Mount Saint Vincent University
Department of Applied Human Nutrition

The effect of total milk protein, casein and whey protein ingestion on blood glucose and insulin in rats

by
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Dairy product consumption is highly associated with reduced postprandial glycaemia. Dairy proteins, including caseins, and whey protein, have been found to reduce postprandial glucose response and increase insulin response. However, the effect of milk protein fractions on short-term blood glucose control is unclear. The objective of this study was to investigate the acute effect of total milk protein (TMP), micellar casein (CN), and whey protein (WP) on blood glucose and insulin in rats. The rationale for this study is based on the fact that dairy products have a different protein composition determined by their natural and added content of milk proteins and therefore the glycaemic control provided by dairy products is particularly determined by their protein composition. The hypothesis of this study was that TMP, CN and WP may have a distinct effect on short-term blood glucose and insulin response.

Methods: in a repeated measure crossover study design, six Wistar-Han rats (4 months old) with jugular vein catheters and vascular access buttons were randomly gavaged (350mg/3ml) with one of the treatments provided in a random order with a total of four treatments received by each rat: WP, CN, TMP, G after 6-hour daylight fasting. Blood samples were collected at 0, 15, 30, 60, 90, and 120 minutes for blood glucose and 0, 15 and 30 minutes for serum insulin.

Results: There was an effect of treatment ($P = 0.0002$), time ($P < 0.0001$) and a treatment by time interaction ($P < 0.0001$) on blood glucose over 120 min. There was no difference between the treatments at the baseline. At 15 min, TMP, CN, and WP led to reduced blood glucose with a significant difference when compared to G. At 30min CN and WP led to reduced blood glucose compared to G ($P < 0.05$) but not TMP ($P = 0.06$). At 30 min, CN led to a higher BG compared to WP ($P < 0.05$). At 60, 90, and 120 min, the effect of protein treatments on blood glucose was not significantly different when compared to G. The blood glucose area under the curve (AUC) after ingestion of WP, CN, and TMP was lower than G ($P < 0.05$). There was an effect of treatment ($P = 0.02$), time ($P < 0.0001$) and a time by treatment interaction ($P < 0.0001$) on serum insulin over 30 min observed. There was no difference between the treatments at the baseline. At 15 min, TMP and CN resulted in lower serum insulin compared to G ($P < 0.05$), while WP was neither different from G nor from TMP and CN ($P > 0.05$). There was an effect of treatment on insulin AUC over 30 min ($P = 0.006$) resulting in a lower serum insulin AUC after protein treatments compared to G ($P < 0.05$).

Conclusion: The ingestion of the major milk protein fraction led to a lower blood glucose and serum insulin compared to the same dose of glucose. Although, TMP, CN and WP followed a similar pattern in their effect on BG and serum insulin, they demonstrate distinct properties at 15 and 30 min after ingestion.

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Chapter 1: Introduction

The human body depends on a tight control of its blood glucose levels to ensure normal body function. Glucose homeostasis is achieved through the opposing and balanced actions of glucagon and insulin (1). Defects of insulin secretion and/or insulin action, result in diabetes mellitus; a metabolic disorder characterized by chronically elevated blood glucose levels, together with disturbances of carbohydrate, fat, and protein metabolism (2). The major physiological stimulant of insulin secretion in humans is the circulating concentration of glucose, and other nutrients (3), including amino acids, such as leucine, lysine, and arginine. These amino acids possess the ability to initiate insulin secretory response even in the absence of glucose and therefore qualify as initiators of insulin secretion (3). Dairy proteins have been reported to contain high proportions of these essential amino acids (4).

Dairy products contain two major milk proteins: whey protein and casein. A total bovine milk protein contains 20% and 80% of whey protein and casein, respectively. They have been identified by various sources to contain high proportions of branched chain amino acids (BCAA) (leucine, isoleucine, and valine) (4), with higher contents found in whey protein (5). However, the secretion and concentration of insulin in blood may be affected by the composition of ingested proteins and thereby the concentration of plasma amino acid. It is known that the ingestion of whey protein and casein are linked with increased insulin secretion in the body (6, 7).

Reports on dairy proteins, especially on whey protein, show beneficial physiological effects in controlling glucose metabolism and food intake as well as improved body weight and adiposity through increased satiety (4, 8). Both whey protein and casein have also been revealed by various studies to have insulinotropic and glucose-lowering properties in both healthy people

and individuals with type 2 diabetes. These studies identified whey protein as the most potent protein (4, 9). However, the direct role of individual milk protein fractions and total milk protein on blood glucose control and insulin secretion remains unexplored. This presents the limitation of their use in functional foods.

The importance of this, has led the current study to investigate the direct effect of total milk protein, whey protein and casein on blood glucose and insulin response in rats.

Chapter 2: Literature Review

2.1 Diabetes: classification, prevalence, comorbidities, and contributing factors.

Diabetes mellitus (DM) is defined by Diabetes Canada as a varied metabolic disorder characterized by the presence of hyperglycemia, ascribed to diminishing of insulin secretion, defective insulin action, or both (10). Incessant diabetes hyperglycemia is commonly related to serious long-term microvascular problems with adverse effects on eyes, kidney, and nerves and an increased risk of developing cardiovascular disease (10).

2.1.1 Classification

Diabetes mellitus is classified into type 1 diabetes mellitus (T1DM), type 2 diabetes mellitus (T2DM), gestational diabetes mellitus (GDM), and other specific types of diabetes (10). The type 1 diabetes (T1DM) is mainly caused by the destruction of the pancreatic beta cells resulting from the inability of the pancreas to secrete insulin which predisposes one to ketoacidosis (10). People at risk of this category of diabetes are often characterized by an autoimmune destruction of the β-cells of the pancreas by genetic backgrounds and environmental factors (11). Type 1 diabetes mellitus (T1DM) can occur at any age, but in most populations, the incidence rate is highest between birth and 14 years old (12)**.** Type 2 diabetes (T2DM) is a result of a combination of resistance to insulin action and an inadequate compensatory insulin secretory response, but with very uncommon cases of ketosis (10). 90 to 95% of all cases of diabetes fall into the category of T2DM, characterized by insulin resistance and subsequent insulin deficiency (13). Gestational diabetes mellitus (GDM) is referred to as impairment in glucose tolerance with onset or first recognition during pregnancy (10). Monogenic diabetes is an uncommon disorder, it is known as a non-insulin dependent and genetically influenced disorder, with an autosomal dominant pattern

of inheritance. It occurs as a result of genetic defects of β-cells function, typically found in young people (<25 years of age) (14).

2.1.2 Prevalence

Diabetes mellitus (DM) is one of the most prevalent chronic diseases in the world, an estimation of 422 million persons aged 18 and above have been diagnosed with diabetes in 2014, while in 2016, diabetes was the direct cause of 1.6 million deaths (15). DM is expected to surge to 552 million by 2030 (16). In Canada, 9% (3,655,000) of the population have been diagnosed with T1DM and T2DM in 2019, with an expected upsurge to 11% (4,785,000) by the year 2029 (17). However, 29% (10,991,000) of people in Canada diagnosed of T1DM, T2DM and prediabetes combined in 2019 are expected to increase to 32% (13,320,000) in 2029 (17).

2.1.3 Comorbidities

Diabetes may exist simultaneously with other primary diseases. The study examining comorbidity in a cohort of community-dwelling older adults with diabetes in Ontario, Canada, found at least one comorbidity in more than 90% of the cohorts population (18). Another common comorbidity of diabetes is hypertension (19), as well as cardiovascular disease, chronic kidney disease, obesity, T2DM-discordant conditions such as chronic obstructive pulmonary disease, and depression (20).

2.1.4 Obesity

Diabetes Mellitus is a common comorbid of obesity. Obesity is a common chronic disorder of excessive body fat (21). It is determined in epidemiological research using the body mass index (BMI), calculated as weight in kilograms divided by height in meters squared (kg/m^2). Obesity is defined as a BMI greater than or equal to 30 kg/m^2 and further divided into subgroups: Class I:

BMI 30–34.9 kg/m^2, Class II: BMI 35–39.9kg/m^2 and Class III: BMI ≥ 40 kg/m^2. The term severe obesity is used for individuals with a BMI ≥ 35 kg/m^2 body mass index [BMI] ≥ 30 kg/m^2) (22).

According to the 2014 Canadian Community Health Survey, more than 5 million adults have obesity and according to the 2015 Canadian Health Measures Survey, 30%, or above one in three adults in Canada have obesity and may require medical support to manage their disease (23). Obesity is one of the leading causes of T2DM and it is positively associated with BMI, although the mechanisms by which obesity increases the risk of developing T2DM are only partly understood. However, the main adverse effect of obesity is on the action of insulin, particularly in liver, muscle and adipose tissue, in which excess of body fat promotes insulin resistance and impairs insulin secretion, thereby resulting in overt T2DM. Also, excess body weight is accountable for one-fourth development of high blood pressure, heart disease, stroke, arthritis, cancer, and other chronic health problems (24). In addition, approximately, one in ten Canadian adults' death (age 20 to 64) is attributed to obesity (23).

2.1.5 Hypertension

Hypertension is defined as a systolic blood pressure at or above 140 mmHg or diastolic blood pressure at or above 90 mmHg, it is associated with a higher risk of cardiovascular diseases, with 2.5 times in women, and 1.6 times greater risk of cardiovascular diseases in men (25). According to Health Canada one in five people (22.7%) of Canadian adults aged 20 years and older have been diagnosed with hypertension (26). Over 75% of people living with diabetes also live with high blood pressure (27), while individuals living with hypertension often independently display signs of insulin resistance (27). Diabetes and hypertension are common, inter-related and share a significant connection in fundamental risk factors (27). The pathogenic relationship between diabetes mellitus and hypertension is bi-directional, as diabetes reflects the impact of

underlying insulin resistance on the vasculature and kidney, while hypertension is characterized by the disruptions in glucose metabolism (28). Insulin resistance states characterized by hyperinsulinemia causes vascular smooth muscle cell proliferation and increased vascular stiffness, which makes individuals with diabetes susceptible to the development of hypertension (29). Furthermore, insulin may explicitly or implicitly damage vasodilation and upsurge oxidative stress and the inflammatory process in the vascular wall (30, 31). Coupled with these effects is the diminished autoregulation of vascular tone, augmented systemic vascular resistance, and elevated levels of blood pressure. Also, the antinatriuretic properties of insulin increase renal retention of sodium and water leading to volume overload and poses risk of developing hypertension (32).

2.1.6 Metabolic syndrome

Metabolic syndrome (MetS) is a constellation of cardiometabolic risk factors that predict chronic disease and all-cause mortality (33-36). It is defined according to the Adult Treatment Panel III criteria, as the presence of three or more of the following criteria in an individual: abdominal obesity (waist circumference > 102 cm for men and > 88 cm for women); elevated plasma triglyceride level (≥ 1.7 mmol/L); decreased high-density lipoprotein (HDL) cholesterol level (< 1.03 mmol/L for men and < 1.30 mmol/L for women); elevated blood pressure (≥ 130/85 mm Hg); or elevated fasting glucose level (≥ 6.1 mmol/L) (37). In 2014, according to the Public Health Agency of Canada, 14.9% of Canadian adults had MetS. Rates were similar in both sexes, but higher in those who are non-Caucasian or overweight or obese (38). In the presence of MetS, the risk of cardiovascular disease is estimated to double, and the risk of type 2 diabetes increases fivefold (34-36, 39).

2.1.7 Diagnostic criteria of diabetes

Impaired blood glucose control denotes the metabolic state between normal blood glucose and diabetes (26). Fasting blood glucose ranges from 3.9–5.5 mmol/L in a person without diabetes (40). Impaired fasting glucose (IFG) is defined by Diabetes Canada as "fasting plasma glucose (FPG) with a range of 6.1 to 6.9 mmol/L" (41). While impaired glucose tolerance (IGT) is present when an individual has blood glucose ranging from 7.8–11.0 mmol/L after a 2hour-plasma oral glucose tolerance test in the presence of an FPG concentration ≥7.0 mmol/L (10). Prediabetes is considered present in an individual with impaired fasting glucose (IFG), impaired glucose tolerance (IGT), or glycated hemoglobin (A_1C) of 6.0% to 6.4%. Each of these places individuals at high risk of developing diabetes and its complications (10). Insulin resistance leads to elevated blood glucose and triglyceride levels and is a characteristic of both metabolic syndrome and T2DM (41).

2.2 The role of the diet in pathogenesis and prevention of diabetes.

Food and eating habits have been found to effectively contribute to the development of T2DM as evident in various studies. For instance, a prospective cohort study investigating the association between glycemic intake (GI), glycemic load (GL), carbohydrate intake, fibre intake and T2DM (42) reported that diets high in carbohydrate, dietary GI, GL, starch, sugar and low in dietary fibre are significant risk factors of T2DM (42). Similarly, previous studies found a strong connection between diet and T2DM (43). In a study conducted with 1,317 subjects with no previous diagnosis of diabetes for 4 years, reported an association between fat intake, T2DM, and impaired glucose tolerance (44). Equally, positive association was found between high energy intake and the T2DM, for instance, in a prospective study investigating the association of white rice consumption and incidence of diabetes. A significant increase in risk was observed in women

with rice intakes of ≥3 bowls (420 g)/d (45). However, studies report that high intake of dietary fibre, whole grain, low GI and GL food may help reduce the risk of T2DM (46-48).

2.3 The causal role of excessive energy and glycemic carbohydrates intake in the development of obesity and diabetes.

Diet is considered essential in the development of type 2 diabetes, mostly because of the effect of high carbohydrate foods on increased blood glucose levels. Previous studies report that excessive energy intake, as well as specific components of diet are linked to T2DM (49-51). An investigation carried out with 500 ethnically diverse schoolchildren for 19 months showed an increased possibility of developing obesity, in response to the consumption of every extra carbonated drink (52). Similarly, sugar-sweetened beverages were linked to risk of developing T2DM (50), especially when consumed in high proportion. In a prospective cohort study, participants who consumed one or more sugar-sweetened beverage (SSB) per day found a greater risk of developing T2DM in 83% of participants compared to those who consumed less than one SSB per month (51). Therefore, this infers that reduction in energy consumption levels with consistent spacing and regularity of food consumption may contribute to blood glucose and weight management (53).

Diets with a high-glycemic-index (GI) or glycemic load (GL) can lead to the higher concentrations of postprandial blood glucose and insulin. This sequentially increases glucose intolerance and risk of development of T2DM (54). In a study conducted with black women, a positive association was found with intake of foods with higher glycemic loads, glycemic index, and the risk of T2DM (55). Consequently, the replacement of high-glycemic-index carbohydrates with low-glycemic-index carbohydrates may effectively impact glycemic control. Similarly, a

study conducted making this replacement found a positive effect on glycemic control in persons with T1DM and T2DM (56).

2.3.1 Contributing factors

Various studies have described lifestyle including diet and physical inactivity as major contributors to the risk of developing T2DM (42, 43). Physical inactivity often involves sedentary behavior which are activities performed with low energy expenditure (57). This lifestyle, may lead to spikes in the blood glucose which can further lead to T2DM and metabolic syndrome (58). In 2017, a study reviewing dietary patterns/habits in T2DM reported a significant relationship between T2DM and physical inactivity (43). Another study carried out with non-diabetic individuals from a high-risk population, over an average 6-year follow-up period found that less active women and men had a higher incidence rate of diabetes in all body mass index (BMI) groups (43). Similarly, a meta-analysis reported that sedentary activities were associated with 112% greater collective relative risk of diabetes (59). This meta-analysis reviewed 10 studies (including 6 prospective studies) that involved 505,045 participants (59) using TV time as case study—large vs small amounts of TV time (different by a few hours per day).

Investigators concluded that sedentary lifestyle has a close relationship with increased risk of cardiovascular disease and all-cause mortality, with emphasis on T2DM (59). Consistently, studies have found that higher levels of physical activity reduce a person's predisposition to diabetes. An investigation carried out on moderate-intensity physical activity in a prospective cohort study showed that participants are less likely to develop diabetes if they achieve suggested levels of moderate activity, compared to their counterparts who live a sedentary life (60). Moreover, physical activity is effective in the reduction of body fat stores and intra-abdominal fat, which is known as a risk factor of insulin resistance (43). Therefore, a moderate loss of 5-10% in

initial weight in T2DM patients or individuals at risk, may substantially improve insulin sensitivity, glycemic control, high blood pressure and dyslipidemia (53).

