Effect of Highbush blueberry consumption on markers of metabolic syndrome

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ARAYA PRANPRAWIT

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Abstract

Background :

Metabolic syndrome (MS) is becoming a major public health challenge worldwide, and is associated with a higher risk of the development of several chronic diseases including type II diabetes. Being physically active would provide the most effective management for metabolic disorders; however, the use of dietary bioactive compounds from various plants has also been proposed as an alternative approach. A number of experimental studies indicate that Lowbush blueberries may be able to reduce symptoms of MS but the evidence for Highbush blueberries, which are commonly consumed, is scarce and their benefits remain doubtful. Therefore, the primary objective of this thesis was to investigate the effect of selected Highbush blueberries grown in New Zealand on their potential for managing metabolic-related disorders in order to provide further knowledge of the role for bioactive compounds from Highbush blueberries.

Method :

The selected eight Highbush blueberry cultivars were initially characterised by measuring total phenolic content using a Folin-Ciocalteu procedure; anthocyanin profiles and chlorogenic acid concentration by HPLC; and antioxidant capacity by the ferric reducing antioxidant power (FRAP) and by 2,2, diphenyl-picrylhydrazyl (DPPH) assays (Chapter 3). Further experiments were then carried out to investigate whether these Highbush blueberries possess any activity against measures of MS *in vitro*. The ability of Highbush blueberries to inhibit α-amylase and α-glucosidase, the enzymes involved in breaking down starch, and their abilities to enhance the growth of beneficial probiotic bacteria, another mechanism associated with improving insulin resistance, were tested in Chapter 4. Finally, the physiological effects of Highbush blueberry consumption on metabolic syndrome biomarkers were assessed *in vivo* using animal models of diet-induced metabolic syndrome (Chapter 5-7).

Results :

Our results demonstrated that selected Highbush blueberries grown in New Zealand contained considerable amounts of polyphenolics and total anthocyanins, and exhibited high antioxidant activities, with 'Burlington' and 'Elliott' cultivars exhibiting the highest total phenolic content (> 3.4 mg GAE/g frozen berries (FB)), total anthocyanins (> 2.2 mg/g FB) and antioxidant capacities (FRAP; > 3.0 mg $FeSO_4$/g FB, DPPH; > 65% inhibition at 5 mg FB). Further i*n vitro* experiments

supported the ability of these blueberries to inhibit α-amylase (10-40% inhibition at 20 mg FB) and α-glucosidase (10-50% inhibition at 25 mg FB); additionally, some blueberry cultivars possessed the ability to increase the growth of the probiotic bacteria *Lactobaccillus acidophilus* by more than 0.5 $\log_{10}$ CFU/mL. However, the extent of these benefits was not closely correlated with total phenolic content ($R^2 < 0.27$), total anthocyanins ($R^2 < 0.23$), or antioxidant capacities (FRAP; $R^2 < 0.42$, DPPH; $R^2 < 0.24$) across all genotypes, indicating that these anti-metabolic syndrome abilities were not simply due to the total bioactives or antioxidant capacities presented in the berries. 'Burlington' and 'Bluecrop', which exhibited strong enzyme inhibition as well as enhanced beneficial probiotic bacterial growth but contained different components of individual anthocyanins, were chosen for further testing *in vivo*. Rats fed a high-fat-high-sugar diet plus 1% freeze-dried whole blueberries (both cultivars) for 8 weeks showed signs of improvement of glucose tolerance and exhibited between 30 and 36% decrease in the degree of insulin resistance (HOMA-IR) as compared to the controls. The blueberries also showed a trend to increase the growth of beneficial bacteria, *Lactobacillus* spp. ($P = 0.20$) and *Bifidobacterium* spp. ($P = 0.15$), in the rats' caecal content. However, no reduction in body weight or fat accumulation was observed with blueberry supplementation. There were no significant differences ($P > 0.05$) in the abilities of 'Burlington' and 'Bluecrop' to modulate any metabolic biomarkers assessed *in vivo*.

Conclusion :

Inclusion of the blueberries into the diet showed promise for management of some markers of metabolic syndrome, in particular the improvement of insulin sensitivity and glucose tolerance. The results of these studies shed some light on the beneficial effect of selected NZ Highbush blueberries against insulin resistance associated with metabolic syndrome.

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Abbreviations

2DG	2-deoxyglucose
2-h PG	2 hours plasma glucose
A1C	hemoglobin A1C or glycated hemoglobin
AACE	American Association of Clinical Endocrinology
ACNs	anthocyanins
ACP	acepromazine
AHA/NHLBI	American Heart Association/Nation Heart, Lung and Blood Institute
AMPK	adenosine monophosphate-activated protein kinase
ANOVA	analysis of variance
AOA	antioxidant activity
ATPIII	National Cholesterol Education Program Adult Treatment Panel III
AUC	area under the curve
BB	blueberry
BMI	body mass index
BW	body weight
CFU	colony forming unit
Cmax	maximum concentration
CONT	starch-based control diet
CRP	C-reactive protein
CVD	cardiovascular disease
db/db	mouse model of diabetes and obesity where leptin receptor is deficient
DEXA	dual-energy x-ray absorptiometry
DNS	3,5-dinitrosalicylic acid
DP	degree of polymerization
DPPH	2,2-diphenyl-1-picrylhydrazyl
DSF	defatted soybean flour
EGCG	epigallocatechin gallate
EGIR	European Group for the study of Insulin Resistance
ER	endoplasmic reticulum
ESR	Environmental Science and Research
FB	frozen berries
$FeCl_3$	ferric chloride

$FeSO_4$	ferrous sulphate
FFA	free fatty acids
FFM	fat-free mass
FISH	fluorescent in situ hybridization
FPG	fasting plasma glucose
FPI	fasting plasma insulin
FRAP	ferric reducing antioxidant power
FW	fresh weight
G6Pase	glucose-6-phosphatase
GAE	gallic acid equivalent
GIT	gastrointestinal tract
GLUT	glucose transporter
HDL-C	high density lipoprotein cholesterol
HF	high-fat
HFD+BB	high fat diet plus blueberry
HFHS	high-fat-high-sugar
HFR	high-fructose
HFR1B	high-fructose diet containing 1% freeze-dried blueberry powder
HFR4B	high-fructose diet containing 4% freeze-dried blueberry powder
HOMA-IR	homeostasis model assessment of insulin resistance
HPLC	High Performance Liquid Chromatography
HS	high-sugar (sucrose)
iBAT	interscapular brown adipose tissue
IC_{50}	inhibitory concentration of 50%
IDF	International Diabetes Federation
IFG	impaired fasting glucose
IGT	impaired glucose tolerance
IL-6	interleukin-6
IL-10	interleukin-10
IRS	insulin receptor substrate
ITT	insulin tolerance test
LFD	low fat diet
LPS	lipopolysaccharide
MRS	Man-Rogaso-Sharpe

MS	metabolic syndrome
Na_2CO_3	sodium carbonate
NAFLD	non-alcoholic fatty acid liver disease
NCEP-ATP III	National Cholesterol Education Program Adult Treatment Panel III
NGSP	National Glycohemoglobin Standardization Program
NHANES	National Health and Examination Survey
NIDDK	National Institute of Diabetes and Digestive and Kidney Diseases
ob/ob	leptin-deficient obese mouse model
OD	optical density
OGTT	oral glucose tolerance test
ORAC	oxygen radical absorbance capacity
PAs	proanthocyanidins
PCA	principal component analysis
PEPCK	phosphoenolpyruvate carboxykinase
PI3K	phosphotidylinositol 3-kinase
PKB/Akt	protein kinase B
*p*NPG	*p*-nitrophenyl α-D-glucopyranoside
PPAR-γ	peroxisome proliferator-activated receptor gamma
QUICKI	quantitative insulin sensitivity check index
ROS	reactive oxygen species
SAPU	Small Animal Production Unit
SD	Sprague-Dawley
T2DM	type II diabetes mellitus
TG	triglycerides
TNF-α	tumour necrosis factor α
TPC	total polyphenolic content
TPTZ	2,4,6-tripyridyl-s-triazine
VHFD	very high fat diet
WAT	white adipose tissue
WC	waist circumference
WHO	World Health Organization
WHR	waist-hip ratio

CHAPTER 1

Introduction

Metabolic syndrome (MS) represents a group of common metabolic disorders that are strongly associated with increased risk for several age-related chronic diseases including type II diabetes. The pathophysiology of MS is not completely understood yet but appears to be largely attributed to insulin resistance and obesity with a progression characterized by inflammation (Eckel et al., 2005). Over the past two decades, the prevalence of metabolic syndrome has increased dramatically in many countries and it undoubtedly is becoming a global public health challenge in the 21st century. The most recent data from the International Diabetes Federation (IDF, 2012) suggested that 366 million people over the world suffer from diabetes, with at least one fourth of the current adult population worldwide having metabolic syndrome. For these reasons, prevention of metabolic disorders has become an important task for primary healthcare physicians because of the relatively high costs for medication used for treatment of MS as well as the high potential for MS to progress to the development of the chronic disease. Therefore, there is a need for alternative therapeutic approaches in addition to medication, and the use of dietary bioactive compounds from plants is one of interest.

Blueberries (*Vaccinium* spp.) are well known to be rich in various bioactive compounds such as anthocyanins, and have been found to have high antioxidant capacities. Interestingly, various members of the *Vaccinium* spp., particularly *V. angustifolium* (Lowbush blueberries), have been reported to be used extensively as a traditional medicine for diabetic treatment by Quebec (Canada) traditional practitioners for many years (Martineau et al., 2006). Furthermore, a number of recent publications describing both *in vitro* and *in vivo* research studies have supported the concept that blueberry-derived polyphenols are a viable complementary treatment for various metabolic disorders (Vuong et al., 2007; Grace et al., 2009; Prior et al., 2010; Vendrame et al., 2013). However, to date, there are very few data on the beneficial role of Highbush blueberries (*V. corymbosum*), the more common type of commercially-grown blueberries, on the management of metabolic syndrome. Therefore, the main objective of the present study was to investigate the effect of selected cultivars of Highbush blueberries grown in

New Zealand on their ability to manage metabolic-related disorders. In this thesis, a series of *in vitro* and *in vivo* experiments were carried out in order to provide further knowledge on the role of polyphenols, in particular anthocyanins as the predominant bioactive compounds in Highbush blueberries, on biomarkers of metabolic syndrome linked to type II diabetes.

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CHAPTER 2

Literature Review

2.1 Metabolic syndrome

2.1.1 Definitions of metabolic syndrome

The metabolic syndrome has been described as a cluster of metabolic abnormalities, generally comprising four core components: impaired fasting glucose, increased abdominal obesity, dyslipidemia (hypertriglyceridemia, low levels of HDL-cholesterol) and blood pressure elevation. These are well-accepted to be strong risk factors for the development of various chronic non-communicable diseases such as cardiovascular disease (CVD) and type II diabetes mellitus (T2DM). Metabolic syndrome itself is also known as syndrome X, syndrome X plus, insulin resistance syndrome, or the deadly quartet (Cameron et al., 2004; Eckel et al., 2005). The term “metabolic syndrome” has attracted scientific and commercial interest for more than two decades since it has been proposed to be a central feature in the development of CVD and T2DM by Reaven in 1988; however, there is still no universally agreed definition and particular cut-off points for diagnosing metabolic syndrome.

Historically, the World Health Organization (WHO), between 1998 and 1999, was the first group to propose a set of criteria in order to provide a tool for clinicians and researchers to diagnose metabolic syndrome. Subsequently, many health-related organizations have also formulated definitions of metabolic syndrome, including the European Group for the study of Insulin Resistance (EGIR) in 1999, the National Cholesterol Education Program Adult Treatment Panel III (NCEP-ATP III) in 2001, the American Association of Clinical Endocrinology (AACE) in 2003, the American Heart Association/Nation Heart, Lung and Blood Institute (AHA/NHLBI) in 2004, and the International Diabetes Federation (IDF) in 2005. Among the currently recommended diagnostic criteria, the definitions of metabolic syndrome given by the WHO, EGIR, NCEP-ATP III and IDF are considered as the most common criteria used for diagnosis of metabolic syndrome (Bruce & Bryne, 2009). These definitions agree on the major components including glucose

intolerance, abdominal obesity, dyslipidemia and hypertension, but do differ in particular details as outlined in Table 2.1.

The definitions given by the WHO and EGIR agree in that they both require the measurement of insulin resistance, which can be determined by an oral glucose tolerance test or ideally by the hyperinsulinemic euglycaemic clamp technique. However, this measurement is time-consuming and labour-intensive, and therefore is suitable as a research tool but is impractical for the clinical setting (Bruce & Bryne, 2009). For this reason, the NCEP-ATP III launched a new definition in 2001; this set of criteria requires only waist circumference, blood pressure, and fasting assessment of blood glucose and lipids, without the measurement of insulin resistance included, so it is more amenable to the clinical setting. However, an issue with using the NCEP-ATP III definition was subsequently discovered with regards to its applicability to various ethnic groups, especially for the obesity criteria. For example, the cut-offs used for obesity in Asians should be different from those used in Europeans. The IDF recognized this and therefore proposed new criteria with ethnicity-specific cut-offs, in an attempt to address the variations between populations and to make the criteria applicable for use in clinical practice worldwide (Eckel et al., 2005).

2.1.2 Prevalence of metabolic syndrome

Since there are several definitions for metabolic syndrome used at present, it is difficult to present the global data on the prevalence of metabolic syndrome in different populations. The relevant studies often differ in their study design, the composition of the population studied (sex, age, race and ethnicity etc.), year that the studies were undertaken, and definitely the definition of metabolic syndrome used. Regardless of these obstacles, the prevalence of metabolic syndrome is high throughout the world. According to the International Diabetes Federation (IDF), approximately 20-25 percent of the world's adult population has metabolic syndrome and these people are likely to have a five-fold greater risk of type II diabetes compared with people without the syndrome (IDF, 2006). Furthermore, the National Health and Examination Survey (NHANES) in 2003-2006 reported that 34% of adults, 20 years of age and over, in the United States met the NCEP-ATP III criteria for metabolic syndrome (Ervin, 2009).

Table 2.1: Definition of metabolic syndrome based on different criteria used

	WHO (1999)	EGIR (1999)	NCEP-ATP III (2001)	IDF (2005)
General requirement	Insulin resistance (defined as 1 of the following) - Diabetes - Impaired fasting glucose - Impaired glucose tolerance - hyperinsulinemia plus ≥ two of the following:	Insulin resistance (defined as insulin levels > 75th percentile of non-diabetic patients) plus two or more of the following:	Three or more of the following:	Central obesity (population specific) plus at least two of the following:
Abdominal obesity	Men: WHR > 0.9 Women: WHR > 0.85 or BMI ≥ 30 kg/m^2	Men: WC ≥ 94 cm Women: WC ≥ 80 cm	Men: WC ≥ 102 cm Women: WC ≥ 88 cm	Men (European): WC > 94 cm (Asian): WC > 90 cm Women: WC > 80 cm
Blood pressure	≥ 140/90 mmHg	≥ 140/90 mmHg	≥ 130/85 mmHg	≥ 130/85 mmHg
Lipids	TG ≥ 150 mg/dL (1.7 mmol/L)	TG ≥ 180 mg/dL	TG ≥ 150 mg/dL (1.7 mmol/L)	TG ≥ 150 mg/dL (1.7 mmol/L)
	HDL-C (men) < 35 mg/dL (women) < 39 mg/dL	HDL-C < 40 mg/dL	HDL-C (men) < 40 mg/dL (women) < 50 mg/dL	HDL-C (men) < 40 mg/dL (women) < 50 mg/dL
Glucose		≥ 110 mg/dL (6.1 mmol/L)	≥ 110 mg/dL (6.1 mmol/L)	≥ 100 mg/dL (5.6 mmol/L)
Other factors	Urinary albumin excretion > 20 µg/min	-	-	-

WHR = waist-hip ratio; BMI = body mass index; WC = waist circumference; TG = triglycerides; HDL-C = high density lipoprotein cholesterol

(Source: modified from Eckel et al., 2005; Bruce & Bryne, 2009 ; Kassi et al., 2011)

Cameron and co-authors have been gathering data about the prevalence of metabolic syndrome in populations worldwide. Figure 2.1 presents the prevalence of metabolic syndrome from various countries using the NCEP-ATP III since this criteria was more appropriate to use in large clinical settings. Where data were provided, the prevalence of metabolic syndrome among men aged 20 years and older has been reported to range from 8% in India to 44% in USA (Native Americans), and in women from 7% in France to 56% in USA (Native Americans). There is also no universal trend in the prevalence of metabolic syndrome when comparing men and women. Some countries such as India, Iran, Oman and Turkey found greater numbers of women than men that met metabolic syndrome criteria, whereas other countries (Australia, France) reported a higher prevalence in men. The reason for this is not clear, but may be due to cultural differences in socioeconomic status, work-related activities and views on body fat (Cornier et al., 2008).

