SELECTION AND EVALUATION OF SUPERIOR APPLE GENOTYPES FOR
POTENTIAL GLYCEMIC CONTROL


By


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Submitted in fulfillment of the requirements
for the degree of Master of Science



at




Dalhousie University
Halifax, Nova Scotia
August 2022




Dalhousie University is located in Mi'kma'ki, the
ancestral and unceded territory of the Mi'kmaq.
We are all Treaty people.

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# ABSTRACT

The tremendous apple biodiversity resulted in varying (poly)phenol levels with potential type 2 diabetes (T2D) protective properties. This project screened 476 apple accessions from Canada's Apple Biodiversity Collection to identify accessions with *in vitro* anti-diabetic activities. 'Daux Belan' (DB), an accession containing high total (poly)phenol content (TPC), was assessed for glucose and lipid metabolism regulation in high fat (HF) diet-induced obese mice. The inhibition of carbohydrate-hydrolyzing enzymes and toxic advanced glycation end-product formation (AGEs) are positively correlated with apple TPC *in vitro*. DB supplementation to mice at 0.15% diet for 18 weeks did not protect against HF diet-induced obesity, glucose intolerance, and hypertriglyceridemia. Overall, apple (poly)phenols exhibited promising hypoglycemic activities *in vitro* by inhibiting carbohydrate-hydrolyzing enzymes, dipeptidyl peptidase-4 enzyme and AGEs. Further research is warranted using cell and pre-clinical animal models to verify the suitability of apple (poly)phenols as a functional food ingredient to manage T2D.

# LIST OF ABBREVIATIONS USED

| | |
|---|---|
| ABC | Apple Biodiversity Collection |
| AGE | Advanced glycation end products |
| ANOVA | Analysis of variance |
| ATP | Adenosine triphosphate |
| AUC | Area under the curve |
| BSA | Bovine serum albumin |
| DB | Daux Belan |
| DIO | Diet-induced obesity |
| DMSO | Dimethyl sulfoxide |
| DPP-4 | Dipeptidyl peptidase-4 |
| EGP | Endogenous glucose production |
| GAE | Gallic acid equivalent |
| GIP | Glucose-dependent insulinotropic polypeptide |
| GLP-1 | Glucagon-like peptide 1 |
| GTT | Glucose tolerance test |
| H & E | Hematoxylin and Eosin |
| $HbA_{1c}$ | Hemoglobin $A_{1c}$ |
| HDL | High-density lipoprotein |
| HF | High fat |
| $IC_{50}$ | Half-maximal inhibitory concentration |
| IP | Intraperitoneal |
| IPGTT | Intraperitoneal glucose tolerance test |
| ITT | Insulin tolerance test |
| HPLC-MS | High-performance liquid chromatography-mass spectrometry |
| LC-qTOF-MS | Liquid chromatography-quadrupole time-of-flight-mass spectrometry |
| LDL | Low-density lipoprotein |
| M. | Malus |
| MTT | Metabolic tolerance test |
| OG | Oral gavage |

| | |
|---|---|
| OGTT | Oral glucose tolerance test |
| PBS | Phosphate buffered saline |
| PNPG | 4-nitrophenyl α-D-glucopyranoside |
| RCS | Reactive carbonyl species |
| SD | Standard deviation |
| SEM | Standard error of the mean |
| SGLT-1 | Sodium-glucose co-transporter 1 |
| SGLT-2 | Sodium-glucose co-transporter 2 |
| T2D | Type 2 diabetes |
| TG | Triglyceride |
| TPC | Total (poly)phenol content |
| Z | Zestar |

# ACKNOWLEDGEMENTS

**CHAPTER 1: INTRODUCTION**

Domesticated apple (*Malus × domestica* Borkh.) is an economically important fruit crop in temperate regions that are consumed worldwide (Duan et al., 2017). The diversity of apple genetic makeup is reflected in over 10,000 documented apple cultivars (Janick & Moore, 1996). However, due to strict selection criteria for redness, firmness, flavor, and storability (Wu et al., 2018) and the repeated crossbreeding of the same few cultivars, modern commercial apple cultivars display significantly narrowed genetic and phenotypic variability (Migicovsky et al., 2021), including total (poly)phenol content (TPC) (Watts et al., 2021). Apple, like many fruits and vegetables, is a significant source of dietary fibre, ascorbic acid, vitamins, and (poly)phenols (Wu et al., 2018). (Poly)phenols are secondary metabolites produced by plants with antioxidative and antimicrobial properties against ultraviolet light and pathogens (Cao et al., 2019). Apples are a primary dietary source of antioxidants in the US, where apple (poly)phenols represent 22% of the dietary (poly)phenol intake (Vinson et al., 2001). Due to their various beneficial roles in human health, the use of certain (poly)phenols as dietary supplements in the prevention of chronic diseases is gaining attention (Kawabata et al., 2019).

The incidence of type 2 diabetes (T2D) is increasing at an alarming rate. In 2021, it is estimated that 537 million adults between the ages of 20-79 have diabetes, with T2D accounting for over 90% of diabetes cases (International Diabetes Federation, 2021). Diabetes prevalence is projected to reach 643 million by 2030 and 783 million by 2045 (International Diabetes Federation, 2021). As the ninth leading cause of mortality, T2D is characterized by decreased insulin secretion due to pancreatic β-cell dysfunction and insulin resistance in target tissues such as liver, muscle, and adipose tissues (Chatterjee et

al., 2017), resulting in the hallmark symptoms of hyperglycemia, hypertension, hyperlipidemia, and glucosuria (Kang et al., 2020). Chronic hyperglycemia can result in microvascular and macrovascular complications that cause blindness, limb amputation, kidney failure, vascular disease, and heart disease (Pippitt et al., 2016). While genetic factors can predispose individuals toward T2D, environmental factors such as obesity, physical inactivity, energy-dense diets, and heavy alcohol consumption play important roles in T2D manifestation (Bellou et al., 2018; Chatterjee et al., 2017; Gurung et al., 2020).

Dietary (poly)phenols in fruits and vegetables can protect against hyperglycemia by maintaining normal glucose metabolism (Rupasinghe et al., 2017). A positive association is demonstrated between apple consumption and lowered risk of T2D (Alperet et al., 2017; Guo et al., 2017; Muraki et al., 2013; Song et al., 2005). In addition to attenuating hyperglycemia, apple (poly)phenol consumption has also been reported to protect against hyperlipidemia by reducing serum/plasma and liver lipid parameters such as triglyceride (TG) and cholesterol in both animal studies (Fathy & Drees, 2016; Li et al., 2019a; Masumoto et al., 2016; Shin et al., 2016; Zou et al., 2020) and human trials (Koutsos et al., 2020; Yuliwati et al., 2020).

## 1.1. Research hypothesis

The hypotheses of this study were: i) apple (poly)phenols exert anti-diabetic properties *in vitro* and *in vivo*, where the anti-diabetic activities positively correlate with (poly)phenol contents, and ii) dietary apple (poly)phenol supplementation in the whole apple form (peel + flesh) ameliorates metabolic impairments in chronic HF diet-fed mice, specifically regarding obesity, glucose tolerance, and lipid metabolism.

## 1.2. Research objectives

The overall objective of this research was to evaluate the potential of fresh apple consumption for the prevention or management of T2D. The specific objectives were to:

1) identify unique apple accessions with anti-diabetic characteristics *in vitro* using inhibition assays of α-amylase enzyme, α-glucosidase enzyme, DPP-4 enzyme inhibition assay, and AGE formation.

2) assess major (poly)phenol constituents of selected apple accessions using targeted high-performance liquid chromatography-mass spectrometry (HPLC-MS) metabolite profiling, and

3) investigate the impact of the chronic dietary intervention of high-(poly)phenol-containing 'Daux Belan' apple powder on glucose tolerance and lipid metabolism using a diet-induced obese mouse model.

**CHAPTER 2: LITERATURE REVIEW**

This literature review is divided into the following four major sections: 1) overview of the apple fruit, 2) glucose metabolism and type 2 diabetes, 3) therapeutic effects of apple (poly)phenols with an emphasis on glycemic control, and 4) experimental animal model of diet-induced obesity and hyperlipidemia. In the first section, a brief history and biodiversity of apples will be introduced, followed by background information on apple (poly)phenols. The second section focused on the role of insulin in glucose metabolism and T2D manifestation. Then, the general mechanisms of action against hyperglycemia by apple (poly)phenols will be discussed. Finally, experimental animal models of diet-induced obesity and hyperlipidemia will be reviewed. It is well known that diet plays an important role in the development and progression of T2D. More specifically, dietary (poly)phenols have shown promising therapeutic properties in this regard. Since apples are a widely available (poly)phenol-rich food that can be easily incorporated into the daily diet, it is of interest to investigate the effectiveness of apple (poly)phenols in the management and prevention of T2D.

## 2.1. Apple (*Malus domestica* Borkh.)

### 2.1.1. Apple history and biodiversity

Apples are easily accessible year-round due to their long-term storability and production in both hemispheres of the globe (Bossi Fedrigotti & Fischer, 2020). Other than being consumed as fresh fruit, apples are versatile in that they can be processed into value-added products such as juice, cider, vinegar, sauce, jam, dried apples, and canned apples (Vidović et al., 2020). Found in the wild throughout Eurasia and North America (Mratinić

& Akšić, 2012), species diversity in terms of form, color, and flavor in Central Asian wild apples is significantly greater than in North American wild apples (Harris et al., 2002). It is established that the wild apple germplasms of *M. sieversii* in central Asia, specifically in the Tien Shan Mountain ranges of Kazakhstan, are the major progenitors of the domesticated apples, *M. domestica* Borkh. (Ha et al., 2021; Vavilov, 1987). Through westward passage along the ancient Eurasian trade route known as the Silk Road, intensive introgression between domesticated apples and wild crab apples (Cornille et al., 2014), particularly the European *M. sylvestris* Mill. (Cornille et al., 2012; Migicovsky et al., 2021), further diversified the genetic makeup of *M. domestica*.

While there are over 10,000 documented apple cultivars, only a few are commercialized (Janick & Moore, 1996). In fact, 'Red Delicious', 'Gala', 'Granny Smith', 'Fuji', 'Golden Delicious', 'Honeycrisp', 'McIntosh', 'Cripps Pink/Pink Lady', and 'Empire' are the nine most produced apple cultivars in the United States (US Apple Association, 2022). Dominant cultivars on the market are mainly propagated from repeated crossbreeding of the selected few varieties, such as 'Cox's Orange Pippin', 'Golden Delicious', 'Red Delicious', 'Jonathan', and 'McIntosh' (Noiton & Alspach, 1996). Commercial apples are selected for their desirable attributes, such as appearance, firmness (Migicovsky et al., 2021), juiciness (Bowen et al., 2019), fruit size (Fang et al., 2017), reduced acidity (Ma et al., 2015) and prolonged shelf-life (Wu et al., 2018). Due to the intense breeding in a consumer-driven approach using crossbreeding of the several same elite cultivars, the genetic variability of commercialized apples has narrowed (Migicovsky et al., 2021). Thus, ancient, heritage, and local varieties that do not meet the modern standard of apple production in terms of productivity, fruit quality, and shelf-life are

selected out of orchard breeding and are diminishing (Anastasiadi et al., 2017). Therefore, despite the tremendous diversity in documented apple cultivars, consumers are only exposed to a small portion of the genetic variation in the supermarket as a consequence of repeated use of a small number of elite varieties (Watts et al., 2021).

### 2.1.2. Apple (poly)phenols

Dietary (poly)phenols found abundantly in plant-foods such as fruits, vegetables, whole grains, cereal, legumes, tea, coffee, wine, and cocoa (Kesavan et al., 2018), are a group of secondary metabolites that are involved in the attraction of pollinators (Koes et al., 1994), deterrent of insects and herbivores (Mierziak et al., 2014), suppression and quenching of reactive oxygen species (antioxidative activity) (Zandi & Schnug, 2022), and protection against ultraviolet radiation and microorganisms in plants (Siemińska-Kuczer et al., 2022). Among plants, (poly)phenols secreted by roots of some plants influence allopathic effects through the inhibition of the germination and growth of nearby plants (Star, 1980). (Poly)phenols also contribute to the taste (e.g. bitterness, astringency) (Pandey & Rizvi, 2009), color (e.g. Orange, red, blue, or purple pigments from anthocyanin in the flower, fruit, and leaves) (Shoji, 2007), and preservation (e.g. antioxidant and antimicrobial properties) (Wu & Zhou, 2021) of food.

Despite the recent narrowing of apples' phenotypic diversity due to intense selection, apples are extremely variable in (poly)phenol content, where the inter-cultivar variation of (poly)phenols can exceed two orders of magnitude (McClure et al., 2019). (Poly)phenol profiles, content, and antioxidant capacity of apples are influenced by cultivar, tissue zones, harvest time, geographical location, and storage conditions (Bahukhandi et al., 2018; Khanizadeh et al., 2007). HPLC data of 15 old and new apple cultivars suggested

that (poly)phenol content is doubled in old cultivars compared with the new cultivars, and antioxidant capacity was up to 30% higher in old cultivars (Kschonsek et al., 2018). Similarly, in a study assessing ascorbic acid (a powerful antioxidant) concentration in mature fruits of 457 apple accessions, ascorbic acid varied greatly between accessions, with wild fruits being higher in ascorbic acid concentrations than domesticated cultivars (Fang et al., 2017). These data are supported by the recent finding that a 30% reduction in (poly)phenol content is observed in commercial apples over the past 200 years (Watts et al., 2021), likely due to active breeding for less astringency and slower enzymatic browning (Kschonsek et al., 2018), though it is important to note that not all apple (poly)phenols are uniformly affected by selective breeding pressure (Farneti et al., 2015). (Poly)phenol compounds such as hydroxycinnamic acids (chlorogenic acid), dihydrochalcones (phloridzin), and stilbenes, as well as organic acids such as malic acid and ascorbic acid, were found in higher levels in wild *Malus* compared with *M. domestica*, while the levels of flavonols and flavan-3-ols were not significantly different between the wild and domestic apple (Farneti et al., 2015). As such, inter-cultivar variation in apple properties related to (poly)phenol content, such as therapeutic effects of apple (poly)phenols, specifically anti-diabetic properties (Rupasinghe et al., 2017), is expected in this study.

### 2.1.2.1. Classification and distribution

Apart from nutrients such as dietary fibre, organic acids, sugars, vitamins, and minerals (Anastasiadi et al., 2017; Wu et al., 2018), apples also contain dietary (poly)phenols as one of the major bioactives. The five major (poly)phenol groups are flavan-3-ols (epicatechin and procyanidins), phenolic acids (chlorogenic acid), dihydrochalcones (phloridzin), flavonols (quercetin glycosides), and anthocyanins

(cyanidin-3-galactoside) (Güneş Bayir et al., 2019; Huber & Rupasinghe, 2009; Kschonsek et al., 2018; Rupasinghe et al., 2017). Among (poly)phenols, flavonoids are one of the most studied classes as they are present widely in the human diet (Sun et al., 2020). Flavonoids are subdivided into six subclasses: flavonol, flavone, flavanone, isoflavone, anthocyanins, and flavan-3-ols (Fraga et al., 2019). Apple (poly)phenol compounds are more concentrated in the peel than in the flesh (Kalinowska et al., 2020; Khanizadeh et al., 2007; Kim et al., 2019; Tsao et al., 2003), and anthocyanins are exclusively found in red-fleshed or red-skinned apples (Starowicz et al., 2020). The (poly)phenol profile and content vary by cultivar and are influenced by many other factors (Francini & Sebastiani, 2013).

## 2.1.2.2.      Dietary intake, bioavailability, and metabolism

Total dietary (poly)phenol intake varies among populations and is largely influenced by the local diet (Del Bo' et al., 2019). The overall intake is estimated to be 1 g/day (Scalbert & Williamson, 2000). In the US, apple (poly)phenols represent 22% of the dietary (poly)phenol intake (Vinson et al., 2001). Apple consumption ranges from 100 to 200 g per day (Starowicz et al., 2020), providing approximately 400 mg of (poly)phenols per portion (Scalbert & Williamson, 2000), depending on the cultivar.

The extent to which (poly)phenol compounds exert health-promoting effects is largely influenced by their bioaccessibility and bioavailability (Polia et al., 2022). Bioaccessibility refers to the amount of compound available for uptake by the intestine, and bioavailability is defined as the amount of compound that is left for systemic circulation after digestion to reach target tissues to perform its biological activities (Polia et al., 2022). (Poly)phenol compound bioavailability is largely dependent on the chemical structure (Brenes et al., 2016). Particularly, the degree of polymerization and structural

complexity play significant roles in the site of absorption. Almost all flavonoids present in plants are in the glycosylated form, meaning they have one or more sugar attachments (Xiao, 2015). Despite the promising therapeutic potential *in vitro*, they exhibit poor oral bioavailability due to their low water-solubility and permeability (Zhao et al., 2019). To increase bioavailability, the attached sugar from glycosylated flavonoids is removed by glycosidases (i.e. β-glucosidases and α-rhamnosidases), turning into flavonoid aglycones in a process called deglycosylation. This process occurs either before ingestion (removed in the food itself or during processing) or after ingestion in the gastrointestinal tract (secreted by gastrointestinal cells or microflora) (Scalbert & Williamson, 2000). Once deglycosylated, flavonoid aglycones undergo conjugation in the intestines and liver, and the resulting conjugated metabolites are released into the systemic circulation (Brenes et al., 2016).

While (poly)phenols with low molecular weights (monomers and aglycones) are absorbed from the small intestine, those of high molecular weights remain intact passing through the upper digestive tract and reaching the colon (Manach et al., 2004). Merely 5-10% of the total (poly)phenol intake is directly absorbed in the small intestine (Brenes et al., 2016), and the remaining majority are transported to the large intestine to be processed by the gut microbiota, and the resulting metabolites are either absorbed into the circulation or excreted with feces (Xiao, 2015). In summary, the many factors affecting the bioavailability of dietary flavonoids include molecular weight, glycosylation to improve permeability, metabolic conversion by enzymes, and interaction with gut microflora (Thilakarathna & Rupasinghe, 2013), as well as inter-individual heterogeneity in all the above-mentioned factors (Manach et al., 2017).

## 2.2. Glucose metabolism and type 2 diabetes (T2D)

### 2.2.1. Role of insulin in glucose homeostasis

The maintenance of glucose homeostasis is achieved by the coordinated effort between the gastrointestinal tract, pancreas, liver, muscle, adipose tissue, adrenal glands, and brain (MacDonald et al., 2021). It is balanced by two counteracting hormones: hyperglycemic glucagon and hypoglycemic insulin. Glucagon is secreted from the α-cells of the pancreatic islets of Langerhans in response to hypoglycemia to increase blood glucose levels, whereas insulin is produced by the β-cells in response to hyperglycemia to lower blood glucose levels (Ojha et al., 2019). In hyperglycemic conditions, glucose transporter protein 2 (GLUT2) facilitates glucose entry into β-cells, which stimulates insulin exocytosis through either the ATP-sensitive $K^+$ channel-dependent pathway (Ashcroft & Gribble, 1999) or by calcium-dependent protein kinase C (Trexler & Taraska, 2017). Following the release, insulin is transported through the portal venous system from the β-cells, where approximately half of the secreted insulin is taken up by the liver, and the other half is then merged into the systemic circulation (Lewis et al., 2021).

Insulin alleviates the pressure of hyperglycemia by suppressing endogenous glucose production (EGP). Directly, insulin in the liver suppresses EGP by inhibiting hepatic glycogenolysis. Indirectly, peripheral insulin suppresses EGP by inhibiting glucagon secretion from the pancreas (Lewis et al., 2021) and inhibiting Forkhead box O1 transcription factor-mediated expression of gluconeogenic genes such as glucose 6 phosphatase, fructose 1,6-bisphosphatase, pyruvate carboxylase, and phosphoenolpyruvate carboxykinase (Oh et al., 2013). Insulin also inhibits fatty acid (FA) breakdown in adipocytes (lipolysis), which reduces the available plasma free fatty acids (FFA) for liver

uptake, thereby reducing gluconeogenesis and thus EGP (Alves-Bezerra & Cohen, 2017). In addition to EGP inhibition, insulin signals glucose disposal by stimulating glucose uptake into peripheral tissues such as muscle and adipose tissues via glucose transporter protein 4 (GLUT4) (Wilcox, 2005). In the muscle, glucose uptake mainly stimulates glucose storage in the form of glycogen (Sylow et al., 2021). In adipose tissues, insulin inhibits lipolysis, stimulates glucose uptake, and promotes lipogenesis (Wilcox, 2005). Impaired insulin sensitivity disrupts the equilibrium between glucose ingestion, production, utilization, and storage that ensures a healthy glucose metabolism (MacDonald et al., 2021), leading to impaired glucose homeostasis that can progress to diabetes mellitus (Mason et al., 2020).

### 2.2.2. T2D pathogenesis and risk factors

Insulin resistance is referred to as a blunted response to insulin-mediated glucose disposal in peripheral tissues (Wilcox, 2005) and is developed as a consequence of increased adiposity and elevation in FFA (Fryk et al., 2021). Peripheral insulin resistance leads to impaired glucose tolerance, which is the prediabetic stage that precedes overt T2D. When unresolved, decreased insulin secretion due to pancreatic β-cell dysfunction and reduced insulin sensitivity in tissues responsible for glucose uptake (i.e. liver, muscle, and adipose tissues) causes failure to inhibit EGP and blood glucose accumulation, thus resulting in hallmark T2D symptoms of hyperglycemia, hypertension, hyperlipidemia and glucosuria (Chatterjee et al., 2017; Kang et al., 2020). T2D can also be accompanied by polyuria, polydipsia, unexplained weight loss, blurred vision, as well as growth impairment, and susceptibility to certain infections (American Diabetes Association, 2013). Detrimental effects of chronic hyperglycemia can include microvascular (eyes, kidneys,

and nerves) and macrovascular (heart and blood vessels) complications that cause blindness, limb amputation, kidney failure, vascular disease, and heart disease (Pippitt et al., 2016), resulting in disablement and reduced quality of life (Goyal & Jialal, 2019).

T2D is a multifactorial disease. Generally, people of non-white ancestry, people with a family history, and the aging population are at greater risk for T2D (Kaku, 2010). While genetic predisposition is a major risk factor, significant environmental variables contributing to the rising frequency of the disease worldwide include obesity, physical inactivity, energy-dense diets (Chatterjee et al., 2017), and microbiome composition (Gurung et al., 2020). As summarized in a meta-analysis, low socioeconomic status, pre-existing medical conditions (e.g., gestational diabetes, metabolic syndrome, premature birth), heavy alcohol consumption, smoking, and air pollution can also play a role in the manifestation of the disease (Bellou et al., 2018). Numerous risk factors mentioned above are associated with lifestyle factors, which highlights the importance of lifestyle modification in the prediabetic stage and T2D remissions.