2.4 Links between diabetes and obesity

Obesity has been associated with the risk of developing T2DM. By 2025 it is anticipated that the prevalence of T2DM related to obesity will increase up to 300 million people globally (61). One of the factors identified as a predisposition for both obesity and T2DM is the genetic factor. It is currently presumed that only those obese individuals who exhibit a genetic failure of the pancreas to recompense for insulin resistance will develop T2DM. Therefore, the presence of "diabetes genes" to enable the limitation of β-cell function is vital in the development of T2DM (62).

Obesity also affects insulin resistance, although the core adverse effect of obesity is on insulin action, particularly in the liver, muscle, and adipose tissues (62). The "glucose fatty-acid cycle" explains that the relationship between obesity and T2DM is based on the observation of competition between glucose and fatty acid oxidation in the heart muscle (63). The augmented supply of non-esterified fatty acids from ample adipose tissue storage contends with glucose utilization, especially in the muscle. Thus, the inhibition of the glycolytic enzymes such as pyruvate dehydrogenase, phosphofructokinase, and hexokinase result in a reduction of glucose oxidation rate and increased glucose concentrations (62), while the associated amplified fatty acid turnover is followed by an increased release of glycerol from adipose tissue which is reused for hepatic glucose production, further enhancing abnormal glucose metabolism. Therefore, a higher availability of fatty acids may be the single most important factor in disrupting the function of insulin in obesity (62).

Elevated free fatty acids were suggested to have a direct effect on insulin action impairment. Obese subjects and those with T2DM have shown a high intramyocellular lipid accumulation, an important feature of the insulin-resistant state (62). Likewise, marked impairment of glucose-stimulated insulin secretion and reduction in insulin biosynthesis is linked to chronic exposure of β-cells to excessive fatty acids (64), while insulin resistance may be caused as a result of defects in mitochondrial fatty acid oxidation which may lead to increased intracellular fatty acid metabolites (fatty acid CoA, diacylglycerol). However, young insulin-resistant offspring of parents with T2DM have shown features of impaired mitochondrial function (65), while obese individuals have been reported to have smaller mitochondria with reduced bioenergetic capacity than lean controls (66). Though studies on this topic are still inadequate, there is growing evidence that a defective mitochondrial function could be a prominent feature of disturbances in both insulin secretion and action (67).

Tumor necrosis factor alpha (TNF-α), a multifunctional cytokine exerts a variety of catabolic effects in the adipose tissue. In obese subjects, the two receptor subtypes of TNF-α are usually overexpressed in the adipose tissue (68-70). It induces inhibition of glucose uptake because of an impairment of insulin signaling and suppression of GLUT 4 expression, decrease of lipoprotein lipase expression and activity, and an increase in lipolysis (71). Also, TNF-α reduces the expression of adiponectin, a protein that is abundantly expressed in fat cells and exerts direct antidiabetic and anti-atherosclerotic actions. This cytokine (TNF-α) causes insulin resistance by stimulating the phosphorylation of insulin receptor substrate 1 (IRS-1) at the serine residue 307 which inhibits the transduction of the insulin signal to downstream elements (72).

The connection between obesity and type 2 diabetes (T2DM) has further been proven through the management of T2DM with weight-loss (73). Also, Diabetes Canada, suggests that

insulin sensitivity and glycemic control can be improved through a moderate weight loss of 5-10% (74).

2.5 Mechanisms of the normal and abnormal physiology of blood glucose control.

2.5.1 Normal physiology of blood glucose control

The ability of the body to control its blood glucose levels is essential in maintaining normal body functions (1). The insulin-expressing β-cells (~60% of adult human islet cells) and the glucagon-expressing α-cells, produced in the pancreas by the body islets (Langerhans), secrete insulin and glucagon respectively, the most relevant hormones involved in glucose regulation among several others. (3). The anatomic arrangement of islet cells differs among species (3). For instance, islets in rodents are primarily composed of β-cells located in the center with other cell types in the periphery while human islets exhibit interconnected α- and β-cells (75, 76). Insulin accelerates the transport of glucose into insulin-sensitive cells and enables its conversion to storage compounds via glycogenesis (conversion of glucose to glycogen) and lipogenesis (fat formation) (77, 78). Secretion of both glucagon and insulin are inhibited by somatostatin (79), and the pancreatic polypeptide regulates the exocrine and endocrine secretion activity of the pancreas (80).

Glucose homeostasis is achieved by the opposing and balanced actions of glucagon and insulin in the pancreas to ensure that blood glucose levels are maintained within a very narrow range of 4-6mM (1). During fasting or in between meals, when blood glucose levels are low, glucagon is released from α-cells to promote hepatic glycogenolysis. It also drives hepatic and renal gluconeogenesis to increase endogenous blood glucose levels (81) during prolonged fasting, whereas, insulin secretion from β-cells is stimulated by higher levels of exogenous glucose levels. In a post-absorptive state, (82) insulin triggers glucose uptake into insulin-dependent muscle and

adipose tissues and promote glycogenesis (1). This lowers blood glucose levels by removing the exogenous glucose from the bloodstream (83, 84).

The glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), known as the incretin hormones are also involved in blood glucose regulation due to their effects on insulin and glucagon (85). Although both GLP-1 and GIP are considered glucose-dependent hormones, they are secreted only when glucose levels rise above normal fasting plasma glucose levels but do not directly stimulate insulin secretion (86). These hormones aid in the stimulation of insulin secretion as they are normally released in response to meals and, by activating certain receptors (G protein–coupled) on pancreatic β-cells. Therefore, when glucose levels are low, GLP-1, and GIP levels (and their stimulating effects on insulin secretion) are diminished (86).

2.5.2 Insulin secretion

The peptide hormone insulin is completely synthesized in and secreted from pancreatic β-cell (87). The gene encoding preproinsulin is located on the short arm of chromosome 11 in humans (88). Preproinsulin is speedily (<1 min) discharged into the cisternal space of the rough endoplasmic reticulum, where proteolytic enzymes immediately cleave the signal peptide, thus generating proinsulin, a precursor of insulin.

Proinsulin is a 9-kDa peptide, containing the A and B chains of insulin (21 and 30 amino acid residues, respectively) joined by C peptide (30 – 35 amino acids). It has a similar structural conformation with insulin. The C peptide aligns disulfide bridges that link A and B chains to enable the molecules correctly fold for cleavage and transports the proinsulin in micro vesicles to the Golgi apparatus. It is then packaged into secretory granules and converted to insulin in the Golgi complex and continues to mature within the secretory granule through the sequential action of two

endopeptidases (prohormone convertases 2 and 3) and carboxypeptidase H (89). This leads to the removal of the C peptide chain, liberating two cleavage dipeptides and yielding insulin (3).

2.5.3 The regulation of insulin secretion

To ensure insulin regulations, β-cells are equipped with mechanisms to detect changes in circulating nutrients, in hormone levels and the activity of the autonomic nervous system (3). Insulin secretion is primarily determined physiologically by the circulating amount of glucose and other nutrients, which includes amino acids and fatty acids. These nutrients have the capacity to induce an insulin secretory response. The β-cells detect changes in the circulating nutrients and release insulin to enable nutrient uptake, metabolism, or nutrient storage by the target tissues as nutrients are absorbed in the gastrointestinal system (3). They also detect a decrease in the circulating nutrients, thereby enabling deactivation of insulin secretion to prevent hypoglycemia. The β-cells' response to nutrient initiators of insulin secretion can be modified by a variety of hormones and neurotransmitters which act to amplify, or occasionally inhibit, the nutrient-induced responses (3). In normal glucose metabolism, the potentiators of insulin secretion have little or no effect on insulin secretion (3). This mechanism prevents inappropriate secretion of insulin, which can cause potentially harmful hypoglycemia. The overall insulin output depends on the relative input from initiators and potentiators at the level of individual β-cells, the synchronization of secretory activity between β-cells in individual islets, and on the coordination of secretion between the hundreds of thousands of islets in a human pancreas (3).

2.5.4 Insulin clearance

Insulin clearance is an important component in the metabolism of insulin as it controls cell responses by reducing the supply of insulin while interacting with certain aspects of insulin action (90). The primary site of insulin clearance is the liver accounting for about 80% of endogenous

insulin extraction under normal physiological conditions, while the remaining from the kidneys and muscles (91). The basic mechanism of hepatic insulin clearance is established by receptor-mediated insulin uptake and insulin degradation in hepatocytes. In the systemic circulation, unprocessed insulin is transmitted to the target peripheral tissue and brain for action and further receptor-mediated uptake and degradation in the periphery (92). While insulin functions exclusively by crossing the blood-brain barrier (BBB) in the central nervous system (CNS), using a saturable transporter, and affecting feeding and cognition through CNS mechanisms that is mainly independent of glucose utilization (93). The endothelial cells in target tissues of extra-hepatic insulin then contribute to the regulation and function of homeostasis of systemic insulin (94, 95).

The rate of insulin clearance decreases in glucose intolerance, obesity, particularly abdominal obesity, hypertension, hepatic cirrhosis, and nonalcoholic fatty liver disease (96). Since the plasma concentration of insulin is mainly determined by its rate of secretion and clearance, evidence suggests that amplified insulin resistance relates to lower insulin clearance (97, 98) particularly among Hispanics and African Americans (96, 99, 100). Nevertheless, reduced insulin clearance has significant physiological functions; for instance, animal models have shown that reduced insulin clearance serves as a compensatory mechanism to preserve β-cell function and to preserve peripheral insulin levels in the states of insulin resistance (101, 102). Reduced insulin clearance and increased insulin secretion results in chronic hyperinsulinemia (99, 103-105) to compensate for resistance to peripheral insulin, especially in persons with obesity (98, 106, 107). Several epidemiological studies have shown that decreasing insulin clearance in obese subjects is more important in compensating for peripheral insulin resistance than increasing insulin secretion (108, 109). Also, dairy intake was identified to reduce insulin clearance in a study conducted to

investigate the effect of a dairy and nondairy snack in both normal-weight (NW) and overweight/obese (OW/OB) children on blood glucose regulation and food intake (FI) (110).

2.5.5 Nutrient-induced insulin secretion

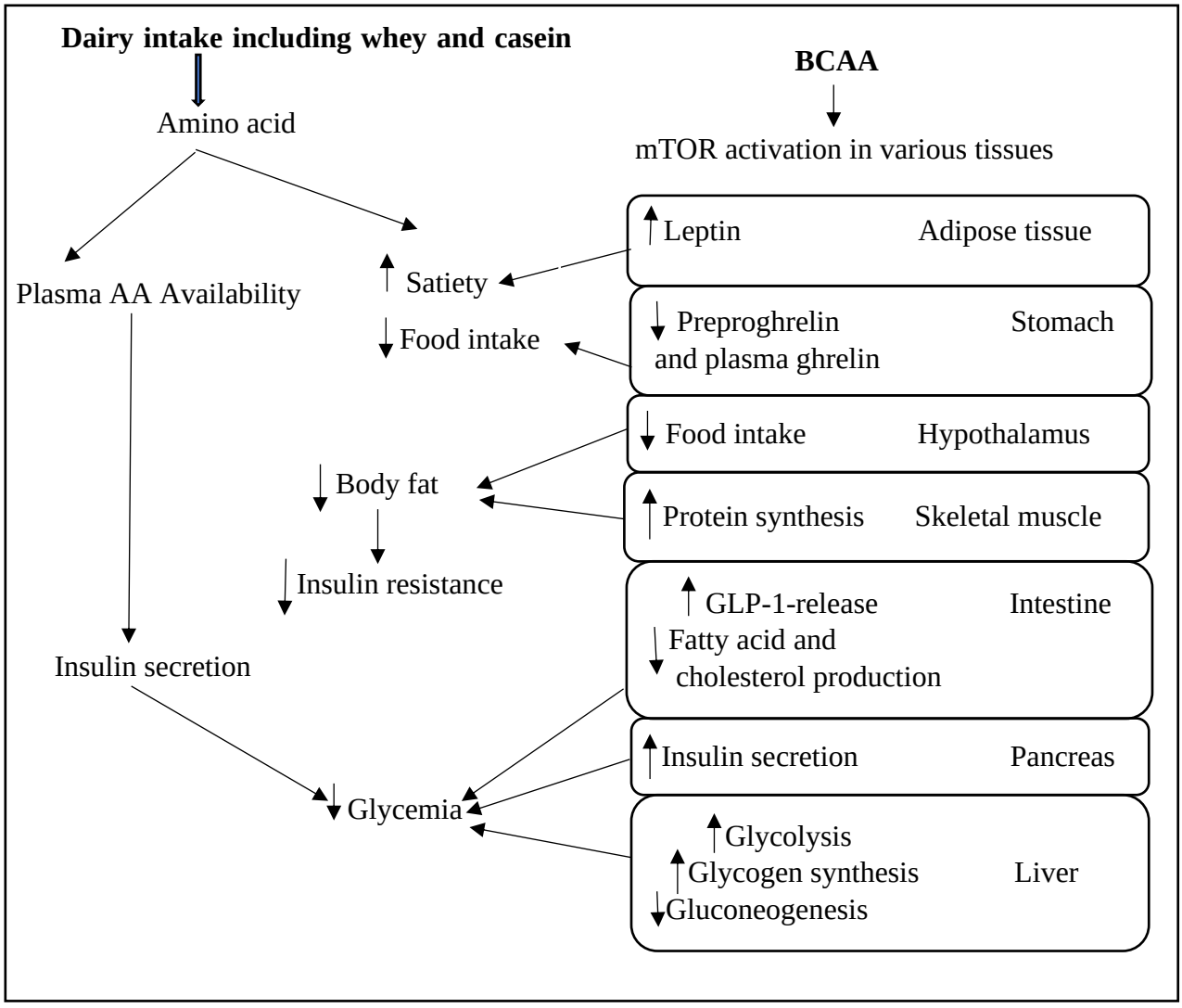
In the β-cells, elevated blood glucose levels after meals are the main stimuli for the release of insulin (82). The circulating blood glucose is transported via high capacity glucose transporters (GLUT); GLUT2 in rodents, GLUT1, 2, and 3 in humans (111, 112). GLUT, which is located on the surface of the β-cells and enables rapid equilibration of extracellular and intracellular glucose concentrations. Once inside the cell, glucose undergoes glycolysis, thereby generating adenosine triphosphate (ATP), resulting in an increased ATP/ADP ratio. This altered ratio leads to the closure of ATP-sensitive K^+-channels (K_{ATP}-channels), thereby causing membrane depolarization, opening of voltage-gated Ca^{2+} channels, Ca^{2+} influx, and exocytosis of insulin granules (1, 113). Channels are opened to ensure the maintenance of the resting potential under non-stimulated conditions, by transporting positively charged K^+-ions down their concentration gradient out of the cell. After closing, the subsequent decrease in the magnitude of the outwardly directed K^+-current causes the membrane to be depolarized. The Ca^{2+} voltage-dependent channels (VDCCs) are then opened. The fusion of insulin-containing granules with the membrane and the subsequent release of their content are triggered by an increase in intracellular calcium concentrations (113). However, the entire secretory cycle is two-phased with the initial step entering approximately 10 minutes after the glucose stimulus. Most of the insulin is released during this first phase while, the remaining insulin is secreted in the slowly increasing second phase that is more sustained and typically persists as long as glucose level is elevated (114). The first phase involves insulin secretory granules situated close to the β-cell plasma membrane. It occurs by responding to a rise in extracellular glucose, islet ATP and cyclic adenosine monophosphate (cAMP) levels surge, and

initiating closure of ATP-sensitive potassium channels; this triggers β-cell membrane depolarization and calcium influx through voltage-sensitive calcium channels; the resulting rise in intracellular calcium contributes to the migration of insulin-containing granules into the β-cell membrane, where they combine/integrate or melt into the membrane with the release of the content of the granules (114). Insulin release in the second phase includes the synthesis of new insulin molecules and ATP-dependent mobilization of granules from the storage pool into the faster discharged pool (114).