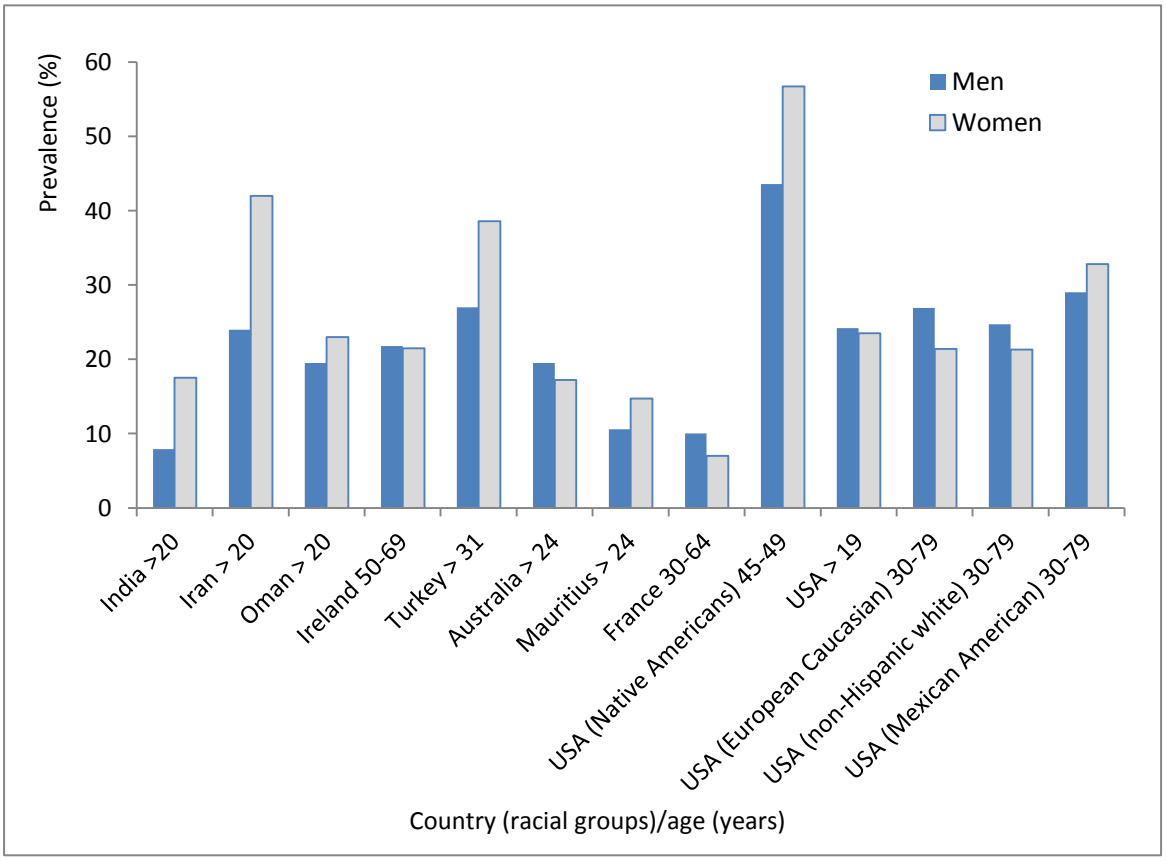


Figure 2.1: Prevalence of metabolic syndrome according to the NCEP-ATP III definition (Source: modified from Meigs et al., 2003; Cameron et al., 2004)

A study from the United States compared metabolic syndrome prevalence among three ethnic groups from the same age group (30-79) and reported that the syndrome appears to be more

common among Mexican Americans compared with either non-Hispanic white subjects or European Caucasian ancestry (Meigs et al., 2003). In New Zealand, a paper published in 2004 reported that Maori and Pacific people living in South Auckland (aged 40-59 years) had a prevalence of metabolic syndrome ranging from 48.5% to 52.8%, which was approximately 2 times higher than the European people (24.6%) (Simmons & Thompson, 2004). Another cross-sectional survey published in 2007 estimating ethnic-specific metabolic syndrome prevalence of 4022 adults aged between 35-74 years conducted in Auckland area showed a consistent result that Maori were twice and Pacific people were two and a half times as likely as others (mostly European) to have metabolic syndrome (Gentles et al., 2007). These findings supported the association between ethnic origin and metabolic syndrome. However, it is important to note that there is also variation in socioeconomic status between groups of ethnic origin, in which Mexican Americans and Maori/Pacific Island people appear to have lower socioeconomic status than white Americans and white New Zealanders, respectively. It has been shown that poverty and prevalence of metabolic syndrome are closely associated (Matthew et al., 2008). Therefore, ethnic origin may be a partial explanation, but their socioeconomic status may also, in part, be behind these differences.

There also was an attempt to compare the prevalence of metabolic syndrome using three different definitions (WHO, NCEP-ATP III, and IDF) in some epidemiological studies. Previous studies demonstrated that the estimated prevalence using the IDF definition appeared to be slightly higher than when the NCEP-ATP III definition was used within the same population (Cameron et al., 2007), and both are often higher than when using the WHO criteria. For example, in the San Antonio Heart study (Lorenzo et al., 2007), the prevalence of metabolic syndrome in white, non-Hispanic men using the WHO, NCEP-ATP III, and IDF definitions was 18.8%, 24.0% and 28.4%, respectively, and for white, non-Hispanic women was 12.1%, 16.8% and 24.7%, respectively. For Mexican-Americans, the prevalence was 28.3%, 29.6% and 40.4% for men, and 27.3%, 30.9% and 38.5 for women when using the WHO, NCEP-ATP III and IDF definitions respectively. The NCEP-ATP III and IDF definitions are similar with respect to criteria for hypertension and dyslipidemia, but cut-offs for obesity and hyperglycemia are lower in the IDF definition, resulting in higher prevalence estimates when using the IDF criteria compared to the NCEP-ATP III definition. On the other hand, the inclusion of insulin resistance (IGT/IFG/DM) as a prerequisite, makes the WHO's criteria more restrictive, and hence may obtain lower prevalence estimates.

Although prevalence estimates of metabolic syndrome are dependent on the definition that is used for determination, major consistent findings are that metabolic syndrome prevalence increases over time and is definitely age-dependent. In the National Health and Examination Survey (NHANES) conducted in 1988-1994, approximately 29% of population studied met the NCEP-ATP III criteria for metabolic syndrome. This rate had increased to 34% in the 2003-2006 NHANES cohort (Kassi et al., 2011). Additionally, these data demonstrated that metabolic syndrome prevalence increases with age, from 7% in people aged 20-29 years to > 40% in the 60-69 year age group (Ford et al., 2002). Another example of the effect of age on metabolic syndrome is also clearly shown in a study of the Iranian population, where the prevalence increased from < 10% for both men and women in the 20-29 year age group to 38% and 67% in the 60-69 year age group for men and women, respectively (Azizi et al., 2003). Similarly, among the French population the prevalence was < 5.6% in the 30-39 year age group, rising to 17.5% in the 60-64 year age group (Eckel et al., 2005).

2.1.3 Conditions contributing to the pathogenesis of metabolic syndrome

The great variation between individuals in susceptibility to metabolic syndrome suggests the pathophysiology is an incredibly complex issue that involves numerous physiological, genetic, and environmental parameters, and we are still a long way from understanding how it all works. However, to date the hallmarks of metabolic syndrome, including insulin resistance and visceral obesity are believed to drive the pathogenic process. In addition, a number of other factors could be involved such as increases in cellular oxidative stress, chronic inflammation, and adipocytokine levels.

2.1.3.1 Glucose homeostasis and insulin resistance

The maintenance of glucose homeostasis is controlled by complex interactions between organs, hormones and the neuronal control system**.** Insulin and glucagon are known to hold leading roles for balancing blood glucose levels, and their effects are antagonistic. Insulin is a hypoglycemic hormone that enhances glucose storage into cells, while glucagon is a hyperglycemic hormone that promotes the release of glucose from the liver (Figure 2.2). In response to a rise in glucose levels after meal ingestion, pro-insulin undergoes posttranslational processing into insulin and C-peptide. This process occurs in the secretory vesicles just before

functional insulin is secreted from the pancreatic beta-cell into blood circulation. Insulin stimulates the uptake of glucose into peripheral tissues, mainly skeletal muscle and adipose cells, and induces storage of glucose in the liver in the form of glycogen (glycogenesis); glycogen can be rapidly released, broken down to glucose and used for energy as needed. As a rule, the body's energy requirements are met first, followed by the formation and storage of glycogen; finally, if excess glucose is still available, it is converted to fat and stored in adipose tissue. Once the blood glucose level returns to the normal range, insulin secretion is suppressed (Marieb & Hoehn, 2013).

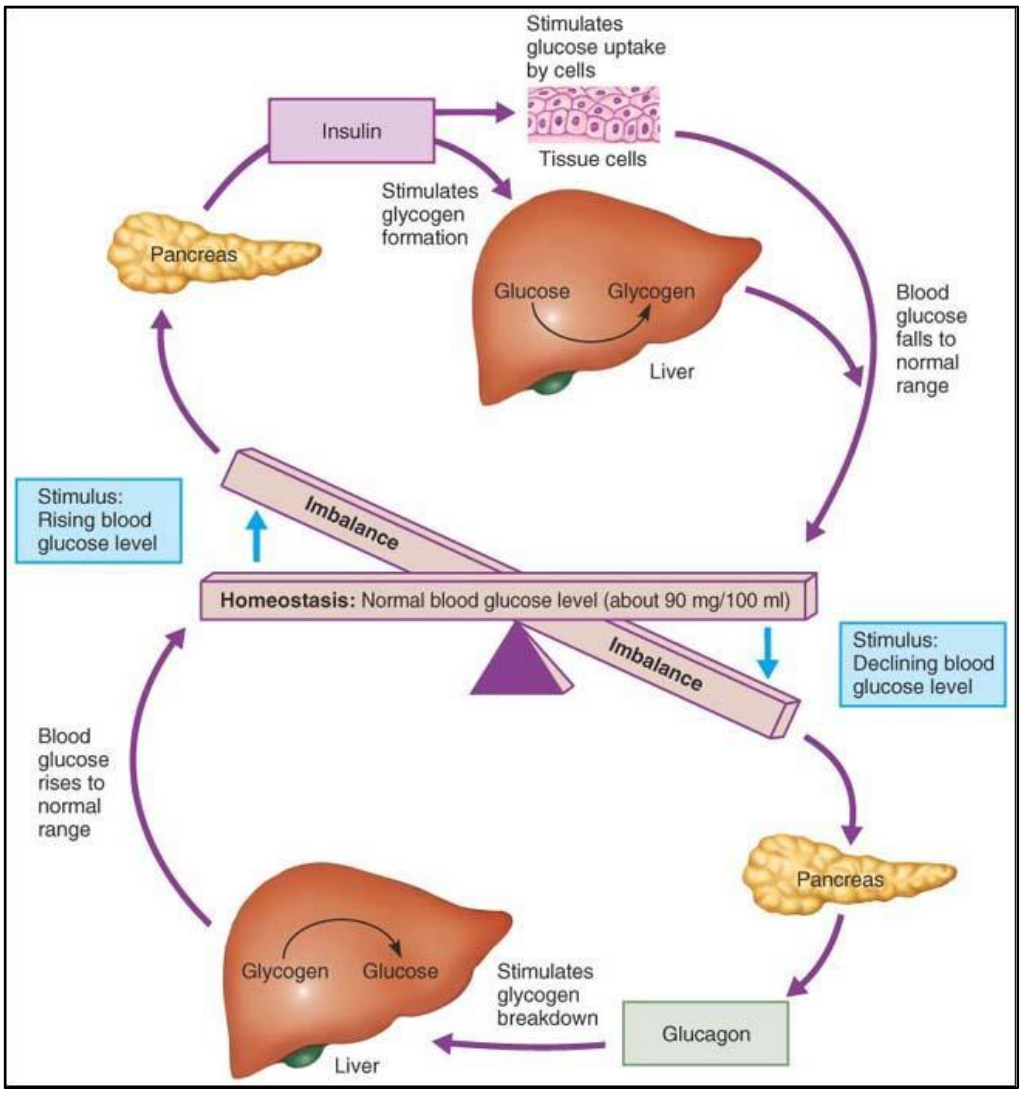


Figure 2.2: Regulation of blood glucose levels by Insulin and glucagon from the pancreas (Source: Marieb & Hoehn, 2013, reprinted by permission of Pearson Education, Inc.)

The insulin-induced stimulation of glucose uptake into muscle and adipose cells requires insulin-stimulated translocation of the glucose transporter (GLUT)-4. Insulin initiates the biological effects by binding to its receptor on the muscle and/or adipose cell surface. The insulin receptor is a glycoprotein belonging to a subfamily of tyrosine kinase receptors, and is composed of two α-subunits and two β-subunits. Once insulin binds to the α-subunits of the insulin receptor, tyrosine kinase activity residing in the β-subunit is then stimulated and catalyses the phosphorylation of a number of cellular proteins such as insulin receptor substrate (IRS)-1 and IRS-2. In the basal state, GLUT-4 is sequestered within secretory vesicles in the cytoplasm. Upon phosphorylation, these vesicles are released from intracellular site, allowing trafficking of GLUT4 to the surface via exocytosis, where the vesicles then dock and fuse with the cell membrane and allow glucose to enter the cell by facultative diffusion (Figure 2.3) (Saltiel & Kahn, 2001). This effect of insulin on the translocation of GLUT-4 is reversible; once insulin levels fall, GLUT-4 is then removed from the cell surface and restored back to secretory vesicles in intracellular sites (Bilous & Donnelly, 2010). Conversely, glucose uptake into liver, intestine, kidney, and brain tissues is not dependent on insulin. Glucose enters the islet beta-cell and hepatocytes via GLUT-2 glucose transporters, whereas GLUT-3 together with GLUT-1 are involved in non-insulin mediated uptake of glucose into the brain (Bilous & Donnelly, 2010).

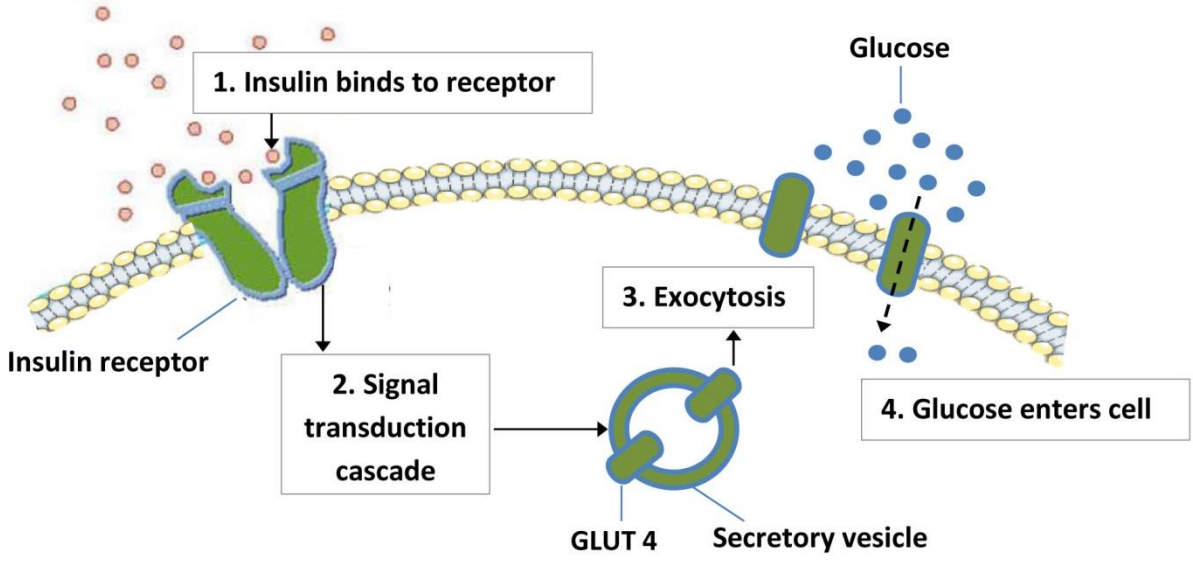


Figure 2.3: Insulin regulation of glucose transport into cells
(Source: modified from Chamber, 2012)

During a fasting state or starvation, declining blood glucose levels cause secretion of glucagon from the pancreas. Glucagon promotes the conversion of liver glycogen to glucose

(glycogenolysis), and synthesis of glucose from non-carbohydrate molecules (gluconeogenesis). The hepatic glucose production causes the release of glucose into the blood circulation and brings blood glucose levels to the normal range (approximately 80-100 mg/dL or 4-6 mmol/L). This process is necessary for maintaining blood glucose within homeostatic levels to ensure an adequate supply of glucose for normal organ function and survival, since the brain and other neuronal tissues use glucose at a constant rate (Bajaj & DeFronzo, 2003).

Insulin resistance

Insulin resistance is a condition in which the target tissues decrease responsiveness to normal circulating levels of insulin (Sesti, 2006). In the insulin-resistant individual, the cell-surface receptor on skeletal muscle, liver and adipocyte cells no longer binds insulin properly and/or responds to the insulin signal, with the consequence of reduced glucose uptake (Hirabara et al., 2012). As a result, the pancreas tries to compensate by increasing insulin secretion for the purpose of maintaining glucose homeostasis. Many people with insulin resistance have increased levels of both glucose and insulin circulating in their blood concurrently (NIDDK, 2008).

Insulin sensitivity, another term used to describe the degree of insulin resistance, is defined as the effectiveness of insulin to reduce circulating glucose by stimulating glucose uptake into cells and/or enhancing hepatic glycogen storage (Trout et al., 2007). It is difficult to distinguish between the opposing terms “insulin resistance” and “insulin sensitivity” since both have been used interchangeably in a number of publications. In general, a significantly reduced level of insulin sensitivity is called insulin resistance, and people with lower insulin sensitivity will have higher insulin resistance. Similarly, impaired insulin sensitivity individuals require higher insulin to help glucose to enter cells and are likely to display overt hyperinsulinemia as well as decreased insulin-mediated glucose clearance.