### 2.2.3.  T2D management and prevention

T2D treatment and prevention strategies include obesity management, diet and lifestyle intervention, and pharmacological therapy (Chatterjee et al., 2017) to achieve a glycated hemoglobin $A_{1C}$ (HbA$_{1c}$) goal of $\leq 6.5\%$ for most people (Garber et al., 2020). Lifestyle modification is defined as increased physical activity in the combination of a low-fat, low-calorie diet aimed at weight loss (Diabetes Prevention Program Research Group, 2002). It is one of the primary therapies in T2D management (Zheng et al., 2018). Lifestyle modification is the most effective preventative method in reducing T2D risk in overweight or obese patients with impaired glucose tolerance across all genders, ethnicities, and

genetic predispositions, and with greater success than a T2D drug, metformin (Diabetes Prevention Program Research Group, 2002). However, the effectiveness of lifestyle intervention and adherence to medication outside of a controlled study is challenged by various economic, social, and environmental barriers (Chatterjee et al., 2017).

In combination with lifestyle modification, the first-line pharmacotherapy of choice is metformin (Chatterjee et al., 2017). Metformin is a weight-neutral drug that does not cause hypoglycemia, and it attenuates insulin resistance by decreasing hepatic glucose output, enhancing peripheral tissue sensitivity, and promoting glucagon-like peptide 1 (GLP-1; an incretin hormone produced in the intestines that acts on various target tissues) secretion  (DeFronzo et al., 2016; MacDonald et al., 2002). When metformin alone is unable to achieve an HbA$_{1c}$ level of $\leq$ 9%, additional glucose-lowering drugs may be prescribed in addition to metformin (Inzucchi et al., 2015). This is referred to as dual therapy. To minimize the occurrence of adverse events, circumstances special to each patient should be considered, including weight, HbA$_{1c}$ level, comorbidities, and hypoglycemia risk (Handelsman et al., 2015). In addition, triple therapy (three different glucose-lowering drugs prescribed at once) is implemented if the individual HbA$_{1c}$ target is not achieved after approximately three months of dual therapy (Inzucchi et al., 2015).

Common drugs used for dual and triple therapy include sulfonylureas, meglitinides, thiazolidinediones, GLP-1agonists, DPP-4 inhibitors, sodium-glucose cotransporter 2 (SGLT-2) inhibitors, $\alpha$-glucosidase inhibitors, and insulin (Apovian et al., 2019; Chatterjee et al., 2017; Habtemariam, 2019; Handelsman et al., 2015). These drugs improve HbA$_{1c}$ levels but have various advantages and disadvantages. Sulfonylureas effectively promote the stimulation of insulin secretion but have a severe risk for hypoglycemia (Garber et al.,

2020) and weight gain (Apovian et al., 2019). Meglitinides act similarly to sulfonylureas with lower hypoglycemia risk but are shorter-lasting and less effective (Apovian et al., 2019). Thiazolidinedione is the only drug that directly improves insulin sensitivity in target organs (Garber et al., 2020), however, weight gain due to fluid retention is a side effect. Incretin therapies include GLP-1 agonists and DPP-4 inhibitors. Injections of GLP-1 agonists promote GLP-1 action which can aid in weight loss (Garber et al., 2020), where GLP-1 is a potent gastric incretin hormone with glucose-dependent insulinotropic and glucagonostatic properties (Sharma et al., 2018). Similarly, DPP-4 inhibitors are weight-neutral agents that enhance GLP-1 levels by inhibiting DPP-4 enzyme action, thereby causing glucose-dependent insulin synthesis and secretion (Garber et al., 2020). SGLT-2 inhibitors are glucosuric, which results in weight loss and reduced systolic blood pressure (Garber et al., 2020). Moreover, inhibition of starch-digesting and glucose-releasing $\alpha$-glucosidase enzymes delays carbohydrate digestion in the small intestines and thus lowers postprandial blood glucose levels (Apovian et al., 2019). While the actions of $\alpha$-glucosidase inhibitors may promote weight loss, nausea, and dose-dependent gastrointestinal side effects (such as diarrhea, flatulence, and bloating) may also accompany. Lastly, initiation of insulin therapy can be considered if the oral agents discussed above do not provide sufficient glycemic control or if severe hyperglycemia is present, where $A_{1c} \geq 10\text{-}12\%$ (Inzucchi et al., 2015), which is inevitable for many diabetic patients. In summary, common problems with pharmacological treatment of T2D can include the risk of hypoglycemia, short duration of action, fluid retention, weight gain, and gastrointestinal discomfort. Overall, the optimal T2D management program consists of a combination of lifestyle intervention, glucose-lowering therapy with individualized

glycemic targets, as well as structured education and self-management programs with psychological support (Chatterjee et al., 2017).

### 2.2.4. Dietary approach to T2D management

A significant association is drawn between T2D and obesity (Verma & Hussain, 2017). Dietary habits play a major role in T2D development and progression (Guo & Ling, 2015), where a high intake of carbohydrates, fat, and sugar, particularly fructose is associated with the development of T2D (Sami et al., 2017). However, certain dietary habits are strongly associated with the prevention and management of the disease (Russell et al., 2016). In recent years, due to their beneficial physiological functions, an increasing number of studies are conducted on the usage of dietary phytochemicals as potential chronic disease management agents (Adyanthaya et al., 2010). Dietary plant (poly)phenols and (poly)phenol-rich foods (e.g. fruits and vegetables) have been reported to decrease blood sugar and are protective against T2D (Rupasinghe et al., 2017), with one of the many effects being inhibitory activities of critical starch digestive enzymes such as α-amylase and α-glucosidase, resulting in the retardation of carbohydrates degradation (Asgar, 2013; Rasouli et al., 2017). A positive association has been demonstrated between fruit consumption and lowered risk of developing T2D, as well as decreased risks of death and development of major vascular complications among diabetic individuals (Du et al., 2017). In summary, given the undesirable side-effects associated with anti-T2D drugs, the demonstration of the potential protective effect of natural fruit-(poly)phenol consumption concerning the management and prevention of T2D by the broad scientific evidence justifies the investigation of T2D prevention using a (poly)phenol-rich apples.

## 2.3. Anti-diabetic effects of apple (poly)phenols

Apples are a good source of dietary flavonoids that have been proposed to have natural anti-diabetic effects, and apple consumption is found to reduce T2D risk by 18% (Guo et al., 2017). One study compared the risk of T2D in women who consumed one apple a day with women who did not and reported that the risk of T2D was reduced by 28%. (Song et al., 2005). Apple (poly)phenols, whether taken individually (Li et al., 2020; Zhao et al., 2019) or in synergy with other fruits and vegetables (Agustinah et al., 2016; Schloesser et al., 2017), have shown promising glycemic controlling properties. Evidence of apple (poly)phenols on blood glucose management are widely reported *in vitro*, revealing their actions on therapeutic targets such as carbohydrate-hydrolyzing enzymes, α-glucosidase, and α-amylase (Gong et al., 2020a; Nkuimi Wandjou et al., 2020; Zhao et al., 2019), and advanced glycation end products (Cianfruglia et al., 2020; Khangholi et al., 2016).

In terms of the reported effects of apple (poly)phenol in animal studies, Egyptian Anna apple juice and apple peel extract were reported as hypoglycemic, hypolipidemic, cardioprotective, antioxidative, and anti-inflammatory in a chemically induced diabetic rat model (Fathy & Drees, 2016). Using diabetic mice, Ogura et al. (2016) reported that the oral administration of procyanidins (the main ingredient of apple (poly)phenols) ameliorated glucose intolerance, insulin resistance, and hepatic gluconeogenesis, as well as suppressed inflammation expression (Ogura et al., 2016). Apple (poly)phenols were also found to inhibit intestinal dietary carbohydrate glucose absorption by inhibiting sodium-coupled glucose transporter 1 (SGLT1)-mediated glucose uptake (Schulze et al., 2014), improving insulin sensitivity (Manzano et al., 2016), trapping AGE (Sampath et al., 2017),

increasing antioxidant capacity *in vivo* (Xu et al., 2019) and reducing fat deposition in obese mice (Tamura et al., 2020) and finishing pigs (Xu et al., 2019). It was noted that the DPP-4 inhibition properties of apple (poly)phenols are under-explored. As for the effect of apple (poly)phenol consumption in T2D patients, apple (poly)phenol supplementation significantly improved glycemic response and glucose tolerance in healthy (Makarova et al., 2015; Prpa et al., 2020; Sansone et al., 2018), borderline diabetic (Shoji et al., 2017), and T2D human subjects (Gheflati et al., 2019; Kausar, 2019). Overall, results from various *in vitro, in vivo*, and clinical research all pointed to the promising anti-diabetic effects of apple consumption (dried, fresh, (poly)phenol extract, and processed products) (Manzano et al., 2016). Therefore, with the consideration of the availability, cost-effectiveness, unique (poly)phenolic composition, and existing scientific evidence, apple (poly)phenols are investigated for *in vitro* and *in vivo* anti-diabetic properties in this study.

### 2.3.1. Carbohydrate-hydrolyzing enzyme inhibition

α-Amylase and α-glucosidase are carbohydrate-hydrolyzing enzymes involved in converting dietary polysaccharides and disaccharides into glucose (Hanhineva et al., 2010). Present in the mouth and small intestines, salivary/pancreatic α-amylase breaks the α-1,4-glycosidic linkages of starch molecules, turning starch into smaller oligosaccharides (Gurung et al., 2013). The oligosaccharides are further hydrolyzed into glucose by α-glucosidase located in the enterocyte brush border for absorption into the blood by glucose transporters (Gong et al., 2020a). α-Amylase and α-glucosidase and inhibitors are marketed as anti-diabetic drugs as they compete with the enzyme activity and slow down carbohydrate digestion, thereby reducing the rate of glucose absorption in the small intestines and preventing the rapid post-prandial blood glucose rise (Eleazu et al., 2018;

Xiao & Hogger, 2014). Various plant-food (poly)phenols have been observed to exhibit carbohydrate-hydrolyzing enzyme inhibitory activities in virtually all (poly)phenols analyzed, including in fruit juices (He et al., 2017), potato (Kalita et al., 2018), black tea (Striegel et al., 2015), legumes (Tan et al., 2017), apple (Ci et al., 2018; Sun et al., 2016), berries (De Silva & Rupasinghe, 2020; Rupasinghe et al., 2017), and olive oil (Figueiredo-González et al., 2019), to name a few. Most studies report (poly)phenols exerting more potent α-glucosidase inhibition than α-amylase inhibition (Figueiredo-González et al., 2019; Striegel et al., 2015; Yilmazer-Musa et al., 2012).

### 2.3.2 Dipeptidyl-peptidase-4 (DPP-4) enzyme inhibition

DPP-4 enzyme is found in almost all human organs and plays numerous roles in various physiological processes (Patil et al., 2015). Concerning glucose homeostasis, DPP-4 naturally degrades glucose-regulating incretin hormones such as glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) (Ríos et al., 2019). These incretin hormones are secreted in response to food ingestion to regulate blood glucose by stimulating insulin secretion, suppressing glucagon release, delaying nutrient absorption, and modulating appetite (Waldrop et al., 2018). The greater spike in insulin secretion in response to an oral load of glucose compared with an intravenous load of glucose is coined the "incretin effect" (Holst et al., 2021). T2D patients exhibit impaired or decreased incretin effect (Holst et al., 2021), which is associated with decreased insulin secretion, increased postprandial glucagon levels, and elevated postprandial glucose (Patil et al., 2015). Thus, the protection of these insulin-secreting hormones is important in the maintenance of glucose homeostasis.

The inhibition of DDP-4 enzymes is an appealing therapeutic target for preserving incretin hormones and prolonging insulin action. Natural DPP-4 inhibition by extracts and bioactives of fruits, vegetables, and many plants including culinary herbs (Bower et al., 2014), haskap berries (De Silva & Rupasinghe, 2020), grape seed-derived procyanidins (Li et al., 2016a) and proanthocyanidins (Casanova-Martí et al., 2018), black currant (Tani et al., 2017), black soybean (Qiu et al., 2018), and yam (Go et al., 2015) has been reported. (Poly)phenols increase GLP-1 concentrations *in vitro* (Les et al., 2018; Tani et al., 2017), in diabetic animal models (Casanova-Martí et al., 2018; Zhang et al., 2014), and overweight/T2D patients (Boix-Castejón et al., 2018; Liu et al., 2014). Increased serum insulin (Qiu et al., 2018) and appetite suppression (Zanzer et al., 2018) by (poly)phenol supplementation have also been reported. Apple (poly)phenols have not been investigated as a potential DPP-4 inhibitor.

### 2.3.3 Inhibition of the advanced glycation end products (AGE) formation

AGE is produced during the glycation process, which occurs when the carbonyl group of reducing sugar (i.e. glucose) binds to the free amino group of an amino acid or lipids in a non-enzymatic reaction (Crascì et al., 2018). This reaction yields irreversible and unstable early glycation products named Schiff bases, which convert to the stable intermediate glycation products, Amadori products, following dehydration and rearrangement, and lastly leads to the formation of AGE (Khan et al., 2019; Khangholi et al., 2016). The glycation process produces reactive carbonyl species (RCS; i.e. glyoxal, methylglyoxal, and 3-deoxyglucosone) (Anwar et al., 2021), and these glycation intermediates can further react with protein to form AGE (Wang et al., 2011). Additionally, exogenous AGE is introduced to the body from the ingestion of foods prepared from high

temperatures cooking methods such as broiling, grilling, roasting, searing, and frying (Goldberg et al., 2004; Uribarri et al., 2010). Toxic accumulation of AGE and AGE intermediates causes dicarbonyl distress, oxidative damage, and inflammation due to cell and tissue dysfunction (Cianfruglia et al., 2020; Spínola et al., 2019), and contributes to aging and chronic diseases such as diabetes and its complications, Alzheimer's disease, kidney disease, and chronic heart failure development (Starowicz & Zieliński, 2019).

AGE formation is increased in hyperglycemic conditions and promotes diabetes complications (Khan et al., 2019). Natural inhibitors such as (poly)phenols suppress AGE formation through several mechanisms (Khangholi et al., 2016), including regulating glycemic control to alleviate hyperglycemia (Kim et al., 2016), scavenging free radicals to protect against carbonyl and oxidative stress (Cianfruglia et al., 2020), metal chelating (Bhuiyan et al., 2017), trapping AGE precursors RCS (Sampath et al., 2016), breaking AGE-related protein cross-linking (Kim et al., 2011), and anti-lipid oxidation (Alam et al., 2015). Thus, the T2D-protective effect of (poly)phenol-rich apples by the inhibition of AGE formation was investigated in this study.

## 2.4. Experimental animal model of diet-induced obesity and glucose intolerance

Animal models are widely used in the discovery, validation, and optimization of novel therapeutics for safe human consumption (Fang et al., 2019). While no single animal model covers all the characteristics of T2D etiology, pathophysiology and complications, animal models can still mirror at least one aspect of the disease (Cefalu, 2006). The various T2D animal models include non-mammalian, large animals, non-human primates, and rodent models. The rodent model can be further divided into monogenic (Lep ob/ob and

Lepr ob/ob mouse), polygenic (New Zealand Obese mouse and TALLYHO/Jng mouse), diet-induced obesity (DIO; polygenic C57BL/6J mouse), and chemical-induced (streptozotocin) models (Fang et al., 2019). Through genetic modification, systemic or tissue-specific up or down-regulation of genes provides valuable insights into the physiological roles potential targets play in glucose metabolism and diabetes manifestation (Bowe et al., 2014). This study utilized the DIO model with C57BL/6 mice to test the potential effects of apple (poly)phenol supplementation on body weight gain and glucose and lipid homeostasis. C57BL/6 mice can easily develop obesity, glucose intolerance, and insulin resistance, collectively referred to as pre-diabetes, when challenged with high-fat feeding, similar to the human T2D etiology (Fang et al., 2019), and male mice tend to display a stronger disease phenotype than female mice (Goren et al., 2004). The DIO model creates rapid weight gain and insulin resistance from increased dietary energy intake and decreased metabolic efficiency (Winzell & Ahrén, 2004). This model is thought to better mirror human obesity than the genetic-modified models since obesity development is manipulated environmentally through diet induction, rather than through genetic manipulation (King & Bowe, 2016), and thus, was the chosen model for this study.

### 2.4.1. Metabolic tolerance tests

Metabolic tolerance tests (MTT) provide simple readouts on systemic impairment of carbohydrate metabolism using blood glucose or insulin levels at fasting state or after stimulation (Nagy & Einwallner, 2018) and are widely used in the screening of glucose intolerance and early insulin resistance for diabetes diagnosis (Benedé-Ubieto et al., 2020). Depending on the animal model and study endpoints, different MTT methodologies include the glucose clamp (DeFronzo et al., 1979), glucose tolerance tests (GTT), insulin

tolerance tests (ITT), pyruvate tolerance test, alanine, and glutamine tolerance tests, lactate minimum test, and metformin tolerance test (See Benedé-Ubieto et al., 2020 for detailed descriptions).

Of the various MTTs mentioned, GTT and ITT are two of the most employed methods. GTT, as the name suggests, provides an overview of glucose tolerance. It involves administrating a glucose load orally (OGTT) or intraperitoneally (IPGTT) after an overnight fast and measuring blood glucose concentration before and across the two hours after glucose administration (Bowe et al., 2014). Healthy glucose metabolism would demonstrate a rapid blood glucose elevation after glucose challenge, peaking at 15 min post-challenge, and gradually decreasing to normal after approximately 60-90 min. This is facilitated by numerous body processes, such as increased insulin secretion, activation of tissue glucose disposal in liver, fat, and muscle tissues (Benedé-Ubieto et al., 2020), inhibition of endogenous glucose production, and regulation of glucose entry in splanchnic organs in the abdominal cavity (stomach, intestines, pancreas, spleen, and liver) to counteract postprandial hyperglycemia and hyperinsulinemia (Kowalski & Bruce, 2014). Impaired glucose tolerance is revealed by increased glucose concentrations or increased cumulative area under the curve (AUC) when compared with the control group. To complement GTT results on glucose tolerance, ITT is often performed. Similar to GTT, ITT monitors blood glucose levels over time in response to exogenous insulin administration (Benedé-Ubieto et al., 2020), providing insights into insulin sensitivity in target tissues such as the liver and skeletal muscle. Insulin resistance is indicated by diminished blood-glucose-lowering rates compared with control.

### 2.4.1.1. Comparisons of GTT glucose administration methods

Oral gavage (OG) and intraperitoneal injections (IP) are the two most common methods for glucose delivery in GTT. OG offers the most physiological route for glucose absorption as glucose is absorbed from the small intestines, draining through the mesenteric vein into the portal vein and hepatic sinusoids (Kowalski & Bruce, 2014). It is the only method of delivery that induces portal vein hyperglycemia and hyperinsulinemia and promotes the release of insulin-stimulating incretin hormones, thereby strongly stimulating glucose uptake and glycogen storage in the liver (Cherrington, 1999). However, the expertise required to perform OG and the stress imposed on the animals can often create greater variability and sometimes cause injury (Benedé-Ubieto et al., 2020). Even though procedure stress and complications can be minimized by the usage of anesthesia, they can alter blood glucose and plasma insulin dynamics and confound results. A common, easier alternative to OG is IP delivery (Al Shoyaib et al., 2019). Injected into the peritoneal cavity, solutes are absorbed into the mesenteric vessels and emptied into the portal vein, merging into the systemic circulation after undergoing first-pass metabolism in the liver (Al Shoyaib et al., 2019; Benedé-Ubieto et al., 2020). The similar pharmacokinetics to that of OG and the ease of performance of the procedure make IP injections a desirable delivery method (Benedé-Ubieto et al., 2020). For simplicity, IP was the route of delivery chosen for GTT in this study.

### 2.4.2. Hyperlipidemia

Apart from impairing glucose metabolism, insulin resistance and T2D also lead to alterations in systemic lipid metabolism, causing hyperlipidemia (Ormazabal et al., 2018). Diabetes-associated hyperlipidemia is characterized by elevated total cholesterol,

triglycerides (TG), lowered high-density lipoprotein (HDL) cholesterol, and a marked increase of small dense low-density lipoprotein (LDL) cholesterol (Hill & Bordoni, 2022; Thapa et al., 2017), all of which strongly increase the risk of developing cardiovascular diseases (Ormazabal et al., 2018). Similar to T2D, the first line of therapy for hyperlipidemia is diet and lifestyle modification, then pharmacological therapies can be added if needed (Hill & Bordoni, 2022). Evidence of hyperlipidemia alleviation has been reported using apple (poly)phenols extracts (Li et al., 2021; Li et al., 2016b; Sekhon-Loodu et al., 2014; Susilowati et al., 2020; Tian et al., 2018; Yao et al., 2014) or apple products, including apple juice (Harsono et al., 2021; Ravn-Haren et al., 2013), fermented apple juice (Li et al., 2022), apple cider vinegar (Kausar et al., 2019), and apple pomace (Umbreen et al., 2020). Therefore, it is hypothesized that supplementation of high-(poly)phenol-containing Daux Belan apple powder would exert benefits on high-fat-induced dyslipidemia.

# CHAPTER 3: (POLY)PHENOLS OF APPLES CONTRIBUTE TO *IN VITRO* ANTI-DIABETIC PROPERTIES: ASSESSMENT OF CANADA'S APPLE BIODIVERSITY COLLECTION

## 3.1. Abstract

The recent trend in sedentary lifestyles and nutritionally dense diets is type 2 diabetes prevalence in many parts of the world. While effective, pharmacologic glycemic management can cause undesirable gastrointestinal side effects and hypoglycemia. Thus, there is a popular appeal in safe and natural glycemic management using dietary (poly)phenols. In this study, (poly)phenol extracts of 476 apple accessions from Canada's Apple Biodiversity Collection (ABC) and six major apple (poly)phenol compounds identified were assessed for *in vitro* anti-diabetic properties against α-glucosidase, α-amylase, dipeptidyl peptidase-IV (DPP-4), and advanced glycation end products (AGE) formation activities. Apple (poly)phenol extracts varied in their anti-diabetic actions in a dose-dependent manner. High (poly)phenol-containing apples demonstrated superior anti-diabetic action and total (poly)phenol contents (TPC) were inversely correlated with the IC$_{50}$ values of α-glucosidase, α-amylase, and AGE formation, but not DPP-4. Major (poly)phenol compounds in apples, such as procyanidin B2, phloridzin, and epicatechin concentrations were significantly correlated with IC$_{50}$ values of α-glucosidase enzyme in the high (poly)phenol-containing apples. High TPC apples are not suitable for marketing as fresh fruit due to bitterness and astringency associated with the high (poly)phenol content; however, these apples show potential to use in the development of value-added functional ingredients or nutraceuticals for blood glucose management. The high TPC apple, 'S23-03-749', a dessert apple advanced breeding line, introduces a novel option for apple supplementation in the whole food form for the dietary management of glycemia.