Similarly, several amino acids stimulate the *in vivo* or *in vitro* secretion of insulin. However, though the majority require the presence of glucose, some such as leucine, lysine, and arginine may stimulate insulin secretion when glucose is absent, and thus qualify as initiators of secretion. Leucine penetrates the islets by an independent sodium transport mechanism and facilitates a biphasic increase in the release of insulin (3). The effects of leucine on β-cell membrane potential, ion fluxes, and insulin secretion are closely related to those of glucose but smaller than those of glucose (115). Consequently, the metabolism of leucine within β-cells enables the reduction of potassium permeability, thereby leading to depolarization and activation of l-type calcium channels through which calcium enters the β-cells and initiates insulin secretion (3). Leucine is also able to trigger the amplifying pathway of insulin secretion in a K_{ATP} channel-independent manner, similar to glucose. The charged amino acids, lysine, and arginine cross the β-cell plasma membrane via a transport system specific for cationic amino acids. The accumulation of these positively charged molecules is generally considered to directly depolarize the β-cell membrane that leading to calcium influx (3). Furthermore, there are reports of improved beta-cell secretion and enhanced physiological surge of post-prandial amino acid concentration after the

ingestion of fast absorbable proteins (example, whey protein) in T2DM patients with tenacious endogenous beta-cell secretion (116).

Figure 2. 1 Chart describing the potential mechanisms by which amino acids in dairy products improve glucose control (117).



2.5.6 Abnormal glucose metabolism

In the transition from normoglycemia to diabetes mellitus, the relative contribution of changes in the appearance or disappearance of glucose is unknown (118). Hepatic insulin resistance leads to insulin restraint of endogenous glucose production after an overnight fast (119) and gluconeogenesis in persons with impaired fasting glucose (IFG) (118). This hepatic insulin resistance is related to a superfluous accumulation of fat in the liver. Also, the rate of glucose utilization becomes normal or augmented after an overnight fast when glucose uptake is mainly independent of insulin and driven by the mass-action consequence of hyperglycemia (119). Therefore, it leads to a clinical consequence, whereby the mechanism designed to lower fasting glucose begins to aim at constraining overproduction of glucose from the liver, rather than further increasing glucose uptake in T2DM (120).

Similarly, after meal ingestion, there is a complete suppression of endogenous glucose production under normal conditions as a result of an increase in insulin and glucose concentrations as well as an associated decrease in glucagon. Nonetheless, in persons with T2DM, this endogenous glucose suppression is unfinished due to resistance in hepatic insulin, impaired insulin, and excessive glucagon secretion (121-123). However, persistent maintenance of postprandial hyperglycemia is mostly caused by continual hepatic glucose production after meal consumption (121). Persons with IFG and additional defects in glucose disappearance and consequent post-prandial hyperglycemia, experience impaired insulin secretion, as a result of higher post-prandial glucose concentration. Consistent impaired insulin secretion will eventually result in overt T2DM (124).

The liver's capacity to store glucose during a meal seems unbroken or just slightly weakened in patients with T2DM (121, 125). Generally, the rate of utilization of glucose is quite

normal, as hyperglycemia itself offsets impaired insulin stimulation of glucose uptake into peripheral tissues through a mass action effect of glucose (126). Furthermore, in both normal subjects and patients with T2DM, the brain uses equal levels of glucose, thus, the incomplete cessation of the production of endogenous glucose must therefore, be the cause of both post-prandial hyperglycemia and fasting hyperglycemia (119). Endogenous glucose production is regulated (inhibited) by insulin which increases hepatic glucose uptake by stimulating glucokinase activity and decreases hepatic glucose release by decreasing the conversion of glucose-6-phosphate to glucose (126).

2.5.7 Pathophysiology of Diabetes Mellitus

The pancreas has an outstanding capacity to adapt to increasing demands on insulin for normoglycemia (such as in obesity, pregnancy, cortisol excess), but a defect in pancreatic islet β-cell function plays a major role in the development of hyperglycemia (127). Abnormal metabolism of insulin occurs in people with diabetes, which has varying causes (118). T1DM occurs as a result of autoimmune destruction of pancreas, yet the fasting hyperglycemia insulin deficiency does not progress until the majority (> 80%) of the β-cells are lost to the fundamental autoimmune process. However, insulin secretion deficiencies are apparent in asymptomatic people years before the development of diabetes (118). Persons who have tested positive to islet antibodies mostly experience a reduction in first-phase insulin secretion in reaction to intravenous glucose injection (118, 128). The level of insulin resistance in T1DM is similar to that of T2DM. The pathophysiologic mechanism of T1DM includes triggering of autoimmunity consequential in one or multiple islet cell autoantibodies linked with gradual β-cell death and loss of β-cell secretory function. This is established by the loss of first-phase insulin release (FPIR), reduced C peptide levels, then glucose intolerance, and finally hyperglycemia (129). Diminished insulin secretion is

typically accompanied by impaired insulin action (119, 130), and the abnormalities of T1DM in insulin action is tissue-specific (130, 131). For example, glucose uptake is typical in cardiac muscles, while the skeletal muscle is normal. However, the insulin binding and action indicates a decrease in adipocytes (132), but normal in fibroblasts (118) of people with T1DM. The insulin is typically delivered via the subcutaneous, rather than the intraportal route, so treatment of T1DM with insulin enhances systemic hyperinsulinemia, which is an indicator of insulin action impairment in humans, (133).

Meanwhile, T2DM occurs as a result of inadequate β-cell secretion of insulin for the glucose load (127) and or decreased tissue responses to insulin (insulin resistance) at one or more points in the complex hormone action pathways (134) causing a progressive deterioration of β-cell function and mass (127). The major abnormalities found in T2DM include absent first-phase and diminished second-phase release in response to hyperglycemia in hyperglycemic clamp experiments (114, 135, 136). There is also a delay in responses to the ingestion of mixed meals, and non-glucose stimuli with a decrease in utmost secretory capacity (136, 137) and abnormal oscillatory pattern.

Impaired insulin secretion is often aggravated by insulin resistance, which is characterized by insulin's failure to decrease plasma glucose levels through suppression of hepatic glucose production and stimulation of glucose utilization in skeletal muscle and adipose tissue (138). This insulin resistance results in the replacement of cellular utilization of fats and protein for energy due to inept glucose utilization. However, the major mechanism leading to progressive loss of β-cell function and mass, are identified as genetic factors and/or several environmental- factors such as malnutrition in utero and early childhood, glucotoxicity, lipotoxicity, obesity, proinflammatory cytokines, leptin, and islet cell amyloid, inadequate stimulation by incretin (114). These factors

differ in each person, but in the case of the genetic factors, the polygenic disorder of gene in T2DM is said to possibly affect the β-cell apoptosis, regeneration, glucose sensing, glucose metabolism, ion channels, energy transduction, microtubules/ microfilaments and other islet proteins essential for the synthesis, packaging, movement, and release of secretory granules (139, 140).

In most situations, insulin resistance is the first obvious defect to be identified in persons with prediabetes. Since the first phase is diminished, insulin secretion may not compensate for insulin resistance. Therefore, in the transitioning phase from normal to impaired glucose tolerance and DM, insulin sensitivity undergoes about a 40% decline and a 3 to 5-fold deterioration in insulin secretion (141). In DM, chronic hyperglycemia may lead to further deterioration of insulin sensitivity and secretion (glucotoxicity) overtime. Several *in vitro* and animal studies have shown that β-cell function is impaired by prolonged elevation of free fatty acid (FFA) (142, 143). There is an indication that fatty acids impede glucose-stimulated insulin secretion, insulin gene expression damage, and promote β-cell apoptosis (144). The FFA has been suggested to only have an adverse effect in the presence of hyperglycemia to β-cell function, but, (increased plasma FFA) not a primary cause of T2DM (144).

In a hyperglycemic state, the fat structure is also anomalous mainly due to enlarged adipocytes being insulin-resistant (in visceral fat) and have reduced capacity for storing fat, resulting in lipid flow into muscles, liver and possibly β-cells, thereby strengthening the muscle and resistance of hepatic insulin, and decreased insulin secretion (138). They are also the primary sources of adipocytokines. Inside liver cells, the higher levels of free fatty acids are converted to triglycerides that accumulate and induce steatosis (or fatty liver) and thereby increase the risk of nonalcoholic steatohepatitis (NASH) and cirrhosis (138).

A rise in glucose levels in people without diabetes is rapidly followed by a rise in insulin secretion that antagonizes the effect of glucagon on the liver (118). By comparison, increased release of hepatic glucose is followed by more increase of glucose in patients with diminished ability to enhance insulin secretion (and therefore, the loss of glucose) to compensate for the rise in concentrations of glucagon (145). However, patients with T2DM when compared with healthy subjects showed increased levels of plasma GIP after meal ingestion (146, 147), GLP-1 levels have shown conflicting results, but mostly modest in patients with T2DM and healthy subjects (114, 148, 149). Nonetheless, the incretin effect is impaired in T2DM (150), thus insulin responses to both GLP-1 (151) and GIP (152) are reduced.

2.5.8 Neurohumoral regulation of T2DM

Various etiologies, such as genetically or environmentally caused T2DM, appear to be of less importance for disease management and prognosis in advanced stages of T2DM. Thus, it is imperative to identify a unique mechanism relevant to the management of T2DM (153). Neurohumoral stimulation has been identified as one of such mechanism, which encompasses autonomic-nerve-system (ANS) dysfunction, i.e. sympathetic activation and vagal deactivation, as well as the activation of hormones such as the renin-angiotensin-aldosterone system (RAAS) (153). RAAS supplies a beneficial effect of the therapeutic angiotensin-II blockade in T2DM by either using the angiotensin-converting-enzyme (ACE) inhibitors or immediate angiotensin-II, subtype-1 (AT1) antagonists (154-156). Primarily, angiotensin-II functions is a sympathetic-nervous-system activator (157) as well as a vagal function deactivator (158). The activation of the RAAS also enables the generation of free reactive oxygen species (ROS) through the NADPH-oxidase mechanism (159), leading to oxidative injury (160) and insulin resistance. Besides, angiotensin-II elicits a pro-inflammatory, and monocytic response. On the contrary, the blockade

of the RAAS prompts a decrease of oxidative stress (161), a reduction of C-reactive protein (CRP) as a marker of inflammation, a restoration of endothelial function, and an upsurge of adiponectin concentration (134). This signifies an adipocyte-mediated amelioration of insulin sensitivity (162).

The sympathetic and parasympathetic nervous system innervates the pancreatic islets of all species. The presence of nerve endings in pancreatic islets and of specific neurotransmitter receptors present in α-and β-cells suggest an autonomic nervous control of glucagon and insulin secretion (144). Glucose-sensing cells located at several anatomical sites—the mouth, the gut, the hepatoportal vein area, the brainstem, and the hypothalamus—are linked together through nervous connections. They ultimately control the activity of the sympathetic and parasympathetic nervous innervation of the endocrine pancreas (163). The sympathetic nervous system plays a leading role in the stimulation of glucagon secretion by activating the α-cell β2-adrenergic receptor. As seen in the sympathoadrenal system activation, a system that stimulates secretion of epinephrine in the blood by the adrenals combines with the nervous secretion of norepinephrine directly at the islet cell level to enhance glucagon secretion (and to inhibit insulin secretion) (163).

Also, catecholamine secretion triggered by insulin-induced hypoglycemia may be suppressed by superior mesenteric ganglionectomy or by capsaicin pain in the hepatic portal vein but not by vagal nerve transection (164, 165). This indicates the direct involvement of these peripheral glucose sensors in the autonomic-nerve control of the secretion activity of the α-cell system. External factors such as ethanol and tobacco ingestion may continue to contribute to oxidative stress (166, 167) or sympathetic-nerve-system activation (168, 169). Furthermore, life-style modifications, such as exercise, diet modification may reinstate ANS function in T2DM or precursor conditions by reestablishing cardiovascular reflexes (170).

2.5.9 Strategies to reduce the effect of T2DM

2.5.9.1 pharmacological treatment

According to Diabetes Canada, many individuals with type 2 diabetes can reach their target blood glucose levels with diet and physical activity guidance. They may also require glucose-lowering medication (171). Glucose-lowering medications for type 2 diabetes include first-line glucose-lowering medication-Metformin: the first choice of medication for T2DM patients; it is known to be safe and works by enhancing the liver and peripheral tissues sensitivity to insulin by activation of AMP-activated protein kinase to make the body effectively use insulin as well cause a decrease in liver glucose production (171). Metformin poses a low risk of hypoglycemia and does not lead to an increase in weight (171).

Second-line glucose-lowering medication-Dipeptidyl peptidase IV (DPP-4) inhibitors, an omnipresent enzyme which acts on incretin hormones, mainly the GLP-1 and GIP (172), which functions to lower blood glucose by increased levels of insulin after meals as well as decreased levels of glucagon and slows gastric emptying. Examples of the DPP-4 inhibitors include alogliptin, linagliptin, saxagliptin and sitagliptin. Similar to metformin, they do not enhance weight gain and are connected to a low risk of hypoglycemia (171). The GLP-1 receptor agonists are injectable medications that become active at the postprandial phase when the blood glucose increases. GLP-1 receptor agonists enhance increased insulin levels, which helps lower blood glucose and glucagon levels. This medication is also effective in slowing digestion as well as reducing appetite. They are related to weight loss and a low risk of hypoglycemia (171). Examples include short-acting exenatide and lixisenatide, long-acting dulaglutide and exenatide as well as extended-release liraglutide. Sodium glucose cotransporter-2 (SGLT2) inhibitors are also second-line medications that function by inhibiting SGLT-2 transport protein to avert glucose reabsorption

by the kidney, but they are associated with weight loss and minimal risk of hypoglycemia. Similarly, insulin secretagogues (meglitinides, sulfonylureas) are second-line medications that enable the pancreas to secrete more insulin. Thiazolidinediones improve insulin sensitivity in peripheral tissues and liver by activation of peroxisome proliferator-activated receptor-gamma receptors (171). Examples of this medication include the canagliflozin, dapagliflozin and empagliflozin.

2.5.9.2 Dietary approach

The dietary approach is a significant strategy for minimizing the risk and varied outcomes of T2DM. Functional foods beyond basic nutritional benefits have been identified to show significant benefits for health promotion and chronic disease risk reduction (173, 174). Besides, recent studies have concentrated on the properties and protective effects of functional foods. Their food sources have been studied *in vitro* and *in vivo* for the treatment of different aspects of DM in bioactive food compounds, and several clinical trials have even verified these advantages in persons with DM (175-177). These effects may be seen in the various food sources, including functional dairy products containing bioactive proteins such as casein and whey protein, immunoglobulins, bioactive peptides, conjugated linoleic acids, lactic acid bacteria and bifidobacterial with functional properties which may help in improving the features of metabolic syndrome, modulate gut microbiota, regulate satiety and food intake, increase dietary fat excretion, reduce adiposity and body weight, increase insulin sensitivity, modulate immune responses in diabetic patients, increase total antioxidant capacity, reduce lipid peroxidation, and reduce HbA1c (178). Due to the various potential benefits of dairy proteins in blood glucose and insulin regulation, there has been significant level of interest in these proteins and the mechanisms they involve in blood glucose control.

2.6 Dairy products and composition

According to the Canadian Food and Drug Regulations "B.08.003. [S], milk or whole milk is defined as normal lacteal secretion obtained from the mammary gland of the cow, genus *Bos* (179). There are various types of milk for human consumption, including goat milk, buffalo milk, sheep milk, yak milk, equine milk, camel milk, and bovine milk which is the most commonly consumed milk (180).

Milk contains two major protein fractions that can be separated by acidification to pH 4.6 (181). The insoluble proteins at this pH are termed caseins, while the soluble proteins are called whey protein or serum proteins. These soluble and insoluble proteins can be divided into six milk-specific proteins: four caseins, α-s1, α-s2-, β-casein, and k-casein at approximately 40%, 10%, 45% and 15% of whole casein. Whey protein contains predominantly β-lactoglobulin (β-Lg) and α-lactalbumin (α-La). It also includes some minor whey proteins, bovine serum albumin (BSA) and immunoglobulins (Ig) (181); constitutes 8% and 6% of the whey proteins in mature bovine milk. The remaining is mostly non-protein nitrogen and trace amounts of some proteins, including about 60 indigenous enzymes (181).