In general, individuals can be categorised as insulin-resistant or insulin-sensitive based on their response to an oral glucose tolerance test (OGTT; Cornier et al., 2008). In humans, the standardized OGTT involved overnight fasting followed by the oral administration of 75 g of glucose. Blood samples are collected for glucose measurement at 0, 30, 60, and 120 minutes after the glucose load. In subjects with normal glucose tolerance, glucose concentrations should be less than 7.8 mmol/L (140 mg/dL) at the end of 120 minutes (Nathan et al., 2007). Since glucose tolerance represents the clearance of glucose load into peripheral tissues by insulin, the

OGTT method reflects only resistance within insulin-responsive tissues, mainly skeletal muscle and adipocytes; it does not reflect the hepatic insulin resistance or whole body insulin resistance/sensitivity (Demir et al., 2008; Home & Pacini, 2008).

The euglycemic hyperinsulinemic clamp is often referred to in the literature as the gold standard for measuring whole body insulin resistance/sensitivity (Trout et al., 2007). However, this method is labour-intensive, expensive, and requires experienced personnel to manage the technical difficulties, thus other indirect methods were consequently developed for estimating insulin resistance/sensitivity. The homeostasis model assessment of insulin resistance (HOMA-IR), a method derived from the mathematical modelling of fasting plasma glucose (FPG) and fasting plasma insulin (FPI), was first proposed by Matthews and co-workers in 1985. HOMA-IR is defined by HOMA-IR = [FPG (mmol/L) x FPI (μIU/mL)]/22.5. The HOMA-IR index has a range of approximately 2 to 15, with higher scores indicating increasing insulin resistance (Trout et al., 2007). For example, in the San Antonio Heart study, the mean HOMA-IR for individuals with normal glucose tolerance was 2.1, for individuals with impaired glucose tolerance was 4.3, and for diabetic patients was 8.3 (Haffner et al., 1997). Quantitative insulin sensitivity check index (QUICKI) is a similar mathematical index for assessing insulin sensitivity. The formula for the QUICKI is 1/[log FPI (μU/mL) + log FPG(mg/dL)]. According to the study of Katz et al. (2000), the mean QUICKI was 0.382 among non-obese subjects, 0.331 in obese individuals, and 0.304 in people with diabetes. Both indices have been shown to correlate well with the results of the euglycemic hyperinsulinemic clamp technique, and have often been used for assessing changes in insulin resistance/sensitivity after treatment (Bonora et al., 2000; Mizrahi et al., 2010). Additionally, these two methods require only one blood sample to be drawn from fasting subjects; however they have the same limitation, in that they provide information only on insulin resistance at the steady (fasting) stage. Therefore HOMA-IR and QUICKI mostly reflect hepatic insulin sensitivity but not the peripheral action of insulin (Abdul-Ghani et al., 2006; Hettihewa et al., 2006). Also, both of these methods appear to have poor sensitivity when used for detecting insulin resistance in individuals with pancreatic beta-cell dysfunction (Mizrahi et al., 2010).

Visceral obesity and dyslipidemia

Adipose tissue serves as an energy reservoir for the body due to its ability to store fat, which can be converted into glucose especially in a fasting condition. A change in the

insulin:glucagon ratio in blood circulation signals lipolysis-regulating hormones (e.g. catecholamines) to break down triglycerides stored in adipose tissue and release free fatty acids and glycerol into the blood circulation. Glycerol is consequently transported to the liver and undergoes gluconeogenesis to form glucose molecules, whereas three molecules of free fatty acids are bound with albumin and transported to the liver, skeletal muscle, or other peripheral tissues depending on where the demands for energy are greatest (Ranallo & Rhodes, 1998). In the liver, free fatty acids are metabolized into acetyl coenzyme A (acetyl CoA) and either enter the Krebs cycle for energy production or serve as a substrate for triglyceride synthesis in the hepatocyte. However, in the case of prolonged starvation, acetyl CoA undergoes ketone formation. The ketone bodies synthesized by liver readily diffuse into the blood-stream and are used peripherally as an energy source by tissues such as the brain, heart, kidneys and skeletal muscle (Laffel, 1999). These substances are not considered a part of the true triglycerides pool of energy, but become necessary when there is a limited availability of glucose (Figure 2.4).

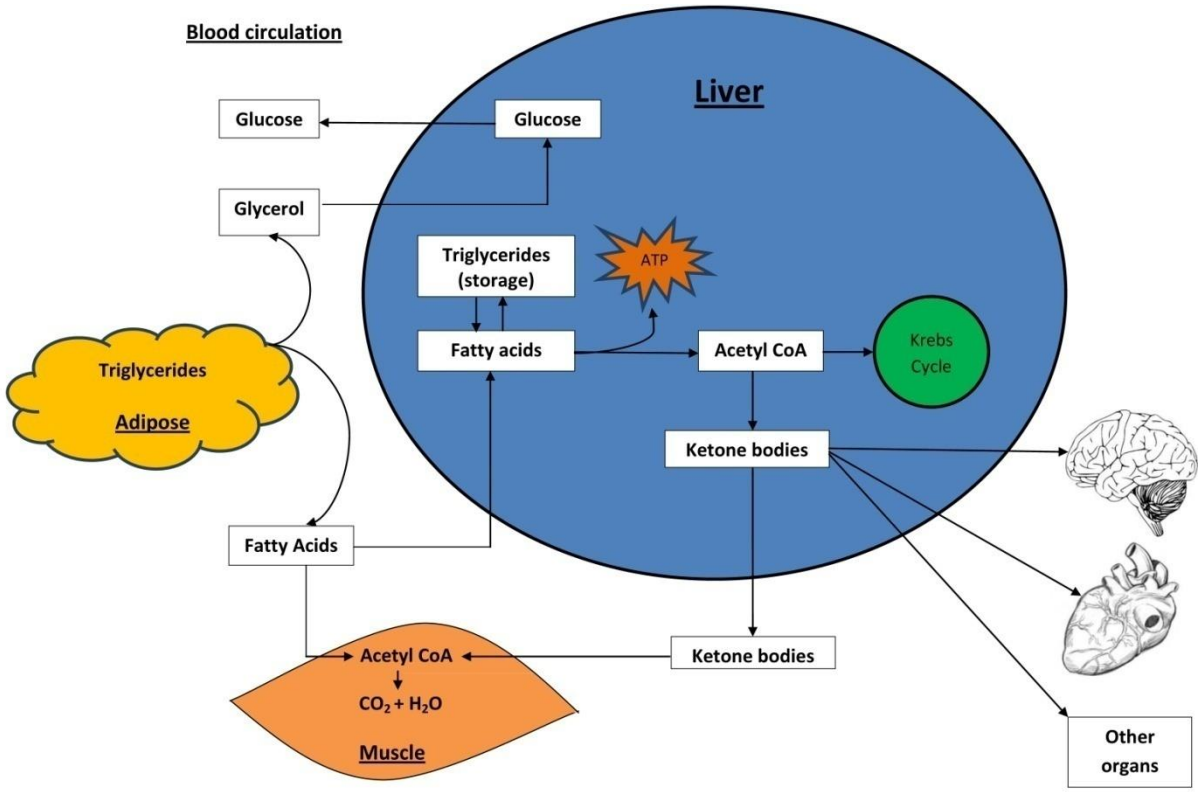


Figure 2.4: Energy metabolism during fasting condition
(Source: modified from Laffel, 1999; Horn, 2008)

It has been shown previously that excessive circulating levels of free fatty acids can lead to impaired glucose metabolism and insulin action, particularly in skeletal muscle and liver (Arner, 2002). In the muscle, based on the Randle cycle, there is competition between glucose and free fatty acids for usage as energy substrates. Therefore, when the level of free fatty acids is high, these substances can be oxidized in preference to glucose, resulting in decreased glucose oxidation (Boden & Shulman, 2002). Free fatty acids may also influence insulin production. Previous data have also indicated that elevated plasma free fatty acids inhibit insulin signaling and decrease GLUT-4 translocation, leading to a reduction in insulin-stimulated muscle glucose uptake (Arner, 2002; Boden & Shulman, 2002). The precise role of elevated free fatty acids linked to hepatic insulin resistance remains unclear. However, it is believed that high levels of free fatty acids may impair insulin action and/or induce hepatic glucose production via gluconeogenesis (Arner, 2002). For these reasons, exposure to high concentrations of circulating free fatty acids could also lead to impaired muscle uptake of glucose, hyperglycemia and insulin resistance.

Importantly, researchers now conclude that there are regional differences in lipolysis activity between adipose tissues, with the visceral fat depot having a much higher rate of lipolysis than subcutaneous fat (Arner, 2002). The mechanism behind the differences appears to be due to the action of lipolysis-regulating hormones. The action of catecholamines (hormones involved in release of free fatty acids from fat cells) has been found to be 10 times greater in adipocytes from the visceral region than from peripheral subcutaneous tissue (Wahrenberg et al., 1989). Additionally, the anti-lipolytic action of insulin is much less pronounced in visceral fat compared with the subcutaneous region (Arner, 2002). Therefore, in obese individuals, the combined effects of an enlarged visceral fat mass and increased rate of visceral lipolysis result in greater plasma free fatty acid levels released into blood circulation compared to lean subjects, and therefore could lead to more severe insulin resistance.

2.1.3.2 Adipocytokines and chronic inflammation

Adipose tissues, the main site of energy storage, produce signaling proteins called adipocytokines, which play a significant role in the regulation of energy homeostasis and metabolism. Many of these adipocytokines, including leptin, interleukin-6 (IL-6), resistin, tumour necrosis factor α (TNF-α), and C-reactive protein (CRP), are known to be pro-inflammatory factors that cause insulin resistance. In contrast, adiponectin and interleukin-10 (IL-10) are anti-

inflammatory agents that help improve insulin sensitivity in peripheral tissues (Montagut, 2009). This section focuses on the two adipocytokines, leptin and adiponectin, which have been studied in this thesis.

Leptin is a pro-inflammatory hormone secreted from adipocytes that plays a major role in inhibiting appetite and regulating energy expenditure. A deficiency of leptin hormone and/or its receptor may result in uncontrolled food intake, increased body weight gain and eventually obesity (Walker et al., 2007). In animal studies, both *ob/ob* (leptin-deficient) and *db/db* (leptin receptor deficient) mice exhibited early hyperglycemia, hyperinsulinemia, obesity and diabetes. Administration of exogenous leptin was able to reverse these parameters in *ob/ob* mice but not in *db/db* mice, as the latter strain of mice lack the leptin-receptor and thus remain unresponsive to leptin. High levels of glucose and insulin stimulate leptin secretion, whereas increases in levels of free fatty acids and the rate of lipolysis inhibit secretion of leptin (Walker et al., 2007).

Not only does the intake of foods stimulate the secretion of leptin, but it has also been demonstrated that adipocytes can alter the production and/or the sensitivity of leptin. It is often found that the serum leptin concentration correlates positively with body fat content and is usually increased in obese individuals. This suggests obese individuals may experience insensitivity to leptin, which consequently leads to more production of leptin and/or leptin resistance. Elevated leptin production by adipose tissue also facilitates the secretion of pro-inflammatory cytokines such as TNF-α and IL-6, which influence each other in promoting more severe chronic inflammation in the insulin responsive tissues (Iikuni et al., 2008).

On the other hand, adiponectin is an anti-inflammatory cytokine, mainly synthesized by adipocytes, which modulates a number of metabolic processes including glucose regulation and fatty acid catabolism (Esteve et al., 2009). In the liver, adiponectin has been found to be associated with inhibition of expression of hepatic gluconeogenic enzymes and to decrease the rate of endogenous glucose production. In skeletal muscle, it enhances glucose transport, improves insulin signal transduction and increases fatty acid oxidation. Therefore, a deficiency in circulating levels of adiponectin significantly impacts energy metabolism consistent with metabolic syndrome (Eckel et al., 2005). Levels of circulating adiponectin are found to be negatively correlated with adipose tissue mass. This inverse relationship is stronger in visceral fat than in subcutaneous fat; for example, obese individuals with a high proportion of visceral fat have decreased adiponectin levels independent of body weight (Taksali et al., 2008). Additionally, the synthesis of adiponectin is found to be increased after weight loss (Esteve et al.,

2009) and impaired in states of calorie excess, which may be associated with leptin resistance (Montagut, 2009).

Although the association between obesity and inflammation in metabolic syndrome is well documented in a number of previous studies, the precise physiological mechanisms involved in initiation of the inflammatory response in obesity are still unknown. One theory proposed by de Luca & Olefsky is that ["the expansion of adipose tissue leads to adipocyte hypertrophy and hyperplasia and that large adipocytes outstrip the local oxygen supply leading to cell autonomous hypoxia with activation of cellular stress pathways"] (de Luca & Olefsky, 2008). This deterioration affects inflammation to cells and the secretion of pro-inflammatory cytokines. Notwithstanding, there has also been an accumulation of evidence indicating a link between the change of pro-inflammatory adipocytokines and insulin resistance. For instance, basal plasma leptin levels were found to be significantly elevated in lean insulin-resistant subjects (4.35 ng/mL) compared to lean insulin-sensitive subjects (1.90 ng/mL), and leptin concentrations in both of these two groups were significantly lower than values in obese individuals (9.27 ng/mL) (Segal et al., 1996). In contrast to leptin, circulating adiponectin levels have been reported to correlate inversely with insulin resistance and to be significantly reduced in obese subjects and individuals with insulin resistance (Kadowaki et al., 2006; Esteve et al., 2009). In addition, prospective and longitudinal studies have found that plasma adiponectin is a predictor of type II diabetes, and that a decrease in the levels of circulating blood adiponectin precedes the onset of development of diabetes (Lindsay et al., 2002; Daimon et al., 2003; Snehalatha et al., 2003; Spranger et al., 2003).

Notably, it has also been documented that some of the inflammatory indexes vary by ethnicity. For instance, the concentrations of C-reactive protein were higher in Indian Asian people than in European white individuals and were found to be associated with a greater increase in visceral obesity and insulin resistance in Indian Asians (Chambers et al., 2001).

2.1.3.3 Oxidative stress

Reactive oxygen species (ROS) and nitrogen molecules are typically produced as natural by-products of biological processes such as mitochondrial respiration and enzymatic oxidation. However, accumulation of these reactive molecules or a reduced capacity for their elimination in tissues such as skeletal muscle and liver leads to the condition of oxidative stress. Oxidative stress may play a role in the pathophysiology of metabolic syndrome, particularly

hypertension and insulin resistance. For example, a study of adipocytes cultured *in vitro* indicated that presence of insulin increased the production of H_2O_2, a chemical substitute for ROS. This is consistent with the observation *in vivo* that hyperinsulinemia decreased the concentration of vitamin E, an antioxidant agent, suggesting that increased levels of insulin may produce oxidative stress (Ceriello, 2000). Furthermore, in subjects with hyperglycemia and impaired glucose tolerance, infusion of glutathione, another antioxidant substance, significantly potentiated the beta-cell response to glucose-induced insulin secretion (Paolisso et al., 1992).

The role of oxidative stress involvement in the regulation of glucose/energy metabolism leading to metabolic syndrome remains debatable. However, one theory holds that the transformation of glucose/free fatty acids into energy through citric acid cycle (Krebs cycle) activity generates an excess of reactive oxygen species (ROS) as a by-product. Over consumption of carbohydrate leads to increased glucose and free fatty acids uptake in cells and causes higher oxidative stress in mitochondria. Cells consequently try to prevent the entrance of energy into mitochondria by inhibiting insulin-stimulated glucose uptake to protect themselves against harmful effects of ROS. However, such action may indirectly result in resistance to insulin in muscle and adipose tissues (Ceriello & Motz, 2004). Alternatively, increased oxidative stress can place strain on endoplasmic reticulum (ER) capacity and trigger ER stress response (Chen, 2006), and excess production of ROS may have toxic effects on the membrane structure of the beta-cells, which in turn may contribute to impaired insulin secretion (Ceriello, 2000).

2.1.3.4 Other contributing factors

The pathogenesis of the development of metabolic syndrome is known to be complex. In addition to the factors described above, many other factors can contribute. Genetic history and physical inactivity are well-documented to be of importance for the development of metabolic syndrome. The presence of microalbuminuria, non-alcoholic fatty acid liver disease (NAFLD), obstructive sleep apnoea, and cigarette smoking have also been evidenced in the literature to be associated with metabolic syndrome (Eckel et al., 2005).

2.1.4 Type II diabetes

When the body compensates for insulin resistance by increasing insulin secretion over a long period of time, pancreatic beta-cells eventually become defective and no longer produce

sufficient insulin to keep up with the body's needs. As a result, there is excess glucose build-up in the bloodstream and sets the stage for type II diabetes. Diabetes patients can have either hyposecretion or hypoactivity of insulin, both of which result in high levels of glucose in the blood circulation after a meal because the glucose is unable to enter the cells in most tissues. Generally, when glucose cannot be utilized as cellular energy, the body reacts inappropriately with the reactions that normally occur in the fasting state to make glucose available to use: glycogenolysis (breakdown of glycogen), lipolysis (breakdown of fat), and gluconeogenesis. This response leads to even higher levels of circulating glucose and consequently, excess glucose begins to be secreted into the urine (glycosuria). In severe diabetes, more fats are mobilized leading to high blood levels of fatty acid (lipidemia) and their metabolites (so-called ketone bodies). The condition in which ketone bodies accumulate in the blood causes a reduction in blood pH (ketoacidosis). If untreated, ketoacidosis can disrupt heart activity, oxygen transport and cause severe depression of the nervous system, and eventually lead to coma and death (Marieb & Hoehn, 2013).