## 3.2. Introduction

Apples are a popular fruit worldwide that is readily available year-round due to their long-term storability and production in both hemispheres of the globe (Bossi Fedrigotti & Fischer, 2020). Despite the tremendous biodiversity in documented apple cultivars, consumers are only exposed to a small portion of the genetic variation in the supermarket as a consequence of repeated use of a handful of popular varieties in breeding (Migicovsky et al., 2021; Watts et al., 2021). Most commonly consumed fresh, apples

provide a good source of nutrients such as dietary fibre, organic acids, sugars, vitamins, and minerals (Anastasiadi et al., 2017; Wu et al., 2018). Apples also contain dietary (poly)phenols, which are secondary metabolites produced by plants with anti-oxidative and antimicrobial properties against ultraviolet light and pathogens (Cao et al., 2019; Siemińska-Kuczer et al., 2022). The five major (poly)phenol groups include flavan-3-ols/procyanidins (epicatechin and procyanidins), phenolic acids (chlorogenic acid), dihydrochalcones (phloridzin), flavonols (quercetin glycosides), and anthocyanins (cyanidin 3-galactoside) (Huber & Rupasinghe, 2009; Kschonsek et al., 2018; Rupasinghe et al., 2017). Due to the beneficial roles of (poly)phenols in human health, there is increasing interest in using dietary (poly)phenol supplementation in the prevention of chronic diseases (Kawabata et al., 2019).

Type 2 diabetes (T2D) is a serious, chronic metabolic disorder that is elevating in global prevalence (Saeedi et al., 2019). Over half a billion adults between the ages of 20 and 79 years suffer from diabetes, with T2D accounting for more than 90% of diabetes cases (International Diabetes Federation, 2021). T2D is characterized by hyperglycemia, hypertension, and hyperlipidemia as a result of insulin resistance or deficiency (Kang et al., 2020), and can further develop into diabetes-related complications if left untreated. In the case of metabolic disorders, specifically T2D, dietary habits have a significant impact on the prevention and management of the disease (Russell et al., 2016). There is a positive correlation between habitual fruit consumption and lowered diabetes prevalence, as well as reduced risks of major complications in diabetic patients (Du et al., 2017), which include cardiovascular diseases, autoimmune diseases, development of fibrosis, inflammation, eye complications, neuropathy, renal failure, infection, and cancer (Sun et al., 2020).

Apples help maintain glucose homeostasis and can reduce T2D risks by 18% (Guo et al., 2017) through (poly)phenolic actions such as improving β-cell function, increasing insulin sensitivity, reducing inflammation and lipotoxicity, lowering hepatic glucose output, and regulating carbohydrate metabolism (Kang et al., 2020). Specifically, dietary (poly)phenols signal for the blockage of critical digestive enzyme activities, including α-amylase and α-glucosidase (Rasouli et al., 2017), thereby delaying glucose absorption into the bloodstream. In addition, the inhibition of dipeptidyl peptidase-4 (DPP-4) by (poly)phenols (Kang et al., 2020) is protective against the breakdown of incretins, which are gut hormones secreted after food ingestion to stimulate insulin production for glycemic control (Nauck & Meier, 2018), hence suppressing postprandial hyperglycemia. Moreover, advanced glycation end products (AGE) are cell-damaging, oxidative-stress-inducing products of the reaction between the carbonyl group of reducing sugars and the amino group of protein, lipids, and DNA (Khangholi et al., 2016). AGE formation is accelerated by prolonged hyperglycemia, and accumulation of AGE can increase the risks of diabetes complications (Khangholi et al., 2016). Other than regulating blood glucose levels to alleviate hyperglycemia, (poly)phenols can exert antiglycation effects by 1) competing with sugars to prevent sugar-protein linkages, 2) acting as antioxidants and free-radical scavengers to ameliorate oxidative stress by trapping or inhibiting reactive carbonyl species formation, 3) chelating metal ions that are linked with AGE formation, 4) breaking AGE protein crosslinks, and 5) blocking AGE interaction with receptors of AGE (Anwar et al., 2021).

The beneficial effects of apples on human health are long recognized, and recently, the genetic diversity of apples has been receiving wider interest (Dar et al., 2019; Marconi

et al., 2018; Omasheva et al., 2018). This present study screened an extensive collection of apple accessions (n = 476), including wild *Malus sievers*ii apples, heritage cultivars, commercial cultivars, and advanced breeding lines to evaluate for inhibition of α-glucosidase, α-amylase, DPP-4, and AGE formation, compared to hyperglycemic agents. Additionally, due to the large sample size, (poly)phenol compound composition was characterized in selected 10 apple accessions from each of the high total (poly)phenol content (TPC) group, commercial apple group, and low TPC group to represent the population. Six major apple (poly)phenol compounds were also evaluated for the same anti-diabetic properties to understand their contribution. Lastly, the relationship between TPC and the inhibition of α-glucosidase, α-amylase, DPP-4, and AGE formation was investigated.

## 3.3. Materials and methods

### 3.3.1. Sample collection and preparation

Apples (n = 476 accessions) from Canada's Apple Biodiversity Collection (ABC; Agriculture and Agri-Food Canada Research and Development Centre, Kentville, Nova Scotia, Canada; 45°04′08″N 64°28′41″W) were collected at the commercial maturity stage during the 2016 harvest (See Watts et al. (2021) for a detailed description of ABC). Fifteen fruits were collected for each accession (planted in duplicate), maintained in cold storage at 3°C for one month, and cored and flash-frozen in liquid nitrogen. For every accession, apple flesh with peel was pooled and ground into a fine powder and stored at -80°C in 50 mL Falcon® tubes.

### 3.3.2. Source of chemicals

For the metabolomic analysis, all reagents were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise stated. Procyanidin B1, B2, and C1 were obtained from Indofine Chemical Company (Hillsborough, NJ), and quercetin 3-glucoside was purchased from Fluka Chemie GmbH (Buchs Switzerland). HPLC grade solvents were obtained from Fisher Scientific (Georgetown, ON). For the anti-diabetic assays, all chemicals used were purchased from Sigma Aldrich (St. Louis, MO), except for the procyanidin B2, cyanidin 3-galactoside, and quercetin 3-$O$-galactoside, which were obtained from Chengdu Alfa Biotechnology (Chengdu, China).

### 3.3.3. Identification and quantification of (poly)phenolic composition in selected apple accessions

To identify and quantify (poly)phenolic composition in ABC apple accessions, 30 selected apple accessions were chosen to represent the population based on their previously reported TPC values (Watts et al., 2021). Ten accessions were selected from each of the following groups: high (poly)phenol group (10 accessions with the highest TPC), commercial group (10 popular commercial dessert and cider apples in North America), and low (poly)phenol group (10 accessions with the lowest TPC).

Apple (poly)phenols were extracted from tissue containing flesh and peel using sonication-assisted 80% methanol (containing 0.1% formic acid). Each apple sample (0.5 g) containing flesh and peel was weighed into a 2 mL microcentrifuge tube and dissolved in 0.7 mL of 80% methanol. The sample was vortexed and sonicated for 20 minutes. Next, the sample was vortexed again and centrifuged for 10 minutes at room temperature at 10,000 × $g$ (Thermal ICE Microlite, Fisher Scientific Company, Ottawa, Ontario). The

supernatant was transferred into a new 2 mL microcentrifuge tube. An additional 0.7 mL of 80% methanol was added to the leftover pellet, and the tube was vortexed and sonicated for another 20 min, then further vortexed, centrifuged (10 min, 13,000 × $g$), and the supernatants from the two extractions were pooled. In a vacuum centrifuge (ThermoFisher), the pooled supernatants were dried, and 1 mL of 10% methanol (0.1% formic acid) was added to the pellet, vortexed, sonicated for 15 seconds, further vortexed for 10 seconds, and finally centrifuged at 10,000 × $g$ for 10 minutes. The supernatants were collected in HPLC vials for injection (Gong et al., 2018). Each accession was extracted in duplicate.

Untargeted apple (poly)phenol identification was performed using an LC-qTOF-MS system consisting of a NanoAcquity UPLC system (Waters Corporation, Milford MA, USA) with a BEH C$_{18}$ 1.7 µm 1.0 × 100 mm column for chromatography separation and an electrospray ionization-equipped mass spectrometer (Synapt XS HDMS; Waters Corporation, Milford MA, USA) was used for mass detection. Data processing was conducted using Progenesis QI (Version 3.0, Nonlinear Dynamics, Waters Corporation, Milford MA, USA). For the identification of apple (poly)phenol compounds, mass features and retention times were validated utilizing reference standard compounds (Chlorogenic acid, cyanidin 3-galactoside, quercetin 3-rhamnoside, quercetin 3-glucoside, phloridzin, epicatechin, catechin, procyanidin B1, B2, and C1).

Quantification of apple (poly)phenol compounds was performed as described by Gong et al. (2018). The same standard compounds listed above were used in the creation of calibration curves, which were used to calculate the absolute amount of each compound. The results were averaged from the two individually extracted replicates.

### 3.3.4. (Poly)phenol extraction for anti-diabetic assays

A fresh set of apple (poly)phenols were extracted in duplicates from the same frozen apple powder containing flesh and peel used for LC-qTOF-MS analysis using sonication-assisted 80% methanol (containing 0.1% formic acid) (Watts et al., 2021). Each apple sample (approximately 0.5 g) containing flesh and peel was weighed into a 2 mL microcentrifuge tube and dissolved in 0.7 mL of 80% methanol. The sample was vortexed and sonicated for 20 min. The sample was vortexed again and centrifuged for 10 min at room temperature at 13,000 × $g$ (Sorvall™ Legend™ Micro 17, Thermo Fisher Scientific Inc., Ottawa, ON, Canada). The supernatant was transferred into new 2 mL microcentrifuge tubes. An additional 0.7 mL of 80% methanol was added to the leftover pellet, and the tube was vortexed and sonicated for another 20 min, then further vortexed, centrifuged (10 min, 13,000 × $g$), and collected for supernatant. The tube was centrifuged once more (10 min, 13,000 × $g$), and the supernatants were pooled. The apple (poly)phenol extracts were stored at -80°C in 2 mL microcentrifuge tubes. Each accession was extracted in duplicates.

### 3.3.5. *In vitro* anti-diabetic assays

Apple extracts were screened for α-amylase, α-glucosidase, DPP-4, and AGE inhibition starting with the accessions with the highest TPC (Folin-Ciocalteu method) until no inhibitory activity was detected (less than three dose-dependent % inhibition data points). The inhibitory activities were expressed as the half-maximal inhibitory concentration (IC$_{50}$) in mg/mL, indicating the concentration of the apple extracts capable of inhibiting 50% of enzyme activity or AGE formation. For the three enzyme inhibitory assays, positive control reactions were carried out according to the procedures outlined, except the volume of apple extracts was replaced by assay buffer. The sample controls

consisted of apple extracts without enzyme and substrate, with the missing volumes replaced by assay buffer.

### 3.3.5.1. α-Amylase enzyme inhibition

The inhibitory assay of the porcine pancreas α-amylase enzyme was performed as previously described (Proença et al., 2019) with slight modifications. The modifications include increasing α-amylase enzyme concentration (0.2 U/mL to 4 U/mL) and 2-chloro-4-nitrophenyl-α-D-maltotrioside (CNPG3) substrate concentration (0.5 mM to 5 mM), as well as using 0.01 M potassium phosphate buffer (PBS) in place of 0.02 M sodium phosphate buffer. Apple test extracts were prepared by evaporating methanol and redissolving in 0.01 M potassium phosphate buffer (PBS; pH 6.8) before diluting to a series of seven concentrations (5-35 mg/mL). Chlorogenic acid (1,500-6,000 µg/mL; 6% final DMSO concentration), cyanidin 3-galactoside (150-4,500 µg/mL; 4% DMSO), epicatechin (900-10,000 µg/mL; 4% final DMSO concentration), phloridzin (3,000-10,000 µg/mL; 10% final DMSO concentration), procyanidin B2 (600-2,400 µg/mL; 10% final DMSO concentration) and quercetin 3-*O*-galactoside (600-2,400 µg/mL; 10% final DMSO concentration) were dissolved in DMSO and diluted in PBS. The diabetes drug acarbose was used as the reference inhibitor, diluted to 0.1, 1, 2.5, 5, 10, 25, 50, and 100 µg/mL in PBS. The test extract or acarbose (20 µL) was added to the enzyme (20 µL; 4 U/mL in PBS; Sigma A3176) in a 96-well foil-covered microplate and pre-incubated at 37 °C for 10 minutes. The substrate, 2-chloro-4-nitrophenyl-α-D-maltotrioside (20 µL; 5 mM in PBS; Sigma 93834), was added to the plate to initiate the reaction and incubated at 37 °C for 30 min. Trisodium phosphate solution (240 µL; 1% w/v; pH 11) was added to stop the reaction. After a 10-second shake by the plate reader, the absorbance was read at 405 nm to quantify

the amount of phenolate ion. The IC$_{50}$ value was determined using GraphPad Prism software, where a dose-response curve was fitted using the log of extract concentrations and percent inhibition. The inhibition percentage was calculated using the formula below:

$$\text{Inhibition (\%)} =$$

$$\frac{(control\ absorbance - control\ blank) - (sample\ absorbance - sample\ blank\ absorbance)}{control\ absorbance - control\ blank} \times 100$$

### 3.3.5.2.    α-Glucosidase enzyme inhibition

The inhibitory assay of the α-glucosidase enzyme of *Saccharomyces cerevisiae* was performed as previously described (De Silva & Rupasinghe, 2020; Watanabe et al., 1997) with slight modifications. The α-glucosidase enzyme concentration was reduced from 1 U/mL to 0.25 U/mL. The apple extracts and reference drug acarbose were prepared the same way as in the α-amylase assay above, except acarbose was diluted to the initial concentrations of 0.01, 0.1, 1, 10, 100, 250, 500, and 1000 µg/mL. The initial concentration ranges of the major (poly)phenol compounds present in apples were as follows: Chlorogenic acid (10-45 µg/mL; 1% final DMSO concentration), cyanidin 3-galactoside (100-425 µg/mL; 4% final DMSO concentration), epicatechin (0.03-300 µg/mL; 1% final DMSO concentration), phloridzin (0.03-300 µg/mL; 1% final DMSO concentration), procyanidin B2 (0.03-300 µg/mL; 2% final DMSO concentration), and quercetin 3-*O*-galactoside (5-130 µg/mL; 4% final DMSO concentration) were dissolved in DMSO and diluted in PBS. α-Glucosidase enzyme (G5003 Sigma; 0.25 /mL) was diluted using 0.01M PBS containing 0.2% w/v bovine serum albumin (BSA). The substrate solution was prepared by dissolving 30.1 mg of 4-nitrophenyl α-D-glucopyranoside (PNPG; N1377

Sigma) in 20 mL of PBS. The stop solution (0.2 M) was prepared by dissolving 1.058 g of sodium carbonate in 50 mL of PBS.

In brief, 120 µL of apple test extract/acarbose and 20 µL of α-glucosidase enzyme solution were plated in a 96-well microplate. The plate was covered with foil and pre-incubated at 37 °C for 15 min. PNPG substrate (20 µL) was added to initiate the reaction. The plate was covered with foil and incubated for 15 mins at 37 °C. Following the incubation, 80 µL of 0.2 M sodium carbonate solution was added to the wells to stop the reaction. After 10 seconds of shaking by the plate reader, the plate was measured at 405 nm for p-nitrophenyl quantification. The $IC_{50}$ value was estimated using non-linear regression analysis in GraphPad Prism where a dose-response curve was fitted using the log of extract concentrations and percent inhibition. Percent inhibition was calculated using the formula below:

$$\text{Inhibition (\%)} = \frac{(control\ absorbance - control\ blank) - (sample\ absorbance - sample\ blank\ absorbance)}{control\ absorbance - control\ blank} \times 100$$

### 3.3.5.3.     DPP-4 enzyme inhibition

The inhibitory assay of the human DPP-4 enzyme was performed as described by De Silva & Rupasinghe (2020) with slight modifications to optimize enzyme and substrate concentrations. Apple (poly)phenol test extracts were prepared in the same manner as described in the α-amylase inhibition assay, except samples were diluted in the DPP-4 assay buffer. The diabetes drug sitagliptin (1-1,000 nM), prepared in the assay buffer (pH 7.8; containing 25 mM HEPES, 140 mM NaCl, 1% BSA, and 80 mM $MgCl_2$) was used as the reference compound. The initial concentration ranges of the major (poly)phenol compounds present in apples were as follows: Chlorogenic acid (24-1,800 µg/mL; 1.5%

final DMSO concentration), cyanidin 3-galactoside (3-360 µg/mL; 4% final DMSO concentration), epicatechin (36-1,800 µg/mL; 1.5% final DMSO concentration), phloridzin (180-3,000 µg/mL; 3.5% final DMSO concentration), procyanidin B2 (36-1,800 µg/mL; 1.5% final DMSO concentration), and quercetin 3-*O*-galactoside (90-960 µg/mL; 1% final DMSO concentration) were dissolved in DMSO and diluted in assay buffer. The test sample or sitagliptin (20 µL) was mixed with the DPP-4 enzyme (20 µL; 3.125 mU/mL in assay buffer) and assay buffer (30 µL) in a black 96-well plate. The substrate (50 µL; 2.5 µM in assay buffer), H-Gly-Pro-AMC, was added to all wells to initiate the reaction. The plate was then covered in foil and incubated at 37 °C for 30 min. After incubation, fluorescence was read using an excitation wavelength of 350 nm and an emission wavelength of 450 nm to quantify the rate of substrate hydrolysis for the determination of enzyme activity. The $IC_{50}$ value is estimated using non-linear regression analysis in GraphPad Prism where a dose-response curve is fitted using the log of extract concentrations and percent inhibition. Percent inhibition was calculated using the formula below:

$$\text{Inhibition (\%)} =$$

$$\frac{(control\ fluorescence - control\ blank\ fluorescence) - (sample\ fluorescence - sample\ blank\ fluorescence)}{control\ fluorescence - control\ blank\ fluorescence} \times 100$$

### 3.3.5.4.    AGE formation inhibition

The inhibitory assay of the AGE formation was performed as previously described (Melo et al., 2015) with slight modifications. Potassium phosphate buffer (PBS; 0.05M) was used in place of sodium phosphate buffer (0.1 M). Apple (poly)phenol test extracts were prepared by evaporating the methanol and re-dissolving in 0.05 M PBS (pH 7.4; containing 100 mM NaCl, 0.02% $NaN_3$ (w/v)) before diluting to the appropriate

concentrations (5-35 mg/mL). Aminoguanidine (1-3,000 μg/mL; dissolved in assay buffer) was used as the reference inhibitor. The initial concentration ranges of the major (poly)phenol compounds present in apples were as follows: Chlorogenic acid (0.03-90 μg/mL; 0.09% final DMSO concentration), cyanidin 3-galactoside (0.03-180 μg/mL; 0.18% final DMSO concentration), epicatechin (0.3-720 μg/mL; 0.72% final DMSO concentration), phloridzin (3-3,600 μg/mL; 3.7% final DMSO concentration), procyanidin B2 (0.03-240 μg/mL; 0.24% final DMSO concentration), and quercetin 3-*O*-galactoside (0.03-150 μg/mL; 0.15% final DMSO concentration) were dissolved in DMSO and diluted in assay buffer. The test sample or reference inhibitor (200 μL), BSA (200 μL; 3 mg/mL) and glucose/fructose solution (200 μL; 200 mM glucose and 200 mM fructose) were added to a 1.5 mL microcentrifuge tube, vortexed, and incubated at 37 °C for seven days (agitated at 170 rpm). After seven days, samples were plated in triplicates (150 μL), and fluorescence intensities were measured using an excitation wavelength of 355 nm and an emission wavelength of 440 nm in black 96-well plates. The $IC_{50}$ value was determined using GraphPad Prism software, where a dose-response curve was fitted using the log of extract concentrations and percent inhibition. Percent inhibition was calculated using the formula below:

$$\text{Inhibition (\%)} = \frac{(control\ fluorescence\ -\ (sample\ fluorescence - sample\ blank\ fluorescence))}{control\ fluorescence} \times 100$$

### 3.3.6. Statistical analyses

In this observational study, the anti-diabetic activities of apple (poly)phenol extracts were measured in duplicates, and the anti-diabetic activities of apple (poly)phenol compounds were measured in triplicates. GraphPad Prism 5 (GraphPad Software Inc., La

Jolla, Calif, USA) was used for enzyme $IC_{50}$ calculations and total (poly)phenolic composition data curation. All other data curation and analyses were performed in R software (R Core Team 2021), with data visualized using the R package ggplot2 (Wickham, 2016).