Table 2. 1 Characteristics of the principal proteins in bovine milk (181)

	Molecular weight	Isoionic Point.	Isoelectric Point	$A^{1\%}$ 1cm	PO_4	Composition in milk (g/L)	Genetic variation
α-s_1-casein	23 615	4.92-5.05	4.44-4.76	10.05	8	12-15	B
	23 542	5.00-5.35	-	10.03			C
α-s_2-casein	25 226	-	-	-	10-13	3-4	A
β-casein,	24 023	5.41	-	-	5	9-11	A^1
	23 983	5.30	4.83-5.07	4.6, 4.7			A^2
	24 092	5.53	-	4.7			B
k-casein	19 038	5.77	5.45-5.77	-	1	2-4	A
	19 006	6.07	5.3-5.8	10.5			B
β-Lactoglobulin	18 363	5.35	5.13	9.6	0	2-4	A
	18 277	5.41	4.2-4.5	10.0-9.6			B
α-Lactalbumin	14 178		4.7-4.9	20.1-20.9	0	0.6-1.7	B

Glycosylated to a variable extent (182). $A^{1\%}$ 1cm- *Absorptivity of a 1% solution measured in a 1-cm light path at 280 nm,*

2.7 Milk proteins, fraction, composition, and amino acid profile.

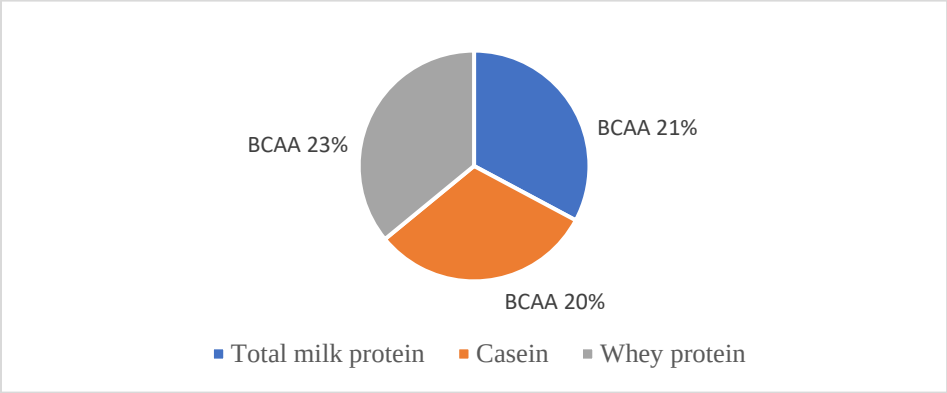
2.7.1 Milk protein

Milk contains 3.3- 3.4 % of total protein, including all nine essential amino acids required by humans (181).

2.7.1.1 Amino acid profile of milk protein

Both whey protein and casein are graded as high-quality proteins based on their amino acid (AA), digestibility, and bioavailability. They have a relatively high proportion of essential AAs (EAAs). They score higher than most other sources of protein when evaluated through various methods including the protein digestibility corrected AA score (PDCASS) (183) and the digestible essential amino acid score (DIAAS) system. Compared to casein, whey protein contains a greater proportion of branched-chain amino acid (BCAA) including leucine, isoleucine, and valine (5). BCAAs alone, and mainly leucine, cause a potent increase in protein synthesis in T2DM (184). Casein contains a higher proportion of histidine, methionine, phenylalanine among other essential amino acids (EAAs) and a greater amount of various non-EAAs including arginine, glutamic acid, proline, serine and tyrosine (183).

Figure 2. 2 Branched chain amino acid content for bovine milk



Proportions % w/w of total amino acids of branched chain amino acids found in bovine proteins, including total dairy proteins, bovine caseins and whey proteins (117, 185)

Table 2. 2 Amino acid composition of milk protein and fractions

Amino acid	Whey protein (g)	Casein (g)
Alanine (g)	1.0	0.6
Arginine (g)	0.5	0.7
Aspartic acid (g)	2.3	1.3
Cysteine (g)	0.7	0.1
Glutamic acid (g)	3.2	4.1
Glycine (g)	0.4	0.3
Histidine (g)	0.4	0.5
Isoleucine (g)	1.2	1.1
Leucine (g)	2.5	1.7
Valine (g)	1.0	1.3
Lysine (g)	2.1	1.4
Methionine (g)	0.4	0.5
Phenylalanine (g)	0.7	0.9
Proline (g)	0.7	2.1
Serine (g)	0.7	1.3
Threonine (g)	0.9	0.8
Tryptophan (g)	0.5	0.2
Total AA (g)	20	20
Total NEAA (g)	10.7	12.1
Total EAA (g)	9.3	7.9
Total BCAA (g)	4.7	4.1

Amounts are shown in g per 20 g of each protein (CN, WP). AA, amino acids; EAA, essential AA; NEAA, non-EAA, BCAA, branched chain AA (186) .

Major fractions of milk protein

Whey protein

Whey protein is defined as the group of milk proteins that remain soluble in the milk serum following casein precipitation at pH 4.6 and 20° C (187). Whey proteins have been reported to contain the highest nutritional quality and found to enhance post-prandial glucose control by both insulin-dependent and independent mechanisms (188). Whey proteins are comprised of multiple globular proteins including beta-lactoglobulin, alpha-lactalbumin, lactoferrin, immunoglobulins, serum albumin, glycomacropeptide, enzymes, and growth factors (4).

Whey protein fractions

Beta-lactoglobulins: The most abundant whey protein in bovine milk is β-lactoglobulin (β-LG). β-LG accounts for over 50 percent of the total whey protein. It has been widely examined due to its nutritional and functional effects on several biological processes (189). It has the ability to bind fat-soluble and amphiphilic molecules and can facilitate the absorption of the fat-soluble nutrients (181). This protein has approximately a quarter (25.1%) of BCAA, alongside bovine serum albumin, and may aid in hydrophobic (fat-soluble) nutrient uptake (190).

Alpha-Lactalbumin*:* α-Lactalbumin (α-LA) is a whey protein that makes up about 22% of the proteins in human milk and about 3.5% of total protein in bovine milk and 17% of whey protein. α-lactalbumin plays a central role in milk production within the mammary gland as part of the lactose synthase complex needed for lactose formation, which serves as a drive to the milk volume (191). It is a major source of bioactive peptides and essential amino acids, such as lysine, tryptophan: a precursor of niacin, thereby, making it an excellent source of niacin equivalents (1 equivalent of niacin = 1mg of niacin or 60mg of tryptophan) (192), it is also a source of BCAA and amino acids containing sulfur, all of which are critical to infant nutrition. α-LA has the potential to be applied as a substitute protein to food products due to its physical characteristics, which include water solubility and heat stability (191). It also has potential as a nutritional supplement to help neurological function and aid sleep in adults due to its unique tryptophan content. (191).

Bovine serum albumin (BSA): Approximately 1–2 % of the protein in bovine milk is BSA, which enters the mammary gland by leakage through intercellular joints. BSA accounts for 50% of bovine blood protein, in which many roles are carried out. It is referred to as a single polypeptide of 582 amino acid residue (193), with 17 cystine residue with a calculated 66000 g/mol. This

protein has a high sulphur amino acid concentration and glutamylcysteine that are precursors to glutathione (194). Glutathione, a natural antioxidant, plays a key role in reducing the effects of oxidative stress (195, 196). Bovine serum albumin also binds long-chain fatty acids and makes them water-soluble (197).

Immunoglobulins (IG) represent 1% of the total milk content, which goes up to 8% of the total whey proteins (193). The main isotype of bovine Ig is the IgG (198, 199), which can be effective in the protection of animals and humans against disease by providing passive immunity (200).

Glycomacropeptides (GMPs) are originated from the proteolysis of κ-casein by milk clotting enzymes (e.g. rennin) but are lost in the liquid whey during separation (processing stage). It has been reported that GMPs stimulates the release of cholecystokinin (CCK), and contribute to satiety (201), however, this effect has not been confirmed in other studies (202).

Lactoferrin (Lf) is a non-heme iron-binding glycoprotein and the most effective metal-binding protein (193). The production of Lf is not limited to milk, as it is found in other body fluids, including saliva, tears, sweat, and semen (203). It consists of 689 amino acid residues with a MW of 77 kW (glycoprotein) and 3 disulfide bonds (193). Lactoferrin performs antioxidant, antibacterial, antiviral, anti-inflammatory, immunomodulatory, (204-206) anti-carcinogenic activity (207) and has been reported to enhance glucose tolerance and induce hypophagia (208).

Whey protein forms

There are three key types of whey proteins gotten in the process of separating whey protein. They are known as whey protein concentrates; whey protein isolates and whey protein powder. Whey protein concentrate contains ~25-89% protein (209), it is formed by milk coagulation with

rennet or acid enzyme, resulting in curd (casein) and whey separation (210). These processes eliminates lactose, ash, and some minerals (210). Whey protein powder is mainly used in the food industry; it has various applications and contains varieties which includes sweet whey protein, acid whey protein (209).

The purest form of protein available is whey protein isolates (209). Whey protein isolate is produced through additional processing of whey, and it contains >90% protein with little quantity of lactose and lipids (209). This is because there is a major elimination of fat and lactose during the processing of whey protein isolate (209). Nonetheless, the whey protein isolate has been reported to effectively yield greater overall insulin response than other two supplements, alpha-lactalbumin enhanced whey, and caseinoglycomacropeptide enhanced whey, but with equal level with the hydrolyzed whey protein (211, 212). Whey protein isolate supplementation has been found to reduce fasting plasma insulin levels and improve insulin sensitivity in overweight and obese persons (213). Several studies have also shown that whey protein hydrolysates can induce insulin and glucose-dependent insulinotropic polypeptide (GIP) secretion to a higher extent than the intact protein (214, 215). Nonetheless, there is no convincing evidence that a particular form of whey protein, is considerably more effective in reducing postprandial hyperglycemia than a different one, rather the palatability is taken into account (212). The hydrolyzed whey proteins, however, are typically less palatable (216) and hence less likely to be used as a functional ingredient.

Casein

Casein is the main component of the protein present in bovine milk and accounts for about 70-80% of its total protein (209). Casein is known as a complete protein containing also calcium

and phosphorous. Casein has a protein digestibility corrected AA score, a PDCAAS rating of 1.23 (generally reported as a truncated value of 1.0) (217).

Casein occurs in milk in the form of a micelle, which is a large colloidal particle. One of the important attributes of casein micelle is its capacity to form a gel or clot in the stomach. This ability to form clot makes the supply of nutrients very effective; the clot also delivers a prolonged gradual release of amino acids into the bloodstream, often lasting several hours (9). Similar to whey protein, casein stimulates insulin secretion which is due to sensitivity to both the composition and concentration of plasma AAs, (6, 7). Total milk protein (that is casein and whey protein) has been reported to alter the absorption of glucose and reduce postprandial blood glucose (4, 218), however, their direct effect on insulin and blood glucose remains unclear.

Casein fractions

α-s$_1$, α-s$_2$-, β-casein, and k-casein

α-s$_1$ and β-casein are known as the two principals of casein. α-s$_1$ constitute 40% of casein in bovine milk and consist of one major and minor components with a single chain polypeptides and same amino acid sequence (181). β-casein is known to be quite complex because of the action of the native milk protease plasmin, *it* constitutes up to 45% of the casein of bovine milk (181). Both the α-s$_1$ and β-casein do not contain cysteine or cysteine residues, whereas the two minor caseins, s$_2$ and *k*-casein, have two intermolecular di-sulfides bonds (219). All caseins are phosphorylated but at varying degrees; the phosphate groups are esterified as serum residue monoesters. In the meantime, casein is the only major protein in the milk that is glycosylated. All caseins, particularly β-casein, contain a high degree of proline, which disrupts α-s$_1$ and β-casein structures; as a result, caseins are very unstructured molecules and are readily susceptible to proteolysis (219).

2.8 The role of dairy intake in diabetes and obesity.

In Canada the terms “milk”, “whey”, “cream”, “butter”, “buttermilk”, “cheese”, “ice cream”, and “yogurt” are protected and reserved exclusively for dairy products (220).

Milk and dairy product consumption has been associated with a reduced risk of type 2 diabetes (221), obesity, and metabolic syndrome (MetS) (222). The relationship between dairy products and blood glucose control has been examined in various studies, including meta-analysis, cohorts, randomized clinical trials, etc., but there seems to be contrasting evidence on the impacts dairy products play in the risk of diabetes and other metabolic disease development (223-228). For instance, a review of various observational studies in different countries investigating the role of dairy products in healthy weight and body composition in children and adolescents found that milk and milk products play a significant role in reducing the risk of obesity (223). Similarly, a study comparing a cohort of 50 children with a history of milk consumption avoidance and a cohort of 200 children who regularly consume milk, revealed that children who avoided milk had smaller skeletons, were shorter, had lower total-body bone mineral content, and lower z-scores for area bone mineral density than the other children who drank milk, and were also of the same age, sex and from the same community (228). Similarly, a case-control study of 53 Puerto Rican children (224) who frequently consumed fruit juice, watched TV daily, maternal BMI, and lower dairy product consumption were associated with obesity. While a study reported a significant inverse association between obesity and dairy product consumption (225).

Investigators claim that the inclusion of dairy products in weight loss diets may be favorable due to its impact in decreasing adiposity while preserving lean mass. In a meta-analysis of randomized control trials, high-dairy calorie-restricted diets led to a significantly greater reduction in body weight, waist circumference, and fat mass, but increased lean mass significantly

more than conventional weight-loss diets (227). Similarly, an increase in milk consumption was associated with a lower waist circumference, systolic blood pressure, and insulin resistance in a study on Argentinean children (225). Two systematic reviews of observational studies also showed that increased dairy consumption may be protective against weight gain (229, 230). A large prospective analysis of more than 120,000 individuals followed for 12–20 years assessed the role of diet (including dairy) and lifestyle on long-term weight gain and found yogurt to be a factor associated with a beneficial impact on weight (231). This study found no association between either weight gain and high or low-fat dairy categories nor cheese and all liquids, except milk, which was associated with weight gain. This effect may be attributed to the presence of fats in milk, but milk also possesses potential protective mechanisms against weight gain, which includes decreased lipogenesis and increased lipolysis (231).

Furthermore, a high calcium ingested with dairy products may interact with fatty acids in the intestine and lead to the formation of insoluble fatty acids and prevent or lower fat absorption (232-234). A systematic review of the randomized control sample confirmed the role of calcium in fat oxidation (235), and that whey protein may also play a role in muscle-sparing and lipid metabolism (213).

The amount of milk consumption has been identified to effectively impact blood glucose tolerance by showing significant effect in reducing the risk of T2DM. In a dose-response analysis, lower risk of 6% in T2DM per each additional daily serving of total dairy (RR 0.94, 95% CI 0.92–0.97) was found (236). Likewise, the association between low-fat dairy consumption and T2DM was identified in a dose-response analysis, by a 10% risk reduction per additional serving (RR-0.90, 95% CI 0.85–0.95) (236). Similarly, in a meta-analysis of four prospective cohort studies on diabetes, milk or dairy consumption also exhibited a protective relationship between type 2

diabetes, with each additional daily serving and led to a significant reduced risk of diabetes (237) In a meta-analysis, the highest consumption of dairy was compared with lowest consumption, while the higher dairy consumption showed a significant reduction in the risk of type 2 diabetes by 14% (combined relative risk (RR) 0.86, 95% confidence interval (CI) 0.79-0.92) compared to the group consuming the of lowest quantity of dairy (236). Furthermore, a systematic review and meta-analysis of observational studies published by Lee (2018) (238), which includes ten cohorts studies and seven cross-sectional studies, compared the highest and lowest categories of dairy product intake. Seven cohort studies with six cross-sectional studies examined the dose-response meta-analysis to determine the association between dairy foods (total dairy foods, milk, and yogurt) and the risk of the metabolic syndrome (239). In this study, it was observed that in a dose-response manner, an increase of 200g/d of total dairy product intake was linked to a lower risk of metabolic syndrome components such as hyperglycemia (seventeen studies), high blood pressure (ten studies), hypertriacylglycerolaemia (five studies) and low HDL-cholesterol (six studies) (239).