Table 2.2: Criteria for the diagnosis of diabetes

Type of test	Criteria
FPG	≥ 126 mg/dL (7.0 mmol/L). Fasting is defined as no caloric intake for at least 8 h.
2-h PG	≥ 200 mg/dL (11.1 mmol/L) during an OGTT. The test should be performed as described by the World Health Organization, using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water.
A1C	≥ 6.5%. The test should be performed in a laboratory using a method that is NGSP certified and standardized to the diabetes control and complications trial reference assay.

In the absence of unequivocal hyperglycemia, these criteria should be confirmed by repeat testing.
FPG = fasting plasma glucose; 2-h PG = 2 hours plasma glucose; A1C = hemoglobin A1c or glycated hemoglobin; NGSP = the National Glycohemoglobin Standardization Program
(Source: Modified from American Diabetes Association, 2013)

Type II diabetes is generally diagnosed when the blood glucose level in the fasting state exceeds 125 mg/dL (7.0 mmol/L) or when the 2-hours glucose load level is above 200 mg/dL (11.1 mmol/L) (see Table 2.2 for the complete diagnostic criteria proposed by the Expert Committee on the Diagnosis and Classification of Diabetes). Prediabetes, a condition in which blood glucose levels are higher than normal but not high enough for a diagnosis of diabetes, is defined by diagnostic cut-off points between 100 and 125 mg/dL (5.6-6.9 mmol/L) for FPG, 140 and 199

mg/dL (7.8-11.0 mmol/L) for OGTT, or 5.7 and 6.4% for A1C. It has been shown that people with identified prediabetes are likely to develop type II diabetes within 10 years; however, the time of progression varies widely between studies and is significantly influenced by the presence of other metabolic disorders such as dyslipidemia and obesity (Després, 2006; NIDDK, 2012).

2.1.5 Management of metabolic syndrome

Weight loss and being physically active are well-accepted to be efficient for managing all metabolic syndrome components, including excessive adiposity, dyslipidemia, hypertension, hyperglycemia and insulin resistance, and thus help to prevent progression to chronic diseases (e.g. diabetes). In addition, the maintenance of normal glucose homeostasis by lower dietary consumption of fats and carbohydrates, and incorporation of healthier eating habits, also help to prevent the pathologies related to impaired glucose metabolism and reduce the risk of developing metabolic syndrome. Accumulated epidemiological data strongly suggest that plant-based diets rich in bioactive compounds are generally associated with the management of metabolic syndrome and chronic diseases. Therefore, diet modification by increased consumption of diets high in phytochemicals may provide an alternative way to decrease the chance of developing metabolic syndrome, and this hypothesis underlines our interest in identifying phytochemical-enriched fruits that could be of benefit in prevention or management of metabolic syndrome.

2.2 Blueberries: composition, bioactive compounds and antioxidant capacities

Blueberries are one of the most popular berries, and are sold in numerous fresh, frozen and processed forms in retail markets. Nearly 160,000 hectares of blueberries are planted worldwide (Strik, 2007), and the planted area is expected to increase due to market interest in their positive health effects. Blueberries were first introduced into New Zealand from North America in 1950 (Poll & Wood, 1985) and their area planted has risen from 239 hectares in 2000 to 522 hectares in 2009 (Scalzo et al., 2013). There are three main types of blueberries cultivated worldwide: Lowbush (wild), Highbush (cultivated) and Rabbiteye. Lowbush (wild) blueberries (*Vaccinium angustifolium*) are originally from North America and Canada, whereas Highbush (*Vaccinium corymbosum*), the most commercialized species, has been planted in many areas of

the world including North America, South America, Europe, Oceania and Asia. Approximately 63% of total Highbush blueberry production over the world in 2005 was sold for the fresh market (Strik, 2007). Unlike Lowbush and Highbush, Rabbiteye blueberries (*Vaccinium ashei*) are endemic to southeastern United States where they are thought to have evolved from the natural hybridization of several native *Vaccinium* species. In New Zealand, most blueberries cultivated are Highbush and Rabbiteye varieties, whereas Lowbush is not grown. Although Rabbiteye can be planted in most areas throughout New Zealand, commercial production is restricted to localities which have sufficient heat in late summer and autumn for full ripening of berries (Graydon, 1999).

2.2.1 Nutrient composition of blueberries

Blueberries are recognised as a good source of dietary fibre and vitamin C. According to the New Zealand food composition database (2013), 1 cup of frozen blueberry (~160 g) contains around 6 g of dietary fiber and 10 mg of vitamin C, which provide approximately one fourth of the recommended dietary intake (RDI) of these nutrients (dietary fiber: 30 g per day for men and 25 g per day for women; vitamin C: 45 mg per day for both men and women; NHMRC, 2006). In addition, blueberries are very low in fat and sodium, however they contain a large portion of sugar.

The nutrient composition of blueberry are summarised in Table 2.3, which would be useful for rough comparison of the macro- and micronutrients found in blueberry between the frozen and freeze-dried samples (the forms of blueberry used in this research). Blueberries comprise of 84% water or 16% dry matter. After freeze-drying, for example, the dietary fibre content could increase from 3.5% to 22%; sugar from around 10% to 60%; protein from 0.6% to 3.8%; and vitamin A and C increase from 2.5% and 6% to 16% and 39%, respectively. These data indicate that, in blueberries, the nutrient compositions of the freeze-dried form are approximately six times higher than those of the frozen samples.

Table 2.3: Nutrient composition of frozen and freeze-dried blueberries

	Nutrients	Frozen blueberry [1] (per 100 g)	Freeze-dried blueberry [2] (per 100 g)	
	Energy	192	1200	KJ
	Water	83.7	N/A	g
	Protein	0.6	3.8	g
	Fat	0.4	2.5	g
	Carbohydrate	9.8	61	g
	Dietary fibre	3.5	22	g
	Sugar	9.7	61	g
	Starch	0.1	0.6	g
Vitamins	Vitamin A	2.5	16	µg
	Beta-carotene	15	94	µg
	Thiamin	trace	N/A	mg
	Riboflavin	trace	N/A	mg
	Niacin	trace	N/A	mg
	Vitamin B6	0.05	0.3	mg
	Vitamin B12	0	0	µg
	Dietary folate	8	50	µg
	Vitamin C	6.2	39	mg
	Vitamin D	0	0	µg
	Vitamin E	0.61	3.8	mg
Minerals	Sodium	2.1	13	mg
	Iodine	trace	N/A	µg
	Potassium	68	425	mg
	Phosphorus	9.3	58	mg
	Calcium	11	69	mg
	Iron	0.4	2.5	mg
	Zinc	0.1	0.6	mg
	Selenium	0	0	µg

[1] Source: New Zealand food composition database, 2013
[2] Freeze-dried data was calculated based on dry matter content of fresh fruits
N/A: Data is not available

2.2.2 Total polyphenolic content

Besides being a source of dietary fiber and vitamin C, blueberries also contain large amounts of non-nutrient phytochemicals, especially polyphenols. These compounds include the anthocyanins, which give the berries their distinctive colour; and act as a visual cue to signal to fruit-eating animals that the berries are ripe. The plant sends this signal at the stage when the seeds are mature so that the berries act as a vehicle to promote dispersal of the seeds (Wills et al., 2007). Other polyphenols accumulate in fruits as natural defence compounds to protect immature fruit from rot-causing organisms such as bacteria and fungi. Once the seeds are

mature, the fruit loses its natural resistance to disease and the berries become much more susceptible to decay. Condensed tannins, hydroxybenzoic acids and hydroxycinnamic acids seem to be of major importance for this mechanism in plants (Häkkinen, 2000).

A wide variety of polyphenolic compounds are found in blueberries including anthocyanins, proanthocyanidins, other flavonoids (kaempferol, quercetin, myricetin), phenolic acids (chlorogenic acid, caffeic acid, ferulic acid, *p*-coumaric acid, gallic acid, protocatechuic acids, syringic acid), and stilbene derivatives (Wu & Kang, 2012). However, blueberries are particularly rich in anthocyanins, proanthocyanidins and the hydroxycinnamate chlorogenic acid (Howard & Hager, 2007).

Table 2.4: Total phenolic content (mg) in different varieties of blueberries, expressed per 100 g fresh weight.

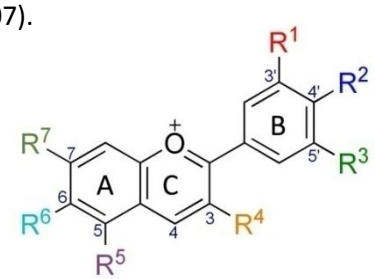
Blueberry types	Cultivars	Solvent	Total phenolics	References
Highbush	Bluecrop	Acetone/water	118	Taruscio et al., 2004
	Jersey		106	
Highbush	Bluecrop	Acetone/water	304	Moyer et al., 2002
	Brigitta Blue		246	
	Duke		274	
Highbush	Bluecrop	Acetonitrile/acetic acid	48	Ehlenfeldt & Prior, 2001
	Brigitta Blue		93	
	Burlington		175	
	Dixi		90	
	Duke		103	
	Elliott		184	
	Jersey		106	
	Reka		78	
Highbush	Bluecrop	Acetonitrile/acetic acid	189.8	Prior et al., 1998
	Jersey		202.8	
	Duke		305.9	
Lowbush	N/A		295-495	
Lowbush	Brunswick	Acetone/water	692	Moyer et al., 2002
Rabbiteye	Bluegem		717	
Rabbiteye	Austin, Brightblue, Brightwell, Climax, Tifblue etc.	Methanol/HCl	270-930	Sellappan et al., 2002
Rabbiteye	Climax, Brightwell, Tifblue, Little Giant	Acetonitrile/acetic acid	230.8-457.5	Prior et al., 1998

In all instances, total phenolic content was assayed with Folin-Ciocalteu method using gallic acid as a standard.

The total polyphenolic contents (TPC) of several cultivars of blueberries were determined in earlier studies and their TPCs are summarised in Table 2.4. The TPC of Highbush, Lowbush and Rabbiteye blueberries ranges from 48 to 318 mg, 295 to 692 mg, and 231 to 930 mg/100 g fresh weight, respectively. The relatively broad ranges of these values are due to differences between both cultivars and environmental factors. In addition, these values are influenced by differences in extraction conditions as well as analytical procedures, making it difficult to compare values between laboratories. However, the general agreement is that among the three different types of blueberries, Lowbush and Rabbiteye blueberries contain overall higher concentrations of total polyphenolics than the Highbush blueberries.

2.2.3 Anthocyanins

Anthocyanins (Greek anthos = flower and kyanos = blue) themselves belong to a larger group of water-soluble plant compounds collectively known as flavonoids. They are responsible for the red, blue and purple colours of the plants in which they occur (Piberger et al., 2011). The basic structure of the anthocyanin molecule consists of two benzoyl rings (A and B) joined by one heterocyclic ring (C) as shown in Figure 2.5. The structural differences between individual anthocyanins are due to the number of hydroxyl groups in the molecule, the degree of methylation of these hydroxyl groups, the type, number and attachment position of the sugar molecules, as well as the type and number of aliphatic or aromatic acid attached to sugar in the molecule (McGhie & Walton, 2007).



	R_1	R_2	R_3
Cyanidin	OH	OH	H
Delphinidin	OH	OH	OH
Malvidin	OCH_3	OH	OCH3
Peonidin	OCH_3	OH	H
Petunidin	OCH_3	OH	OH
Pelargonidin	H	OH	H

Figure 2.5. Structures of anthocyanidins commonly found in berry fruits (Source: modified from Wu et al., 2006)

More than 550 different types of anthocyanins have now been identified in plants. In berry fruits, the most frequently found are in the form of mono-, di- and triglycosides, where glycosides are usually substituted at C3. They form conjugates with sugar molecules, in particular glucose, galactose, rhamnose, arabinose and xylose (Szajdek & Borowsha, 2008). The anthocyanins in berry fruit have been commonly identified using High Performance Liquid Chromatography (HPLC). Blueberries are unique amongst berry fruit, in that, there are approximately 15 major anthocyanin compounds in blueberries, a far wider range than found in any other fruit. Blueberry anthocyanins consist of: 3-monoarabinosides, 3-monogalactosides, and 3-monoglucosides of cyanidin, delphinidin, peonidin, petunidin, and malvidin (Table 2.5).

According to Cho et al. (2004), the percent distribution of anthocyanins was shown to vary among blueberry species. The percentage distribution of monomeric anthocyanins was approximately 27 to 40% of delphinidin, 22 to 33% of malvidin, 19 to 26% of petunidin, 6 to 14% of cyanidin, and 1 to 5% of peonidin, while the distribution of acylated anthocyanin ranged from non-detectable to 9%. In terms of the percent distribution of anthocyanin glycosides, galactosides account for 60 to 67%, arabinosides 26 to 32%, and glucosides 2 to 29%. Their research also noted that the anthocyanin components in blueberries are present predominantly in the skin and outer layer of the pulp.

Anthocyanins readily degrade in the presence of oxygen, light, elevated temperatures and enzymes, and pH especially has a significant effect on their stability. In aqueous solutions, anthocyanins occur as a dynamic equilibrium of four related molecular structures: flavylium cation, quinonoidal, hemiacetal, and cis- and trans-chalcone. The red flavylium cation is the most stable and abundantly exists in acidic solution (pH < 3), which is naturally present in fruit. As the pH increases, the rapid loss of a proton of the flavylium cation leads to the blue quinonoidal structure, while a much slower hydration of this flavylium form leads to the generation of colorless hemiacetal. Then, tautomerization of the hemiacetal form results in the formation of cis- and trans-chalcone forms, which are the end products of this reaction (McGhie & Walton, 2007). Therefore, it is likely that the flavylium cation form will exist in the stomach due to low pH and the other three forms will occur in the gastrointestinal tract (GIT) and plasma, which are neutral to mildly alkaline environments.

Table 2.5: Individual anthocyanins presented in Highbush blueberries quantified by HPLC

Peak	Individual anthocyanin components
1	Delphinidin 3-galactoside
2	Delphinidin 3-glucoside
3	Cyanidin 3-galactoside
4	Delphinidin 3-arabinoside
5	Cyanidin 3-glucoside
6	Petunidin 3-galactoside
7	Cyanidin 3-arabinoside
8	Petunidin 3-glucoside
9	Peonidin 3-galactoside
10	Petunidin 3-arabinoside
11	Peonidin 3-glucoside coeluted with malvidin 3-galactoside
12	Peonidin 3-arabinoside coeluted with malvidin 3-glucoside
13	Malvidin 3-arabinoside

Source: Lohachoompol et al. (2008); Ogawa et al. (2008).

The consumption of anthocyanins in humans has been estimated to be 12.5 mg/day/person in the United States (Wu et al., 2006), while consumption in Europe has been estimated to be 20 mg/day/person (Bordonaba, 2011) and even higher in Finland with a value of 82 mg/day/person (De Pascual-Teresa & Sanchez-Ballesta, 2008). However, estimated consumption has been reported in another paper to be as high as 180-215 mg/day in the United States (Zafra-Stone et al., 2007). So far the metabolism of anthocyanins remains not completely understood; however, based on current knowledge, anthocyanins after consumption can be absorbed in the small intestine (appeared to be at the jejunum) before passing through the liver, where a portion of the absorbed anthocyanins may also be metabolised by methylation and glucuronidation reactions. Intact anthocyanins and metabolites then enter the systemic circulation before being either transported to various organs and tissues or excreted via the urine (McGhie & Walton, 2007).

After ingestion of anthocyanins the maximum concentration in plasma (Cmax) is reached between 45 min to 4 h and excretion is complete within 6–8 h, indicating rapid metabolism and absorption in the body (McGhie & Walton, 2007; De Pascual-Teresa & Sanchez-Ballesta, 2008). Anthocyanins that are not absorbed from the small intestine move into the colon, where they are exposed to a substantial microbial population and degraded into aglycones and phenolic acids (McGhie & Walton, 2007). Initial studies on anthocyanin metabolism suggested that

anthocyanins can be absorbed intact without cleavage of the sugar to form the aglycones because the same anthocyanins that were consumed were also present in the plasma and urine (Matsumoto et al., 2001; McGhie et al., 2003). However, later studies also detected additional anthocyanins-like peaks, suggesting a portion of anthocyanins may also be subject to extensive transformation *in vivo*, and the methylated and glucoronide conjugates of anthocyanins have been shown to be the two major forms of metabolites presented in urine (Wu et al., 2004b). Furthermore, several earlier studies also demonstrated that the chemical structures of aglycones and type of sugar substitution have a significant impact on the absorption and excretion of individual anthocyanins (McGhie et al., 2003; Wu et al., 2004b; He et al., 2006). When considering the concentrations of anthocyanins in the plasma as an indicator of absorption, relatively more galactosides are absorbed than glucosides and arabinosides, respectively, whereas among the aglycones, the absorption is higher for the delphinidins and cyanidins than for the malvidin anthocyanins (Ichiyanagi et al., 2006).