## 3.4. Results

### 3.4.1. Anti-diabetic properties of apples in Canada's Apple Biodiversity Collection

Apple accessions from the ABC displayed variability in their dose-dependent anti-diabetic action against α-glucosidase, α-amylase, DPP-4, and AGE formation activities (Figure 3.1). Generally, α-glucosidase inhibition by the apple extracts was the most prominent compared to the other three parameters measured, given that α-glucosidase activity was suppressed by the greatest number of apple accessions. On the other hand, AGE formation inhibition was the parameter that was inhibited by the least number of apple accessions. An $IC_{50}$ value is the half-maximal inhibitory concentration and is used to measure the efficacy of a drug/compound. It represents the concentration of a drug/compound required to suppress a biological process by half (Aykul & Martinez-Hackert, 2016), where the lower the value, the more potent the drug/compound is. The $IC_{50}$ values of the 362 apple accessions that exerted α-glucosidase inhibition at the tested concentrations ranged from 7.1 mg/mL ('Marachel') to 364 mg/mL ('Blauacher'), with an over 50-fold difference between the highest and the lowest $IC_{50}$ values (Figure 3.1A). As well, $IC_{50}$ values of the 72 apple accessions that exerted α-amylase inhibition at the tested concentrations ranged from 4.3 mg/mL ('Crollon') to 68 mg/mL ('Crow Egg'), indicating a 16-fold difference between the highest and the lowest $IC_{50}$ values (Figure 3.1B). The $IC_{50}$

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values of the 24 apple accessions that exerted DPP-4 inhibition at the tested concentrations ranged from 7.0 mg/mL ('Kaz 96 08-01P-11') to 21 mg/mL ('Grise Dieppoise'), with a 3-fold difference between the highest and the lowest $IC_{50}$ values (Figure 3.1C). Lastly, the $IC_{50}$ values of the 27 apple accessions that exerted AGE formation inhibition at the tested concentrations ranged from 5.2 mg/mL ('Marachel') to 36 mg/mL ('Bongkushu (880626)'), with a 7-fold difference between the highest and the lowest $IC_{50}$ values (Figure 3.1D).
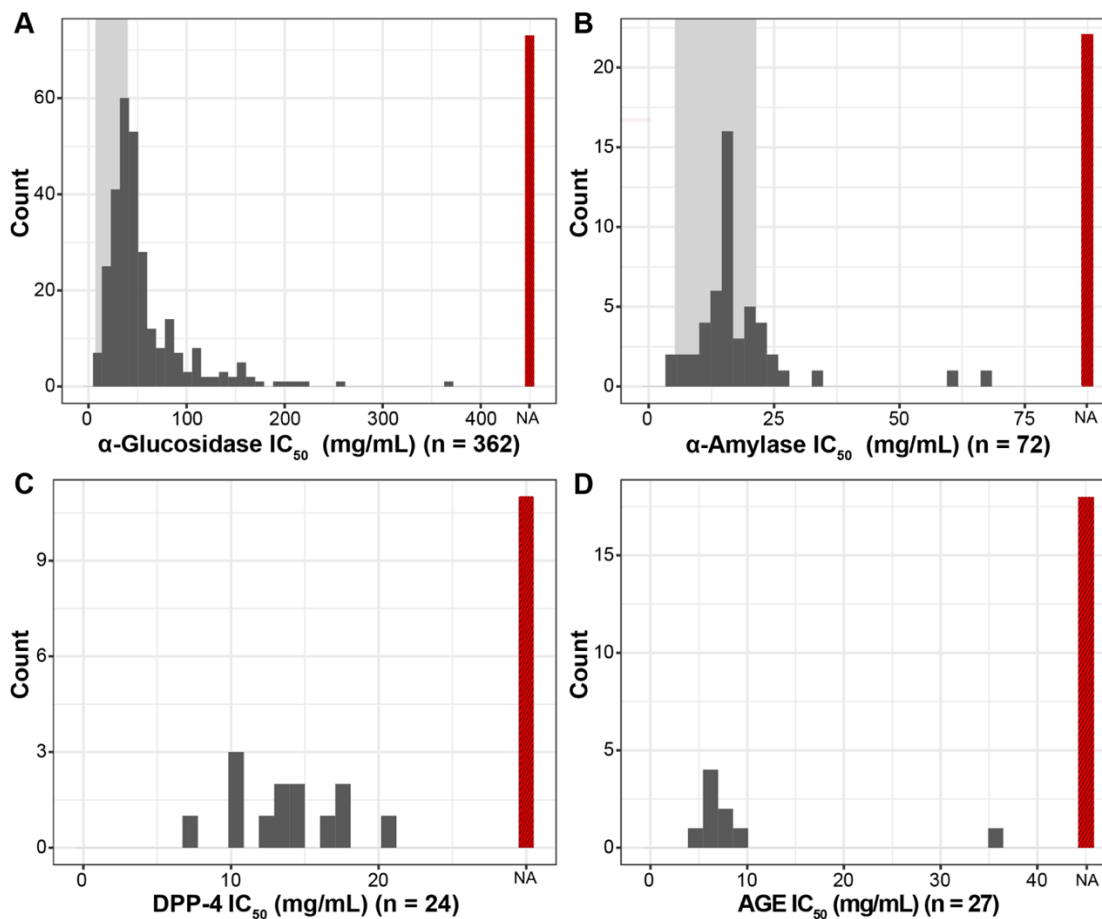
**Figure 3.1:** Distribution of *in vitro* anti-diabetic properties (IC$_{50}$) of apple accessions from Canada's Apple Biodiversity Collection - 2016 Harvest. (A) α-Glucosidase IC$_{50}$ values of 362 apple accessions; (B) α-Amylase IC$_{50}$ values of 72 apple accessions; (C) Dipeptidyl peptidase-4 (DPP-4) IC$_{50}$ values of 24 apple accessions; (D) Advanced glycation end products (AGE) formation IC$_{50}$ values of 27 apple accessions. Shaded areas in (A) and (B) indicate the range of IC$_{50}$ values for the 10 apple accessions with the highest total (poly)phenol content values. The red, striped bar on the right end of each sub-figure represents the counts of accessions with no detectable anti-diabetic activity. NA, no activity.

Here we are presenting in further detail the anti-diabetic $IC_{50}$ values of the 10 apple accessions with the highest TPC, referred to as the high TPC group (TPC values were previously determined by Watts et al. 2021 using the Folin-Ciocalteu method) (Table 3.1). The high TPC group can be categorized into three subgroups as follows according to apple origin, and use (Appendix A): the wild *M. sieversii* accessions initially collected from Kazakhstan ('Kaz 95 18-05', 'Kaz 95 08-06 ID 20622063', 'Kaz 95 08-06 ID 3147', 'Kaz 95 07-05', and 'Kaz 95 18-02P-20'), European cider cultivars ('Coat Jersey', United Kingdom; 'Gros Frequin', 'Daux Belan', and 'Marachel', France), and the dessert advanced apple breeding line developed from the AAFC Kentville breeding program ('S23-03-749').

**Table 3.1:** Mean $IC_{50}$ values (mg/mL) for α-glucosidase, α-amylase, dipeptidyl peptidase-4 (DPP-4), and advanced glycation end products (AGE) formation of the whole fruit extracts of high TPC group of apple accessions compared with reference drugs.

| Apple accession | α-Glucosidase $IC_{50}$ | α-Amylase $IC_{50}$ | DPP-4 $IC_{50}$ | AGE $IC_{50}$ |
|---|---|---|---|---|
| 'Kaz 95 08-06 ID 3147' | 15.4 | 10.3 | nd | nd |
| 'Kaz 95 18-05' | 39.9 | 19.1 | 14.5 | nd |
| 'Gros Frequin' | 12.1 | 7.7 | 12.4 | 7.8 |
| 'Kaz 95 08-06 ID 20622063' | 15.9 | 12.3 | nd | 7.1 |
| 'Kaz 95 07-05' | 17.7 | 9.7 | 10.8 | 9.6 |
| 'Coat Jersey' | 13.2 | 15.2 | 13.8 | 6.1 |
| 'S23-03-749'[†] | 10.7 | 10.5 | 12.9 | 5.6 |
| 'Kaz 95 18-02P-20' | 14.5 | 21.5 | 10.3 | 5.9 |
| 'Daux Belan' | 10.9 | 14.4 | 17 | 5.9 |
| 'Marachal' | 7.1 | 5.3 | 10.3 | 5.2 |
| Reference drug | 0.21 | 0.0058 | 0.00044 | 0.024 |

Note: Mean values were calculated by averaging two replications. Acarbose was the reference drug used for both α-glucosidase and α-amylase inhibition assay. Sitagliptin and aminoguanidine were the reference compounds for DPP-4 and AGE inhibition, respectively. nd, not detected. [†]Advanced breeding lines from AAFC-Kentville Research Station (NS, Canada).

The anti-diabetic properties of the high TPC group were compared with reference drugs (Table 3.1). The α-glucosidase $IC_{50}$ value of the clinical drug commonly prescribed

to slow down carbohydrate digestion, acarbose (0.21 mg/mL), was 34 times lower than the lowest $IC_{50}$ value of the apple extracts ('Marachel'; 7.1 mg/mL). Among the 10 apple extracts, 'Marachal', 'S23-03-749' and 'Daux Belan' showed the strongest α-glucosidase inhibitions. As for α-amylase inhibition, the $IC_{50}$ value of acarbose (0.0058 mg/mL) was over 900 times lower than the lowest $IC_{50}$ value of the apple extracts ('Marachel'; 5.3 mg/mL). Among the 10 apple extracts, 'Marachel', 'Gros Frequin', and 'Kaz 95 07-05' showed the strongest α-amylase inhibition. Moreover, for DPP-4 inhibition, the $IC_{50}$ value of the insulin-release-enhancing drug, sitagliptin (0.00044 mg/mL), was over 20,000 times lower than the lowest $IC_{50}$ value of the apple extracts ('Marachel' and 'Kaz 95 18-02P-20'; 10.3 mg/mL). Finally, the $IC_{50}$ value of the AGE-inhibiting drug, aminoguanidine, was over 200 times lower than the lowest $IC_{50}$ value of the apple extracts ('Marachel'; 5.2 mg/mL). Among the 10 apple extracts, 'Marachel' and 'S23-03-749' exhibited the strongest AGE inhibition. Overall, the reference drugs exerted stronger anti-diabetic action in all four assays with their lower $IC_{50}$ values than the apple extracts. Among the top 10 apple extracts, 'Marachel', the French cider cultivar with the highest TPC, and 'S23-03-749', the dessert advance breeding accession, exerted the strongest inhibition capacities in all four assays.

We found significant correlation between TPC and anti-diabetic properties of apple (poly)phenol extracts, where medium to strong inverse associations were observed for the relationships between TPC and α-amylase $IC_{50}$ (rho = -0.499; $P$ = 2.699e-4), α-glucosidase $IC_{50}$ (rho = -0.672; $P$ = 2.978e-39), and AGE $IC_{50}$ values (rho = -0.895; $P$ = 1.099e-3) (Figure 3.2), indicating the positive associations of TPC with inhibitory activity against the carbohydrate-hydrolyzing enzymes and protein glycation. In contrast, the correlation

between TPC and DPP-4 enzyme $IC_{50}$ values of apple (poly)phenol extracts was not statistically significant ($P = 0.263$).
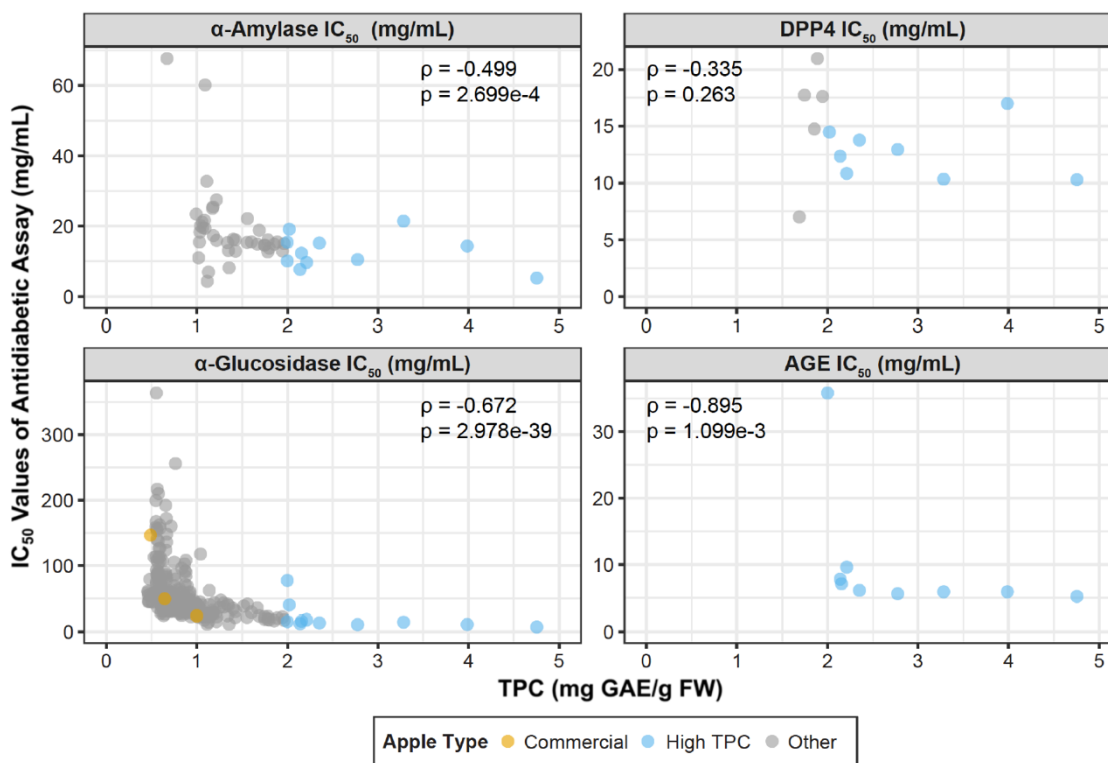


**Figure 3.2:** Spearman correlation between $IC_{50}$ values for α-amylase, α-glucosidase, dipeptidyl peptidase-4 (DPP-4), and advanced glycation end products (AGE) formation inhibition by whole fruit extracts of apple accessions and their total (poly)phenol content measured by Folin-Ciocalteu method.

### 3.4.2. (Poly)phenolic composition of selected apples from Canada's Apple Biodiversity Collection

Given the positive correlations between TPC and anti-diabetic properties of apple extracts established in three of the four parameters tested, we performed (poly)phenol

profile characterization using LC-qTOF-MS in 30 selected apple cultivars (Appendix A). A total of 27 compounds were identified, which can be categorized into six groups of (poly)phenols: phenolic acids (chlorogenic acid, dicaffeoylquinic acid), flavonols (morin, quercetin glycosides (quercetin 3-*O*-galactoside, quercetin 3-rutinoside, quercetin 3-*O*-glucoside, quercetin 3-D-xyloside and quercetin 3-rhamnoside.), anthocyanins (cyanidin 3-galactoside), dihydrochalcones (phloretin, phloridzin, phloretin 2-*O*-xylosylglucoside, phloretic acid and 3-hydroxyphloretin 2'-*O*-glucoside), free flavan-3-ols ((-)-epicatechin, D-(+)-catechin, (-)-catechin gallate and epigallocatechin 3-caffeate and), and oligomeric proanthocyanidins (procyanidin A1, A2, B1, B2, B3, C1, C2, arecatannin B1, and epicatechin-(4β->8)-epicatechin-(4β->8)-catechin) (Figure 3.3). Among these (poly)phenol classes, chlorogenic acid, phloretin glycosides (phloridzin and phloretin 2-*O*-xylosylglucoside), quercetin glycosides, cyanidin 3-galactoside, epicatechin, and procyanidin B2 and C1 are identified as the major subclasses of (poly)phenols present in the apples (Appendix B). Apple (poly)phenol compound composition and concentration vary significantly between accessions (Figure 3.3).
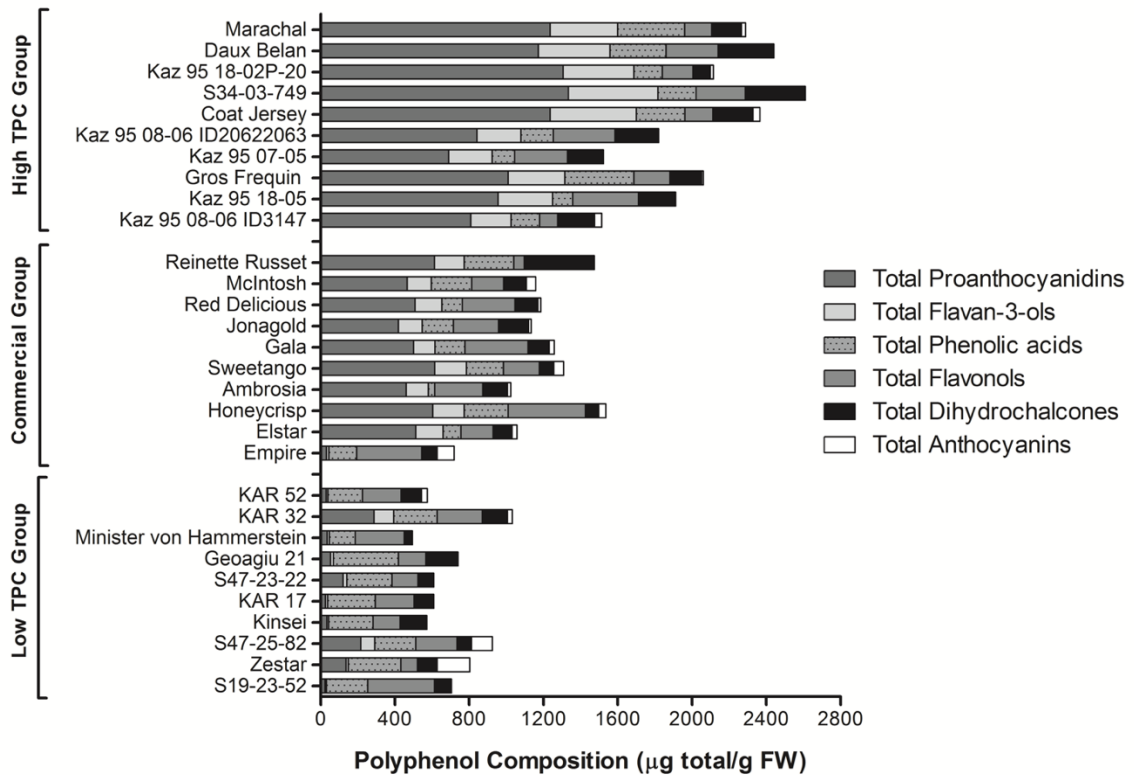
**Figure 3.3:** Total (poly)phenolic composition of the high TPC group (n = 10), commercial cultivars (n = 10), and low TPC group (n = 10). FW, fresh weight.

In the high TPC group comprising wild *M. sieversii* apples, cider apples, and a dessert apple, TPC values ranged from 1.51 ('Kaz 95 08-06 ID 3147') to 2.61 mg total/g FW ('S23-03-749') (Appendix A). The high TPC group was predominantly comprised of total oligomeric proanthocyanidins (51% of TPC) (Figure 3.3), of which, procyanidin C1 represented 14% of TPC and procyanidin B2 represented 13% of TPC (Appendix B).

The commercial group was comprised of mainly dessert apples (Appendix A). TPC values ranged from 0.72 ('Empire') to 1.54 mg total/g FW ('Honeycrisp') in the commercial group. Two major groups of (poly)phenols detected were total proanthocyanidins (41% of TPC) and total flavonols (21% of TPC) (Figure 3.3). In

particular, quercetin glycosides (17% of TPC) and chlorogenic acids (11% of TPC) are the major subclasses of apple (poly)phenols present in this group (Appendix B).

The low TPC group consisted entirely of dessert apples, with TPC values ranging from 0.49 ('Minister von Hammerstein') to 1.03 mg total g/FW ('KAR 32') (Appendix A). This group contained predominantly total phenolic acids (34% of TPC) and total flavonols (29% of TPC; Figure 3.3), where chlorogenic acid and quercetin glycosides made up 34% and 23% of TPC, respectively (Appendix B).

TPC values of the selected 30 accessions previously quantified spectrophotometrically using the Folin-Ciocalteu method (Watts et al., 2021) were compared with the TPC values obtained through the hyphenated chromatographic technique using LC-qTOF-MS (Figure 3.4). Using the Folin-Ciocalteu method, the high TPC group contained significantly more TPC than both commercial apples and the low TPC group ($P$ = 3.2e-11). In comparison, LC-qTOF-MS quantification of TPC revealed statistically significant differences in the mean TPC in all three groups of apples ($P$ = 6.2e-11). Despite the differences, TPC values from both methods were significantly correlated (r = 0.903; $P$ = 2.4e-7) (Appendix C).
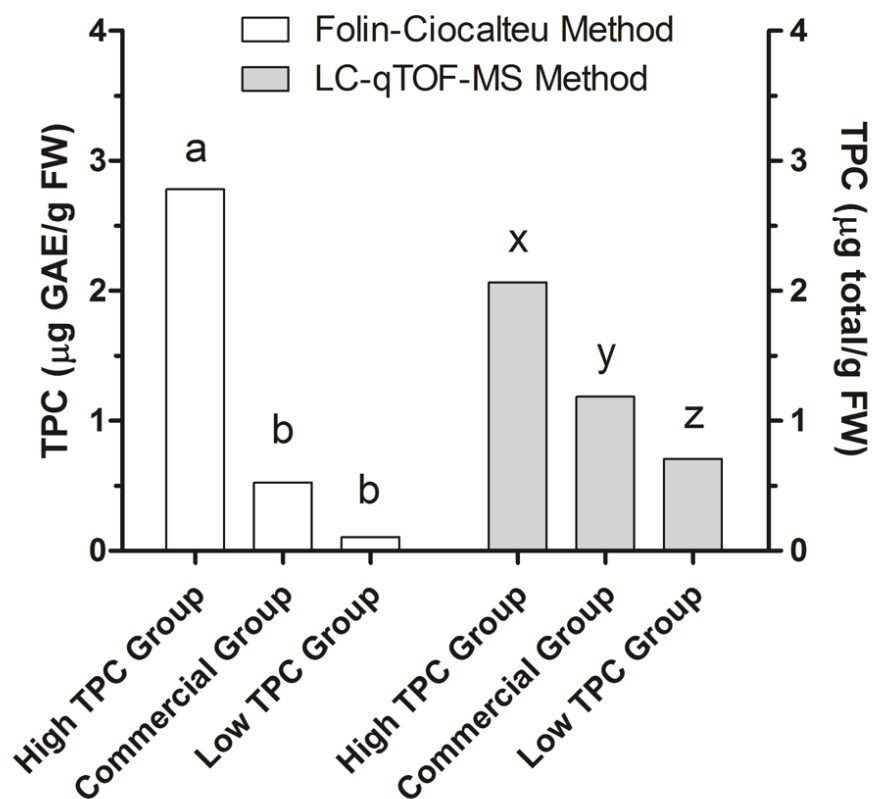
**Figure 3.4:** Mean total (poly)phenol contents (TPC) of the high TPC (n = 10), commercial (n = 10) and low TPC (n = 10) groups quantified using the Folin-Ciocalteu method and the LC-qTOF-MS method. FW, fresh weight. Tukey pairwise comparisons (α = 0.05) were performed, where different letters were assigned to means that are significantly different. A and b letter groupings were assigned to mean TPC quantified using the Folic-Ciocalteu method, while x, y, and z letter groupings were assigned to mean TPC quantified using the LC-qTOF-MS method.

### 3.4.3. Anti-diabetic properties of major apple (poly)phenol compounds

Based on the (poly)phenolic composition results, we selected one significant (poly)phenol compound with the highest concentration out of each (poly)phenol class to

test for anti-diabetic activities: procyanidin B2 for proanthocyanidins, epicatechin for flavan-3-ols, quercetin-3-$O$-galactoside for flavonols, phloridzin for dihydrochalcones, cyanidin 3-galactoside for anthocyanins and chlorogenic acid for phenolic acids.
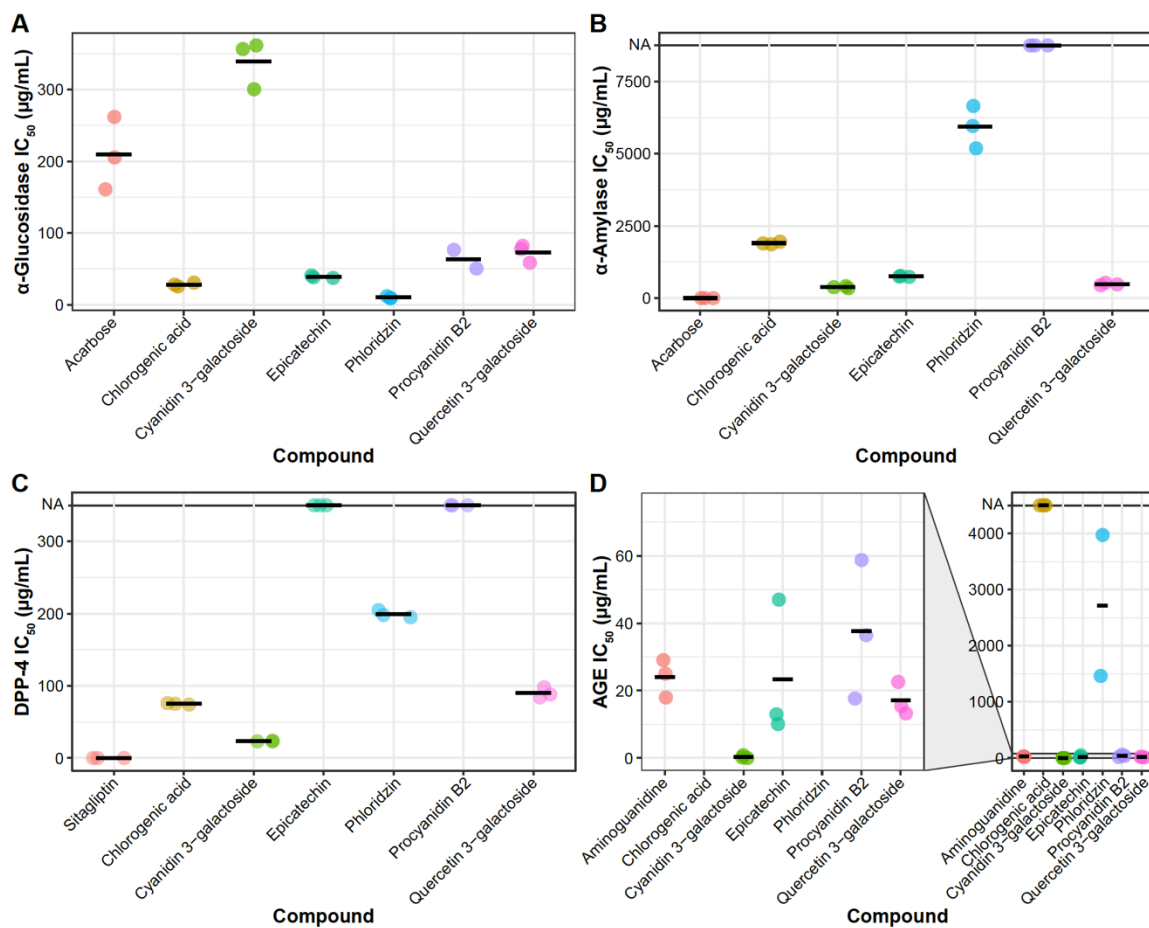
**Figure 3.5:** IC$_{50}$ values of α-glucosidase (A), α-amylase (B), dipeptidyl peptidase-4 (DPP-4) (C), and advanced glycation end products (AGE) formation (D) inhibition by six major (poly)phenol compounds (purity ≥ 97%) present in apples compared with reference diabetes drugs (α-amylase and α-glucosidase: acarbose; DPP-4: sitagliptin; AGE: aminoguanidine). NA, no activity. The black horizontal line for each compound indicates the mean activity level for that compound. Panel (D) shows a zoom-in to indicate for activity levels from 0 to 75 μg.