While, an increase to 200g/d of milk intake (seven studies) was related to a 12% lower risk of abdominal obesity, an increase of 100g/d of yogurt intake (nine studies) was associated with a 16% lower risk of hyperglycemia (RR=0·84; 95 % CI 0·70, 0·98). Also, a meta-analysis comparing the highest and the lowest category of dairy product intake, found that the total dairy product intake showed a lower risk of all components of the metabolic disease, abdominal obesity and hypertriacylglycerolaemia (238). Yogurt intake was also related to a lower risk of hyperglycemia and abdominal obesity. In a dose-response analysis, one-serving increment of dairy food consumption and one-serving increment/day of milk and yogurt were related to 9%, 13%, and 18% lower risk of metabolic syndrome (MetS) (238). Similarly, the cohort studies in this meta-

analysis were reported to be inversely associated with the development or susceptibility of metabolism disease (238). One-serving increments of total dairy food consumption were related with a 9% lower risk of the MetS. In addition, one-serving increment/day in milk and yogurt consumption was related to a 13 and 18% lower risk of the MetS, respectively (238).

Other meta-analyses (239, 240), including observational studies analyzing the relationship between dairy products and diabetes, found that the consumption of dairy products is associated with a lower risk of developing T2DM. Similarly, a recent cohort study (241) investigating the relationship between dairy food intakes and the development of prediabetes and T2DM stated that lower risk is associated with developing prediabetes after consumption of total, low fat and high-fat dairy among those with normal glucose status at baseline. Likewise, among those with pre-diabetes at baseline, there was a 70% reduced risk of developing diabetes with ≥14 serving/week for high-dairy fat compared to <1 serving/week for high-dietary fat (241).

The animal study with 4 weeks old Goto–Kakizaki (GK) rats investigated the effect of α-lactalbumin on impaired glucose tolerance and the mechanism of action of α-lactalbumin against impaired glucose tolerance. This study observed a significant decrease in blood glucose levels in GK rats' group at 30 and 60 min at 10 weeks administration of α-lactalbumin, compared with that of the control group (Wistar rat group) (242). This study (242) reported that glucose intolerance was improved with the administration of α-lactalbumin which may be linked to adiponectin. It, therefore, suggests that consumption of α-lactalbumin is useful in reducing the risk of T2DM (242).

2.9 Milk proteins and their role in the regulation of blood glucose control.

In Western countries, such as Canada, dietary modification and weight loss are main management options, but the best nutritional advice and prescription diets on enhanced glycemic control continue to be a major source of controversy (8). Meanwhile, there have been many premises connected to dairy products, milk, and milk proteins in the prevention and management of type 2 diabetes and other chronic and metabolic diseases (8). Milk is known to be the most complex food of nature, which can be ascribed to its nutritional composition comprising of nutrients essential for life's sustenance and appropriate development (8). Moreover, they are important sources of amino acid, enhancing absorption of nutrients and trace element (212) and providing a range of physiological functions of bioactive peptides (243, 244), which were discovered to be of great impact on health and disease. Amongst the various components of dairy milk, protein is the most copious component associated to advantageous effects on numerous chronic disease risk factors (4) and reduced risk of T2DM (43, 60), while the naturally occurring lipids and carbohydrates in dairy products are likely to be known as neutral factors (53, 245-247). Diary protein can be derived from milk, cheese, and yogurt, or they can be consumed as supplements in isolated or concentrated forms (56). A study conducted to investigate the effect of dairy and non-dairy snacks on glycemia in over weight and obese boys, reported that dairy snacks effectively reduce glycemia and increase circulating insulin levels (110). Consumption of casein or whey protein in adequate amounts have health effects that vary compared to when the same protein is consumed as components of whole-dairy products (4).

Casein is established to be slowly absorbed by the body compared to whey protein (9). This lower absorption is associated with low pH conditions in the stomach, which enables the clotting of casein and delays gastric emptying (8) which may lead to higher satiating effect over time and reduce the postprandial increase in plasma amino acids (248). In a study of isoenergetic

preloads, skimmed milk containing both whey protein and casein was shown to decrease food intake more than total dairy protein consumption alone (249). Casein also shows insulinotropic properties like whey protein but has less potent insulin secretagogue than whey protein. However, casein hydrolysis speeds up digestion, synthesis of amino acids (AAs), enhance insulin secretion, availability of branched-chain amino acids (BCAA), essential amino acids, and total plasma amino acids in circulation, in comparison to casein micellar (4, 248). Continuous consumption of 35g casein/day (over 12 weeks) led to an increase in the concentration of plasma C-peptide of overweight adolescents (250), indicating increased insulin secretion. Also in a study of T2DM overweight population, hyperglycemic prevalence decreased within 24 hours (251) after consumption of casein hydrolysate (~30 g) and leucine (~10 g) beverage intake for breakfast, lunch, and dinner. Moreover, casein may also impact postprandial glucose. A study on whey protein and/or casein ingestion in subjects with T2DM found a significant increase in insulin response and blood glucose disposal, thus recording a decrease in postprandial rise in blood glucose, which is associated to carbohydrate intake, after 24-hour co-ingestion of casein and carbohydrates intake in T2DM subjects (8). Casein dose may also be linked to blood glucose control, as hydrolyzed casein in doses above 12g per day enhances insulin and glucose response caused by carbohydrate when compared to lower doses (252) and intact casein in T2DM subjects. Meanwhile, in long-term T2DM patients, a 40 g higher dose of casein hydrolysate in each major meal did not improve the 24-hour prevalence of hyperglycemia associated with long-term T2DM β-cell deficiency (253). However, investigations have demonstrated casein's different properties and possible mechanisms of action on blood glucose and insulin, but the direct effect of these proteins on blood glucose and insulin, is unclear.

Meanwhile, whey protein has demonstrated to be the most insulinogenic fraction of milk proteins (6). A study examining mixed meal effect of whey protein isolate, micellar casein, or free amino acid control meal, with an average protein dosage of 0.7 g protein/kg BW of approximately 40–55 g protein per meal (116), found significantly higher concentrations of essential amino acid (EAA) (P<0,0001) and branched-chain amino acid (BCAA) in whey proteins than those of micellar casein in the 3-hour postprandial glucose result. This further emphasizes the insulinogenic properties of whey protein, as the BCAA is known to be more insulinogenic, than other amino acids (254), more so, whey protein is rich in branch-chain amino acids, specifically leucine (255). Amino acids affect postprandial insulin differently and are likely to interact with protein to cause various postprandial insulin reactions (255). Besides, whey protein has been associated with a decrease of postprandial glycemia in a dose-dependent manner. For instance, in a study where 18g of whey protein were administered to individuals with T2DM for breakfast or lunch, results showed a higher insulinotropic response, circulation levels of the gut peptide glucose-dependent insulinotropic polypeptide (GIP), and suppression of postprandial glycemia compared to an isoenergetic non-dairy protein (lean ham and lactose) (256). Also, ingestion of 55 g whey protein before consumption of carbohydrate (CHO) or with a CHO lunch resulted in postprandial glucose suppression in T2DM patients (257), while promoting an increase in insulinotropic, and gut peptide (GIP and cholecystokinin, CCK) responses (257). This outcome is linked to the effect whey protein has on the release of incretin hormones in the guts (257, 258) such as glucagon-like-peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP), which is released from the gut after the consumption of food to stimulate insulin secretion from pancreatic β-cells glucose-dependently (259, 260) and the pattern of whey protein digestion and/or absorption (116, 258,

261). However, the protein dose (55g) ingested in this study is higher than a habitual intake and thus may not be replicable in the development of functional foods.

Furthermore, lower insulin levels after breakfast have been observed, as seen in the study by Frid et al. in 2005 (256), where the effects of 18 g whey protein or lean ham + lactose added to both a high-glycemic breakfast (white wheat bread) and lunch meal (instant mashed potatoes and meatballs) in 14 subjects (8 male and 6 female) aged 27–69 years were investigated (256). This study reported 3h AUC insulin secretion to be 31% higher after consumption of whey protein for breakfast than ham intake but showed no difference in glucose concentrations between groups (256). An investigation with lunch found a 57% increase for 3h AUC insulin secretion after protein intake, while also significantly reducing 3h AUC glucose concentrations by ∼21%.

Timing of whey protein consumption before a meal may affect postprandial glycemia (262). In a study investigating the effect of timing on whey protein (WP) intake and insulin response, using 55g WP, 30 minutes before consumption of the treatments (high glycemic meal vs protein with high glycemic meal vs high glycemic control meal) (257). This study found that insulin secretion, GLP-1, and GIP iAUC, were all higher when subjects consumed whey protein before the meal ($P < 0.05$), and when they consumed it with the meal ($P < 0.005$) in comparison with the control meal. GLP-1 was also considerably higher at the postprandial level of 90 minutes when the whey protein was consumed 30 minutes before a meal (257). Besides, whey protein was also reported to slow the rate of gastric emptying as shown in a study where gastric emptying was slower when whey protein was ingested 30 minutes before the meal than it was when whey protein was eaten with a meal or not consumed at all (8). Nevertheless, how total milk protein and individual milk protein fractions directly affect the blood glucose and insulin is still indistinct.

2.9.1 Effect of milk protein on blood glucose in animals

Milk proteins and fractions have also been found to effectively improve glucose tolerance in studies conducted using animals (208, 263-265). A study comparing high protein diets enriched with whey protein isolate (15% whey protein isolate + 15% egg albumen), α-lactalbumin (15% α-lactalbumin + 15% egg albumen), or lactoferrin (15% lactoferrin + 15% egg albumen), on food intake, energy expenditure, and glycemic control, using diet-induced obese rats. This study found a significant decrease after intraperitoneal glucose tolerance tests (IPGTT) at 4 weeks in blood glucose with whey protein, lactoferrin, lactalbumin, and lactoferrin + whey protein (pair-fed) at 30, 60 and 120 min, as well as a 35% reduction in area under the curve (AUC), compared to control (208). Meanwhile at 8 weeks, blood glucose decrease was only found with lactalbumin at 30, 60, and 120 min (208). Similarly, in a study investigating the impacts of diet enrichment with whey protein isolate, whey protein hydrolysate, α-lactalbumin, and casein on diabetes progression in Zucker Diabetic Fatty rats (ZDF) and normal Wistar rats (263). The HbA1c levels were reported to reduce in all milk protein fractions in the ZDF rats, most especially in the casein fraction and an increase in glucose tolerance was also reported after the addition of all milk protein types were given to ZDF rats, specifically in the casein group. However, in the normal rats, whey protein and casein showed a more significant effect than others in the delay of diabetes progression as seen in the oral glucose tolerance test (OGTT) and HbA1c results (263). Also, a study conducted for eight (8) weeks to determine the effects of dietary whey protein, casein, and a combination of the two (2) on energy balance, hormones, glucose metabolism found that, combination of whey proteins and casein diets have lesser impacts on glucose tolerance compared to whey proteins and caseins individually, with whey protein having the most effective impacts (265), which is linked to likely reductions in weight, adipose mass, and plasma leptin concentrations (265).

Milk proteins and fractions were also revealed to decrease meal-induced blood glucose, improve energy balance through varying impacts on food intake, energy expenditure, and gut hormone secretion in diet-induced obese rats. Compared with the protein diet, lactoferrin decreased concentrations of fasting and postprandial glucose. Also fasting plasma insulin concentration also decreased and improved the quantitative insulin sensitivity check index (QUICKI) after consumption of whey protein, lactalbumin, lactoferrin compared to normal protein intake (208).

Varying timing effect has been observed on different fractions, for instance: the lactalbumin only decreased blood glucose at 120-min compared to (normal protein) control. Similarly lactoferrin decreased plasma insulin at 120 min compared to control, whey protein, and pair-fed, and resulted in a greater improvement in QUICKI than whey protein and control (208). Similarly, whey protein, lactalbumin, lactoferrin, and pair-fed lactoferrin with whey protein decreased fasting plasma leptin concentrations at time 60 and 120 min compared to control. Also, whey protein, lactalbumin, and lactoferrin decreased circulating GIP at 60 and 120 min compared to control and pair-fed (208). It confirms that timing is a significant factor in changes that milk proteins have on blood glucose.

Protein dose has been identified as a factor to effectively impact blood glucose and insulin secretions. A 26% dose of whey protein and a 26% dose of casein had more effect on glucose tolerance, and resulted to a greater plasma membrane glucose transporter 4 (GLUT4)–to-total GLUT4 ratio in skeletal muscle, compared to the combination of a 13% dose of whey protein +13% dose of casein (265). This may affirm the possible effect of dosage on glucose tolerance and insulin secretion.

A study examining the impacts of diets enriched with whey protein and casein or a combination of the whey protein and casein in obese OP-CD rats was found to have prolonged hypophagic effects, this effect may impact weight loss and cause a decrease in body fats, which are also risk factors of increased blood glucose, besides, hypophagic effects were mostly seen in the whey protein treatment (WH; 40% protein energy: 26% whey protein + 14% egg white), and casein treatment rats (CA; 40% protein energy: 26% casein + 14% egg white), than in combined diets (WHCA; 40% protein energy: 13% whey protein + 13% casein + 14% egg white) (265).

The research to date confirms milk protein; whey protein and casein to have an insulinotropic effect and reduce glycemia. Nonetheless, an important question was raised whether milk protein, whey protein or the BCAA may have a detrimental effect on health leading to hyperinsulinemia. However, a study conducted by Gheller et al (110) proved that the intake of Greek yogurt led to increased insulin but not to increased C-peptide, thus, led to the conclusion that the increase in insulin is due to reduced insulin clearance after high dairy protein food. Consequently, we wanted to see whether ingestion of milk proteins alone (in the absence of glucose) can similarly lead to a higher insulin level. Therefore, we decided to investigate if the major milk protein fractions have a distinct pattern on their effect on blood glucose and insulin using a small dose of milk protein known to reduce blood glucose in humans (350mg of proteins which is an equivalent of the 10g human dose).

Chapter 3: Objective, hypothesis, and research question.

3.1 Objective

The overall objective of the study was to investigate the acute effect of the intragastric injection of major milk protein fraction on short-term blood glucose and insulin response in rats.

Specific objective are as follows:

1. To investigate the acute effect of total milk protein, micellar casein, and whey protein on blood glucose over 120 min in Wistar-Han rats.

2. To investigate short-term insulin response, after ingestion of total milk protein, micellar caseins and whey proteins over 30 min in Wistar-Han rats.

3.2 Hypothesis

The magnitude of insulin and blood glucose response would depend on the type of protein. The total milk protein, micellar casein, and whey protein may have a distinct effect on short-term blood glucose and insulin response.

3.3 Research question

1. How does the short-term ingestion of different milk proteins affect blood glucose in Wistar-Han rats over 120 min?

2. Does milk protein fraction have varying significant effect on insulin over 30 min in Wistar-Han rats?

Chapter 4: Methodology

4.1 Study design

A randomized repeated measure crossover study design was used to assess the acute effect of commercial TMP, micellar CN, and WP isolates on blood glucose (BG) and insulin response in six (6) Wistar Han-rats. In each cage, two animals were housed at 21ºC. The same treatments were randomly assigned in a different sequence to each rat using the excel random number generator. This approach is effective in providing a more accurate measurement of the treatment outcome.

This experiment was performed following the protocols of Dalhousie University Committee on Laboratory Animals (UCLA). Animals undergo humane handling and restraint in accordance with the ethical guidelines established by the Canadian Council on Animal Care (CCAC). After the experiment, animals were humanely euthanized.

Animals

Ethics approval for this study was obtained from the Dalhousie University Committee on Laboratory Animals (UCLA). This study followed the ethical consideration established by the Canadian Council on Animal Care (CCAC).

Six Wistar-Han rats with jugular vein catheters and vascular access buttons, 4 months old sourced from Charles River were used for this study; previous evidence-based studies were conducted using 5-7 rats in total (266, 267), the Wistar-Han rats was used because it is a general multipurpose model and it is safe and provides efficacy in research related to humans (268). Upon the arrival of these animals, a vascular access button (VAB) was fitted on them. Their catheters were flushed by the locking solution containing heparinized glycerol in a 50:50 ratio. The rats were labeled, housed by two in a cage at 21ºC with 12 hours dark/light cycle, and allowed to adapt

for 7 days before the commencement of experiments. During this period, they were fed a standard chow diet and their body weights were also measured. Furthermore, during the period of incubation, the catheter was maintained by following the sampling procedure provided by Charles River. This procedure includes cleaning of the incision site using a surgical skin disinfectant, after which the vascular access button was removed, followed by removal of the plug, which was thoroughly cleaned with a sterile alcohol wipe. The locking solution was removed with a sterile syringe, if the first attempt fails, the animal will be repositioned, as body position can contribute to poor flow, but if additional attempts fail, a small amount of saline was injected with a second sterile syringe to clear the line and allow for the removal of the lock solution. Blood samples were drawn with a sterile syringe, the catheter was flushed using a sterile syringe, with double the catheter's dead volume of saline (269).