Although there are limited data available in the literature regarding the metabolism of anthocyanins, the most consistent finding is that the bioavailability of anthocyanins is relatively poor, with approximately 0.1% of the ingested dose usually appearing in the plasma or urine (Kalt et al., 2008). One reason may be that anthocyanins are not efficiently hydrolyzed by β-glucosidase in the gastrointestinal tract, but this explanation has not been proven yet. Since only small amounts of anthocyanins and their metabolites are presented in plasma and urine, the evidence to date suggests that anthocyanins may be slightly modified by gastric conditions in the stomach and small intestine, but possibly extensively modified by gut microflora in the colon. However, it is also important to note that anthocyanins undergo rearrangement in response to pH. As mentioned earlier, the red flavylium cation, the most stable form that predominates at pH 1-3, is unlikely to exist lower down in the gastrointestinal tract and in the bloodstream because of the basic environment. It may be possible that anthocyanin structures in the hemiacetal, quinonoids and chalcone forms, which are more likely to be present *in vivo* after absorption and metabolism, would not be readily detected by the current HPLC methods of analysis, which are mostly based on the stable flavylium cation form (McGhie & Walton, 2007), resulting in artificially low amounts being detected in the blood and urine. Sensitive methods for determining the alternative molecular structures of anthocyanins (hemiacetals, quinonoids and chalcones) have not been available so far. In addition, definitive data on all of the possible metabolites of anthocyanins are limited and these are still needed for better understanding of the bioavailability of anthocyanins.

2.2.4 Other bioactive compounds

Phenolic acids

In addition to anthocyanins, the phenolic acids are another group of important polyphenolic compounds found in blueberries. These compounds are predominantly present in two forms: hydroxybenzoic acids and hydroxycinnamic acid, which are mainly found in conjugated forms as glycosides and esters. The main benzoic acid derivatives are gallic acid, salicylic acid, vanillic acid and ellagic acid, while among the group of cinnamic acid derivatives, are *p*-coumaric acid, caffeic acid and ferulic acid. Chlorogenic acid (ester of caffeic acid and quinic acid) (Figure 2.6) has been reported to be the major hydroxycinnamic acid as well as the most abundant phenolic acid found in blueberries (Taruscio et al., 2004). It is responsible for the tart taste of the fruits. In the presence of polyphenol oxidase, chlorogenic acid can be easily oxidized and changed into brown-coloured compounds.

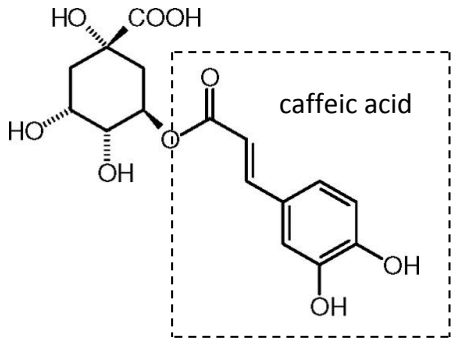


Figure 2.6: Structure of chlorogenic acid (modified from Wu & Kang, 2012)

Proanthocyanidins

Proanthocyanidins (PAs), commonly known as condensed tannins, represent a group of oligomeric and polymeric flavan-3-ols, which can also be found in physiologically-relevant quantities in blueberries. The size of proanthocyanidins are normally described by defining the degree of polymerization (DP): 1 unit for monomer (DP = 1), 2-10 unit for oligomers (DP = 2-10) and > 10 units for polymer (DP > 10) (Rodriguez-Mateos et al., 2012). Different types of proanthocyanidins (PAs) have been identified; PAs consisting exclusively of (epi)catechin are named procyanidins, whereas PAs that contain (epi)afzelechin or (epi)gallocatechin subunits are called propelargonidins or prodelphinidins, respectively. According to Gu et al. (2004), the total PA concentration in cultivated Highbush blueberries is 180 mg/100 g fresh weight (monomer 4

mg; oligomers 47 mg; polymers 129 mg), while Lowbush contain 332 mg PAs/100 g fresh weight (monomer 3 mg; oligomers 69 mg; polymers 260 mg). Procyanidin is the dominant PA component found in both Highbush and Lowbush blueberries.

Other flavonoids

In addition to anthocyanins which are the major flavonoids present in blueberries, flavonols and their glycosides have also been reported. Three major types of aglycones present are kaempferol, quercetin and myricetin, which differ in the number of hydroxyl groups on the B ring (Figure 2.7); the sugar moieties attached to the aglycone include glucose, galactose, rhamnose, arabinose and rutinose (Wu & Kang, 2012).

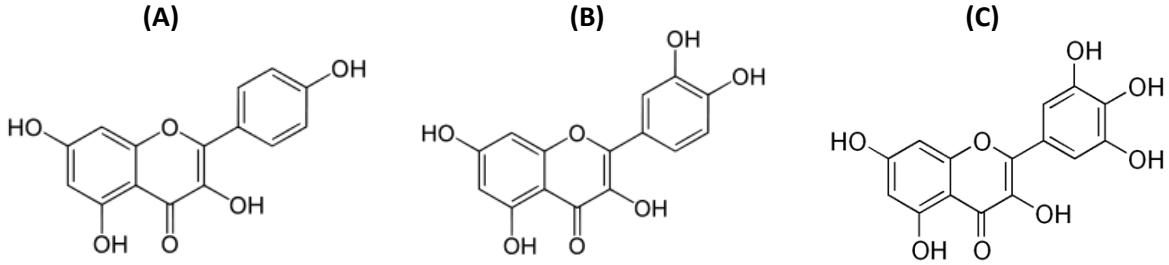


Figure 2.7: Structures of flavonols in blueberries. Kaempferol (A); Quercetin (B); and Myricetin (C). (Source: Wu & Kang, 2012)

Similar to the total phenolic content, the concentrations of anthocyanins and other bioactive compounds present in blueberries vary significantly due to a number of reasons, for example genotypes, agronomic variation, stage of fruit maturation and solvents used for extraction. Table 2.6 summarises the concentrations of major bioactive compounds in three types of blueberries; Highbush, Lowbush and Rabbiteye as reported by Gu et al. (2004) and Howard & Hager (2007). In addition, a study by Prior et al. (1998) reported the average total anthocyanins concentrations in Lowbush (n=7), Rabbiteye (n=6), and Highbush (n=8) were 139, 124, 129 mg/100 g fresh weight (FW), respectively. In a recent study by Rodriguez-Mateos et al. (2012), the concentrations of total anthocyanins, flavonols oligomers, and chlorogenic acid over all six genotypes of Highbush and one genotype of Lowbush blueberries ranged from 128 - 187 mg, 35 - 111 mg, and 34 - 114 mg per 100 g FW, respectively. Based on available data, it appears that Lowbush blueberries have higher concentrations of anthocyanins as well as other

phytochemicals compared to Rabbiteye and Highbush blueberries, although some genotypes of Highbush and Rabbiteye showed higher values.

Table 2.6: Total concentration of various bioactive compounds (mg/100 g FW) in blueberries

Blueberry	Number of studies	Number of genotypes	Total anthocyanins	Chlorogenic acid	Procyanidins	Total flavonols
Highbush	11	85	20-269	42-158	180	9-40
Lowbush	3	17	91-260	59-110	332	N/A
Rabbiteye	3	22	13-515	N/A	N/A	3-17

N/A: Data is not available
(Source: Gu et al., 2004; Howard & Hager, 2007)

2.2.5 Antioxidant capacity

Blueberries (*Vaccinium* species) have been shown to have high antioxidant capacity. Among twenty-four fruits investigated *in vitro*, berry fruits including blueberries were found to have the highest total antioxidant capacity compared to others (Wu et al., 2004a). According to Prior et al. (1998), total antioxidant capacity as measured by oxygen radical absorbance capacity (ORAC) of different blueberry varieties ranged from 13.9 to 45.9 µmol Trolox equivalents/g of fresh berries, with Lowbush possessing higher mean antioxidant capacity than Highbush species. As with total anthocyanins and total phenolics content, antioxidant activity was dependent on species, cultivars and agricultural conditions as well as the size of berries, with small-sized berry cultivars having the highest antioxidant activity per gram (Remberg et al., 2007).

Blueberries owe their antioxidant activities mainly to their phenolic compounds including phenolic acids, condensed tannins and especially anthocyanins. The contribution of phenolic classes to antioxidant activity in blueberry extracts was studied by Zheng & Wang (2003). The authors collected HPLC fractions from each chromatrographic peak and measured the ORAC of each peak. Their findings showed that anthocyanins account for 56% of the whole-fruit extract ORAC, whereas chlorogenic acid and flavonols accounted for 21% and 23%, respectively. Additionally, a number of studies point to the linear correlation between the antioxidant activity and the total concentrations of phenolics as well as anthocyanins in blueberries (Ehlenfeldt & Prior, 2001; Cho et al., 2004; Taruscio et al., 2004). In particular, in certain individual anthocyanins found to contribute significantly, the number and/or the positions of the hydroxyl

group on the B ring of anthocyanin molecules were important for their antioxidant capacity. The radical scavenging activity has been reported to be stronger with more hydroxyl groups on the B ring (figure 2.5); therefore, the ranking order is delphinidin (3',4',5'-OH) > cyanidin (3',4'-OH), petunidin (4',5'-OH) > malvidin, peonidin, peralgonidin (4'-OH) (Ogawa et al., 2008). In addition to specific anthocyanins, among the groups of phenolic acids, cinnamic acid derivatives such as caffeic acid and chlorogenic acid are found to be more active antioxidants compared to benzoic acid derivatives (Zheng & Wang, 2003).

Although earlier evidence showed the apparent low bioavailability of anthocyanins, several *in vivo* studies have demonstrated that dietary consumption of blueberries significantly increased total antioxidant capacity in serum, which may lead to protection against free radicals and oxidative stress in *vivo*. According to Molan et al. (2008), rats gavaged with extracts from two cultivars of Rabbiteye blueberry (Centurion and Maru, 1 ml/day) for 6 days showed significantly increased serum total antioxidant activity as measured by ferric reducing antioxidant power (FRAP) compared to water-gavaged control groups, indicating that blueberry extracts may have the ability to elevate antioxidant potential. These findings are in keeping with the study of Kay & Holub (2002), who conducted human trials to determine whether the consumption of Lowbush blueberries would enhance postprandial serum antioxidant status in healthy subjects. The authors concluded that freeze-dried blueberries (100 g) had the ability to improve total antioxidant status as represented by a 15.0 % increase in serum $ORAC_{total}$ above the control group. Additionally, after consumption of freeze-dried wild blueberry powder with a high fat meal, anthocyanin compounds could be identified in human serum and correlated with an increase in serum ORAC antioxidant capacity (Mazza et al., 2002). Unlike the earlier studies cited, Pedersen et al. (2000) showed that consumption of 500 ml of blueberry juice (obtained from local retailers) did not increase total antioxidant capacity, which was measured using the FRAP assay, in the plasma of healthy volunteers. These contradictory results might be due to the different forms of berries used in the studies, the unequal doses of berries which were supplied to the subjects, or different methodologies used for the total antioxidant analysis. Because of inconsistencies in the reports of the effect of consumption of blueberries rich in anthocyanins on the total antioxidant activity, in particular, it still remains unclear whether an increase in plasma antioxidant status induces downstream alterations in physiology; therefore, further research is still needed in this area.

2.3 Possible mechanisms of action of dietary polyphenols on metabolic syndrome- related pathologies

2.3.1 Impact on starch hydrolysis enzymes

Carbohydrates, in particular starch (consisted of amylose and amylopectin), are the major source of energy in modern human diets. The digestion of starch is mainly due to the action of salivary α-amylase and pancreatic α-amylase, which hydrolyse α-1,4-glycosidic linkages in both amylase and amylopectin and release oligosaccharides (maltose, maltotriose and α-limit dextrins). Further digestion in the small intestine brush border is accomplished by the activity of α-glucosidase (maltase), which hydrolyzes the terminal α-1,4-linked glucose linkages, while the branch-point residues with an α-1,6 bond are hydrolyzed by α-dextrinase (isomaltase) to release monosaccharide glucose before being absorbed into the blood circulation (Gropper et al., 2009). It is well accepted that increased digestion of starch and absorption of glucose induces postprandial hyperglycemia, which could lead to hyperinsulinemia and the development of insulin resistance. α-amylase is an enzyme mainly responsible for digestion of dietary starch into maltose, while α-glucosidase represents the enzyme for further hydrolyzing maltose to glucose. For these reasons, inhibition of the activity of these two enzymes could lead to the reduction of glucose release and absorption in the small intestine, which has been reported to be one of the primary approaches to suppress postprandial hyperglycemia and may help to delay the onset of insulin resistance (Hanhineva et al., 2010).

A variety of polyphenolic compounds has been shown to inhibit α-amylase and α-glucosidase activities *in vitro*. A range of polyphenols including flavonoids (anthocyanins, flavonols, catechins), phenolic acids (chlorogenic acids, caffeic acids), and tannins (proanthocyanidins and ellagitannins) are found in a variety of fruits such as berries (e.g. strawberries, raspberries, blueberries and cranberries), other fruits and vegetables (e.g. grapes, eggplants), green and black tea, and red wine (McDougall et al., 2005; Schäfer & Högger, 2007: Kwon et al., 2008: Cheplick et al., 2010; Hogan et al., 2010: Koh et al., 2010). However, few studies have been carried out on the impact of blueberry polyphenols on these enzymes. Johnson et al. (2011) investigated α-amylase and α-glucosidase inhibitory abilities of different blueberry cultivars grown in southern Illinois (USA) and reported that all Highbush extracts showed similar or higher abilities to inhibit these digestive enzymes compared to acarbose, a therapeutic drug used for control of type II diabetes. Activity ranged from 91.8-103.3% for α-amylase and from 103.2 to

190.8 % for α-glucosidase, compared to the positive control acarbose, which was considered as 100% inhibition. More specifically, a report the following year compared α-glucosidase inhibitory effect between peel and flesh tissues of 33 blueberries (29 Rabbiteye, 2 *V.ashei* hybrid derivatives and 2 Highbush blueberries) and indicated that the α-glucosidase inhibitory effect in blueberry fruits is mainly derived from the peel (20.2-98.1% per 100 μg DW) rather than from flesh tissue (10.2-28.9% per 100 μg DW) (Wang et al., 2012).

It is also important to highlight that, in the past decade, a number of studies attempted to compare the activities of polyphenol-rich extracts from various fruits in order to identify the bioactive compounds that influence the inhibition of α-amylase and α-glucosidase. McDougall et al. (2005) demonstrated that extracts from strawberry and raspberry, which contain considerable amount of soluble tannins, were effective inhibitors of α-amylase. Conversely, the extracts most effective in inhibiting α-glucosidase were those from blueberries and blackcurrants, which contain high amounts of anthocyanins. These findings are consistent with the study of Grussu et al. (2011), who demonstrated that anthocyanins were not crucial for the inhibition of α-amylase because both red (with anthocyanins) and yellow (without anthocyanins) raspberries showed equal inhibitory capacities. Furthermore, Boath et al. (2012) found that a proanthocyanin-rich fraction extracted from rowanberries had a low level of α-glucosidase inhibition compared to the whole rowanberry fruit, suggesting that proanthocyanin components are not influential in the inhibition of α-glucosidase. A very recent study by Johnson et al. (2013) also indicated that the anthocyanin-enriched fraction was a more potent inhibitor of α-glucosidase than the proanthocyanidin-enriched fraction, as indicated by a lower concentration of anthocyanins than proanthocyanidins needed to inhibit α-glucosidase by 50% (IC_{50}). All of these results appear to indicate that procyanidins are effective compounds to inhibit α-amylase, whereas inhibition of α-glucosidase is mainly due to the anthocyanin compounds.

2.3.2 Impact on cellular function and gene expression

A number of *in vitro* studies conducted recently assessed the impact of polyphenols on cell physiology as well as on the expression of genes related to glucose and lipid metabolism, allowing the researchers to explore the specific biological mechanisms behind their effects. Glucose, after being derived from carbohydrate digestion, is primarily controlled by three major organs: pancreas, peripheral tissues and the liver. To decrease hyperglycemia and hyperinsulinemia, which is the cornerstone of anti-insulin resistance linked to metabolic

syndrome, bioactive compounds in plants, besides their ability to inhibit starch hydrolysis enzymes, might exert other beneficial effects by (1) modulating pancreatic function by increasing proliferation of pancreatic beta-cells mass or protection of beta-cells from glucose toxicity, (2) enhancing the uptake of glucose from the blood into peripheral target tissues, or (3) increasing glycogen storage (glycogenesis) and suppressing glucose production in the liver (Hanhineva et al., 2010) (figure 2.8).

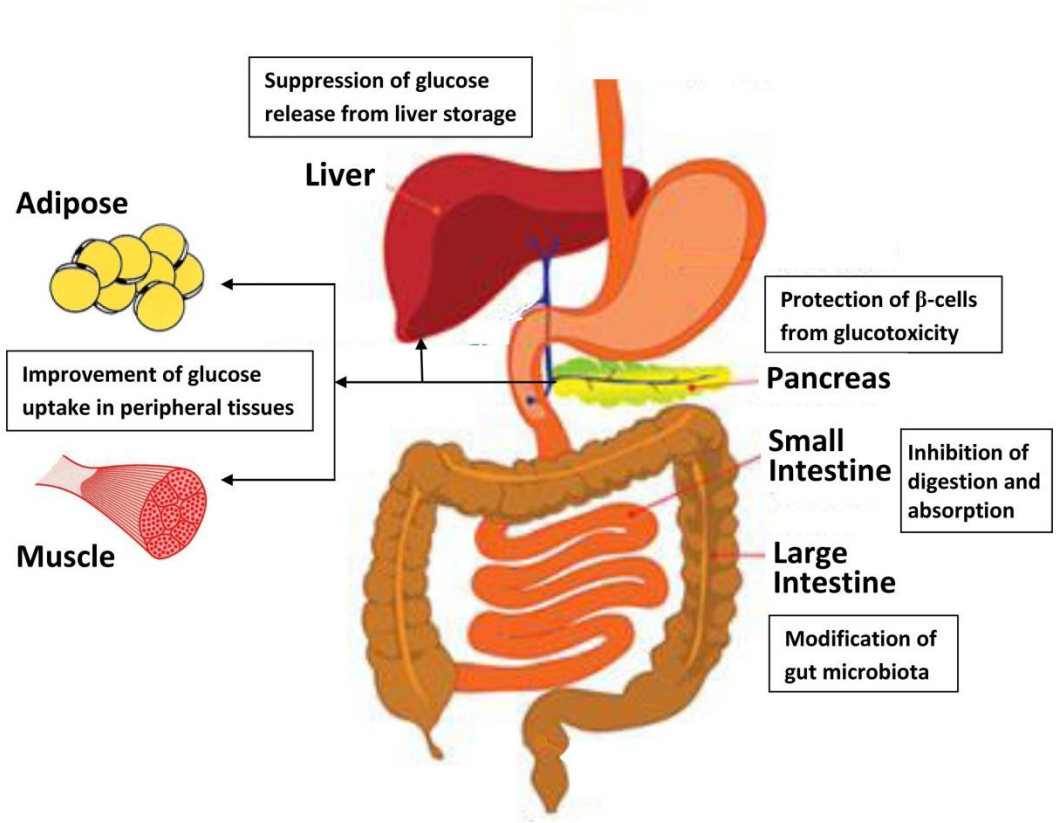


Figure 2.8: Possible site of action of polyphenols on carbohydrate and glucose metabolism (Source: modified from Hanhineva et al., 2010).