All six (poly)phenol compounds inhibited α-glucosidase activity *in vitro* (Figure 3.5A). Phloridzin ($IC_{50}$ = 10 µg/mL), chlorogenic acid ($IC_{50}$ = 28 µg/mL), epicatechin ($IC_{50}$ = 39 µg/mL), procyanidin B2 ($IC_{50}$ = 64 µg/mL) and quercetin 3-*O*-galactoside ($IC_{50}$ = 73 µg/mL), but not cyanidin 3-galactoside ($IC_{50}$ = 340 µg/mL), displayed lower $IC_{50}$ values than the reference drug, acarbose ($IC_{50}$ = 0.21 mg/mL), with phloridzin ($IC_{50}$ = 10 µg/mL) having been the strongest α-glucosidase inhibitor among the compounds tested.

α-Amylase activity (Figure 3.5B) was inhibited by cyanidin 3-galactoside ($IC_{50}$ = 377 µg/mL), quercetin 3-*O*-galactoside ($IC_{50}$ = 485 µg/mL), epicatechin ($IC_{50}$ = 749 µg/mL), chlorogenic acid ($IC_{50}$ = 1,905 µg/mL), and phloridzin ($IC_{50}$ = 5,935 µg/mL), but not procyanidin B2. The drug acarbose had the strongest inhibition of α-amylase activity ($IC_{50}$ = 0.58 µg/mL).

Among the six compounds assessed, cyanidin 3-galactoside ($IC_{50}$ = 23.1 µg/mL) was the most effective DPP-4 inhibitor, followed by chlorogenic acid ($IC_{50}$ = 75 µg/mL), quercetin 3-*O*-galactoside ($IC_{50}$ = 90 µg/mL) and phloridzin ($IC_{50}$ = 199 µg/mL) (Figure 3.5C). Inhibition was not detected in epicatechin and procyanidin B2. The reference drug, Sitagliptin, exhibited stronger DPP-4 enzyme inhibition than all the compounds ($IC_{50}$ = 0.044 µg/mL).

Chlorogenic acid did not inhibit the formation of AGE at the tested concentrations while phloridzin ($IC_{50}$ = 2,716 µg/mL) showed weak inhibition compared with other compounds (Figure 3.5D). In comparison, cyanidin 3-galactoside ($IC_{50}$ = 0.35 µg/mL), again, displayed the most inhibitory activity among the 6 compounds, followed by quercetin 3-*O*-galactoside ($IC_{50}$ = 17.1 µg/mL), epicatechin ($IC_{50}$ = 23 µg/mL), reference compound aminoguanidine ($IC_{50}$ = 24 µg/mL), and procyanidin B2 ($IC_{50}$ = 38 µg/mL).

Lastly, by correlating the six major apple (poly)phenol concentration and two carbohydrate-hydrolyzing enzymes $IC_{50}$ for the high TPC group, we determined that procyanidin B2 (rho = -0.75; $P$ = 0.018), phloridzin (rho = -0.71; $P$ = 0.028), and epicatechin (rho = -0.68; $P$ = 0.035) concentrations were significantly negatively correlated with α-glucosidase $IC_{50}$, while chlorogenic acid concentrations (rho = -0.61; $P$ = 0.066) and α-glucosidase $IC_{50}$ were only marginally negatively correlated (Appendix D). In contrast, none of the six compounds tested were significantly correlated with α-amylase $IC_{50}$ in the high TPC group.

## 3.5. Discussion

Prolonged uncontrolled hyperglycemia experienced by T2D patients can lead to serious and deadly secondary complications. Prior studies have demonstrated the promising potential of apple (poly)phenols in regulating glucose homeostasis by inhibiting carbohydrate-hydrolyzing enzymes, α-amylase, and α-glucosidase (Agustinah et al., 2016; de Oliveira Raphaelli et al., 2019; Gong et al., 2020b; Li et al., 2019b), yet results on DPP-4 and AGE inhibition by apple (poly)phenols remain scarce. With the knowledge of limited diversity and reduced (poly)phenol content in commercial apple cultivars over the last two centuries (Watts et al., 2021), we screened the ABC, an orchard including wild *M. sieversii* accessions from Kazakhstan, heritage cultivars, commercial apples, and advanced novel breeding lines for the anti-diabetic characteristics of α-amylase, α-glucosidase, DPP-4, and AGE inhibition. Here we present evidence that apple (poly)phenols inhibit all four therapeutic targets *in vitro*.

### 3.5.1. Anti-diabetic properties of apples in Canada's Apple Biodiversity
### Collection

Inhibition of carbohydrate-hydrolyzing enzymes reduces the amount of glucose entering the bloodstream by suppressing the cleavage of oligosaccharides into glucose, thereby managing post-prandial hyperglycemia (Ayua et al., 2021). α-Glucosidase inhibition was observed in the greatest number of apple accessions, which indicates that apple (poly)phenol-rich extracts are more active against α-glucosidase than α-amylase (Figure 3.1A & B). This finding coincides with the literature (Ci et al., 2018; Yilmazer-Musa et al., 2012), where (poly)phenols demonstrate greater α-glucosidase inhibitory capacity than α-amylase. The variability in carbohydrate-hydrolyzing enzyme inhibition can be explained by the previously reported 100-fold difference in TPC across the ABC apple accessions (Watts et al., 2021). Also, the dose of (poly)phenols, the range of (poly)phenols, (Barbosa et al., 2010), post-harvest preservation conditions (Adyanthaya et al., 2010), and the molecular structure of (poly)phenol compounds present in a specific apple (Cao et al., 2022) could all contribute to the wide range of inhibitory activity against the carbohydrate-hydrolyzing enzymes across the ABC apples. The negative association between TPC and α-glucosidase $IC_{50}$ values (Figure 3.2) suggests TPC and α-glucosidase inhibition is positively correlated, which is congruent with the literature (Adyanthaya et al., 2010; Barbosa et al., 2010). However, contrary to our findings, previous work by Barbosa and colleagues did not report a significant correlation between TPC and α-amylase inhibition (Barbosa et al., 2010, 2012). This means apples higher in TPC have strong α-glucosidase inhibitory potential that is beneficial in delaying carbohydrate digestion, which is an advantage that should be prioritized in future nutraceutical plant breeding.

Glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP) are incretin hormones in the gut secreted after a carbohydrate-rich meal and are responsible for approximately half of the post-meal insulin secretion (Röhrborn et al., 2015). Due to these incretin hormones, glucose provided orally will elicit a larger insulin secretion than glucose that was given intravenously. This phenomenon is coined the "incretin effect" (Nauck et al., 1986). As incretin hormones are naturally broken down by DPP-4 enzymes, the inhibition of DPP-4 enzymes is a desirable therapeutic target to protect and increase the bioavailability of insulin-secreting incretin hormones, thereby prolonging insulin action, decreasing glucagon concentration, and lowering blood glucose and decelerating gastric emptying (Röhrborn et al., 2015). In this study, we found that apple (poly)phenol extracts containing peel and flesh exerted weak DPP-4 inhibition at the concentrations tested (Table 3.1). Our preliminary finding of the lack of correlation between TPC and DPP-4 inhibition suggests that selecting for high TPC apples would be a poor predictor of DPP-4 inhibition capacity and that based on the limited evidence so far, high TPC apples are not necessarily useful for the enhancement of the incretin effect through the protection of insulinotropic incretin hormones (Figure 3.2). However, it is important to note that the DPP-4 inhibitory activity by apple (poly)phenols should not be dismissed as the concentration/dose of apple (poly)phenol extract as well as (poly)phenol degradation in the extract during storage could have played a role in the weak DPP-4 inhibition observed in this experiment. DPP-4 inhibition has been demonstrated using various plant and dietary sources of (poly)phenols, including (poly)phenols from haskap berries (De Silva & Rupasinghe, 2020), olive oil (Carnevale et al., 2017), grape-seed-derived procyanidins (González-Abuín et al., 2012), seed extracts of *Castanospermum austral* (Bharti et al., 2012), culinary herbs (Bower et al.,

2014) and rosebud extract powder (Kato et al., 2016). To our knowledge, this is the first study on DPP-4 inhibition by apple (poly)phenol extracts, and more studies are needed to further elucidate any influence apple (poly)phenols may have on the incretin effect.

Advanced glycation end products (AGE) are formed during the non-enzymatic glycation process, where the carbonyl group of reducing sugars reacts with the amino group of proteins, lipids, or DNA (Khangholi et al., 2016). AGE accumulation damages cell functions and contributes to the progression and manifestation of micro and macroangiopathic diabetes complications. Only nine apple accessions tested showed detectible AGE $IC_{50}$ values, suggesting weak AGE inhibition by apple (poly)phenol extract containing both peel and flesh (Figure 3.1D). A study compared a variety of commercial fruit juices and reported apple juice showed the least anti-glycation activity (Dorsey & Greenspan, 2014). The significant negative correlation between TPC and AGE $IC_{50}$ values indicates the positive relationship between TPC and anti-glycation activity (Figure 3.2), which means the nutraceutical breeding approach focusing on increasing TPC can improve the anti-glycation functional attributes of apples that is beneficial in preventing cell damage that eventually leads to diabetes complications. It is worth mentioning that 'S23-03-749', as a dessert breeding accession, also showed one of the lowest $IC_{50}$ values of 5.6 mg/mL. The advanced breeding program at the Agriculture and Agri-Food Canada Research and Development Centre (Kentville, Nova Scotia, Canada) targets apple improvement against common diseases. Typically, high TPC is associated with a bitter and astringent taste (Kschonsek et al., 2018), as well as rapid browning of the flesh upon contact with oxygen through the action of (poly)phenol oxidase (Can et al., 2014), all of which are undesirable traits selected out of fresh-eating apples in a consumer-driven breeding approach (Alvarez

et al., 2021). This unexpected finding indicates that 'S23-03-749' could be a suitable breeding material for introducing higher TPC into commercial lines for potential glycemia management and diabetes complication prevention.

### 3.5.2. (Poly)phenolic composition of selected apples from Canada's Apple Biodiversity Collection

Wild apple germplasms (*Malus sieversii*) from the Tian Shan Mountain ranges of Kazakhstan are recognized as major progenitors of the domesticated apple (*Malus domestica* Borkh.) (Ha et al., 2021; Vavilov, 1987). However, over millennia of cultivation, domesticated apples now contain 68% less (poly)phenols than their ancestors, on top of other phenotypic differences such as a 3.6-fold increase in weight and 43% reduction in acidity (Davies et al., 2022). Heritage apples and modern commercial lines are both domesticated apples used for cooking and fresh eating purposes. Heritage apples are past cultivars released before the 20th century that are no longer available in the current commercial market (Amyotte et al., 2017; Bowen et al., 2019). Modern commercial apples, on the other hand, became available during the 20th century, and many share common ancestry with heritage apples due to repeated crossbreeding of the same few varieties (Amyotte et al., 2017; Noiton & Alspach, 1996). Metabolomics-assisted breeding allows breeders to harness technologies such as LC-qTOF-MS to understand the fascinating diversity in apple (poly)phenol content and composition, which, when utilized in combination with the knowledge of anti-diabetic properties of apple (poly)phenols, can better equip apple breeders to incorporate human health benefits into their crops to produce apples with functional attributes that provide health benefits on top of nutritional benefits. It is widely reported that procyanidins are the predominant apple (poly)phenols identified

in both the peel and the flesh (Anastasiadi et al., 2017; Khanizadeh et al., 2007; Tsao et al., 2003; Wojdyło et al., 2008). This is consistent with our findings in the high TPC group, and in the commercial group as well but in lesser quantities (Figure 3). Most cider and wild apples are smaller in size (Anastasiadi et al., 2017), whereas dessert cultivars are selected over the years to produce larger fruits (Busatto et al., 2019). As (poly)phenol compounds are more concentrated in the peel than in the flesh (Kalinowska et al., 2020; Khanizadeh et al., 2007; I. Kim et al., 2019; Tsao et al., 2003), the larger fruits of dessert cultivars mean more flesh, where the flesh makes up approximately 90% of the fruit weight, thereby leading to diluted (poly)phenols in dessert cultivars compared with cider and wild apples (Busatto et al., 2019). Limited (poly)phenol composition studies are performed on apple extracts containing a mixture of peel and flesh, and most research examine TPC in peel and flesh separately. Our reported TPC values in peel and flesh extracts of popular commercial apples such as 'gala' (1188 µg/g FW), 'red delicious' (1186 µg/g FW), and 'McIntosh' (1160 µg/g FW) are similar to values reported by Ceymann et al. (2011), which are 1370, 1330, and 1300 µg/g FW, respectively.

In the low TPC group, phenolic acids and flavonols are found in higher proportions than proanthocyanidins (Figure 3.3). This is congruent with previous reports of higher chlorogenic acid content in cultivated apples compared with wild apples (Liao et al., 2021). Quercetin glycosides are mainly present in the peel with low concentrations in the flesh, and cyanidin 3-galactoside are exclusively found in red apple peel (Khanizadeh et al., 2007; Sanoner et al., 1999; Tsao et al., 2003). The higher percentages of quercetin glycosides and cyanidin 3-galactoside in the commercial and low TPC groups compared with the high TPC group (Appendix B), could be explained by the intentional selection for the aesthetics

of larger fruit size (more apple peel) and redder fruit colour in dessert apples (Busatto et al., 2019).

### 3.5.3. Anti-diabetic properties of major apple (poly)phenol compounds

The health-promoting and anti-diabetic properties of the six major apple (poly)phenol compounds, namely chlorogenic acid (Meng et al., 2013), cyanidin 3-galactoside (Liang et al., 2021), epicatechin (Nie & Cooper, 2021), phloridzin (Khanam et al., 2022), procyanidin B2 (Luan et al., 2014; Yin et al., 2015), and quercetin (Bule et al., 2019), have been widely documented in the literature. These apple (poly)phenol compounds displayed the same trend in carbohydrate-hydrolyzing enzyme inhibition as the results from the apple (poly)phenol extracts where the compounds showed greater $\alpha$-glucosidase inhibition capacity than $\alpha$-amylase inhibition (Figure 3.5A & B). As phloridzin, procyanidin B2 and epicatechin are the only three compounds out of six that correlated with $\alpha$-glucosidase inhibition in this study (Appendix D), these are the compounds focused on in the discussion.

Phloridzin, as a (poly)phenol compound characteristic of apple and particularly concentrated in russet-skinned cultivars (Gutierrez et al., 2018), is well-known for its anti-diabetic properties in controlling blood glucose by competitively inhibiting sodium-glucose co-transporter 1 (SGLT-1) on the brush border membrane of the small intestines, thereby reducing glucose absorption from the intestines into the bloodstream (Schulze et al., 2014). The potent $\alpha$-glucosidase inhibitory action observed in this study by phloridzin (Figure 5A) means, as an $\alpha$-glucosidase inhibitor, it could also delay the enzymatic breakdown of carbohydrates into glucose in the small intestine before absorption by SGLT-1 (Akmal & Wadhwa, 2022). In contrast, phloridzin was found to be a weak $\alpha$-amylase

inhibitor (Figure 5A & B), which is in congruence with previous reports (Li et al., 2019b; Sun et al., 2016). Despite numerous reports of phloridzin as both an effective reactive dicarbonyl species trapping agent (Sampath et al., 2016; Shao et al., 2008), an inhibitor of AGE formation (Zielinska et al., 2019), which are harmful intermediate products of the AGE formation process, phloridzin exerted weak inhibition of total AGE formation (Figure 5D).

Epicatechin and procyanidin B2 (epicatechin dimer) belong to the proanthocyanidins class of (poly)phenols (Rauf et al., 2019). Proanthocyanidins occur in abundance in foods such as fruits, nuts, seeds and wine. Their diverse health effects are reflected in their anti-diabetic properties, particularly concerning the improvement of postprandial hyperglycemia (Justino et al., 2022; Sulaiman, 2014), protection of insulin-secreting pancreatic β-cells (Ding et al., 2013), and alleviation of cell-damaging oxidative stress through inhibition of AGE (Justino et al., 2022). Supported by the numerous health benefits from the literature and the positive correlation between α-glucosidase inhibition and concentrations of phloridzin, epicatechin and procyanidin B2 (Appendix D), increasing concentrations of these compounds in apples for improved blood glucose control could be a valuable target in nutraceutical plant breeding.

Alleviation of glycemic dysregulation through blood sugar control has been widely demonstrated in apples including (poly)phenol extracts of immature apples (Gong et al., 2020b; Li et al., 2019b), (poly)phenol extracts or (poly)phenol fractions of commercially mature apples (De Oliveira et al., 2019; Ogura et al., 2016), apple juice (Fathy & Drees, 2016; Johnston et al., 2002), apple cider (Agustinah et al., 2016), apple cider vinegar (Iman et al., 2016; Kausar et al., 2019), and apple pomace flour (Gorjanović et al., 2020).

Although apple peels are more concentrated in (poly)phenol compounds than apple flesh (Kalinowska et al., 2020; Khanizadeh et al., 2007; Kim et al., 2019; Tsao et al., 2003), studying both apple peel and flesh for anti-diabetic properties is a more accurate representation of the effects of fresh apple consumption. It is important to note that the same seven concentrations were tested across the apple accessions and these concentrations may have been too diluted to exert detectible anti-diabetic activity in some accessions. As the study was conducted over two years, (poly)phenol degradation during freezer storage could also play a role in influencing the anti-diabetic properties. While the $IC_{50}$ values of apple (poly)phenol extracts containing peel and flesh in this study were higher than the reference inhibitors in all anti-diabetic assays performed, high (poly)phenol accessions, specifically 'Marachel', consistently demonstrated its potential for nutraceutical consideration in the form of purified compounds or extracts by its highest inhibitory activity across the experiments. Finally, the identification of 'S23-03-749' as a high (poly)phenol-containing dessert apple with anti-diabetic potential introduces the possibility of higher-(poly)phenol-containing apples in the fresh apple market, which typically consists of closely related, lower-(poly)phenol-containing commercial apples (Migicovsky et al., 2021; Watts et al., 2021), an unintended result of selecting for aesthetics and desirable post-harvest traits (Busatto et al., 2019).

## 3.6. Conclusion

In conclusion, *in vitro* screening of ABC apples indicates anti-diabetic potential in apples of high (poly)phenol content, specifically in inhibiting carbohydrate-hydrolyzing enzymes to reduce blood glucose levels, inhibiting DPP-4 enzymes to increase postprandial insulin secretion, and inhibiting AGE formation to lower diabetes complication risks. Most

high TPC apples are not suitable for the fresh market due to the astringent taste associated with the high TPC but show potential as apple-derived value-added ingredients or nutraceuticals. However, a high TPC dessert apple such as 'S23-03-749' strengthens the rationale of apple supplementation in the whole food form for the dietary management of blood glucose levels in health-conscious consumers. Further research is in progress to evaluate the effect of chronic high TPC apple ('Daux Belan') supplementation in a diet-induced obese and glucose intolerance mouse model, to determine the ability of apple (poly)phenol in modulating glycemic dysregulation *in vivo*.

# CHAPTER 4: EFFECT OF HIGH-(POLY)PHENOL-CONTAINING DAUX BELAN APPLE SUPPLEMENTATION ON DIET-INDUCED OBESITY AND GLUCOSE INTOLERANCE IN C57BL/6 MICE

## 4.1. Abstract

Type 2 diabetes, characterized by prolonged hyperglycemia, is often accompanied by hyperlipidemia. The influence of apple (poly)phenols on energy metabolism in high fat (HF) diet-induced obese mice remains controversial. In addition to basic nutrient components, whole apple (peel and flesh) supplementation provides other bioactive components such as fiber and pectin. This study examined the effect of dietary supplementation of high-(poly)phenol-containing 'Daux Belan' apple (DB; 6.2 mg GAE/mouse/day; 0.15% (poly)phenol) in the form of freeze-dried powder on glucose and lipid metabolism in male HF-fed C57BL/6 mice, with the low-(poly)phenol-containing 'Zestar' apple (Z; 0.4 mg GAE/mouse/day) used as apple control. Obesity, glucose intolerance, hypertriglyceridemia, and hepatic lipid vacuolation were induced by HF feeding while circulating cholesterol levels remained unchanged. DB apple supplementation did not protect against HF-induced body weight gain, hyperglycemia, hepatic triglyceride level elevation, and hepatic lipid vacuolation at the tested dosage. Future studies should be conducted with increased DB dosage and employ DB (poly)phenols supplemented in the form of extracts or sugar-free powder.

## 4.2. Introduction

T2D is a multifactorial chronic disorder that disrupts carbohydrate, protein, and fat metabolism. The combination of obesity and obesity-triggered insulin resistance strongly contributes to the development of T2D (Chobot et al., 2018). T2D is characterized by prolonged hyperglycemia that is caused by a wide array of metabolic imbalances, including insufficient insulin secretion by pancreatic β-cells, impaired glucose uptake due to insulin resistance in peripheral tissues, enhanced glycogenolysis, upregulated gluconeogenesis, and altered insulin-signalling pathway in insulin target tissues (Shahwan et al., 2022). Lifestyle modifications such as increased physical activity, reduced body weight, and increased intake of dietary fiber, whole grains, fruits and vegetables, as well as reduced

saturated fat are protective against progression into T2D in patients with slightly higher than normal blood glucose levels (Uusitupa et al., 2019).

