFICATION

This is to certify that

<u>Devanshi Desai</u>

has participated in the practical training session :

THE RAT:
RECOMMENDED TECHNICAL PROCEDURES

- *<u>Video presentation</u> covering humane handling and restraint methods, sexing of adult and neonatal rats, identification of individual animals, anesthesia techniques, and recommended procedures for blood collection and injections*
- *Discussion on characteristics of healthy rodents*
- *<u>Practical training</u> in humane handling and restraint of rats*
- *Administration of injectable anesthetic and monitoring anesthetic depth*
- *Intraperitoneal, subcutaneous, intramuscular and intravenous injections*
- *Blood collection (saphenous and intracardiac sites)*

Date: <u>March 9, 2020</u>

Jennifer Devitt

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Jennifer Devitt
Training Coordinator
jennifer.devitt@dal.ca
494-8507

Appendix C Rat Randomization sheet

Each rat will be treated with 350 mg Whey protein (WP), α- Lactalbumin (α-LA), β- lactoglobulin (β-LG), Glycomacropeptide (GMP), Micellar casein (MC), Total Milk protein (TMP) dissolved in a glucose solution and Glucose control starting at 13:00

	Rat 1	Rat 2	Rat 3	Rat 4
Day 1	WP	α-LA	β-LG	GMP
Day 5	α-LA	β-LG	GMP	MC
Day 9	β-LG	GMP	MC	TMP
Day 13	GMP	MC	TMP	Glc
Day 17	MC	TMP	Glc	WP
Day 21	TMP	GLC	WP	α-LA
Day 25	Glc	WP	α-LA	β-LG

Group 2: Each rat will be treated with 350 mg WP, α-LA, β-LG, GMP, MC, TMP dissolved in a glucose solution and glucose control starting at 13.00

	Rat 5	Rat 6	Rat 7	Rat 8
Day 2	WP	α-LA	β-LG	GMP
Day 6	α-LA	β-LG	GMP	MC
Day 10	β-LG	GMP	MC	TMP
Day 14	GMP	MC	TMP	Glc
Day 18	MC	TMP	Glc	WP
Day 22	TMP	GLC	WP	α-LA
Day 26	Glc	WP	α-LA	β-LG

Group 3: Each rat will be treated with 350 mg WP, α-LA, β-LG, GMP, MC, TMP dissolved in a glucose solution and glucose control starting at 13.00

	Rat 9	Rat 10	Rat 11	Rat 12
Day 3	WP	α-LA	β-LG	GMP
Day 7	α-LA	β-LG	GMP	MC
Day 11	β-LG	GMP	MC	TMP
Day 15	GMP	MC	TMP	Glc
Day 19	MC	TMP	Glc	WP
Day 23	TMP	GLC	WP	α-LA
Day 27	Glc	WP	α-LA	β-LG

Group 4: Each rat will be treated with 350 mg WP, α-LA, β-LG, GMP, MC, TMP dissolved in a glucose solution and glucose control starting at 13.00

	Rat 13	Rat 14	Rat 15	Rat 16
Day 4	WP	α-LA	β-LG	GMP

Day 8	α-LA	β-LG	GMP	MC
Day 12	β-LG	GMP	MC	TMP
Day 16	GMP	MC	TMP	Glc
Day 20	MC	TMP	Glc	WP
Day 24	TMP	GLC	WP	α-LA
Day 28	Glc	WP	α-LA	β-LG

Appendix D Insulin ELISA Protocol

Crystal Chem
High Performance Assays

CATALOG# 90060

Ultra Sensitive rat Insulin ELISA Kit

Catalog number

Ultra Sensitive Rat Insulin ELISA 90060

Intended use

A high quality enzyme immunoassay for the quantification of rat insulin in fluid, plasma, and serum.

Test principle

Crystal Chem's Ultra Sentitive Rat Insulin ELISA Kit is based on a sandwich enzyme immunoassay using only a 5 µL sample to produce same day results. The kit can be run using an ultrasensitive low range, wide range, or high range screening method to yield a wide dynamic range with just one kit.

Specifications

Sample Types	Serum, Plasma, and Fluid
Assay Time	Same Day Procedure
Range	Low Range: 0.1-6.4 ng/mL Wide Range: 0.1 - 12.8 ng/mL High Range: 1 - 64 ng/mL
Sample Size	5 µL
Sensitivity	0.05 ng/mL
Precision	CV < 10%

Specificity

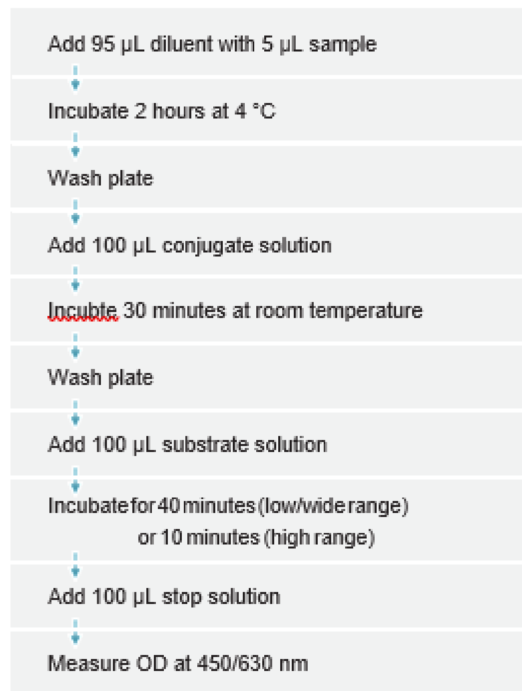
Rat Insulin 100%
Mouse Insulin 100%*
Human IGF-I .... Not detected
Human IGF-II .... Not detected

*Can vary from lot to lot. See insert in kit.

Highlights

- ✓ Kits use only 5 µL sample
- ✓ Very sensitive (0.05 ng/mL)
- ✓ Run different ranges using the same kit
- ✓ Works with multiple sample types
- ✓ Complete the full test in < 3.5 hours

Summary of protocol



See kit insert or email us for a complete protocol