A study on the effects of dietary phenolic acids on pancreatic function, for example, was carried out with epigallocatechin gallate (EGCG) and rutin in rat insulinoma pancreatic beta-cells (RIN m5F). It was observed that secretion of insulin was dramatically suppressed when the cells were exposed to a high concentration of glucose (33 mM for 48 h), but treatment with EGCG (10 µM) or rutin (0.1 µM) was able to increase the insulin secretion from approximately 0.3 nM (in non-treated cells) to between 0.5 and 0.6 nM. In addition, their results indicated a significantly higher relative cell mass in the ECGC and rutin-treated cells after a 120 h high glucose incubation. These findings suggested that these two compounds were able to preserve and/or increase pancreatic

beta-cell mass and to effectively enhance insulin secretion even under high glucose conditions in which secretion of insulin can be suppressed (Cai & Lin, 2009). In another study carried out with anthocyanins, insulin secretion reached the lag phase when rodent pancreatic beta-cells (INS-1 832/13) were exposed *in vitro* to glucose at a concentration of 10 mM; treating the cells with anthocyanins was able to enhance the insulin secretion by 1.4 fold compared to untreated cells, suggesting a role for anthocyanins in the protection of beta-cells against glucose toxicity. It was also shown that the number of hydroxyl groups in ring B of anthocyanins played a significant role in their ability to modulate insulin secretion. Among the different anthocyanidins studied, the most prominent insulin-releasing agents were delphinidin-3-glucoside and cyanidin-3-galactoside (Jayaprakasam et al., 2005).

Alternatively, dietary polyphenols may enhance glucose metabolism by stimulating glucose uptake in peripheral target tissues, mainly skeletal muscle and adipocytes. Data supporting the hypothesis that glucose uptake ability may be modulated by dietary polyphenols is based on the translocation of glucose transporters, in particular GLUT4, which is the only insulin-sensitive GLUT, as well as the expression of signaling pathway mediators required for insulin action and glucose uptake (Hanhineva et al., 2010), such as insulin receptor substrates (IRS), phosphotidylinositol 3-kinase (PI3K) and/or AMP-activated protein kinase (AMPK). Additionally, cell-based assays with rat skeletal muscle (L6 or C2C12) and/or adipose cell lines (3T3-L1) are commonly used to investigate the effects of phenolic compounds on peripheral glucose uptake.

In a study by Prabhakar & Doble (2009), two plant phenolic compounds, chlorogenic acid and ferulic acid, were assessed for their ability to induce uptake of 2-deoxyglucose (2DG) into L6 myotubes. Cholorogenic acid and ferulic acid at a concentration of 25 µM increased the 2DG uptake by 3.03 and 2.97 fold, respectively, compared to untreated control, an effect comparable to metformin and 2,4-thiazolodinedione, the two common commercial oral hypoglycemic drugs. Moreover, the authors found that expression of GLUT4 and peroxisome proliferator-activated receptor gamma (PPAR-γ), a regulator of adipogenesis, were elevated in chlorogenic acid-treated cells, while expression of GLUT4 and PI3K transcripts were significantly enhanced in ferulic acid-treated cells. This suggests that while both chlorogenic acid and ferulic acid enhance glucose uptake into cells by increasing GLUT4 expression, only the ferulic acid exerts an effect on GLUT4 expression via PI3K-dependent pathway, whereas chlorogenic acid may work via a PI3K-independent route. In another study, Zhang et al. (2010) determined the role of EGCG from green tea on glucose uptake in dexamethasone-induced insulin resistance using rat L6 muscle

cells. Dexamethasone was found to inhibit GLUT4 translocation and reduce phosphorylation of AMPK and protein kinase B (PKB, also called Akt), a crucial downstream target of PI3K activation. However, 24-hour treatment with 20 or 40 µM EGCG attenuated the effect of dexamethasone, as indicated by the significant increase in GLUT4 translocation to plasma membrane and the improvement of insulin-stimulated glucose uptake by 80% and 105%, respectively. The authors also found that EGCG was able to increase the AMPK and Akt phosphorylation, suggesting that the AMPK and PI3K/Akt signaling pathway may be responsible for the EGCG-stimulated GLUT4 translocation. Some plant based extracts used in traditional medicine have also been assessed. For instance, four isoflavonoids (genistein derivatives) extracted from *Tetracera scandens* (a Vietnamese medicinal plant) exhibited significant glucose uptake activity in both basal and insulin-stimulated L6 rat skeletal muscle cells in a dose-dependent manner. This suggested that AMPK activation and the mRNA expressions of GLUT4 and GLUT1 appear to be involved in the mechanism behind glucose uptake stimulation (Lee et al., 2009).

The anti-hyperglycemic action of polyphenolic compounds from plants might impact liver function by increasing the activity of glucokinase to improve glycogenesis and/or suppressing hepatic glucose production via glycogenolysis and gluconeogenesis. A potential mechanism involving polyphenols and liver glucose output has been identified by Wolfram et al. (2006) using the rat hepatoma H4IIE cell line exposed to EGCG at 50 or 100 µmol/L. Real-time PCR analysis showed that EGCG decreased the expression of two gluconeogenic genes, phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase), in a time- and dose-dependent manner. Also, a genome-wide DNA microarray assay revealed an effect of EGCG on the expression of genes involved in glucose metabolism. Notably, genes involved in gluconeogenesis (glucose-6-phosphatase and fructose-1,6-bisphophatase) were downregulated, while genes involved in glycolysis (e.g. phosphofructokinase) were upregulated. The same authors also found an increase in mRNA expression of glucokinase in the liver of *db/db* mice fed with EGCG for 7 weeks.

Collins et al. (2007) studied the effect of EGCG on glucose production via gluconeogenesis and the expression of key gluconeogenic genes (PEPCK and G6Pase) using hepatocytes isolated from C57BL/6J mice. EGCG at ≤ 1 µM inhibited hepatic glucose production in a similar manner to insulin by blocking expression of PEPCK and G6Pase genes. Similarly to EGCG, the ability of other phenolic compounds to elevate the activity of glucokinase and/or decrease hepatic gluconeogenesis PEPCK and G6Pase has also been reported in both *in vitro* and animal studies;

active phenolic compounds include isoflavones (genistein and daidzein) from soy (Ae Park et al., 2006; Choi et al., 2008) and flavonoids (hesperidin and naringin) from citrus (Jung et al., 2006).

With regards to polyphenolic extracts from blueberries, Martineau et al. (2006) carried out a variety of cell-based bioassays to investigate the anti-diabetic activity of ethanol extracts from roots, stems, leaves and fruits of wild blueberries. The extracts of root and stem increased glucose transport by 15-25% in C2C12 murine muscle cells, and by 75% in 3T3 murine adipocyte cells. Fruit extracts significantly increased the proliferation of β-cells by 280%, as measured by ^{3}H-thymidine incorporation in β TC-tet murine pancreatic β cells. Additionally, the fruit extract also protected against glucose toxicity by decreasing apoptosis by 20 - 33 % in PC12 pheochromocytoma cells. The authors therefore demonstrated that the extracts of wild blueberry (*V. angustifolium*) possess active components with insulin-like properties.

Another *in vitro* study was carried out by Vuong et al. (2007), who studied the effect of fermented Lowbush blueberry juice on glucose uptake and adipogenesis using muscle and adipocyte cell-based assays. Blueberry juice was fermented with the *Serratia vaccinii* bacterium, which is known to increase the phenolic content and antioxidant activity. The fermented juice increased glucose uptake by 48% in C2C12 murine myotubes and by 142% in 3T3-L1 murine adipocytes in both the presence and absence of insulin, whereas chlorogenic acid and gallic acid, two other major phenolic compounds, had no effect. Furthermore, 6 hour treatment with fermented juice also stimulated the phosphorylation of AMP-activated protein kinase (AMPK) by 1.9-fold in C2C12 cells and 3.2-fold in 3T3-L1 cells, and significantly inhibited triglyceride level during adipogenesis of 3T3-L1 cells. The authors concluded that the blueberry juice fermented with *S. vaccinii* exhibited promising anti-hyperglycemic effects *in vitro*. However, the cell-based studies do not replicate the complex processes operating on ingested berries, and they absolutely require further animal studies to test for beneficial effects physiologically *in vivo*.

2.3.3 Impact on intestinal microbiota

Microbiota in intestine may exert an important effect on the role of polyphenols in controlling metabolic disorders, since these compounds are poorly bioavailable. Two major pathways have been proposed with regard to how the gut microbiota affects obesity and insulin resistance: (1) gut microbiota affect host energy expenditure and storage, and (2) gut microbiota

are involved in the development of low-grade inflammation linked to insulin resistance (Tilg et al., 2009; Delzenne & Cani, 2010).

The human intestine contains numerous microorganisms, which consist of approximately 10^{14} bacteria including up to 500-1000 different species (Cani et al, 2008). Recent research indicates that this bacterial population has a role in the development of fat mass and energy harvest. A possible mechanism involves non-digestible food components, mostly polysaccharides, being transformed into digestible sugars or short chain fatty acids through fermentation by gut microflora, providing energy for both bacteria and host. This hypothesis was drawn from the studies of Bäckhed et al. (2004), who demonstrated that lean axenic mice, colonized with gut microbiota derived from *ob/ob* mice, increased their body weight rapidly by 60% and developed insulin resistance within two weeks. The authors suggested that the mechanism could be an increase in specific gut microbiota found in obese mice, which are able to increase the energy extracted from non-digestible foodstuff in the intestine and provide extra energy to the host, resulting in an elevation of glucose in blood circulation and increased body mass. Consequently, several studies using rRNA gene sequencing, quantitative real time PCR, and fluorescent in situ hybridization (FISH) have demonstrated a strong association between the composition of gut microflora and obesity linked to metabolic syndrome and diabetes. The bacteria involved were mainly Bacteroidetes (*Bacteroides* and *Prevotella*), Firmicutes (*Clostridium*, *Enterococcus*, *Lactobacillus*, and *Ruminococcus*), Actinobacteria (*Bifidobacterium*) and Proteobacteria (*Helicobacter* and *Escherichia*).

Ley et al. (2006) demonstrated that the proportions of Bacteroidetes and Firmicutes, the predominant groups of bacteria in the intestinal tract, differed between lean and obese *ob/ob* mice. Genetically obese (*ob/ob*) mice were 50% lower in Bacteroidetes but higher in Firmicutes than their lean counterparts. Murphy et al. (2010) also confirmed a progressive increase in Firmicutes and a reduction in Bacteroidetes in *ob/ob* mice as well as high-fat-fed mice compared to lean controls. These results are consistent with a subsequent human study. Children living in rural Africa who consumed a plant polysaccharide-rich diet showed depletion of Firmicutes and increased levels of Bacteroidetes, mainly *Prevotella* and *Xylanibacter*, in their fecal microbiota compared to European children who consumed a diet with a lower fiber content. This finding indicated that *Prevotella* and *Xylanibacter* are energy harvesting microbiota, and the African children had adapted to maximize energy extraction from a diet rich in fiber by increasing levels

of these particular bacteria in their intestines (De Filippo et al., 2010; Tremaroli & Bäckhed, 2012).

Insulin resistance is associated with low-grade inflammation, and evidence shows that an increased level of plasma endotoxin or lipopolysaccharide (LPS) from bacteria promotes the production of pro-inflammatory cytokines such as TNF-α and IL-6, which negatively affect glucose tolerance and lead to the development of insulin resistance (D'Aversa et al., 2013). LPS is a major component of the outer membrane in Gram negative bacteria, which can be released into circulation after destruction of the bacteria cell wall. LPS levels in the blood closely correlate with changes in the ratio of Gram-negative to Gram-positive bacteria present in the intestinal microbiota (Cani et al., 2008). In general, the gut epithelium acts as a barrier to prevent LPS translocation from the intestinal lumen to the blood circulatory system. However, several factors can affect this protective function and promote a leaky gut, such as stress (Mazzon & Cuzzocrea, 2008), radiation (Hill et al., 1997), bowel disease infection (Liu et al., 2005), non-steroidal anti-inflammatory drugs (Ito et al., 2013) and alcohol consumption (Enomoto et al., 2001). Intestinal hyperpermeability leads to elevated LPS levels in the circulation and promotes low-grade inflammation. In addition, chronically high dietary fat intake alters the ratio of Gram-negative to Gram-positive in the gut, decreasing Gram-positive bifidobacteria and significantly increasing plasma endotoximia-induced inflammation. This has been found to be positively correlated with increased fasting glycemia, insulinemia and weight gain (Cani et al., 2007a).

Cani et al. further observed that modifying the composition of intestinal microbiota through supplementation with antibiotics or prebiotics was able to restore bifidobacteria and thus effectively reduce metabolic endotoxemia and the level of intestinal LPS in high-fat-fed mice (Cani et al., 2007b; Cani et al., 2008). Bifidobacteria, a dominant member of the intestinal microbiota, has been shown to improve mucosal barrier function and reduce endotoxin levels. Possible mechanisms could be due to (1) these particular strains promote a healthier microvillus environment by increasing villus height and crypt depth, leading to a thicker mucosal layer in the gut, and (2) they do not degrade intestinal mucus glycoproteins like the pathogenic bacteria do and hence prevent LPS translocation (Cani et al., 2009). In *in vitro* epithelial monolayer studies, other probiotic strains including *Lactobacillus rhamnosus* and *Lactobacillus casei* have also been reported to protect against *Escherichia coli*-induced loss of epithelial barrier function (Parassol et al., 2005; Johnson-Henry et al., 2008). In humans, Kalliomaki et al. (2008) demonstrated a higher number of *Bifidobacterium* spp. in fecal contents of normal weight children compared to

overweight children. Conversely, the number of *Staphylococcus aureus* was also found to be lower in children who maintain normal weight than obese children, indicating that *Bifidobacterium* spp. may provide a protective effect, whereas *S. aureus* may act as a trigger for the low grade inflammation that contributes to obesity development. This is in agreement with the study of Collado et al. (2008), who observed a significantly higher number of *Bacteroides* spp. and *S. aureus*, but lower number of *Bifidobacterium* spp., in overweight pregnant women compared with normal weight individuals.

A compositional change in intestinal microbiota has also been demonstrated in type II diabetic patients. The proportions of Firmicutes and Clostridia were significantly reduced, whereas the number of Betaproteobacteria was increased in diabetic patients compared to the normal subjects. Interestingly, the ratio of Bacteroidetes to Firmicutes as well as the numbers of Betaproteobacteria were positively correlated with the concentration of glucose in the plasma (Larsen et al., 2010).

Since there appears to be a link between intestinal microflora and obesity related to metabolic syndrome and insulin resistance, modifying the composition of gut microbiota by incorporation of beneficial bacteria, in particular bifidobacteria and lactobacilli, has been proposed. The use of pre- and probiotics has increased recently as an alternative approach to reverse host alteration in the composition of gut microbiota. Polyphenols in blueberries have been reported to have prebiotic properties in both *in vitro* and *in vivo* studies. Sutherland et al. (2009) demonstrated that organic and aqueous extracts of Highbush blueberries (both neutral and acidic conditions) enhanced the growth of selected probiotic bacteria (*Lactobacillus reuteri*, *Lactobacillus rhamnosus*, and *Bifidobacterium lactis*). An aqueous blueberry extract at pH 7 also inhibited the growth of some pathogenic bacteria (*Escherichia coli* 0157:H7 and *Escherichia coli* LF82). At the same time, Molan et al. (2009) found that extracts of Rabbiteye blueberries ('Centurion' and 'Maru') significantly promoted the growth of *Lactobacillus rhamnosus* and *Bifidobacterium breve* under *in vitro* conditions. These two extracts also significantly influenced the population size of *L. rhamnosus* and *B. breve* observed in rats after being orally gavaged with 4 ml of the extracts for 6 days. The results indicated that blueberry extracts could modify the composition of bacteria by increasing the numbers of beneficial microbes, leading to an improvement in gut health. One study has also been conducted in humans. Consumption of a wild blueberry drink for six weeks significantly elevated the number of bifidobacteria in the caecum, whereas no differences were observed between the blueberry group and the placebo group for *Bacteroides*

spp., *Prevotella* spp., *Enterococcus* spp., and *Clostridium coccoides* (Vendrame et al., 2011). All of these findings confirm the prebiotic properties of bioactive compounds from blueberries, which may provide beneficial effects to improve glucose homeostasis and/or normalise the inflammatory response. However, this hypothesis needs further confirmation *in vivo*.