Apple is a popular fruit among consumers for its availability and affordability. It is also a good source of dietary (poly)phenols (Rupasinghe et al., 2013). *In vitro* anti-diabetic action by apple (poly)phenols is well-documented, particularly in inhibiting carbohydrate-hydrolyzing enzymes, such as α-glucosidase (De Oliveira et al., 2019; Li et al., 2019b) and α-amylase enzymes (Li et al., 2019b; Zhao et al., 2019), that metabolize polysaccharides into glucose (Hanhineva et al., 2010), and as well as inhibiting glucose transporter proteins that facilitate glucose uptake, such as SGLT-1 and GLUTs (Castro-Acosta et al., 2016). In various strains of mice and rats, apple (poly)phenols improved HF-induced body weight gain (Boqué et al., 2013; Cho et al., 2013; Li et al., 2019a; Masumoto et al., 2016; Zou et al., 2020), blood glucose dysregulation (Masumoto et al., 2016; Sampath et al., 2016; Schloesser et al., 2017; Shin et al., 2016; Zou et al., 2020), and hyperlipidemia (Cho et al., 2013; Li et al., 2019a; Masumoto et al., 2016; Ohta et al., 2006).

Doses of 600 and 1,200 mg total apple (poly)phenol/day were hypoglycemic in borderline diabetic people and healthy people, respectively (Castro-Acosta et al., 2017; Shoji et al., 2017). In obese subjects, (poly)phenol-rich diets (2,861 and 2,903 mg/day) improved glucose regulation and insulin sensitivity (Bozzetto et al., 2015). However, studies have also reported no association with T2D protection at total dietary (poly)phenol doses of 1,200 mg/day or higher (Del Bo' et al., 2019). The imperfect concordance of the recommended daily (poly)phenol intake for T2D prevention and management in the literature complicates the establishment of a reference intake amount, likely due to heterogeneity of the studies and limitations in the estimation of (poly)phenol intake (Del

Bo' et al., 2019). Thus, based on the evidence in the literature, the therapeutic dose was set at 1,200 mg total apple (poly)phenol/day/human in this work, translating to 6.2 mg GAE/mouse/day at the diet dose of 0.15% apple (poly)phenol. The *in vitro* anti-diabetic properties of high-(poly)phenol-containing 'Daux Belan' (DB) apple have been demonstrated in our previous work. Therefore, in this study, the effect of DB whole apple (peel and flesh) powder supplementation on T2D was investigated using a diet-induced obese and glucose-intolerant mouse model.

## 4.3. Methods

### 4.3.1. Animals

Thirty-two male mice (8 weeks old; Charles River Laboratories, Montreal, QC, Canada) were singly housed in a controlled environment (12 h day/night cycle, lights on between 7:00 to 19:00) with *ad libitum* access to food and water at Carleton Animal Care Facility of Dalhousie University, Halifax, NS, Canada. Following one week of adaptation to the chow diet, the mice were randomly divided and subjected to the following four diets (n = 8) for 18 weeks: 1) chow, 2) high fat (HF), 3) HF supplemented with high-(poly)phenol-containing DB apple powder (HF + DB), and 4) HF supplemented with low-(poly)phenol-containing 'Zestar' apple powder (HF + Z). The design and protocols for the animal experiments were approved by the Dalhousie University Committee on Laboratory Animals (Protocol # 21-005) (Appendix E). The 3 Rs (reduction, replacement, and refinement) were followed to minimize animal suffering and to reduce the number of animals. The timeline of procedures is outlined in Figure 4.1.
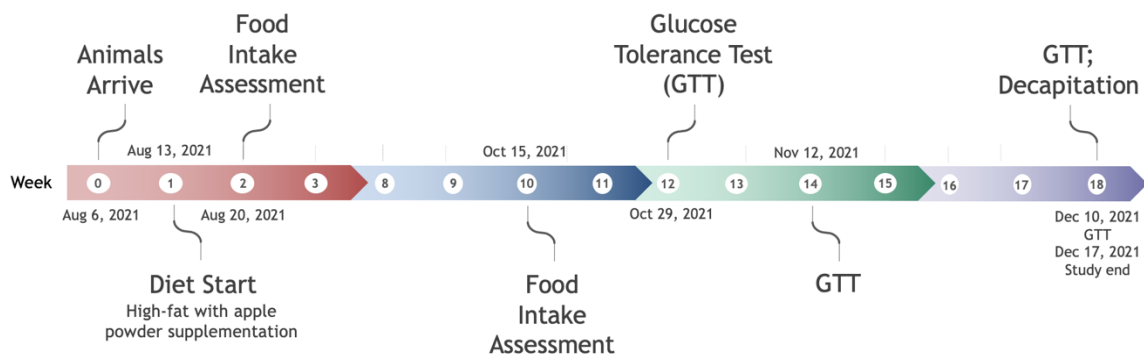
**Figure 4.1:** Timeline of procedures for the mouse study.

### 4.3.2. Preparation and compositional analysis of apple powders

The apples were harvested from the Apple Biodiversity Collection (Agriculture and Agri-Food Canada Research and Development Centre, Kentville, NS, Canada; 45°04′08″N 64°28′41″W) at appropriate maturity based on previously published data (Watts et al., 2021). Six days following respective harvest dates, apples stored at 4°C were cored, sliced, and immediately flash-frozen using liquid nitrogen. Frozen apple slices were freeze-dried (Dura-Stop Digital Control Stoppering Tray Dryer with Dura-Dry MP Microprocessor Control Corrosion Resistant Freeze-Dryer, FTS Systems[TM]; Marbletown, NY, USA) at -40°C for two days, then at 10°C for four days. Freeze-dried apple slices were ground into a fine powder and stored at -80°C in sealed Ziploc bags until the commencement of the feeding trial. Freeze-dried Z and DB samples were analyzed for nutritional composition, where calories (by calculation), moisture (drying under vacuum method; AOAC 950.46b), protein (block digestion method; AOAC 981.10), fat (acid hydrolysis method; AOAC 922.06), ash (direct method; AOAC 920.153) and carbohydrate content (by calculation) were quantified. (Poly)phenolic compositions of Z and DB were characterized by the LC-qTOF-MS method (Unpublished) (Table 4.1).

**Table 4.1:** Proximate and (poly)phenolic composition of freeze-dried 'Daux Belan' and 'Zestar' apple powder.

| | Daux Belan | Zestar |
|---|---|---|
| **Proximate Composition (%)** | | |
| Moisture | 7.4 | 10 |
| Protein | 2.0 | 1.7 |
| Fat | 2.0 | 1.7 |
| Ash | 1.4 | 1.5 |
| Carbohydrates | 87 | 85 |
| Total sugars | 67 | 69 |
| Glucose | 15 | 4.6 |
| Fructose | 36 | 36 |
| Lactose | <0.4 | <0.4 |
| Sucrose | 16 | 28 |
| Maltose | <0.4 | <0.4 |
| Calories | 375 | 363 |
| **(Poly)phenolic Composition (%)** | | |
| Chlorogenic acid | 16 | 36 |
| Cyanidin 3-galactoside | 0.34 | 23 |
| Quercetin glycosides | 12 | 9.5 |
| (-)-Epicatechin | 16 | 0.13 |
| D-(+)-Catechin | 3.6 | 1.4 |
| Phloridzin | 6.7 | 4 |
| Phloretin-2′-$O$-xyloglucoside | 9.4 | 9 |
| Procyanidin C1 | 19 | 2 |
| Procyanidin B2 | 16 | 15 |
| Total (poly)phenol Content (ug/g FW) | 1784 | 778 |

All data are expressed in percentages (%) unless indicated otherwise in brackets. Apple (poly)phenolic compositions were characterized using the LC-qTOF-MS method. FW, fresh weight.

### 4.3.3. Preparation of apple powder-incorporated high fat (HF) diet

The average weight of a human adult and an eight-week-old male C57BL/6 mouse were assumed to be 60 kg (Nair & Jacob, 2016) and 25 g (The Jackson Laboratory, 2021), respectively. The therapeutic dose determined for this study was 1,200 mg gallic acid equivalents (GAE)/day/60 kg adult, therefore the human dose for this study is 20 mg GAE/day/kg adult. Based on the human dose, the mice equivalent dose was calculated using the following equation: Human dose (mg/kg) = Animal dose (mg/kg) × (Animal Km factor/Human Km factor); where the human and mouse Km factor (average body weight/body surface area) were 37 and 3, respectively (Nair & Jacob, 2016). As such, the animal (poly)phenol dose for this study was calculated to be 247 mg GAE/kg body weight mouse/day. The 0.15% diet dose of (poly)phenols was determined using the following equation: Diet dose (mg (poly)phenols/kg diet) = [single daily dose (mg (poly)phenols/kg BW/day) × body weight (g body weight/animal)]/daily food intake (g diet/day).

Freeze-dried DB (0.266 g dry weight/mouse/day) was incorporated into HF powder (Research Diet Inc., New Brunswick, NJ, USA.; D12451; 45 kcal % fat) using a whisk in a clean metal bowl, and aseptic water was added as a binder.  The mixture of apple powder and HF powder was then weighed and divided into equal portions by weight, formed into pellets by pressing with a measuring teaspoon. The pellets were then stored at 4°C in a Ziploc bag for up to a week until feeding. With the assumption that a mouse consumes 4 g of food daily (Johns Hopkins University Animal Care and Use Committee, n.d.), the HF + DB group and HF + Z group were receiving (poly)phenols from apple powders incorporated at equal weights at the approximate dosages of 6.2 mg GAE/mouse/day and 0.4 mg GAE/mouse/day, respectively. Apple powder weights were adjusted monthly

according to body weight gain to maintain the apple (poly)phenol dosage supplemented. Percentages of calories provided by protein, carbohydrate, and fat in chow and HF diets were presented in Table 4.2. Detailed composition of chow (Lab Diet®, St. Louis, MO, USA; 5P04 - Prolab RMH 3500; 15.5 kcal % fat) and HF diets are presented in Appendix F and Appendix G, respectively.

**Table 4.2:** Percentages of calories provided by protein, carbohydrate, and fat in chow and high fat (HF) diet.

| Calories provided by (%) | Chow | High Fat |
|---|---|---|
| Protein | 26.2 | 20 |
| Carbohydrate | 58.3 | 35 |
| Fat | 15.5 | 45 |

### 4.3.4. Body weight and feed intake analysis

Mice were weighed weekly. At week two, feed intake analysis was performed in all 32 single-housed animals for seven consecutive days. On day one, the weights of the feed hopper containing feed were obtained. From day two to seven, the weights of the feed hopper containing leftovers and the weights of the fresh feed given were monitored to calculate the amount of food consumed by the mice. Any feed dropped in the bedding was picked up and weighed.

### 4.3.5. Intraperitoneal glucose tolerance test (IPGTT)

At the beginning of weeks 12, 14, and 18, all mice (n = 32) were subjected to IPGTT. The body weights of the mice were recorded, then food was removed for a 16-hour overnight fast with *ad libitum* water provided. Post-fast body weight was measured and

used for glucose dosage calculation (2 g/kg). Pricking the tail vein with a needle and massaging the tail for a drop of blood, the basal blood glucose measurement was taken using a glucometer and glucose test strip (Accu-chek® Guide, Roche Diabetes Care, Mississauga, ON, Canada). Thereafter, an IP injection of 20% glucose was administered and blood glucose concentrations were measured again at 15-, 30-, 60-, and 120-minutes post-injection. Immediately after the last time point at 120 minutes, food and water was provided. Blood glucose measurements were reported in mM and plotted for a time course of the blood glucose response curve against an exogenous glucose bolus and an area under the curve (AUC) was generated.

### 4.3.6. Animal euthanasia

At the end of the 18-week study, mice were euthanized by decapitation using a small animal guillotine (Braintree Scientific, Inc., MA, USA) following a four-hour fast. Blood glucose concentration was determined using a glucometer (Accu-chek® Guide, Roche Diabetes Care, Mississauga, ON, Canada). Whole blood was collected in EDTA-coated tubes and spun at $2,000 \times g$ for 10 min for the collection of plasma. A piece of liver was placed in 10% formalin for histopathology analysis and the rest of the liver was snap-frozen in liquid nitrogen. Animal plasma and tissues were stored at -80°C until further analysis.

### 4.3.7. Plasma and liver triglyceride (TG) and liver cholesterol content

Plasma TG (FUJIFILM Wako Shibayagi Corporation, Gunma, JP; 632-50991), liver TG (Cayman Chemical, MI, USA; 10010303), and liver total and free cholesterol

contents (Abcam, Cambridge, UK; ab65359) were quantified using colorimetric assay kits according to the manufacturer's instructions.

### 4.3.8. Liver histopathology

Fresh liver tissues were fixed in 10% formalin for 48 hours at room temperature and stored in 70% alcohol at 4°C until further processing. Fixed tissues were trimmed into appropriate size and shape, embedded in paraffin wax, and sliced at 5 µm thickness. Hematoxylin and Eosin (H&E) staining of paraffin-embedded sections was performed as per commercial kit instructions (Abcam, Cambridge, UK; ab245880). Images of the liver slides were captured using the Pannoramic Midi Digital Slide Scanner (3DHISTECH Ltd., Budapest, Hungary). Visualization and enumeration of lipid vacuoles in the liver cross-sections were performed using SlideViewer software (3DHISTECH Ltd., Budapest, Hungary), while the area of lipid vacuoles in the liver cross-sections was analyzed in the ImageJ software (National Institute of Health, MD, USA) using Adiposoft plug-in.

### 4.3.9. Statistical analyses

The plasma and liver of six animals from each treatment were assayed and all data were expressed as mean ± standard error of the mean (SEM). (Poly)phenol dosage results were expressed as mean ± standard deviation (SD). Statistical analyses were conducted using one-way ANOVA followed by Tukey's multiple comparisons test ($\alpha = 0.05$). The number of liver lipid vacuoles was expressed as median ± SEM and statistical analyses were carried out using the Kruskal-Wallis test. Data analyses and curation were performed in GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA).

## 4.4. Results

### 4.4.1. Effect of apple supplementation on food intake, (poly)phenol intake, and body weight

Food intake data collected at week 2 (Figure 4.2A) showed that HF-fed mice consumed significantly less food compared with chow-fed mice ($P < 0.001$), and neither high-(poly)phenol DB nor low-(poly)phenol Z supplementation influenced food intake in the HF groups, indicating that apple powder supplementation did not interfere with feed palatability. At week 2, the mean daily (poly)phenol consumption was $6.6 \pm 0.47$ mg GAE/mouse/day for DB and $0.5 \pm 0.05$ mg GAE/mouse/day for Z (Figure 4.2B). At week 10, the mean daily (poly)phenol consumption was $6.0 \pm 0.59$ and $0.4 \pm 0.07$ mg GAE/mouse/day for DB and Z, respectively. Despite variability between mice within diets, the differences in mean (poly)phenol intake was not statistically significant ($P > 0.05$) from the expected DB (6.2 mg GAE/mouse/day; 0.15% diet) and Z (0.4 mg GAE/mouse/day) dosage at both week 2 and week 10 (Fig 4.2B), meaning that the amount of apple (poly)phenols the mice consumed were consistent with the expectation. Obesity was induced in HF and HF + Z groups starting at week 6, and in HF +DB group at 7 weeks, signified by significant increases in body weights compared to the chow group (Figure 4.2C & D). Apple powder supplementation did not protect against HF-induced obesity since body weight and body weight gain were comparable among all HF groups.
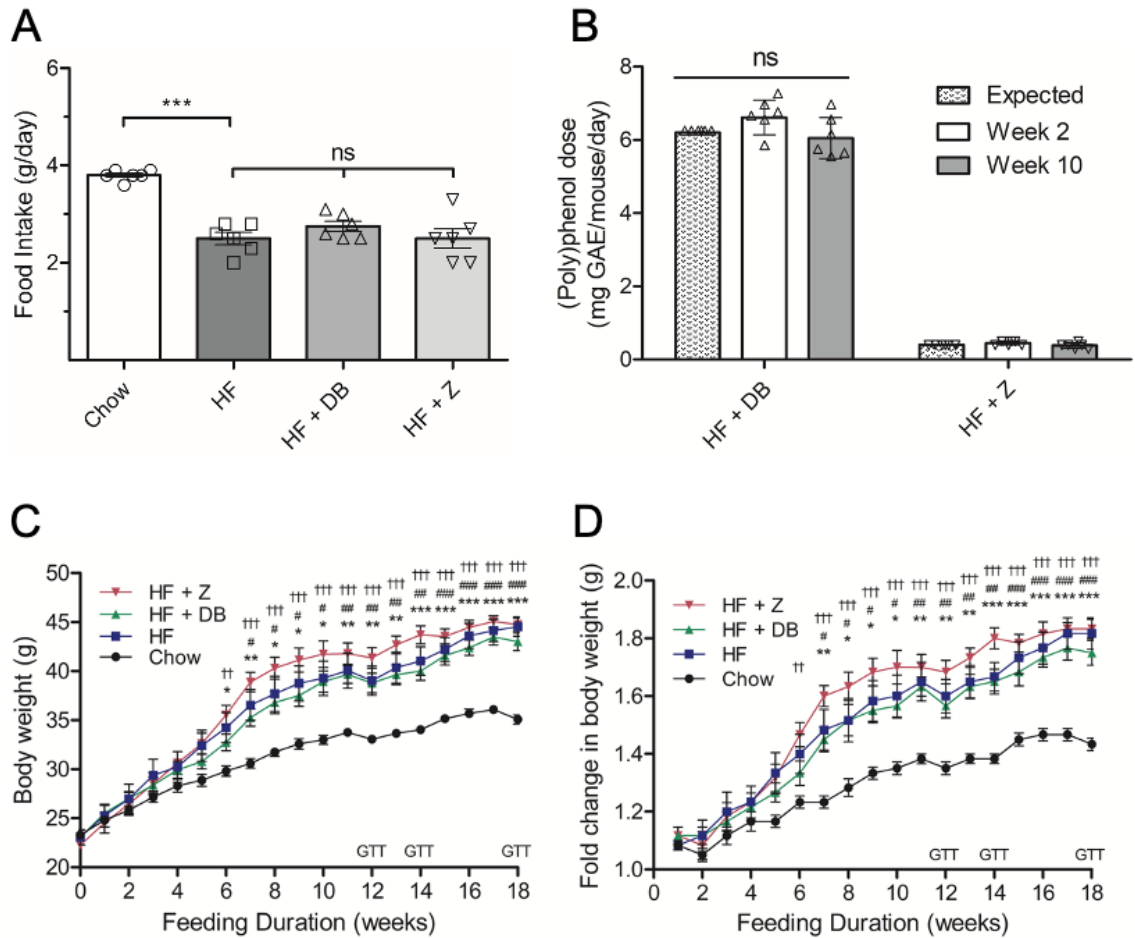
**Figure 4.2:** Effects of high-(poly)phenol 'Daux Belan' apple powder supplementation on food intake and body weight in high-fat-fed male C57BL/6 mice. (A) Food intake. (B) (Poly)phenol dosage was measured at week 2 and week 10. (C) Body weight. (D) Body weight gain expressed as fold change of starting body weight. For A-D, n = 6. Statistical analyses were performed using one-way ANOVA followed by Tukey's multiple comparisons test ($\alpha = 0.05$). Significance codes were assigned as followed:*$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ for chow vs HF; #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$ for chow vs HF + DB; †$P < 0.05$, ††$P < 0.01$, †††$P < 0.001$ for chow vs HF + Z. NS; not significant.

### 4.4.2. Effect of apple supplementation on glucose tolerance

Before week 18, glucose tolerance was similar between all groups (data not shown). At week 18, HF-fed groups had elevated fasting blood glucose levels (Figure 4.3A) and increased glycemic response to IPGTT (Figure 4.3B & C) compared to the chow-fed group. Fasting glycemia, as well as glucose levels and area under the curve (AUC) during IPGTT were comparable between all HF groups, demonstrating that apple powder supplementation did not influence HF-induced changes in glucose homeostasis.
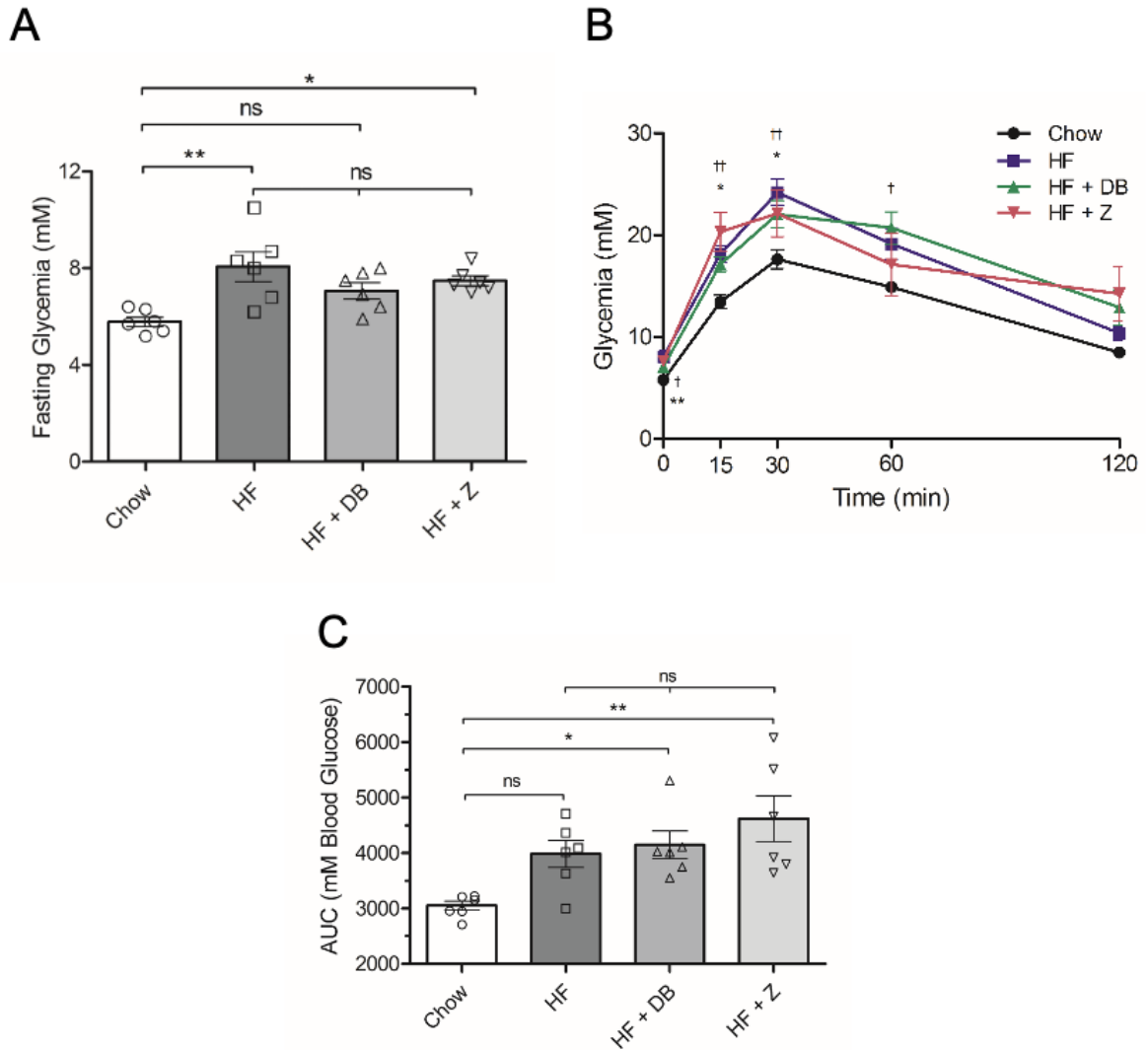
**Figure 4.3:** Effects of high-(poly)phenol 'Daux Belan' apple powder supplementation on glucose homeostasis in high-fat-fed male C57BL/6 mice. (A) Week 18 fasting blood glucose. (B) Week 18 glucose tolerance test and (C) associated area under the curve. For A-C, n = 6. Statistical analyses were performed using one-way ANOVA followed by Tukey's multiple comparisons test ($\alpha = 0.05$). Significance codes were assigned as followed: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ for chow vs HF; #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$ for chow vs HF + DB; †$P < 0.05$, ††$P < 0.01$, †††$P < 0.001$ for chow vs HF + Z. NS; not significant.