Treatments

Milk Protein Isolate 90% (MPI 90) (Milk Specialties Global), micellar casein (CN) was sourced from (Milk Specialties Global), whey protein (WP) isolate from (Agropur Inc) and protein-free control (Glucose solution).

The protein samples and glucose solution was prepared at the Centre of Applied Research (CAR) Mount Saint Vincent University. 350mg of each TMP, CN, WP was measured respectively to reflect human dosage of 10g which is calculated based on allometric scaling as described below. The glucose solution was measured to give 350 mg glucose equivalent of 352 mg of glucose powder which is also approximately half of the dose of glucose used in an oral glucose tolerance test in rodents (2g/kg) and poses no added risk to the animals (270). The solution was made with 36ml water at a temperature of approximately 30ºC to ensure that the solution is well dissolved,

then 3ml samples were taken in a 3ml BD syringe and refrigerated. The milk protein solution was used at the animal facility of the Charles Tupper Building, Dalhousie University.

Table 4. 1 Treatments

Protein	Manufacturer	Product info	Calculation
Total milk protein	Milk Specialties Global	Non-commercial Isolated milk protein concentrate (90%) (MPI 90)	1mg of MPI= 0.87 of protein. 0.87* 403mg= 350mg
Casein (CN)	Milk Specialties Global	Caseinate-Micellar casein	1mg of CN= 0.86 of protein. 0.86*408= 350mg
Whey protein (WP)	Agropur Inc	Whey protein isolate.	1mg of WHI90= 0.87 of protein. 0.87*402mg= 349.7 ~ 350 mg.

Dosage calculation

The dosage was calculated using the allometric scaling; by calculating the animal equivalent dose (AED) based on the body surface area by multiplying the human dose (mg/kg) by the K_m ratio (AED mg/kg = Human dose mg/kg * K_m ratio), the K_m ratio is known as the correction factor (K_m), it is estimated by dividing the average body weight (kg) of species to its body surface area (m^2). after which the AED is divided by the reference body weight of the rats (271).

In this case, the human dosage was = 10000mg, while the reference human body weight was 60kg; 10000/60kg=166.667mg/kg, while the k_m ratio for rat reference is 7.

Rat AED= 166.666*7=1166.667mg/kg.

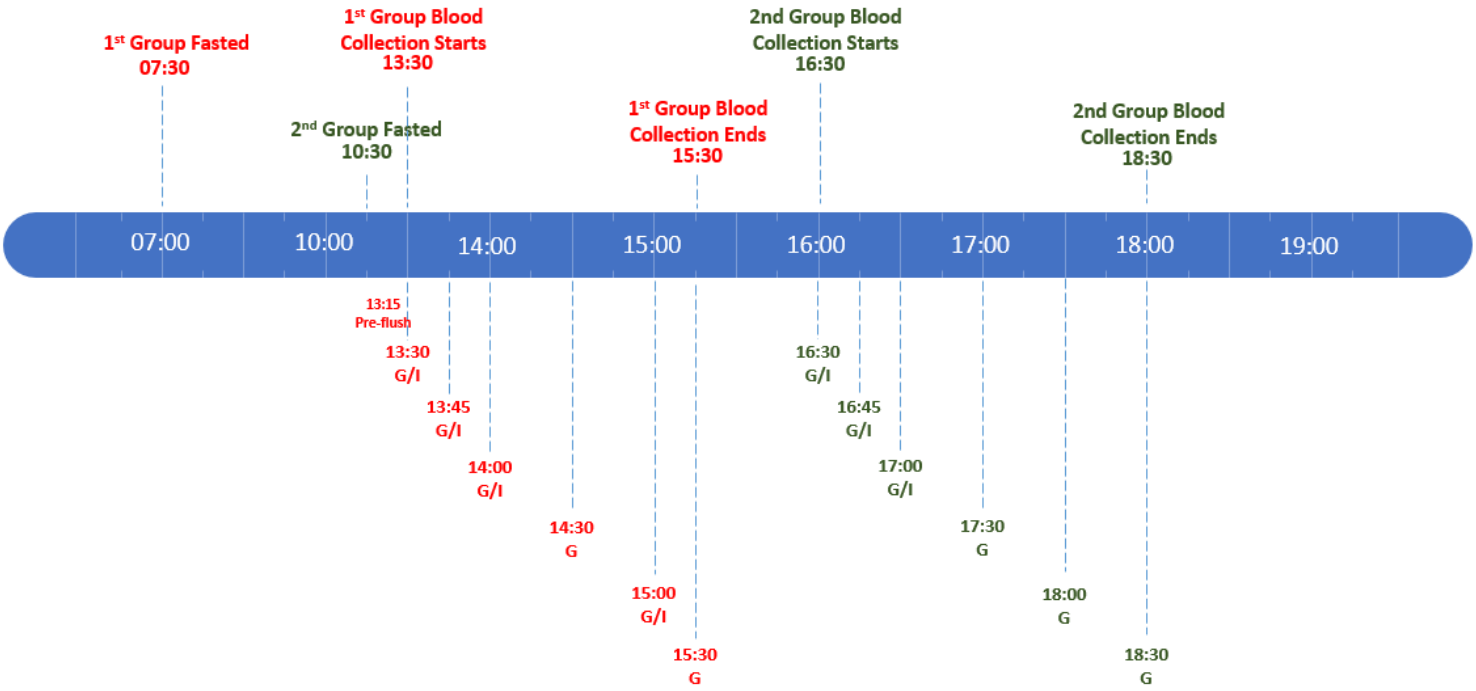
Dose= 1166.667/0.3kg= 350mg

4.2 Study protocol

Six rats with inserted jugular vein catheters were labeled, housed by two in a cage, and adapted for 7 days. After the acclimation period, they were randomly assigned to the treatments. All rats were divided into two groups, three rats in each: after a 6-hour daylight fasting, group 1 started on day 1 at 1 pm, group 2 on day 1 at 4 pm, group 1 also on day 2 at 1 pm, and group 2 also on day 2 at 4 pm. There was a two-day washout between the consecutive sessions. Each rat received the same treatment (one per session), but their order was determined by randomization. Overall, there were four treatments administered through sessions with two days apart between the sessions for 12 days. The entire duration of the study was 19 days, including seven days for the initial adaptation of rats to ULCA and 12 days for the experiment.

At each session (day), blood samples were collected from rats using PNP3MS 1™ Injector connected to a 1mL syringe through Luer Slip Tip. The catheter was flushed immediately after the blood sample is collected with 2x the dead space (~ 90µL) and then locked with the dead space volume (~45µL). The dead space volume was provided by CRL when the rats were shipped. 70µL of whole blood was drawn at the baseline, and at 15 and 30 min after gavage for blood glucose and insulin. Another 10µL of whole blood was collected at each 60, 90, and 120 min after gavage. It is estimated that 50µL of whole blood were retracted with each flush of the catheter. The total volume of blood collected at each session is equal to 290µL. After the final blood collection, animals were euthanized according to the guidelines of the Canadian Council on Animal Care (CCAC).

Figure 4. 1 One-day study protocol



Gavage

The solution with milk protein fractions was administered by gavage because gavage delivers a more reliable dosage and volume. It is also a faster overall absorption of toxic or unpalatable compounds compared to feed delivery (272). The rats with jugular vein catheters were taken from the cage and carefully wrapped with a towel to restrain for gavage. The FTP-15-78-50 plastic feeding tube was attached to the BD 3mL Luer-Lok Tip Syringe containing the treatments and the tube was inserted directly into the stomach of the rats from the mouth to administer the treatments.

Hemocue standards

Blood glucose was measured using the HemoCue® Glucose 201+ System from Quest diagnostic company Lake Forest, California, USA. Before blood collection, the HemoCue® standards was tested using the HemoCue® standards (gh00s), HemoCue® cuvette, HemoCue® Glucose 201+

System, Parafilm™ M Wrapping Film, and Kimtech Science™ Kimwipes™ Delicate Task Wipers: the standards and cuvettes were left at room temperature for 15 minutes before using, the HemoCue® Glucose 201+ System was turned on and the cuvette reader will be opened to dispense 1 drop of the low standard onto Parafilm™ M Wrapping Film, then samples will be collected using the HemoCue® cuvette by touching it to the drop at an approximate 450 angle and held until the cuvette is filled, Kimwipes™ was used to wipe off extra blood on the sides and bottom, the cuvette will be placed inside the open HemoCue® Glucose 201+ System to make sure that it says ready, then HemoCue® Glucose 201+ System was read and it is important to check to ensure the value is within the reference range found in the box with the HemoCue® standards.

Blood collection

Blood was collected directly from the catheter at 0, 15, and 30min for insulin, and at 0, 15, 30, 60, 90, and 120min for glucose, which According to Charles River the jugular vein catheter benefits preclinical and research studies because it allows easy and repeated intravenous access without the need of anesthesia since the blood removal process causes little to no pain (273). The vascular access button was removed, then the catheter tip was sterilized with alcohol pads and wait for 15 seconds for the alcohol to evaporate, the PNP3MS PinPorts™ Injector will be attached to the BD 1mL TB Slip Tip Syringe to access the catheter to draw blood. Firstly, the syringe was used to remove locking solution, removal of locking solution was confirmed once blood was seen, in cases where the first attempt failed, the animal was repositioned and repeated. However, in the situation when this failed, then a small amount of saline (10μL) using a sterile syringe was used to remove the locking solution. A sterile syringe was used to remove blood for glucose analysis (10μL) and insulin analysis (50μL). After blood collection, the catheter was flushed using a

PNP3MS PinPorts™ Injector attached to BD 1mL TB Slip Tip Syringe, with double the catheter's dead volume of saline and locked with a locking solution.

Blood glucose test (3.9 to 5.5 mmol/L)

The glucose was measured using the HemoCue® analyzer (Hb 201+ System) because they provide lab accuracy and are very easy to use. The blood collected was moved from the blood collection station to the HemoCue® station, A pipetter and pipette tips were used to extract blood from the PNP3M used in blood collection, the blood was gently released on the parafilm to provide a hydrophobic surface to collect blood on, then the cuvettes were used to collect blood from the parafilm by touching it to the blood at an approximate 450 angle and held until the cuvette is filled, the Kimwipes™ was used to clean excess blood on the cuvettes. Afterward, the cuvettes were placed in the already opened HemoCue® analyzer for reading, the analyzer must indicate "ready" before it is closed.

Centrifuge

Immediately after blood collection, the blood was inverted 2 times in the syringe, then transferred into labeled microvettes and inverted 10 times to ensure equal volume, after which they were transferred to the benchtop centrifuge with refrigeration (Mega star 3.0, VWR, Germany) (274). After the process of centrifuging whole blood, plasma separation and plasma aliquoting took place. Microtubes were used to collect the plasma from the Microvettes. Two pipettes were used: a 250uL and 20uL. The 250uL was set for approximately 100uL and used to remove the plasma from Microvette while the 20uL pipette tip is used to transfer two 20uL (40 in total) aliquots of plasma into a separate microtube. After which a small centrifuge was used, to allow the aliquoted plasma to settle down in the microtube. The plasma collected was immediately frozen at -80°C.

Insulin

Blood collected at 0, 15, and 30min for insulin was measured with Ultra-Sensitive Rat Insulin ELISA (catalog #90060, Crystal Chem, IL, USA). The Ultra-Sensitive Rat Insulin ELISA kit is effective in the quantitative determination of insulin in rat serum, plasma, and cell culture media. The wide range assay (0.1 – 12.8 ng/mL) was used for analysis. Prior to use, all reagents were brought to room temperature (18-25°C) and stored at 2-8°C immediately after use. Preparation of reagents, working rat insulin, assay procedures, and concentration determination was done following the instructions of the section J. Wide Range Assay (0.1 – 12.8 ng/mL) in Catalog #90060. Absorbance was measured within 30 minutes using a plate reader. (Measure A450 values and subtract A630 values) at a wavelength of 450 **(Appendix 1).** The mean absorbance for each set of duplicates standards or samples was carried out using an excel spreadsheet by plotting the mean absorbance value for each standard on the Y-axis versus the corresponding standard rat insulin concentration on the X-axis **(Appendix 2).**

4.3 Data analysis

The results are expressed as means ± SD for the indicated number of observations. Values of $P \leq 0.05$ were considered statistically significant. The effects of treatment, time, and treatment by time interaction were analyzed by the two-way repeated measure ANOVA followed by Tukey-Kramer post hoc test using GraphPad Prism version 8.0 for Windows; GraphPad Software, San Diego, California USA. Also, the area under the curve (AUC) for insulin and glucose was calculated using the GraphPad Prism version 8.0 for Windows; GraphPad Software, San Diego, California USA. Comparative analysis between treatments for both insulin and BG was done through Tukey's multiple comparison test.

Chapter 5: Results

5.1 Characteristics of laboratory rats

Six healthy male Wistar Han rats 4 months old with an average weight of 373± 35g were used in this study. There was no significant weight loss or increase in rats when rats' weight was compared before and after the experiment.

Table 5. 1 Body weight of rats.

	Rats# weight (kg)					
Sessions	1	2	3	4	5	6
One	334	363	406	348	375	367
Two	338	362	407	348	375	370
Three	339	361	408	345	378	372
Four	342	358	413	347	378	370

Data indicates rats' weight (kg) in each session, n=6

Average rats' weight

Rat#	Average Weight(kg) ± SD
1	338 ± 3
2	361 ± 2
3	409 ± 3
4	347 ± 1
5	377 ± 2
6	370 ± 2

Data indicates rats' weight throughout the study; mean ± SD

5.2 Blood glucose

There was an effect of treatment ($P = 0.0002$), time ($P < 0.0001$) and a treatment by time interaction ($P < 0.0001$) on blood glucose over 120 min. There was no difference between the treatments at the baseline. At 15 min, TMP, CN, and WP led to reduced blood glucose with a significant difference when compared to G **(Figure 5.1).**

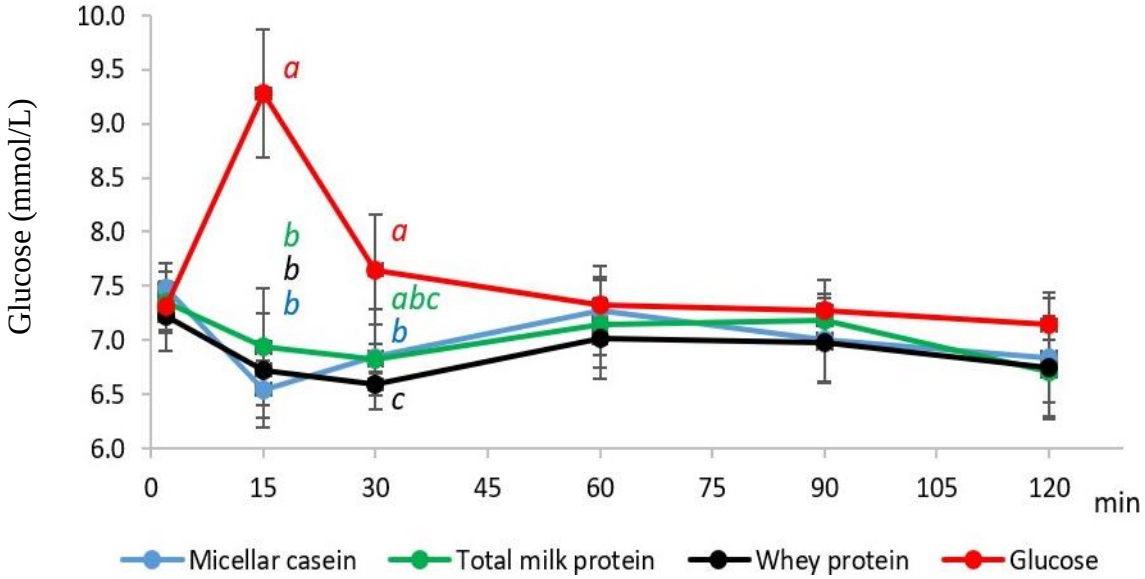
At 30min CN and WP led to reduced blood glucose compared to G ($P < 0.05$), but not TMP ($P = 0.06$) **(Figure 5.1**). At 30 min, CN led to a higher BG compared to WP ($P < 0.05$). At 60, 90, and 120 min, the effect of protein treatments on blood glucose was not significantly different when compared to G **(Figure 5.1)**.