2.4 Health benefits of blueberry on metabolic syndrome

Blueberry fruits are thus well-documented to be enriched in polyphenolic compounds and to exhibit high antioxidant activity (Prior et al., 1998; Wu et al., 2004a). Blueberry extracts have also been shown to influence glucose/energy metabolism through several mechanisms including inhibition of carbohydrate digestion and glucose absorption in the intestine, protection of pancreatic beta-cells from glucose toxicity, improvement of glucose uptake in peripheral tissues, suppression of glucose release from liver storage, and modulation of beneficial microbiota in the intestine (Figure 2.8). Each of these bioactivities could provide benefits associated with the management of metabolic syndrome. Studies with regards to the potential health benefits of polyphenolic compounds, especially those from the extracts from blueberry, on metabolic parameters have been carried out to confirm their physiological benefits in animals and humans, and the data are summarized below.

2.4.1 Evidence from animal studies

To date, only a small number of studies has been carried out to investigate the beneficial effects of bioactive compounds extracted from blueberries on management of metabolic syndrome. However, considerable evidence is available on both preventive and treatment effects of other polyphenolic-rich fruits, vegetables and traditional plants, and these are summarized in Appendix I.

C57BL/6J mice fed a high fat diet (commonly 45% or 60% calories from saturated fat) are commonly used as a diet-induced obesity model, since this mouse strain is well-documented to be sensitive to dietary obesity. For a diet-induced insulin resistance model, Sprague-Dawley (SD) rats or Wistar rats fed a 60-66% fructose diet are widely used (Gajda et al., 2007). It is very difficult to identify the optimal dose of polyphenols needed for metabolic health benefits since the dose of dietary polyphenolic used varies widely between published studies. It is noteworthy

that in examining potential health beneficial effects over a short period of time, a number of studies have used higher doses of polyphenolic compounds than would be found in human daily consumption. However, it is not necessarily true that a higher dose of polyphenols provides more desirable health benefits; for example, Hininger-Favier et al. (2009) found similar beneficial effects when feeding Wistrar rats with 1 g or 2 g of green tea solids per kg of high-fructose diet on oxidative stress, insulin sensitivity and the expression of genes involved in glucose uptake and insulin signaling. Also, Khanal et al. (2010) demonstrated that incorporation of a medium level (6.6 g/kg diet) of cranberry powder into a high-fructose diet produced a better metabolic response in SD rats than lower (3.3 g/kg diet) and higher (33 g/kg diet) levels. In addition, previous evidence has revealed that high doses of polyphenols may actually lead to toxicity effects (Lambert et al., 2010). It is also interesting to highlight that the length of the study period required to have a beneficial effect on metabolic syndrome in animal studies varied considerably, from as short as 2 weeks as demonstrated in the study of Kim et al. (2010b), to 3 months in the study of Braz De Oliveira et al. (2010).

To study the effect of polyphenols from blueberries, Vuong et al. (2009) conducted animal studies to confirm *in vitro* results. The authors demonstrated that biotransformed Lowbush blueberry juice using *S.vaccinii* bacterium incorporated in drinking water of KKA^{y} mice for 3 weeks significantly reduced weight gain in the mice. Additionally, the juice protected the mice from developing glucose intolerance and diabetes. These results showed that the biotransformed blueberry juice possessed anti-diabetic potential in an *in vivo* model.

Prior and colleagues (2008; 2009) carried out a series of studies in the C57BL/6J mouse model to assess anti-obesity effects of Lowbush blueberries in the form of freeze-dried berries or purified anthocyanins (ACNs). After 8 weeks, mice fed a high fat diet (60% calories from fat) plus ACNs from blueberries exhibited lower body weight gain and total body fat compared to the high-fat control. However, 10% freeze-dried whole blueberries added into either a low fat (10% calories from fat) or high fat (45% calories from fat) diet did not alter weight gain or total body fat. The difference observed was unlikely to be due to the concentration of anthocyanins, since total anthocyanin intake provided to the freeze-dried-fed group (3.75 mg/day/mouse) was higher than in the purified ACNs-fed animals (2.82 mg/day/mouse). The researchers concluded that purified anthocyanins were more effective than the freeze-dried form in preventing obesity; however, the rationale behind the difference is not clear.

A subsequent study was carried out using juice, another form in which blueberries are consumed, compared with purified blueberry anthocyanins. Mice were fed either blueberry juice or purified blueberry anthocyanins with a low-fat (10% calories from fat) or high fat (45% calories from fat) diet. Similar to the earlier finding, purified blueberry ACNs at the dose of 0.2 mg/mL (0.49 mg/day/mouse) normalized the impact of the high-fat diet on diet-induced obesity by decreasing the percentage of body fat as well as epididymal adipose tissue weights to levels matching those of mice fed with low-fat diets. Furthermore, consumption of purified anthocyanins from blueberries (0.2 mg/mL) also improved β-cell function (HOMA-BCF) and significantly decreased the fasting serum glucose concentration of high-fat-fed mice. While blueberry juice (5.26 mg ACNs/day/mouse) was not as effective as the purified anthocyanins in preventing obesity, they exhibited a significant effect on the reduction of serum leptin relative to the high-fat control. Interestingly, the higher dose of purified ACNs (1.0 mg/mL or 1.79 mg/day/mouse) was not effective in decreasing any parameter related to obesity (Prior et al., 2010). This observation is consistent with the studies of Khanal et al. (2010) in which the authors observe that the effect of phytochemicals on metabolic syndrome is, possibly, not a dose-dependent response.

In other work, Grace et al. (2009) used the diabetic C57BL/6J mouse model to study the anti-diabetic activity of different anthocyanin-containing extracts in Lowbush blueberries. The result showed that gavaging mice with a phenolic-rich extract or anthocyanin-enriched fraction formulated with Labrasol at a dose of 500 mg/kg body wt decreased blood glucose levels by 33% and 51%, respectively, in diabetic mice compared to those that received the anti-diabetic drug metformin (27% at 300 mg/kg). The authors concluded that the hypoglycemic activity was greater in the anthocyanin compounds than the phenolic fraction. Pure delphinidin-3-*O*-glucoside and pure malvidin-3-*O*-glucoside at 300 mg/kg body weight were also assessed. Malvidin-3-*O*-glucoside, but not delphinidin-3-*O*-glucoside, possessed a glucose-lowering effect. This result indicates that individual anthocyanins may have a specific role and understanding the composition of blueberries is therefore critical, rather than just identifying their total anthocyanin concentration. However, it should be noted that Labrasol, a microemulsifying drug used for increasing the bioavailability, may also contribute to the hypoglycemic activity found in this study.

Recently, Vendrame et al. (2013) investigated the ability of wild blueberries to treat proinflammatory status as measured by TNF-α, IL-6, and CRP levels in the obese Zucker rats.

Treatment with 8% (w/w) wild blueberry-enriched diet for 8 weeks significantly decreased plasma concentrations of TNF-α (-25%), IL-6 (-15%), and CRP (-13%), and increased adiponectin (+22%). In addition, the expression of CRP was down-regulated in liver (-25%), while TNF-α and IL-6 were down-regulated in both liver (-59% and -65%) and adipose tissues (-52% and -64%). However, wild blueberry consumption did not provide a significant effect on adiponectin mRNA expression.

DeFuria et al. (2009) investigated the effect of whole blueberry freeze-dried powder (1:1 blend of *Vaccinium ashei* 'Tifblue' and *Vaccinium corymbosum* 'Rubel') on adipose tissue inflammation and insulin resistance. Blueberry powder (4% w/w) was incorporated in a high fat diet (60% calories from fat) and fed for 8 weeks to male C57BL/6J mice. The results showed that blueberry powder attenuated oxidative stress (as indicated by increased glutathione peroxidise gene expression) and protected against inflammation of adipose tissue by attenuating upregulation of inflammatory genes (i.e. TNF-α, IL-6), which could lead to decreasing insulin resistance and combating obesity-associated pathology. In another study, the same freeze-dried powder (1:1 Tifblue and Rubel) at the dose of 2% (w/w) in a high-fat diet (45% calories from fat) and fed to Zucker Fatty rats was found to decrease serum triglycerides, fasting insulin, HOMA-IR, and glucose area under the curve. Furthermore, blueberry powder intake also altered skeletal muscle and adipose tissue PPARs activity, and affected PPAR transcripts involved in fat and glucose metabolism. However, except for the reduction of triglycerides, these effects were not observed in Zucker Lean rats fed with blueberries (Seymour et al., 2011).

In a study of Kim et al. (2010a), the peels of Rabbiteye blueberries (Tifblue) were tested for their effects on inflammatory gene expression in liver and adipose tissues. Syrian Golden hamsters were fed a high-fat diet (37% calories from fat + 0.15% cholesterol) containing either 8% (w/w) dried whole blueberry peels, 2% dried ethanol extract of peels, or 6% residue from extracted peels. All blueberry peel diets not only significantly decreased plasma VLDL-chol and total cholesterol, but also increased fecal lipid excretion. The expression of hepatic gene *CYP7A1*, which is involved in the initial step of bile acid synthesis, was also found to be up-regulated by all blueberry diets; however, no changes in inflammatory gene expression were observed in adipose tissues.

To my knowledge, only one study has been published on cultivated Highbush (*V. corymbosum*) blueberries. In this study, blueberry polyphenols (BB) were stabilized in defatted soybean flour (DSF) in order to concentrate the anthocyanins and other polyphenols as well as eluting sugars

from the blueberries. Obese and hyperglycemic C57BL/6J mice were provided *ad libitum* with a very high-fat diet (61% calories from fat) supplemented with either BB-DSF or DSF without blueberries for 13 weeks. BB-DSF significantly reduced the impact of a very high-fat diet-induced metabolic syndrome as indicated by a reduction in body weight, fasting blood glucose and serum cholesterol as well as an improvement in glucose tolerance in mice fed with BB-DSF compared with the DSF controls (Roopchand et al., 2013).

2.4.2 Evidence from human studies

Similarly, there are only a few intervention studies reporting the effects of blueberry consumption on metabolic biomarkers in humans, and the results so far are relatively inconclusive, possibly due to considerable inter-individual variation and the complexity of the human biological system. The first report on the effect of daily supplementation with blueberry bioactives on whole-body insulin resistance was conducted by Stull et al. in 2010. Using a double-blind, randomized, placebo-controlled clinical study design, thirty-two obese, insulin-resistant men and women received for 6 weeks a smoothie either with 45 g of blueberry bioactives (made from a 1:1 mixture of Tifblue and Rubel whole freeze-dried blueberries) or without blueberry but equal in other nutritive values. Biomarkers associated with insulin sensitivity and metabolic syndrome were measured. The insulin sensitivity, measured by insulinemic-euglycemic clamp, was significantly improved in the blueberry-supplemented group compared to the control group. However, no significant changes in total body weight, adiposity, serum glucose, lipid profile (triglycerides, cholesterol, LDL and HDL-cholesterol) or inflammatory biomarkers (TNF-α, CRP and MCP-1) were observed.

In another study, a randomized controlled trial was carried out to evaluate the effect of blueberry consumption on features of metabolic syndrome (blood pressure, serum glucose and lipid profile), lipid peroxidation, and inflammation in obese subjects with metabolic syndrome. Forty-eight participants were randomized into two groups; the treatment group consumed a beverage made from freeze-dried blueberries (50 g of freeze-dried weight from a mixture of 1:1 Tifblue and Rubel) daily for 8 weeks, whereas the controls received an equivalent amount of water. Decreases in blood pressure (both systolic and diastolic) were greater in the treatment group (-6 and -4%, respectively) than in the control subjects. Additionally, plasma oxidized LDL and serum malondialdehyde decreased more in the blueberry group (-28 and -17%, respectively)

than in the placebo group. However, no significant differences were observed in serum glucose concentration, lipid profiles, or biomarkers of inflammation (Basu et al., 2010).

Giongo et al. (2011) found an increase in biological antioxidant potential values (as measured with a FRAP assay) and a slight reduction in some serum markers related to inflammation including CRP, ceruloplasmin, complementary factors 3 and 4, in overweight and obese children after consumption of fresh blueberries or blueberry puree (375 g/week) for 8 weeks. These beneficial effects were greater in the group that consumed fresh blueberries compared to the group that ate the puree. It is also interesting to note that no differences in the glycemic response or satiety index were observed between the treatment (100 g of blueberries served with pancakes) and the control (pancakes) group when blueberries were added to a starch–based meal (Clegg et al., 2011). The authors hypothesized that the reported beneficial impact of polyphenolic compounds in blueberries on glycemic response observed in other studies was possibly due to the testing of carbohydrates in the form of sugars (monosaccharides or disaccharides), but blueberry polyphenols may be unable to bind with the amylose and amylopectin fractions of starch, thus explaining the lack of a reduction in glycemic response observed in their study.

2.5 Aim and research objectives

As discussed in the above literature review, blueberries and their extracts appear to possess an anti-metabolic syndrome bioactivity, which has been demonstrated in a number of *in vitro* assays and *in vivo* studies. However, almost all of the previous studies in this area have been carried out using Lowbush or Rabbiteye blueberries rather than the Highbush blueberries that are available in New Zealand and more familiar to consumers.

Highbush blueberries are known to have lower concentrations of antioxidants in general, and anthocyanins in particular, compared to Lowbush and Rabbiteye blueberries. This raises the questions of whether Highbush cultivars possess similar positive attributes, and which cultivars of these berries planted in New Zealand have the potential to be used for the management of metabolic syndrome. Additionally, not every individual anthocyanin exhibits anti-metabolic syndrome activity to the same degree, and some may work synergistically. These issues need more clarification.

Therefore, to gain knowledge and understanding with regards to the possible anti-metabolic syndrome benefits of New Zealand Highbush blueberries, this study aims to investigate the effect of selected Highbush blueberry varieties grown in New Zealand on antioxidant status, gut microbiota, and their effectiveness on the management of metabolic syndrome by using a series of *in vitro* assays and *in vivo* animal trials. More specifically, this proposed research addresses the following four objectives:

1. To determine the anthocyanin concentration, total phenolic content and antioxidant capacity of eight varieties of Highbush blueberries grown in New Zealand (Chapter 3).

2. To determine the abilities of the eight varieties of Highbush blueberries to inhibit starch hydrolysis enzymes as well as enhance the growth of beneficial probiotic bacteria using *in vitro* methods (Chapter 4).

3. To examine the effect of Highbush blueberry intake on total antioxidant status, intestinal microbiota, insulin resistance and associated metabolic risk factors in rats with diet-induced metabolic syndrome (Chapter 5 and 7).

4. To identify individual anthocyanins of Highbush blueberries in order to further determine whether any of these compounds are responsible for the fruits' collective anti-metabolic syndrome benefits (Chapter 3, 4 and 7).

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CHAPTER 3

Phenolic composition and antioxidant activity of Highbush blueberries

Abstract :

It is well accepted that the health benefits of blueberry consumption are in part due to their phytochemical concentration. Eight varieties of Highbush blueberries (*Vaccinium corymbosum*) grown in New Zealand were assessed for their bioactive compounds including anthocyanin profiles, concentrations of chlorogenic acid, total phenolic content and antioxidant activity. For anthocyanin and chlorogenic acid determination, blueberries were extracted using acidified water, whereas for total phenolics and antioxidant activity, three different solvents were compared: mixed solvent (acetone: methanol: water: formic acid, 40:40:20:0.1% v/v), acidified water (water plus 5% formic acid), and water alone. The results showed that there were significant differences ($P < 0.05$) between blueberry genotypes for these measurements. Across all eight varieties, 'Elliott' and 'Burlington' had the highest concentrations of total anthocyanins (>2.2 mg/g frozen berries (FB)), chlorogenic acid (>1.0 mg/g FB), total phenolic content (>3.4 mg GAE/g FB), and antioxidant capacity as determined by the ferric reducing antioxidant power (FRAP) (>3.0 mg $FeSO_4$/g FB) and by 2,2, diphenyl-picrylhydrazyl (DPPH) assay (>65% inhibition), whereas 'Reka' appeared to show the lowest values (total anthocyanin 0.8 mg/g FB, total phenolics 2.7 mg GAE/g FB, FRAP 2.6 mg $FeSO_4$/g FB, and DPPH 42% inhibition), except for chlorogenic acid, for which 'Duke' contained the lowest concentration (0.5 mg/g FB). Solvents used for the extraction had a significant effect ($P < 0.05$) on total phenolics and antioxidant activities, with mixed solvent extracts showing the highest total phenolic content and antioxidant capacity, followed by acidified water, and water alone. Significant correlations ($P < 0.01$) were also observed between total phenolic content and antioxidant capacities across all genotypes suggesting that the phenolic compounds are likely to be the major contributors to the antioxidant activity in blueberries. The results obtained from this study serve as fundamental

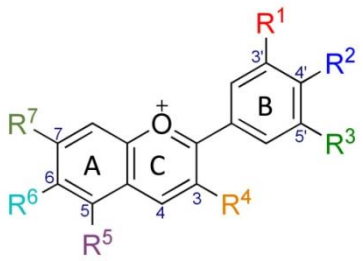
information of predominant bioactive compounds in selected Highbush blueberries grown in New Zealand for use in further studies.