### 4.4.3. Effect of apple supplementation on plasma and hepatic lipid parameters

Plasma TG levels were comparable across all four diet groups (Figure 4.4A). Liver TG content increased 0.6-fold in the HF group but was comparable between HF, HF + DB, and HF + Z groups (Figure 4.4B). HF diet did not increase liver cholesterol levels (Figure 4.4C) and total and free liver cholesterol levels were not altered by DB nor Z supplementation in the HF diet ($P > 0.05$). Apple power supplementation did not protect against HF-induced hepatic triglyceride elevation.
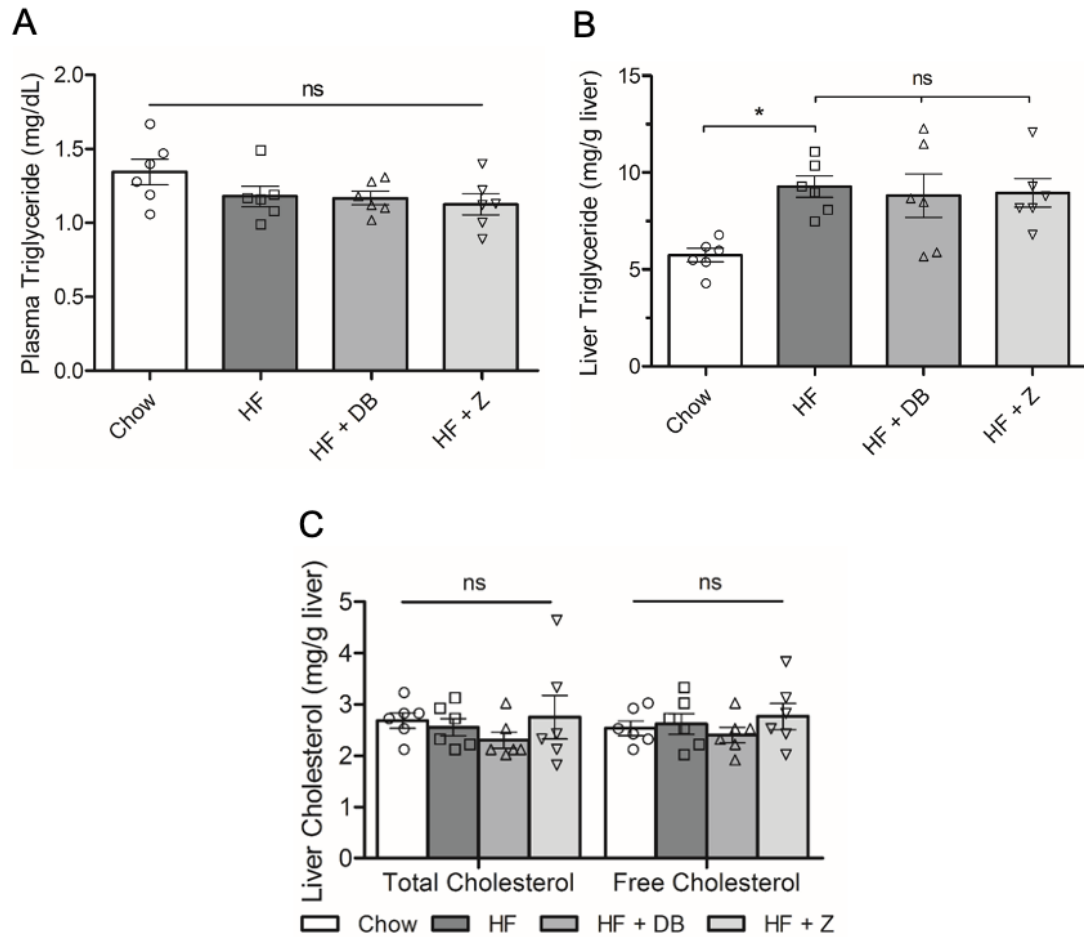
**Figure 4.4:** Effect of high-(poly)phenol 'Daux Belan' apple powder supplementation on (A) plasma triglyceride, (B) liver triglyceride, and (C) and liver total and free cholesterol content in high-fat-fed male C57BL/6 mice. Statistical analyses were performed using one-way ANOVA followed by Tukey's multiple comparisons test ($\alpha = 0.05$). Significance codes were assigned as followed: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. NS; not significant.

### 4.4.4. Effect of apple supplementation on liver histopathology

Chow-fed mice showed round central hepatocyte nuclei with no lipid vacuolation (Figure 4.5A). HF-fed mice developed significant hepatic lipid vacuolation which compressed hepatocytes and their nuclei (Figure 4.5B). Apple powder supplementation in

the HF diet did not significantly repress hepatic lipid vacuolation (Figure 4.5C-D). The number and size of liver lipid vacuoles (Figure 4.4D size of liver lipid vacuoles (Figure 4.4E) were similar across all HF groups ($P > 0.05$). Apple powder supplementation did not protect against HF-induced hepatic lipid vacuolation.
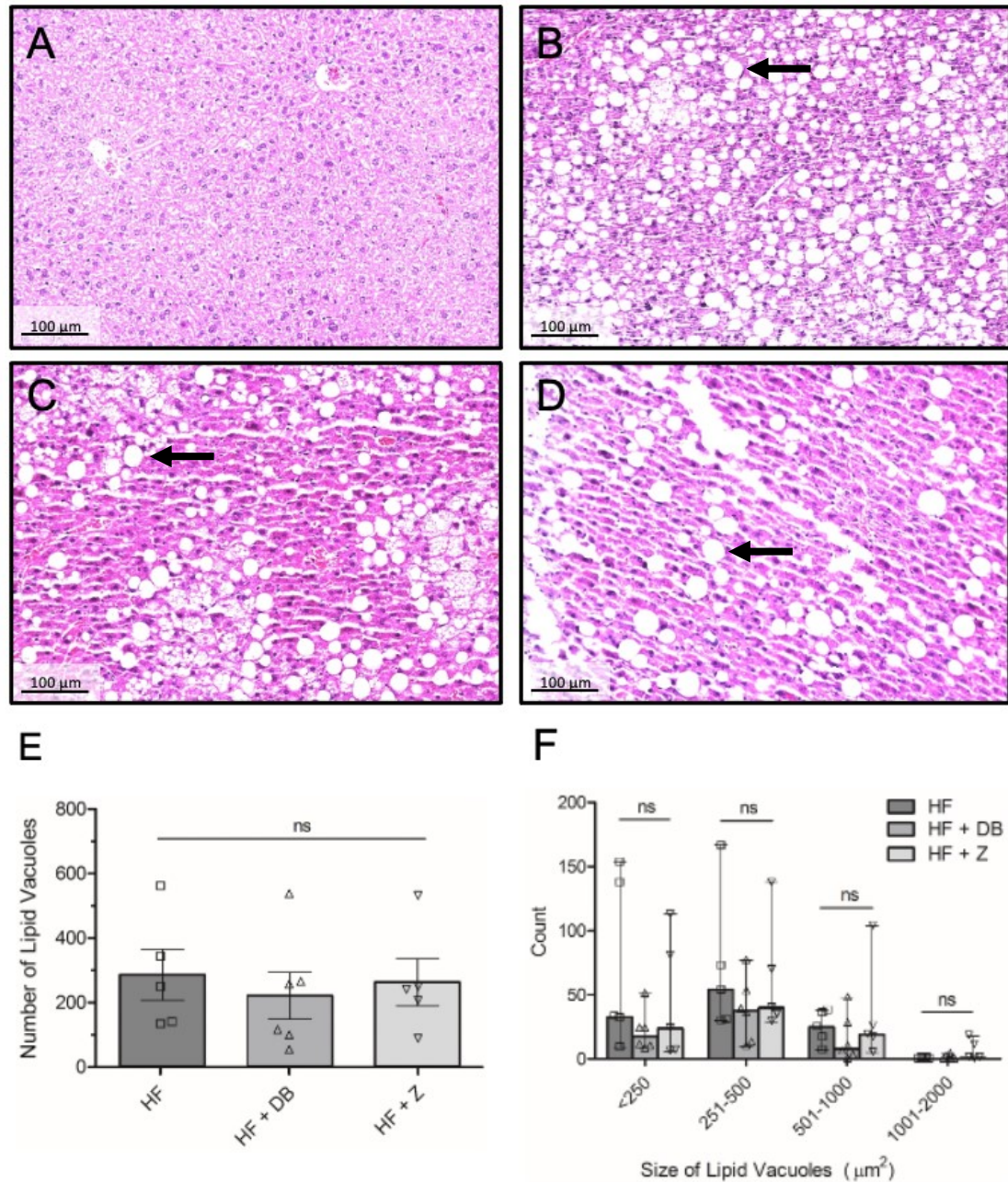
**Figure 4.5:** Liver histopathology examination (H & E staining; scale bar = 100 μm; 20X magnification) of hepatic lipid vacuolation in male C57BL/6 mice. (A) Chow, (B) high fat, (C) high fat + high (poly)phenol 'Daux Belan' apple powder, and (D) high fat + low (poly)phenol 'Zestar' apple powder. Round vacuoles indicated by the black arrow represent lipid deposition in high fat-fed mice that were washed out during tissue

processing. The number of liver lipid vacuoles (E) and size distribution of liver lipid vacuoles (F) in high-fat-fed male C57BL/6 mice. Statistical analyses were performed using one-way ANOVA followed by Tukey's multiple comparisons test ($\alpha = 0.05$). NS; not significant.

## 4.5. Discussion

There is limited scientific literature that explored the effect of apple (poly)phenols on glucose and lipid metabolism in HF-induced obese C57BL/6 mice. Only apple (poly)phenol extracts (Li et al., 2019a; Schloesser et al., 2017; Tamura et al., 2020; Zou et al., 2020) and cloudy apple juice (Han et al., 2021) have been studied. This work is the first to evaluate apple (poly)phenol supplementation in whole fruit powder form. The supplementation of apples in the whole fruit form (peel and flesh without the core) is advantageous because, in addition to providing (poly)phenolic compounds, apples contain other valuable T2D-protective bioactive constituents such as insoluble fibre (Alongi et al., 2019) and pectin (Ravn-Haren et al., 2013), and consumption of dietary fiber also promotes satiety (Flood-Obbagy & Rolls, 2009).

As expected, HF feeding induced obesity in mice (Figure 4.2B), however, apple powder supplementation did not affect body weight gain. The effect of apple (poly)phenol supplementation on body weight gain in HF-fed mice remains controversial. Supplementation of apple (poly)phenol extracts from unripe Fuji apples at dosages of 0.5% diet (Zou et al., 2020) and 5% diet (Tamura et ahas2020) have shown to reduce body weight gain in HF-fed male C57BL/6 mice. On the other hand, Schlosser et al. (2017) have reported no significant impact of 5% apple/kale extract supplementation on body weight in HF-fed female C57BL/6 mice. TPC decreases as the fruit matures, meaning unripe

apples are higher in TPC than ripe apples (Wojdyło & Oszmiański, 2020). It is conceivable that the usage of unripe fruit (poly)phenols rather than mature whole fruit influences body weight gain.

Impaired fasting glucose and impaired glucose intolerance increase the risk of T2D (Goyal et al., 2022), and elevated TG and cholesterol are important indicators of obesity (De Moura e Dias et al., 2021).  It is possible that the animals in this study were insensitive to dyslipidemia, as indicated by the lack of elevation in hepatic cholesterol levels induced by HF feeding over 18 weeks. Using the same model of diet-induced obesity (DIO), improved glucose tolerance (Schloesser et al., 2017; Tamura et al., 2020; Zou et al., 2020) and decreased serum TG and cholesterol levels by apple (poly)phenol (Li et al., 2019a; Zou et al., 2020) has been documented.  However, some are reporting decreased blood TG but not cholesterol levels by apple (poly)phenols (Han et al., 2021; Soleti et al., 2020). Dose-dependent reduction in serum cholesterol and TG by apple (poly)phenol is observed using Kunming mice (Fu et al., 2021). Here, we report that DB supplementation at a 0.15% diet dose did not influence obesity, and glucose and lipid homeostasis in HF-fed male C57BL/6 mice.

This study is subjected to several limitations that could be addressed in future research. First, apple (poly)phenol content is known to differ by growing season and stage of maturity (Kårlund et al., 2014). With the study focusing on the effect of DB apple powder supplementation at a fixed dosage from a single ripeness stage of commercial maturity, any observation is only attributed to the specific concentration of DB apple (poly)phenols tested. Human cell models can be employed to test multiple dosages in future research to understand any dose-dependent effects of DB apple powder supplementation

on glucose and lipid metabolism (Liaw et al., 2016). Second, while inbred C57BL/6 mice essentially have identical genetics, they still display significant inter-individual variability in susceptibility to DIO and related phenotypes due to variability in HF diet consumption (De Francesco et al., 2019). In this study, the lack of differences in lipid profile in HF-fed compared to chow-fed mice suggests possible insensitivity to DIO-induced dyslipidemia in this cohort of animals. As such, studying more than one cohort may provide a more complete picture. Third, the Lab Diet® laboratory rodent chow diet is used as a control against the HF diet obtained from Research Diets® in this study (see diet composition comparison in Appendix F & G). It is possible that a low-fat control diet from the same HF diet supplier may offer a more accurate comparison of metabolic alterations due to HF feeding (Research Diets, Inc., 2011). Lastly, whole apple supplementation has been found to reduce flavan-3-ols bioaccessibility and thus its serum concentration in minipigs when compared with extract supplementation (Monfoulet et al., 2020). Strategies to enhance the bioaccessibility and bioavailability of (poly)phenol compounds in whole apple powder can be considered to improve diet intervention outcomes (Ribas-Agustí et al., 2018; Thilakarathna & Rupasinghe, 2013). Future research can investigate the effect of higher dosages of DB apple powder, or DB supplemented as (poly)phenol extracts, sugar-free powders, and in combination with other bioactives.

**4.6. Conclusion**

In conclusion, high-(poly)phenol-containing DB apple supplementation in the whole fruit form as freeze-dried powder did not attenuate HF-induced body weight gain, glucose intolerance, hepatic hypertriglyceridemia, and hepatic lipid vacuolation at the dosage of 6.2 mg GAE/mouse/day (at 0.15% diet). Suggestions for future studies include

utilizing human cell models to observe dose-dependent changes in lipid parameters, testing higher dosages of apple power, (poly)phenol extracts alone or in combination with other fruits, vegetables, or protein bioactives over a shorter study period, studying the effects of sugar-free apple powder supplementation, and employing a different food matrix to improve the bioaccessibility of apple (poly)phenols. Further research is needed to confirm the effect of high-(poly)phenol DB supplementation in the whole fruit form on alleviating glucose intolerance and to reveal its effect on adiposity and lipid metabolism.

**CHAPTER 5: GENERAL DISCUSSION AND CONCLUSION**

**5.1. Anti-diabetic activities of apple (poly)phenols *in vitro* and *in vivo***

In the first experiment (Chapter 3), the *in vitro* screening of 478 apple accessions from Canada's ABC is performed to identify apple accessions with anti-diabetic potential and to assess the major (poly)phenol constituents of selected apple accessions by HPLC-MS. Given the previously reported 100-fold differences in TPC across the ABC population (Watts et al., 2021), along with the finding that the inhibition of α-glucosidase, α-amylase, and AGE formation was correlated with TPC, the large variation in anti-diabetic properties of apple peel and flesh (poly)phenol extracts within the ABC population was expected. HPLC-MS analysis revealed high concentrations of phloridzin, epicatechin and procyanidin B2 in apples containing significantly high TPC. Considering the positive correlation identified between phloridzin, epicatechin, and procyanidin B2 concentrations and α-glucosidase inhibition, as well as the numerous health-promoting properties (Ding et al., 2013; Justino et al., 2022; Sulaiman, 2014) by these (poly)phenols, a TPC-focused breeding approach to increase the concentrations of these bioactive compounds in apples, can be suggested as a valuable target in apple breeding aiming at developing T2D-protective nutraceutical attributes such as delaying carbohydrate digestion and reducing postprandial blood glucose spike (Ayua et al., 2021). High TPC apples such as 'Marachal' and 'Daux Belan' show potential applications in developing value-added functional ingredients or nutraceuticals for blood glucose management. 'S23-03-749', which is a dessert apple advance breeding line containing high TPC, provides a potential avenue to introduce high TPC apple into the fresh fruit market that is currently filled with low TPC cultivated apples (Davies et al., 2022).

In the second experiment (Chapter 4), the effect of high-(poly)phenol-containing DB apple powder consisting of peel and flesh (a 0.15% apple (poly)phenol; 6.2 mg GAE/mouse/day) and low-(poly)phenol-containing Z apple powder (apple control; 0.4 mg GAE/mouse/day) on glucose tolerance and lipid parameters in diet-induced obese male C57BL/6 mice was investigated. DB supplementation at the tested dosage did not influence body weight, glucose tolerance, liver TG, and hepatic liver vacuolation. Although inbred C57BL/6 mice are susceptible to DIO and are widely used in obesity research, they have been observed to display large variation in the initial basal fat mass, which is a significant predictor of body weight gain when later introduced to HF-feeding (Yang et al., 2014). In addition, susceptibility to DIO in C57BL/6 mice can also be influenced by various circumstances in the intra-uterus environment, early post-natal stage, and adulthood (De Francesco et al., 2019). Considering these factors that influence DIO susceptibility, potential counteracting approaches can include repeating the study in multiple cohorts of animals and distinguishing between HF-tolerant and intolerant animals, which can be accomplished by increasing the number of animals to offset variability while maintaining statistical power or by performing a median-based split on food intake data (Brenachot et al., 2018). Another possible reason for not observing the significant impact of DB supplementation on the measured parameters is the dosage of apple (poly)phenols employed. This work is the first study to explore whole apple powder supplementation in a DIO model using C57BL/6 mice. The DB dose of 62 mg GAE/mouse/day (a 0.15% apple (poly)phenol), which is equivalent to 1,200 mg GAE/adult/day, was employed in this study. Both 600 and 1,200 mg total apple (poly)phenol/day are reported effective hypoglycemic doses in borderline diabetic people and healthy people, respectively. Despite the use of

allometric scaling in dose extrapolation from human to mouse in this study, where the human dose is extrapolated to an animal equivalent dose based on the body surface area normalization method (Nair and Jacob, 2016), the positive outcomes observed in reported human studies were not replicated in the animals in this study. Further, intact (poly)phenols supplemented by whole apples that were not absorbed in the small intestines require biotransformation into metabolites for utilization by gut microbiota in the colon (Ozdal et al., 2016). It is reported that mice contain different gut bacteria genera and species than humans (Ley et al., 2005), suggesting potential differences in (poly)phenol metabolism between mice and humans and that it is possible that mice are not a very good model system for (poly)phenol metabolism. However, limited scientific literature report anti-obesity effects of 0.5% (Zou et al., 2020) and 5% (Tamura et al., 2020) unripe Fuji apple (poly)phenol extract supplementation in the DIO model of C57BL/6 mice, but not 5% apple/kale extract (Schloesser et al., 2017). As such, DB dose can be increased in future works to investigate potential glycemia protective actions as suggested by *in vitro* findings.

## 5.2. Recommendations for future research

Several future research directions can be taken to further elucidate the T2D protective properties of high (poly)phenol apples. First, *in vitro* and *in vivo* anti-diabetic properties of apple (poly)phenols were tested using apples from the 2016 and 2021 harvest seasons, respectively. It is well-known that the concentration and composition of (poly)phenol compounds differ between harvest seasons (Kårlund et al., 2014). Thus, it is recommended that future studies examine accessions from more than one harvest season and use apples from the same harvest season for comparison of *in vitro* and *in vivo* findings for better comprehension. Second, apple (poly)phenol concentrations peak during early

development and decrease as the fruit matures (Wojdyło & Oszmiański, 2020). Immature apples have been demonstrated to inhibit carbohydrate-hydrolyzing enzymes *in vitro* and *in vivo* (Gong et al., 2020b; Li et al., 2019b; Li et al., 2020; Zhao et al., 2019). Therefore, future studies can be directed to investigate high TPC accessions such as 'Marachel', 'Daux Belan', and 'S23-03-749' at earlier maturity stages and increased dosage concentrations for potential anti-diabetic effects. Third, to combat the large inter-individual variability in DIO susceptibility in inbred C57BL/6 mice (De Francesco et al., 2019), and possible random instances of dyslipidemia resistance as suspected in this work, it is recommended to study metabolic changes by apple supplementation to an HF diet using more than one cohort of animals. Fourth, direct apple supplementation in the whole food form has been reported with reduced flavan-3-ols bioaccessibility (Monfoulet et al., 2020). As such, the full potential of apple (poly)phenol supplementation can be investigated using various food processing strategies to enhance bioavailability (Ribas-Agustí et al., 2018). Lastly, apple (poly)phenol supplementation in the extract form, as sugar-free powders, or in combination with other fruits, vegetables or bioactive proteins are also potential areas to explore. In conclusion, the preliminary data confirmed the thesis hypothesis that apple (poly)phenols modulate enzymes and processes that reduce glycemia *in vitro*, suggesting the potential use of apple (poly)phenols as value-added food and nutraceutical ingredients. These findings need to be confirmed using well-designed cell and pre-clinical experimental animal models before making recommendations on the T2D protective effects of apple (poly)phenols for subsequent studies involving human participants.