Table 5. 2 Mean blood glucose response

Treatment	Mean ± SD					
	0 min	15 min	30 min	60 min	90 min	120 min
Casein	7.5 ± 0.2[a]	6.5 ± 0.3[b]	6.8 ± 0.1[b]	7.3 ± 0.4[a]	7.0 ± 0.4[a]	6.8 ± 0.5[a]
Total milk protein	7.3 ± 0.3[a]	6.9 ± 0.5[b]	6.8 ± 0.5[abc]	7.1 ± 0.4[a]	7.2 ± 0.2[a]	6.7 ± 0.3[a]
Whey protein	7.2 ± 0.3[a]	6.7 ± 0.5[b]	6.6 ± 0.1[c]	7.0 ± 0.4[a]	7.0 ± 0.4[a]	6.7 ± 0.5[a]
Glucose	7.3 ± 0.2[a]	9.3 ± 0.6[a]	7.6 ± 0.5[a]	7.3 ± 0.3[a]	7.3 ± 0.3[a]	7.1 ± 0.3[a]

Data are means ± SD, n = 6. Abbreviations: C= casein, M= total milk protein, W= whey protein, G= glucose. Effect of time (P<0.0001), treatment (P=0.02), and a time by treatment interaction (P<0.0001). Values with different letters are significantly different (P<0.05).

Figure 5. 1 Blood glucose 0-120 min.

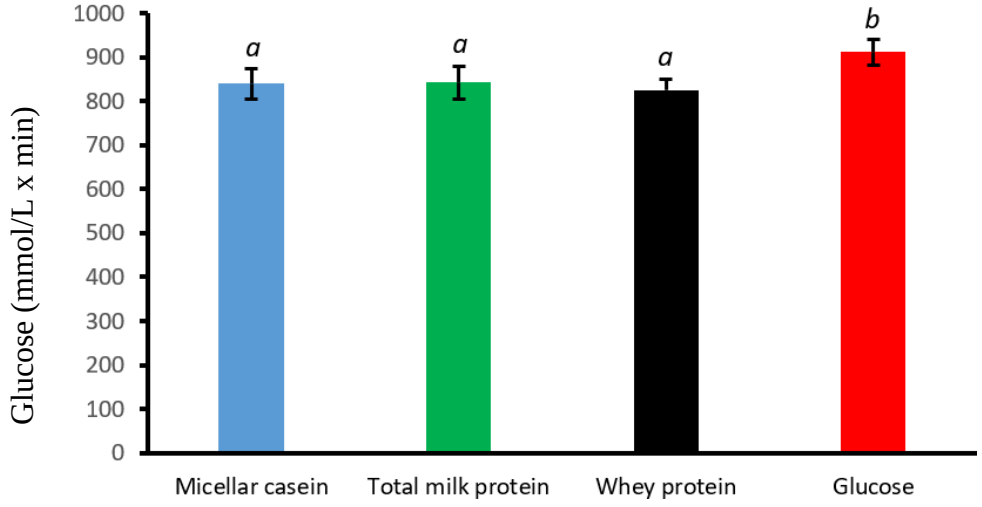


Mean ± SD. Two-way ANOVA (treatment and time) with Tukey's-Kramer post-hoc test. Effect of time (P<0.0001), treatment (P=0.0002), and a time by treatment interaction (P<0.0001). Values with different letters are significantly different (P<0.05).

5.3 Blood glucose AUC

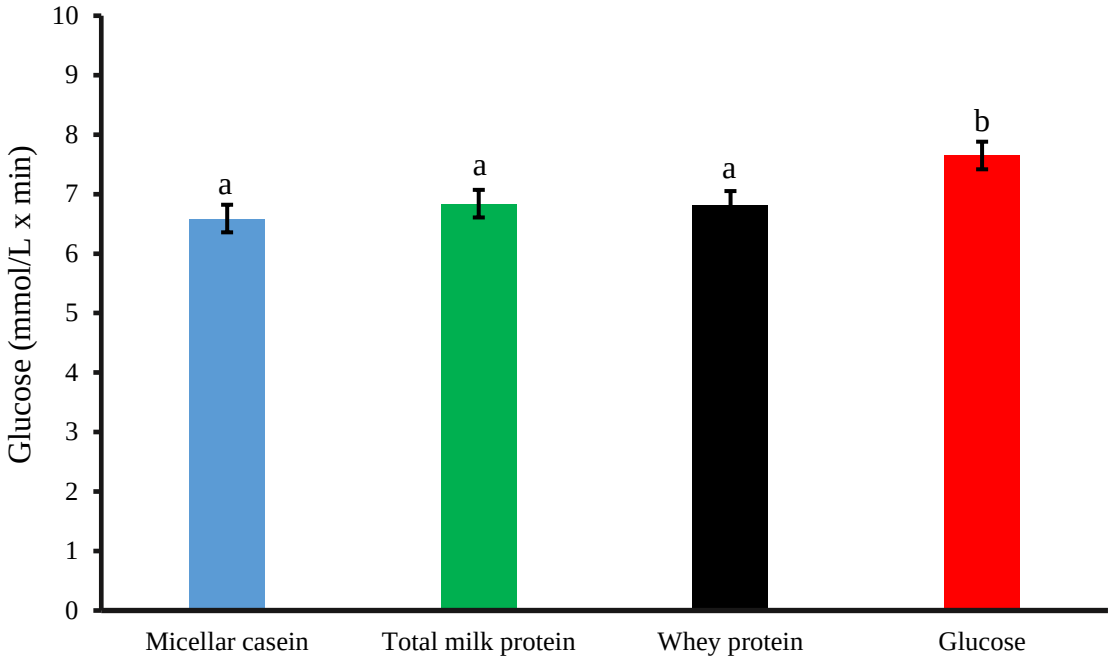
The effect of treatment on blood glucose AUCs over 120min (P = 0.001). The blood glucose after ingestion of WP, CN, and TMP was lower than G (Figure 5.2) ($P < 0.05$). There was no difference between WP, CN, and TMP on blood glucose AUC 0-120 min ($P > 0.05$). Similarly, blood glucose AUC at 0-30 min after ingestion of WP, CN and TMP showed significant difference ($P < 0.05$) when compared with G. All protein treatments when compared WP, CN and TMP were not significantly different on blood glucose AUC 0-30 min ($P > 0.05$).

Figure 5. 2 Blood glucose AUC (0-120 min)



Mean ± SD. One-way ANOVA with Tukey's-Kramer post-hoc test. Effect of treatment (P=0.001). Values with different letters are significantly different (P<0.05).

Figure 5.3 Blood glucose AUC (0-30 min)



Mean ± SD. One-way ANOVA with Tukey's-Kramer post-hoc test. Effect of treatment (P=0.001). Values with different letters are significantly different (P<0.05).

5.4 Serum insulin

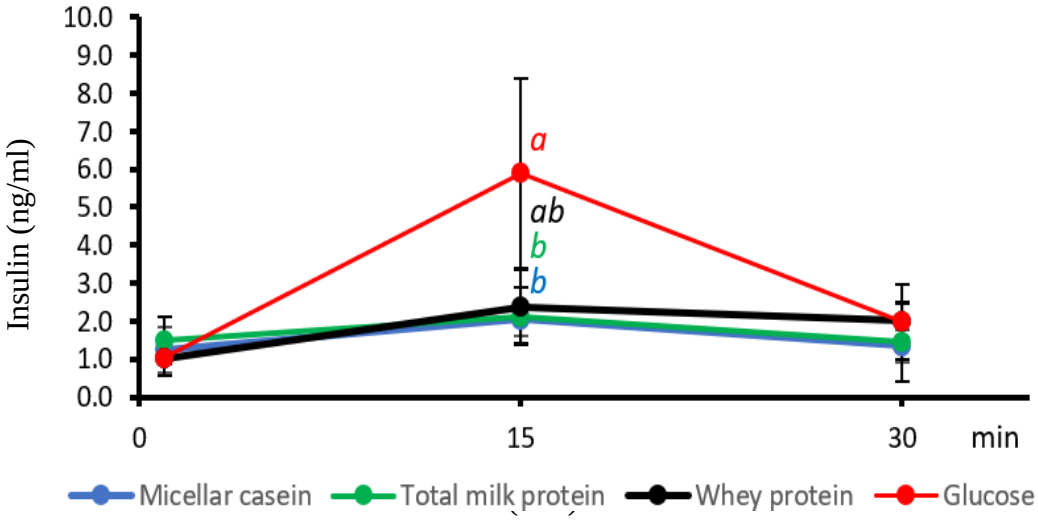
There was an effect of treatment ($P = 0.02$), time ($P < 0.0001$) and a time by treatment interaction ($P < 0.0001$) on serum insulin over 30 min observed. There was no difference between the treatments at the baseline. At 15 min, TMP and CN resulted in lower serum insulin compared to G ($P < 0.05$), while WP was neither different from G nor from TMP and CN ($P > 0.05$) (**Fig 5.4).**

Table 5. 3 Mean serum insulin response

Treatment	Mean ± SD		
	0	15	30
Casein	1.3 ± 0.6[a]	2.2 ± 0.4[b]	1.5 ± 0.4[a]
Total milk protein	1.5 ± 0.6[a]	2.1 ± 0.8[b]	1.5 ± 1.1[a]
Whey protein	1.0 ± 0.1[a]	2.4 ± 1.0[ab]	2.0 ± 0.4[a]
Glucose	1.0 ± 0.5[a]	5.9 ± 2.5[a]	2.0 ± 1.0[a]

Data are means ± SD, n = 6. Abbreviations: C= casein, M= total milk protein, W= whey protein, G= glucose. Effect of time (P<0.0001), treatment (P=0.02), and a time by treatment interaction (P<0.0001). Values with different letters are significantly different (P<0.05).

Figure 5. 3 Serum insulin (0-30 min)

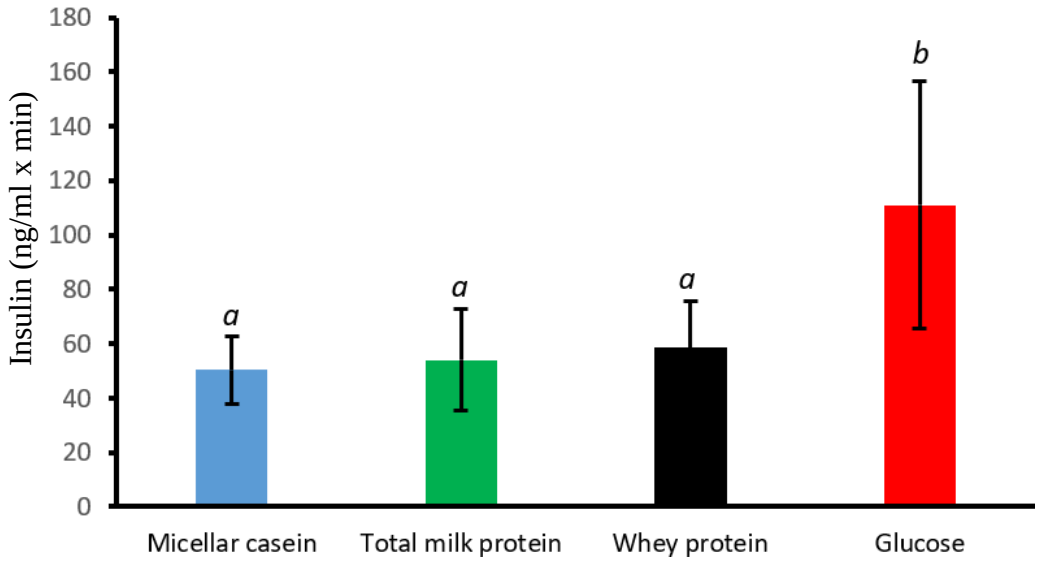


Mean ± SD. Two-way ANOVA (treatment and time) with Tukey's-Kramer post-hoc test. Effect of time (P<0.0001), treatment (P=0.02), and a time by treatment interaction (P<0.0001). Values with different letters are significantly different (P<0.05).

5.5 Serum insulin AUC

There was an effect of treatment on insulin AUC over 30 min ($P = 0.006$) resulting in a lower serum insulin AUC after protein treatments compared to G ($P < 0.05$). When compared, WP, TMP, and CN did not vary substantially in their effect on insulin AUC.

Figure 5. 4 Serum insulin AUC (0-30 min)



Mean ± SD. One-way ANOVA with Tukey's-Kramer post-hoc test. Effect of treatment (P=0.0006). Values with different letters are significantly different (P<0.05).

Chapter 6: Discussion

This study is the first to evaluate the effect of milk protein fractions and total milk protein on blood glucose control and insulin response in rats. Milk protein has been identified to possess glucose lowering and insulinotropic properties. However, most studies have explored the effect of whey protein and casein, but not the effect of total milk protein alongside its fractions on blood glucose and insulin. Therefore, we decided to explore this effect and identify the most potent milk protein and determine the time frame that provides the most improvement in blood glucose.

One of the major findings in this study was that blood glucose levels reduced significantly ($P < 0.05$) at 15 and 30 mins when compared to G, while a reduction in insulin was observed over 30 mins **(Fig. 5.1)** after ingestion of TMP, CN and WP. This result is consistent with previous studies indicating that protein, especially milk protein (275, 276) explicitly reduces glycemic control compared to carbohydrate alone. The possible mechanism of action attributed to this effect is the insulinotropic activity of milk protein, as it has been identified to acutely improve glycemic control by stimulating insulin secretion and reducing plasma glucose levels (248). This stimulating effect could be mediated through bioactive peptides or by specific amino acids (277) released during digestion. Amino acids are known as the potent stimulators of insulin release, especially the branched-chain amino acids which have been established to possess more insulinogenic effect than others (6). Another possible pathway is the secretion of the enterogastric, incretin hormones (glucose-dependent insulinotropic polypeptide (GIP) and glucagon like peptide 1(GLP-1). This hormone exerts its activity by binding to GIP and GLP-1 receptors, which are expressed on pancreatic β-cells to induce insulin-containing granule exocytosis (278), thus increasing glucose-stimulated insulin secretion. Previous research reported a decrease in postprandial glycemia, appetite, food intake and increase in circulating insulin levels, (279-281) following consumption

of different forms of dairy products. However, the exact mechanism by which blood glucose levels are lowered are yet to be established, although amino acids and bioactive peptides derived from milk proteins are thought to change the physiological milieu, which includes delayed gastric emptying and augmentation of incretin and insulin responses, consequently leading to reduced postprandial glucose levels (282). This effect is consistent with previous research that attributes high protein content in Greek yoghurt as the reason for the decrease in postprandial glycemia (280), while ascribing the outcome to whey protein content in Greek yoghurt. However, whey protein has been reported as the more insulinogenic fraction of milk protein and described to be more effective in lowering blood glucose, while this assertion maybe true, in the current study, WP only significantly lowered BG than CN at 30 min, but at 15min, CN appeared to have a lower mean blood glucose (**Table 5.1)** than WP, although there was no significant difference. Nonetheless, the effect of WP at 30 min may be associated with the possible elevated concentrations of specific insulinogenic amino acids as well as bioactive peptides, either originally present in whey protein or formed during digestion.

Surprisingly, the TMP did not significantly lower blood glucose levels when compared to G at 30 mins, it was also not significantly different at 30 min when compared with WP. The effect of total milk protein when compared with protein fractions varies in reports. Studies have reported that ingestion of whey protein together with casein slows down absorption and may allow greater absorption of BCAA and possibly lead to greater effect on blood glucose unlike whey protein, which is easily absorbed by the body, and may lead to reduction of intestinal absorption of BCAA (283). Yet, another study reported that, diets enriched in whey protein, casein, and their combination (whey protein + casein) exerted differential effects on energy balance and glucose metabolism, while the whey protein+ casein group had lower impact on blood glucose compared

to whey protein (265). Similarly, milk with increased protein content (9.3% milk protein) and reduced casein-to-whey protein ratio (40:60) resulted in lower peak glucose responses in the pre-meal period, at 30 and 45 min (284). This effect can be associated with the previous reports that total milk protein is less effective than the isolation of its fractions (4). Although this was not marginally proven in the present study.