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# APPENDIX

## Appendix A

General information of apple accessions and total (poly)phenol content (TPC) of the high TPC group (n = 10), commercial group (n = 10), and low TPC group (n = 10).

| Accession | Use | Country of origin | TPC by FC (mg GAE/g FW) | TPC by LC-qTOF-MS (mg total/g FW) |
|---|---|---|---|---|
| **High TPC Group** | | | | |
| 'Marachal' | Cider | France | 4.75 | 2.29 |
| 'Daux Belan' | Cider | France | 3.99 | 2.44 |
| 'Kaz 95 18-02P-20' | Wild | Kazakhstan | 3.28 | 2.11 |
| 'S23-03-749'† | Dessert | Canada | 2.77 | 2.61 |
| 'Coat Jersey' | Cider | United Kingdom | 2.35 | 2.37 |
| 'Kaz 95 08-06 ID 20622063' | Wild | Kazakhstan | 2.31 | 1.82 |
| 'Kaz 95 07-05' | Wild | Kazakhstan | 2.21 | 1.52 |
| 'Gros Frequin' | Cider | France | 2.14 | 2.06 |
| 'Kaz 95 18-05' | Wild | Kazakhstan | 2.02 | 1.91 |
| 'Kaz 95 08-06 ID 3147' | Wild | Kazakhstan | 2.00 | 1.51 |
| **Commercial Group** | | | | |
| 'Reinette Russet' | Cider | France | 1.00 | 1.47 |
| 'McIntosh' | Dessert | Canada | 0.64 | 1.16 |
| 'Red Delicious' | Dessert | United States | 0.61 | 1.19 |
| 'Jonagold' | Dessert | United States | 0.59 | 1.20 |

| | | | | |
|---|---|---|---|---|
| 'Gala' | Dessert | New Zealand | 0.52 | 1.19 |
| 'Sweetango' | Dessert | United States | 0.43 | 1.31 |
| 'Ambrosia' | Dessert | Canada | 0.43 | 1.03 |
| 'Honeycrisp' | Dessert | United | 0.41 | 1.54 |
| 'Elstar' | Dessert | Netherlands | 0.37 | 1.06 |
| 'Empire' | Dessert | United States | 0.26 | 0.72 |
| **Low TPC Group** | | | | |
| 'KAR 52'[†] | Dessert | Canada | 0.19 | 0.57 |
| 'KAR 32'[†] | Dessert | Canada | 0.18 | 1.03 |
| 'Minister von Hammerstein' | Dessert | Germany | 0.14 | 0.49 |
| 'Geoagiu 21' | Dessert | Romania | 0.12 | 0.74 |
| 'S47-23-22'[†] | Dessert | Canada | 0.09 | 0.61 |
| 'KAR 17'[†] | Dessert | Canada | 0.09 | 0.61 |
| 'Kinsei' | Dessert | Japan | 0.07 | 0.57 |
| 'S47-25-82'[†] | Dessert | Canada | 0.06 | 0.93 |
| 'Zestar' | Dessert | United States | 0.06 | 0.80 |
| 'S19-23-52' | Dessert | Canada | 0.05 | 0.70 |

Note: Apple phenotypic data on world, use, country and TPC by FC (mg GAE/g FW) are obtained from (Watts et al., 2021). TPC by LC-qTOF-MS (mg total/g FW) data is unpublished.

[†]Advanced breeding lines from AAFC-Kentville Research Station (NS, Canada)

## Appendix B

Major (poly)phenol compound composition of the high TPC group (n = 10), commercial cultivars (n = 10), and low TPC group (n = 10). FW, fresh weight.
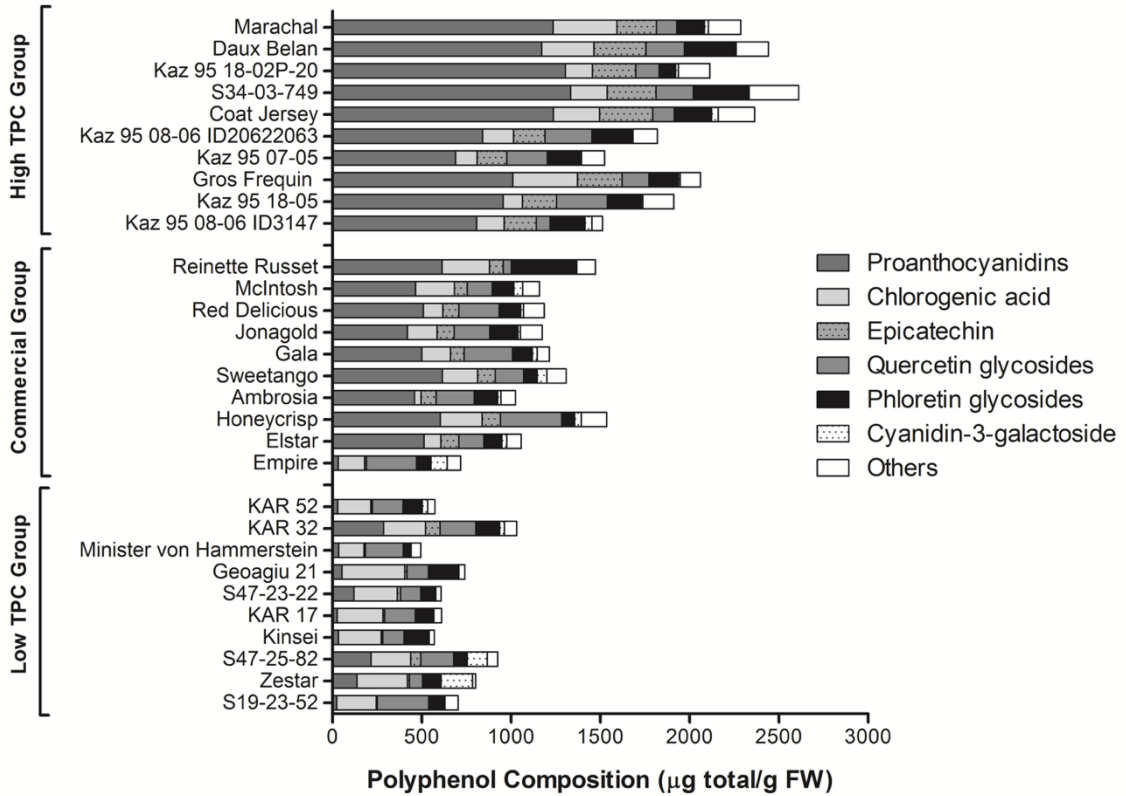
**Appendix C**

Pearson correlation between total (poly)phenol content (TPC) determined by Folin-Ciocalteu and LC-qTOF-MS method for the high TPC group (n=10), commercial cultivars (n=10), and low TPC group (n=10). FW, fresh weight.
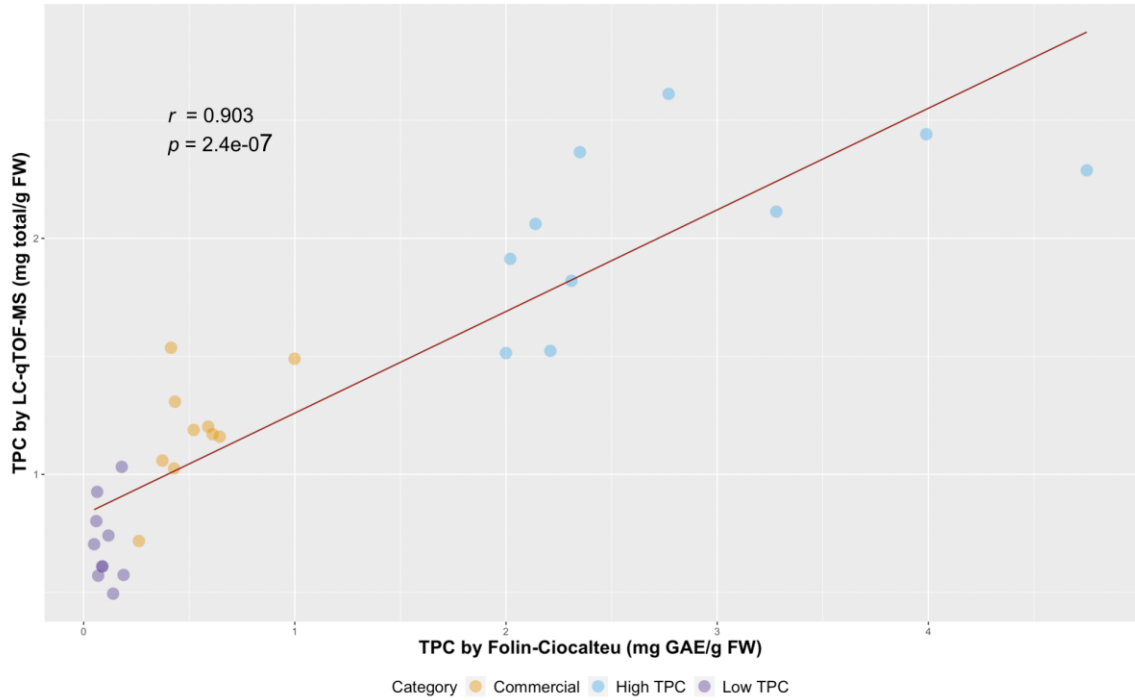
# Appendix D

Spearman correlation between concentrations of six major apple (poly)phenol compounds and $IC_{50}$ values of two carbohydrate-hydrolyzing enzymes in the high TPC group (n = 10) of apple accessions.
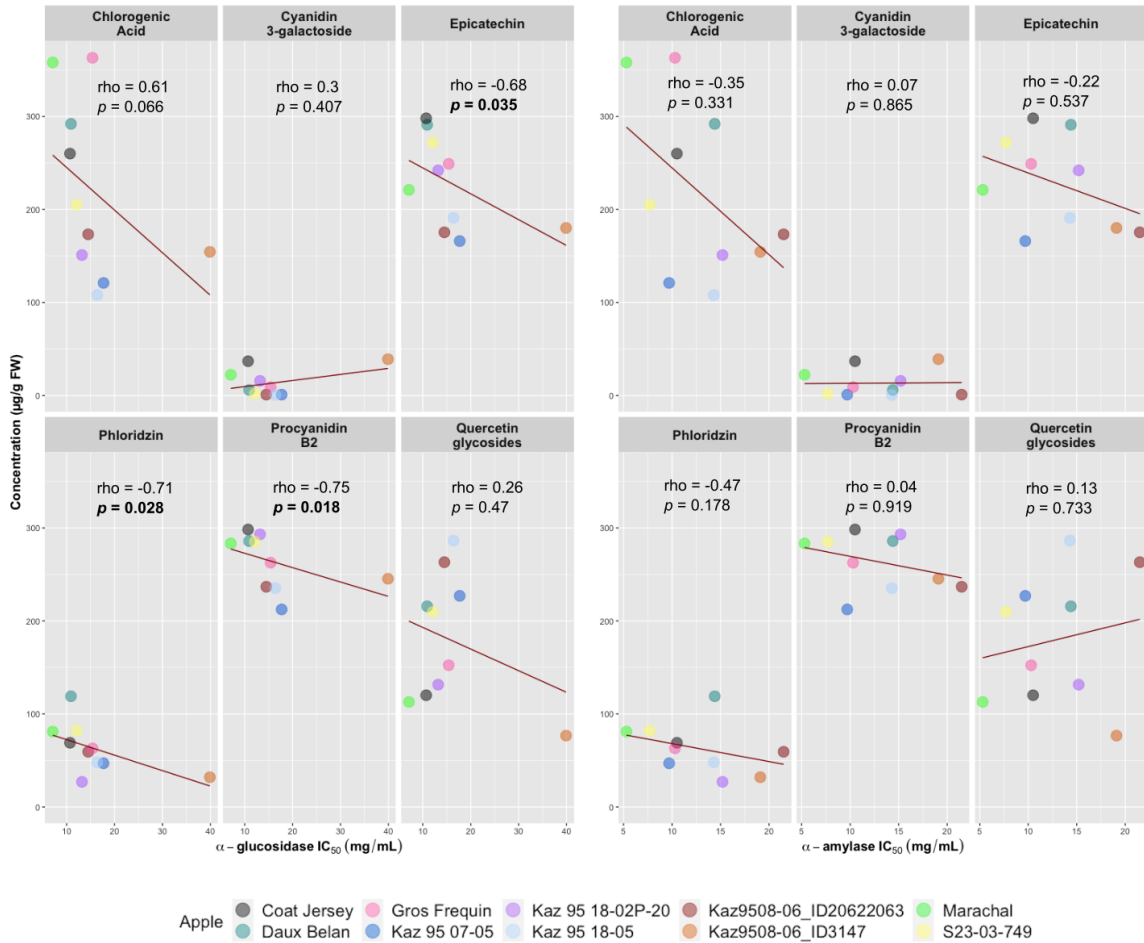
## Appendix E

Screenshot of animal study approval

**Date:** Friday, May 21, 2021 at 11:52 AM
**Subject:** Notice of Protocol Amendment Approval RE: 21-005

**DALHOUSIE UNIVERSITY, UNIVERSITY COMMITTEE ON LABORATORY ANIMALS**

**NOTICE OF PROTOCOL AMENDMENT APPROVAL**

PROTOCOL NUMBER: 21-005

EXPIRY DATE: March 01, 2022

INVESTIGATOR: Dr Vasantha Rupasinghe

CATEGORY/LEVEL: D - (experiments which cause moderate to severe distress or discomfort)

TITLE OF STUDY: (21-005) Selection and evaluation of superior apple genotypes for potential health benefits

Event/file #: 1033572 - 10066816

Amendment is approved.

Thank you,

Jennifer Wipp, Coordinator
University Committee on Laboratory Animals
Dalhousie University
1355 Oxford Street
902.494.1270 | UCLA@dal.ca | WEBSITE: https://www.dal.ca/dept/animal-ethics.html

# Prolab® RMH 3500, Autoclavable     5P04*

## DESCRIPTION

Prolab® Rat/Mouse/Hamster 3500 is an autoclavable diet formulated for growth and reproduction. It is the companion product of Prolab® RMH 3000. Protein, mineral and vitamin levels have been fortified and carefully balanced so that maximum food values are maintained after sterilization. It can also be pasteurized or sterilized without clumping. This diet is formulated using the unique and innovative concept of Constant Nutrition®, paired with the selection of highest quality ingredients to assure minimal inherent biological variation in long-term studies.

### Features and Benefits
- Constant Nutrition® formula helps minimize nutritional variables
- Processed and packaged for autoclaving
- Supports optimum growth and efficient reproduction performance of rats, hamsters and mice
- Fortified with extra nutrients to compensate for losses during autoclaving
- Processed with silicon dioxide to reduce sticking and clumping

### Product Forms Available
- Oval pellet, 10 mm x 16 mm x 25 mm length (3/8"x5/8"x1")

## GUARANTEED ANALYSIS

Crude protein not less than . . . . . . . . . . . . . . . . . . . . . . . . .22.0%
Crude fat not less than . . . . . . . . . . . . . . . . . . . . . . . . . . . . .5.0%
Crude fiber not more than . . . . . . . . . . . . . . . . . . . . . . . . . .5.0%
Ash not more than . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .6.0%
Added minerals not more than . . . . . . . . . . . . . . . . . . . . . .2.5%

## AUTOCLAVING SUGGESTIONS

To autoclave the pellets, place on trays, in small bags, or in larger bags, to a depth of no more than 3 inches. When steam autoclaved, the pellets swell and exert force on adjacent pellets. Confinement by a bag or container creates additional pressure, which may result in sticking. *Assay before and after autoclaving:* Conditions of sterilization must be determined for each autoclaving unit. Microbiological evaluation should be done to insure sterilization is achieved. It is best to assay the diet before and after sterilization to determine nutrient losses.

## INGREDIENTS

Ground wheat, soybean meal, wheat middlings, ground yellow corn, fish meal, soybean hulls, soybean oil, alfalfa meal, calcium lignin sulfonate, calcium carbonate, brewers dried yeast, salt, dicalcium phosphate, silicon dioxide, DL-methionine, L-lysine, manganous oxide, magnesium oxide, ferrous sulfate, zinc oxide, copper sulfate, calcium iodate, cobalt carbonate, vitamin A acetate, cholecalciferol, dl-alpha tocopheryl acetate, vitamin B₁₂ supplement, riboflavin, nicotinic acid, calcium pantothenate, menadione dimethylpyrimidinol bisulfite (source of vitamin K), folic acid, pyridoxine hydrochloride, thiamin mononitrate, biotin, choline chloride.

## FEEDING DIRECTIONS

Prolab® RMH 3500, Autoclavable is designed for growth and reproduction of rodents. It contains all the nutrients that are required for growth, lactation, and reproduction. This diet should be fed free choice in a self-feeder. Keep a constant supply of fresh water available.
*Rats*– All rats will eat varying amounts of feed depending on their genetic origin. Larger strains will eat up to 30 grams per day. Smaller strains will eat up to 15 grams per day. Feeders in rat cages should be designed to hold two to three days supply of feed at one time.
*Mice*–Adult mice will eat up to 5 grams of pelleted ration daily. Some of the larger strains may eat as much as 8 grams per day per animal. Feed should be available on a free choice basis in wire feeders above the floor of the cage.
*Hamsters*–Adults will eat up to 14 grams per day.
**Important:** A feeding program is only as effective as the management practices followed.
**Caution:** Store in a dry, well ventilated area, free of pests and insects. Do not use moldy or insect-infested feed.
12/11/09

## CHEMICAL COMPOSITION[1]

### Nutrients[2]
| | |
|---|---|
| Protein, % | 22.5 |
| Arginine, % | 1.32 |
| Cystine, % | 0.29 |
| Glycine, % | 1.12 |
| Histidine, % | 0.53 |
| Isoleucine, % | 1.16 |
| Leucine, % | 1.66 |
| Lysine, % | 1.35 |
| Methionine, % | 0.51 |
| Phenylalanine, % | 1.01 |
| Tyrosine, % | 0.61 |
| Threonine, % | 0.83 |
| Tryptophan, % | 0.30 |
| Valine, % | 1.15 |
| Serine, % | 1.16 |
| Aspartic Acid, % | 2.31 |
| Glutamic Acid, % | 5.07 |
| Alanine, % | 1.20 |
| Proline, % | 1.63 |
| Taurine, % | 0.03 |
| **Fat (ether extract), %** | **5.9** |
| **Fat (acid hydrolysis), %** | **6.7** |
| Cholesterol, ppm | 265 |
| Linoleic Acid, % | 2.72 |
| Linolenic Acid, % | 0.34 |
| Arachidonic Acid, % | 0.00 |
| Omega-3 Fatty Acids, % | 0.65 |
| Total Saturated Fatty Acids, % | 1.19 |
| Total Monounsaturated Fatty Acids, % | 1.29 |
| **Fiber (Crude), %** | **4.8** |
| Neutral Detergent Fiber[3], % | 15.0 |
| Acid Detergent Fiber[4], % | 6.4 |
| **Nitrogen-Free Extract (by difference), %** | **50.1** |
| Starch, % | 31.3 |
| Glucose, % | 0.1 |
| Fructose, % | 0.1 |
| Sucrose, % | 0.8 |
| Lactose, % | 0.0 |
| **Total Digestible Nutrients,%** | **79.4** |
| **Gross Energy, kcal/gm** | **4.10** |
| **Physiological Fuel Value[5], kcal/gm** | **3.43** |
| **Metabolizable Energy, kcal/gm** | **3.19** |

### Minerals
| | |
|---|---|
| **Ash, %** | **6.7** |
| Calcium, % | 1.17 |
| Phosphorus, % | 0.76 |
| Phosphorus (non-phytate), % | 0.51 |
| Potassium, % | 0.82 |
| Magnesium, % | 0.21 |
| Sulfur, % | 0.24 |
| Sodium, % | 0.27 |
| Chlorine, % | 0.47 |
| Fluorine, ppm | 20 |
| Iron, ppm | 400 |
| Zinc, ppm | 120 |
| Manganese, ppm | 130 |
| Copper, ppm | 13 |
| Cobalt, ppm | 0.27 |
| Iodine, ppm | 0.99 |
| Chromium, ppm | 1.5 |
| Selenium, ppm | 0.28 |

### Vitamins
| | |
|---|---|
| Carotene, ppm | 2.4 |
| Vitamin K (as menadione),ppm | 1.8 |
| Thiamin Hydrochloride, ppm | 90 |
| Riboflavin, ppm | 15 |
| Niacin, ppm | 80 |
| Pantothenic Acid, ppm | 31 |
| Choline Chloride, ppm | 1500 |
| Folic Acid, ppm | 2.9 |
| Pyridoxine, ppm | 6.4 |
| Biotin, ppm | 0.33 |
| B₁₂, mcg/kg | 73 |
| Vitamin A, IU/gm | 30 |
| Vitamin D₃ (added), IU/gm | 2.2 |
| Vitamin E, IU/kg | 75 |
| Ascorbic Acid, mg/gm | — |

### Calories provided by:
| | |
|---|---|
| Protein, % | 26.201 |
| Fat (ether extract), % | 15.459 |
| Carbohydrates, % | 58.340 |

*Product Code
1. Formulation based on calculated values from the latest ingredient analysis information. Since nutrient composition of natural ingredients varies and some nutrient loss will occur due to manufacturing processes, analysis will differ accordingly.
2. Nutrients expressed as percent of ration except where otherwise indicated. Moisture content is assumed to be 10.0% for the purpose of calculations.
3. NDF = approximately cellulose, hemi-cellulose and lignin.
4. ADF = approximately cellulose and lignin.
5. Physiological Fuel Value (kcal/gm) = Sum of decimal fractions of protein, fat and carbo-hydrate (use Nitrogen Free Extract) x 4,9,4 kcal/gm respectively.

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**Appendix G**

Detailed high fat diet composition

| Ingredient (%) | High Fat |
|---|---|
| Casein | 23.31 |
| L-Cystine | 0.35 |
| Sucrose | 20.60 |
| Lodex 10 | 11.65 |
| Corn Starch | 8.48 |
| Cellulose | 5.83 |
| Lard | 20.68 |
| Soybean Oil | 2.91 |
| Mineral Mix S10026B[a] | 5.83 |
| Choline Bitartrate | 0.23 |
| Vitamin Mix V10001C[b] | 0.12 |
| FD&C Red Dye #40 | 0.01 |

[a] The mineral mix is composed of 17.98% sucrose, 33% potassium citrate, 26% calcium phosphate, 11% calcium carbonate, 5.18% sodium chloride, 5.15% magnesium sulfate, 0.84% magnesium oxide, 0.42% ferric citrate, 0.25% manganese carbonate, 0.11% zinc carbonate, 0.039% chromium potassium sulfate, 0.021% copper carbonate, 0.006% ammonium molybdate, 0.004% sodium fluoride, 0.001% sodium selenite, and 0.001% potassium iodate.

[b] The vitamin mix is composed of 78.4% sucrose, 10% vitamin E, 3% niacin, 2% biotin, 1.6% pantothenic acid, 1% vitamin D3, 1% vitamin B12, 0.8% vitamin A, 0.7% pyridoxine HCl, 0.6% riboflavin, 0.6% thiamine HCl, 0.2% folic acid, and 0.1% menadione sodium bisulfite.

Note: Diet composition data obtained from Research Diets, Inc.

https://www.researchdiets.com/formulas/d12451