Insulin response was higher after the ingestion of glucose treatment as opposed to the milk protein treatments. This substantial effect on insulin after ingestion of G is due to the elevated levels of blood glucose. This is because Insulin secretion from the pancreas respond to elevated blood glucose, although, the function of insulin is to reduce blood glucose levels through increase in glucose uptake in muscle and adipose tissue, as well as promoting glycolysis and glycogenesis in liver and muscle. Nevertheless**,** WP's effect on insulin was similar to that of G at 15 min (**Fig 5.4**), and a higher blood insulin mean **(Table 5.2)** compared to TMP and CN. This correlates with previous study wherein whey protein resulted in higher postprandial insulin response when compared to other three protein sources (casein, gluten or cod). This effect was attributed to the more rapid appearance of amino acids in plasma when derived from whey protein (285). This faster amino acid presence subsequently, elicits a faster and more accentuated insulin and glucagon response. However, the effect of treatment on insulin AUC when compared was not different from one another, at 15 and 30 min. This outcome may be due to the absence of carbohydrate co-ingested with milk proteins, because studies have previously reported that co-ingestion of whey protein with carbohydrates (lactose) results in a higher insulin response than cheese (casein) or milk (whey protein and casein), respectively (6), when compared to the ingestion of carbohydrate or protein alone. This, then indicates a synergy between oral protein and glucose (286, 287).

Therefore, studies maybe required to investigate the co-ingestion of TMP, CN & WP to achieve further substantial evidence.

The protein dosage is considered to be an effective factor in assessing the impact of protein on the regulation of blood glucose and insulin response. While some studies have calculated that the far more effective dose is ~25-50g of protein (8), another study established 10g of milk protein as effective in improving glycemic control and insulin secretion (188, 288). Besides, the optimal dose for improving glycemic control in humans have not been determined, therefore 10g protein dose was used in the current study because of their proven effectiveness and their conceivable usage as a protein substitute in meals, aimed at improving glycemic control without substantially raising total protein intake. For example, 4.5g dose of whey protein stimulated insulin secretion, similarly 9g dose of protein improved glucose responses (289). This effect coupled with the result of our studies suggests that lower doses of protein are as well effective in reducing glycemic responses. However, in the development of functional foods, this aspect is very significant and could need further studies in humans.

Milk protein's impact on blood glucose and insulin over time have been associated with the time of consumption. Studies have described milk proteins, especially whey protein to be more effective when consumed 30 min before a meal (8, 257). A study conducted over an 8h postprandial period, reported that the initial insulin response in the first 30 min was significantly higher after whey protein hydrolysate was added to a meal (211). Likewise, consuming whey protein before a meal effectively enhanced glycemic response. Milk protein preload given 30 min before a meal slowed gastric emptying when compared to either a nutrient-free preload or ingestion of whey protein with the meal (257, 262). Whey protein preload consumed before a carbohydrate diet or with a carbohydrate diet has been established to possess the capacity to stimulate incretin

hormone secretion and slow gastric emptying in subjects with type 2 diabetes. Also, significant increase in insulin after consumption of dairy treatment compared to non-dairy treatment was observed only at 15 and 30 min in a two-hour period (279). This effect was seen in this study, as the effect of TMP, WP, and CN was seen over 30 min on blood glucose and insulin. Therefore, effect of treatment by time interaction observed in this study may imply that meal consumption after 30 mins may enable marked reduction in postprandial glycemia in people with T2DM (257).

In summary, all protein treatments resulted in a lower BG AUC compared to glucose control over 120 and 30 min. However, at 30 min, CN resulted in a higher BG compared to WP while TMP resulted in intermediate response not significantly different from G or from the other milk protein fractions. There was no difference between the TMP, CN and WP on insulin AUC over 30 min. 10g protein dose proved effective in stimulating insulin secretion and glycemic responses. At 15 min, WP did not reduce insulin significantly when compared with TMP and CN. At 30 min, the insulin response after all treatments returned to baseline. The beneficial effect of milk protein is observed over 30 min of ingestion. Future studies would be needed to elucidate how individual milk protein fractions affects the regulation of blood glucose and insulin secretion when co-ingested with glucose

6.1 Limitations & Strength

A potential limitation is the limited volume of blood collected, which prevented further analyses such as that of glucagon and other hormones. Another constraint experienced was the failure of catheter in rats, as this led to automatic disqualification of animal from study.

The major strength of this study was applying a randomized crossover design that is typically used in human clinical trials. This allowed to minimize the biological variability and compare the data within the same rats. Another strength was a use of catheterized animals resulting in the minimally invasive repeated blood collection procedure. As a result of this, animals experienced less pain and stress. Similarly, housing animals as dyads assisted rats' health and wellbeing. All these put together reduced confounding covariates in the study. Lastly, this study helped to understand how milk proteins influence the regulation of blood glucose and insulin and acts as a precedent for more studies to explore their use in functional food products in order to reduce metabolic risk factors.

6.2 Conclusion

The ingestion of the major milk protein fractions led to a lower blood glucose and serum insulin compared to the same dose of glucose. Although TMP, CN and WP followed a similar pattern in their effect on BG and serum insulin, they demonstrated distinct properties at 15 and 30 min after ingestion.

These findings introduce new perspectives in the nutritional and technological evaluation of milk products and encourage utilization of these substances for production of food and new health promoting products. More studies related to the mechanisms by which these proteins exert their effects in humans are required to achieve further substantial evidence.

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Appendices

Appendix 1: Insulin Assay

Bio-Tek Instruments

Assay:INSULIN
Wavelength:450

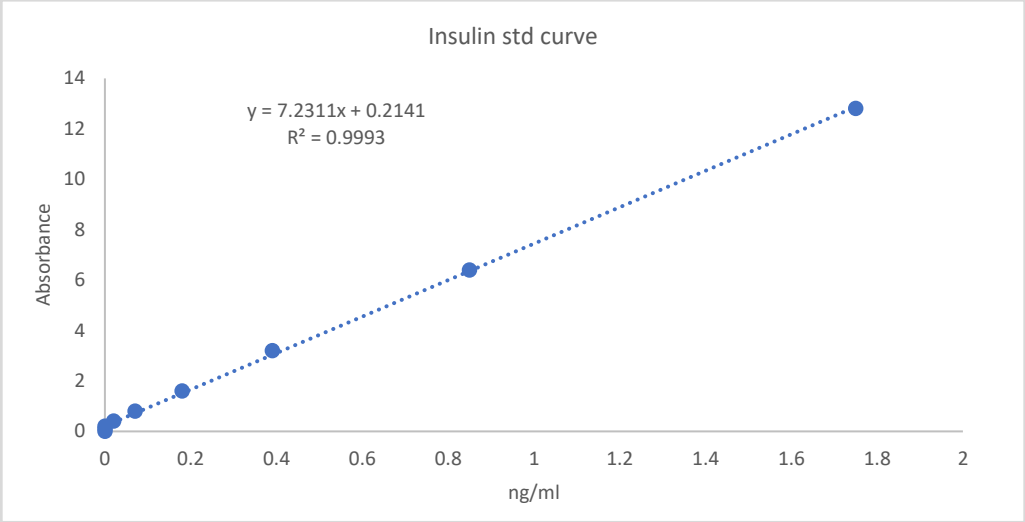
Date:02/20/20
Time:03:38:33
Temp:

Lot:
Operator:
Plate ID:2

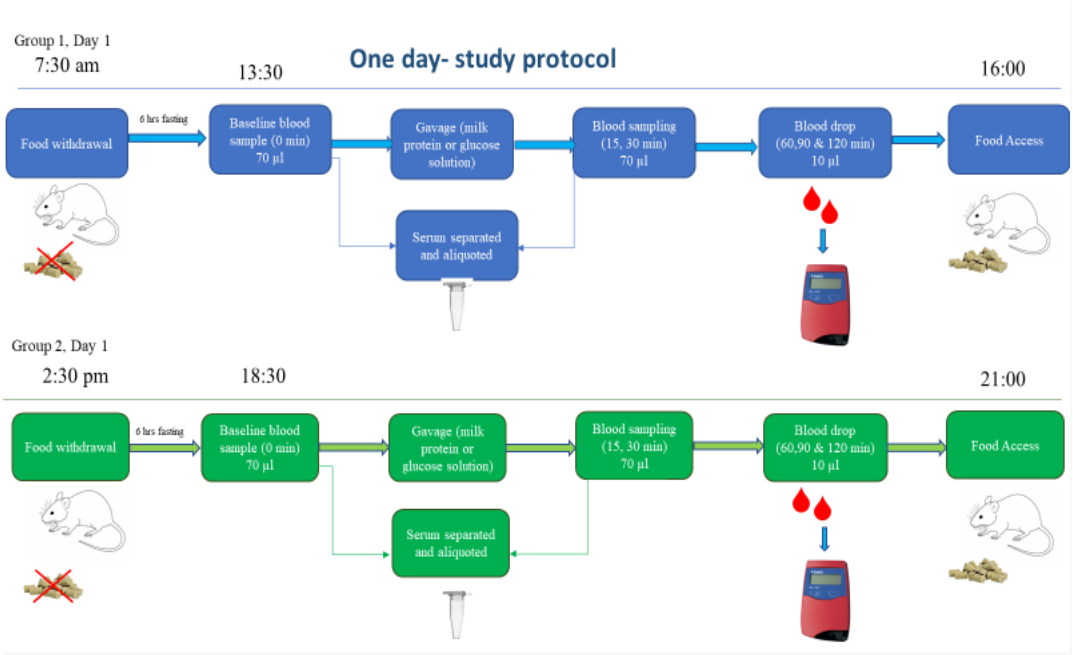
COMMENTS

	1	2	3	4	5	6	7	8	9	10	11	12
A	CALL CalcOD Well RSLT	0.056 SMP1	0.101 SMP9	1.669 SMP17	0.249 SMP25	0.252 SMP33	0.214 SMP41	0.198 SMP49	0.469 SMP57	0.248 SMP65	0.155 SMP73	0.217 SMP81	0.000 SMP89
B	CALL CalcOD Well RSLT	0.057 SMP2	0.113 SMP10	1.801 SMP18	0.281 SMP26	0.295 SMP34	0.220 SMP42	0.205 SMP50	0.520 SMP58	0.252 SMP66	0.147 SMP74	0.205 SMP82	0.000 SMP90
C	CALL CalcOD Well RSLT	0.060 SMP3	0.212 SMP11	0.328 SMP19	0.381 SMP27	0.207 SMP35	0.166 SMP43	0.265 SMP51	0.206 SMP59	0.137 SMP67	0.259 SMP75	0.112 SMP83	0.000 SMP91
D	CALL CalcOD Well RSLT	0.062 SMP4	0.229 SMP12	0.307 SMP20	0.379 SMP28	0.206 SMP36	0.168 SMP44	0.253 SMP52	0.287 SMP60	0.150 SMP68	0.245 SMP76	0.121 SMP84	0.000 SMP92
E	CALL CalcOD Well RSLT	0.065 SMP5	0.437 SMP13	0.288 SMP21	0.286 SMP29	0.158 SMP37	0.292 SMP45	0.146 SMP53	0.330 SMP61	0.319 SMP69	0.145 SMP77	0.219 SMP85	0.000 SMP93
F	CALL CalcOD Well RSLT	0.065 SMP6	0.490 SMP14	0.274 SMP22	0.319 SMP30	0.161 SMP38	0.310 SMP46	0.152 SMP54	0.382 SMP62	0.321 SMP70	0.158 SMP78	0.227 SMP86	0.000 SMP94
G	CALL CalcOD Well RSLT	0.075 SMP7	0.805 SMP15	0.200 SMP23	0.131 SMP31	0.331 SMP39	0.196 SMP47	0.277 SMP55	0.305 SMP63	0.191 SMP71	0.211 SMP79	0.579 SMP87	-.00 SMP95
H	CALL CalcOD Well RSLT	0.081 SMP8	0.830 SMP16	0.162 SMP24	0.131 SMP32	0.332 SMP40	0.209 SMP48	0.280 SMP56	0.309 SMP64	0.196 SMP72	0.213 SMP80	0.718 SMP88	-.00 SMP96

Appendix 2: Insulin curve



Appendix 3


Group 1, Day 1
7:30 am
13:30
One day- study protocol
16:00
Food withdrawal
6 hrs fasting
Baseline blood sample (0 min) 70 µl
Gavage (milk protein or glucose solution)
Blood sampling (15, 30 min) 70 µl
Blood drop (60,90 & 120 min) 10 µl
Food Access
Serum separated and aliquoted
Group 2, Day 1
2:30 pm
18:30
21:00
Food withdrawal
6 hrs fasting
Baseline blood sample (0 min) 70 µl
Gavage (milk protein or glucose solution)
Blood sampling (15, 30 min) 70 µl
Blood drop (60,90 & 120 min) 10 µl
Food Access
Serum separated and aliquoted

Appendix 4

DALHOUSIE UNIVERSITY
Inspiring Minds

PRACTICAL TRAINING CERTIFICATION

This is to certify that

Temilola Olowookere

has participated in the practical training session :

THE RAT:
RECOMMENDED TECHNICAL PROCEDURES

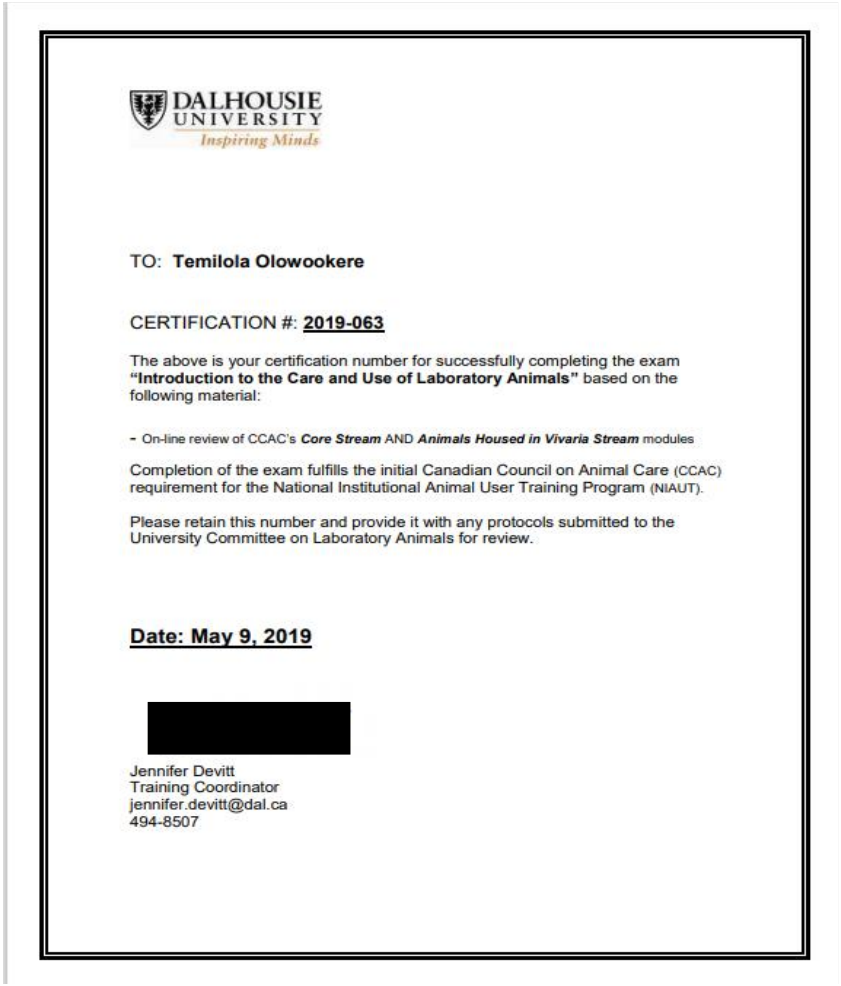
- *Video presentation covering humane handling and restraint methods, sexing of adult and neonatal rats, identification of individual animals, anesthesia techniques, and recommended procedures for blood collection and injections*
- *Discussion on characteristics of healthy rodents*
- *Practical training 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: May 10, 2019

[redacted]

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

Appendix 5



DALHOUSIE UNIVERSITY
Inspiring Minds

TO: **Temilola Olowookere**

CERTIFICATION #: **2019-063**

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: May 9, 2019

[redacted]

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