Introduction :

A number of experimental and epidemiological studies have provided good evidence that consumption of blueberries may reduce the risk of various oxidative-related diseases such as cancer (Yi et al., 2005), cardiovascular disease (Basu et al., 2010), diabetes (Stull et al., 2010), and hypertension (Cassidy et al., 2011). These beneficial effects have been attributed to the relatively high polyphenolic concentrations of blueberries, which act as antioxidants by scavenging reactive oxygen and nitrogen species, as well as chelating redox-active transition metal ions (Aruoma, 1999). Due to their significant antioxidant capacities, which may contribute importantly to a variety of health benefits, blueberry polyphenols have been of great interest to scientists as well as consumers until now.

The Highbush blueberry, like other *Vaccinium* species, is enriched in polyphenols, notably anthocyanins, procyanidins, and hydroxybenzoic acids, most prominently chlorogenic acid. In particular, blueberries are known for their complex anthocyanin pattern, which is the most varied composition of anthocyanins compared to other fruits (Lohachoompol, 2008). There are fifteen major anthocyanins found in Highbush blueberries consisting of 3-monogalactosides, 3-monoglucosides, and 3-monoarabinosides of cyanidin, delphinidin, malvidin, peonidin, and petunidin (Figure 3.1). Furthermore, some varieties of Highbush blueberries have been identified as containing substantial amounts of acylated anthocyanins (Goa & Mazza, 1994).

High performance liquid chromatography (HPLC) is a common technique used to quantify anthocyanins in foods including berries. Substantial literature also suggests that methanol, ethanol or acetone in acid conditions are the most efficient solvents for extraction for anthocyanin analysis because these solvents provide high extract yields, consequently delivering extracts with high antioxidant activity (Garcia-Viguera et al., 1998; Naczk & Shahidi, 2004; Dai & Mumper, 2010). Therefore, such solvents have been widely used in earlier studies involving determination of anthocyanins and antioxidant activity (Wang et al., 2000; Connor et al., 2002a; Lohachoompol et al., 2008).



	R_1	R_2	R_3
Cyanidin	OH	OH	H
Delphinidin	OH	OH	OH
Malvidin	OCH_3	OH	OCH3
Peonidin	OCH_3	OH	H
Petunidin	OCH_3	OH	OH

Figure 3.1: Structure of common anthocyanins in blueberries (Wang et al., 2000)

Although blueberries have been widely cultivated in New Zealand, published research on New Zealand blueberries regarding determination of their beneficial bioactive compounds is relatively limited (Scalzo et al., 2009; Birt, 2011 Scalzo et al., 2013). In particular, little attention has been paid to the chlorogenic acid concentration in blueberries. Furthermore, it has been well documented that various factors can influence the polyphenolic contents and antioxidant activity of blueberries, including genotypes, environmental variation, stage of fruit maturity, and season of harvesting (Connor et al., 2002b; Kalt et al., 2003). Therefore, it was necessary to determine the actual phenolic concentrations in the Highbush blueberries being considered for use in this research. Furthermore, these values can also vary greatly depending on the solvent used for the extraction (Sulaiman et al., 2011; Wijekoon et al., 2011). Although solvents like acidified methanol or acetone are recommended, these toxic solvents are not appropriate in creating extracts intended to be fed to animals or humans. For this reason, eight genotypes of NZ Highbush blueberry in the present study were mainly extracted using water in order to more closely mimic animal/human consumption. Subsequently, anthocyanin profiles, chlorogenic acid concentrations, total phenolic content and antioxidant activity of the blueberries were then identified. Furthermore, the efficacy of different solvent extraction (pure water/ acidified water/ mixed solvent) on total phenolic content and their antioxidant capacity was also compared.

Materials and Methods :

Chemicals and standards

Cyanidin-3-glucoside chloride, chlorogenic acid, gallic acid, and ferrous sulphate ($FeSO_4$). Folin-Ciocalteu's phenol reagent, sodium carbonate (Na_2CO_3), 2,4,6-tripyridyl-s-triazine (TPTZ), ferric chloride ($FeCl_3$), sodium acetate, 2,2'-diphenyl-1-picrylhydrazyl (DPPH), and hydrochloric acid were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). HPLC-grade methanol and formic acid were purchased from Thermo Fisher Scientific (Palmerston North, New Zealand). Ultrapure water obtained from a Milli-Q system (Millipore, Milford, MA) was used for the preparation of the reagents and blueberry extracts.

Raw materials

Highbush blueberries (*Vaccinium corymbosum*) of the eight varieties 'Bluecrop', 'Brigitta', 'Burlington', 'Dixi', 'Duke', 'Elliott', 'Jersey' and 'Reka', were the generous gift of Mamaku Blue blueberry orchard, Rotorua, New Zealand. All samples were cultivated in the same environmental growing condition, harvested at commercial maturity and stored at -20^{o}C until used.

Anthocyanin and Chlorogenic acid analysis

The anthocyanin and chlorogenic acid components were analyzed by high performance liquid chromatography (HPLC) following the method of Wang et al. (2000) with some modifications. Briefly, all cultivars were prepared by grinding 50 g of frozen blueberries in a food processor (Breville, MiniWizz Processor WT400, Australia). 15 g of each well-blended sample was mixed with 30 ml of 5% formic acid. Then, the mixture was homogenized by vortexing for 3 min, and centrifuged at 10,000 rpm (Allegra TM 64 R Centrifuge, Beckman Coulter, CA, USA) for 10 min. The supernatant was collected and then filtered under vacuum with Buchner funnel lined with two sheets of Whatman No.4 filter paper. After that, the residue was rinsed with 5% formic acid until the filtrate was made up to 50 ml. Approximately 1 ml of the extract was then filtered again through a 0.2 µm PVDF filter and transferred into HPLC vials. The HPLC system consisted of a Shimadzu HPLC CTO-20A (Shimadzu Corp., Kyoto, Japan) coupled with an auto-sampler (SIL-20AC) and a photo-diode array (PDA) detector SPD-M20A. Anthocyanins were separated on a Phenomenex Luna C18 (2) 150 × 4.6 mm (5 µm) reverse phase column (Phenomenex, North Shore City, NZ). The solvents used were 5% (v/v) aqueous formic acid (solvent A) and 100% HPLC grade methanol (solvent B). The flow rate was set at 1 ml/min, and the total running time was 70

min, with a linear gradient profile as follows: 0-1 min, 14% B; 1-10.24 min, 14-17% B; 10.24-35.28 min, 17-23% B; 35.28-64.59 min, 23-47% B; 64.59-66.59 min, 47-14% B. Quantification of anthocyanins was based on peak areas determined at 520 nm and compared to the absorbance of cyanidin 3-O-glucoside chloride, which was used as the standard. Quantification of chlorogenic acid was based on the peak area of a chlorogenic acid standard determined at 280 nm. For each cultivar, three replicate samples were analyzed. The berry pomace left over on the filter paper after the Buchner funnel filtration was then freeze-dried as a powder and kept at -20^{o}C until use for further analysis of total phenolics and total antioxidant activity. To determine the mean blueberry weight of each cultivar, 30 ripe berries were weighed individually.

Determination of total phenolic content and antioxidant activity

All eight Highbush blueberry cultivars as mentioned above were prepared either extracted with solvents or freeze dried as a powder.

For solvent extraction, three different solvents were used including 100% water, water plus 5% formic acid or mixed organic solvent (acetone: methanol: water: formic acid 40:40:20:0.1% v/v). Water-only extracts were prepared by grinding 50 g of frozen berries with 50 ml of Milli-Q water using a small food processor (Breville, MiniWizz Processor WT400, Australia). The mixture was centrifuged at 10,000 rpm for 10 min and the supernatant was then collected and used either for total phenolic content (TPC) or antioxidant activity analysis immediately or stored at -20^{o}C until analysed. Mixed solvent extracts were kindly provided by Ms. Natasha Birt and Dr. Giorgio Tibaldi (Massey University, Palmerston North, New Zealand), who analyzed the anthocyanin concentration in mixed solvent blueberry extracts using HPLC, while 5% formic acid blueberry extracts were prepared using the same procedure as for HPLC as mentioned above.

For freeze-dried samples, approximately 20 g of crushed whole berries or pomace were prefrozen on trays at -30^{o}C overnight, and then freeze-dried using a freeze-dryer model FD18LT (Cuddon Ltd., New Zealand). Briefly, freeze-dryer plates were cooled to -35^{o}C. Once loaded with samples, the plate temperature was maintained at -35^{o}C and the pressure at 2 mbar for 3 hours before increasing the temperature of the plates to 20^{o}C and holding at this condition until the product was dry. When analyzed, freeze-dried blueberry samples from both whole berries and berry pomace were dissolved in Milli-Q water and analyzed at different concentrations (2, 5, 10, 15 and 20 mg/mL for TPC and FRAP assays, and 1, 2, 5 and 8 mg/mL for DPPH analysis).

Total phenolic content (TPC)

Total phenolics were measured using a Folin-Ciocalteu procedure as used by Molan et al. (2008). Briefly, 12.5 μl of blueberry extract was added to 250 μl of 2% sodium carbonate solution (Na_2CO_3) in a 96-well microplate and allowed to react for 5 min at room temperature. Subsequently 12.5 μl of 50% Folin-Ciocalteu phenol reagent (diluted 1:1 in Milli-Q water) was added and incubated for 30 min at room temperature. Absorbance of blueberry extracts and a matching blank were spectrophotometrically measured at 650 nm using a microplate reader (ELx808 BioTek Instruments Inc, USA). A standard curve was prepared using different concentrations (0-1000 μg/mL) of aqueous gallic acid solution. Total phenolics were calculated as mg gallic acid equivalent (GAE) per g of sample based on a standard curve.

Ferric Reducing Antioxidant Power (FRAP) assay

The FRAP assay was carried out according to the previous method of Benzie & Strain (1996) and Molan et al. (2009) with some modification. In brief, FRAP reagent consisted of 300 mmol/L acetate buffer (pH 3.6), 10 mmol/L TPTZ (2,4,6-tripyridyl-s-triazine) in 40 mmol/L hydrochloric acid, and 20 mmol/L ferric chloride (III) solution at the ratio of 10:1:1 (v/v/v), respectively. The sample extract (8.5 μl) was added to 275 μl of 50% FRAP reagent (diluted 1:1 in Milli-Q water) in a 96-well microplate and incubated at 37^{o}C for 30 min, then the absorbance was measured spectrophotometrically at 595 nm using a microplate reader (ELx808 BioTek Instruments Inc, USA). Readings from blank wells containing matching concentrations of blueberry extract in Milli-Q water were subtracted from test well readings to correct background absorbance. A standard curve was prepared using various concentrations (0-6000 μmol/L) of $FeSO_4 \cdot 7H_2O$. The antioxidant activity was calculated based on the ability of extracts to reduce ferric (III) iron to ferrous (II) iron, and results were expressed as mg ferrous ion (II) equivalent per g of sample.

Scavenging of diphenyl-picrylhydrazyl (DPPH)-radicals

The ability of the blueberry extracts to scavenge DPPH-radicals was determined using the earlier method of Van Amsterdam et al. (1992) with slight modifications (Molan et al., 2009). Briefly, 25 μl of blueberry extracts were added to 250 μl of 0.2 mM DPPH in 95% ethanol in a 96-well microplate and incubated in the dark at room temperature for 30 min to allow the reaction to progress to completion before reading the absorbance at 550 nm using a microplate reader (ELx808 BioTek Instruments Inc, USA). Readings from blank wells containing matching

concentrations of blueberry extract in Milli-Q water alone were subtracted from test well readings to correct background absorbance. The absorbance reading was compared to the control, which contained 25 µl of water instead of the extract. The antiradical activity of samples was calculated as a percentage of DPPH decolouration relative to a control using the following equation:

$$\% \text{ inhibition} = \frac{(\text{absorbance of control} - \text{absorbance of sample extract})}{\text{absorbance of control}} \times 100$$

Statistical analysis

Analyses of anthocyanins and chlorogenic acid were performed in triplicate, whereas total phenolic content and antioxidant capacity were measured in 3 replicate wells in duplicate experiments. Data are expressed as mean ± S.E. Statistical analysis was performed either by one-way or two-way analysis of variance (ANOVA) where appropriate. Tukey's test was used for mean comparisons and the differences were considered statistically significant at $P < 0.05$. Linear regression analysis (with coefficient of determination, R^2) was performed to determine the correlation between two variables and principal component analysis (PCA) was performed to detect clustering of anthocyanin composition among the blueberry varieties. All statistical tests were analyzed using the SAS program for Windows version 9.2 (SAS Institute Inc., Cary, NC, USA).

Results and Discussion :

Anthocyanins and chlorogenic acid

The concentrations of total anthocyanins of eight acidified water blueberry extract cultivars are reported in Figure 3.2A. The results indicate that the concentrations of total anthocyanin varied substantially between the varieties. 'Burlington' and 'Elliott' exhibited the highest concentrations of total anthocyanins (2.4 mg/g and 2.2 mg/g frozen berries (FB), respectively) among the tested cultivars, whereas 'Reka' showed the lowest amount (approximately 0.8 mg/g FB). The concentrations of anthocyanins in 'Burlington' and 'Elliott' were significantly higher ($P < 0.05$) than the other six varieties; and there was no significant difference of total anthocyanins concentration between 'Reka', 'Brigitta' and 'Bluecrop'.

The total anthocyanin concentration of all blueberry cultivars reported here was lower than those reported by other researchers. For example, the concentrations reported for 'Bluecrop', a commercially available variety of Highbush species, were 0.9 mg/g FB (Prior et al., 1998), 1.0

mg/g FB (Goa & Mazza et al., 1994), 1.1 mg/g FB (Connor et al., 2002b), 1.4 mg/g FB (Cho et al., 2004) and 1.8 mg/g FB (Ehlenfeldt & Proir, 2001). The major difference is almost certainly due to the different solvent used for the extraction since acidified organic solvents like HCl or acetic acid in acetonitrile or methanol were used for preparation of sample extracts in earlier studies whereas acidified water was used in this research . As mentioned earlier, such organic solvents or their combination are more efficient for anthocyanin extraction because they can destroy cell membranes, concurrently dissolve and stabilize the anthocyanins, and allow an efficient extraction (Naczk & Shahid, 2004). Brambilla et al. (2008) carried out a comparison between organic solvent and acidified water extraction of some varieties of Highbush blueberry and reported that on average the anthocyanin concentration was 32% higher when extracted with organic solvents compared to an acidified water extraction. Other differences are possibly also due to growing conditions, berry maturities as well as different extraction conditions, making it difficult to compare the actual phytochemical values between studies.

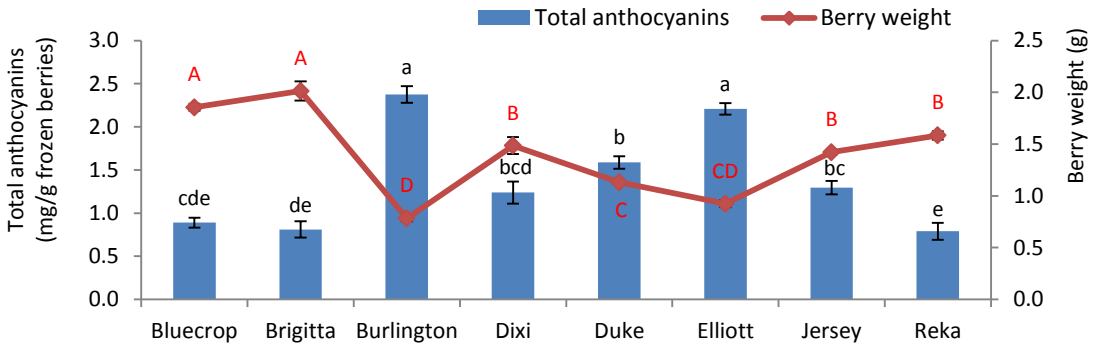


Figure 3.2A: Total anthocyanin concentrations and average berry weight of the eight Highbush blueberry varieties. Data expressed as mean ± S.E. of triplicate independent experiments (N = 3) for total anthocyanins, and ten replicates of the three independent sets (N = 30) for berry weight. Difference superscript letters indicate significant differences between cultivars ($P < 0.05$).

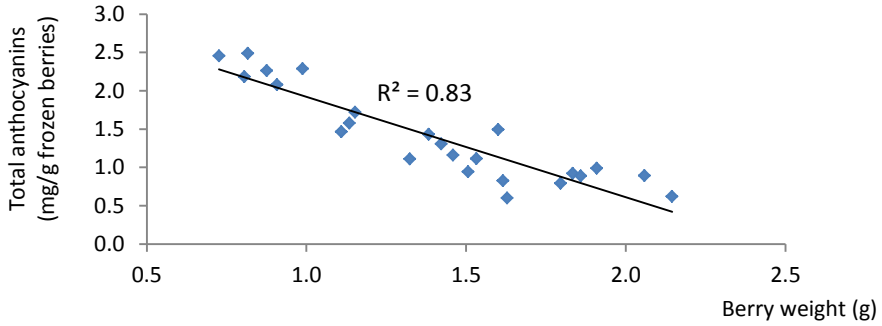


Figure 3.2B: Linear correlation between the total anthocyanin concentrations and berry weights in the eight water plus 5% formic acid blueberry extracts.

Regardless, the results presented here are in agreement with the study carried out by Ehlenfeldt & Prior (2001), the only study so far to report on all eight varieties studied here. 'Burlington' and 'Elliott' contained higher concentrations of total anthocyanins than 'Duke', which are higher than 'Bluecrop', while 'Reka' exhibited the lowest values among all eight cultivars tested. Notably, total anthocyanin concentration were found to be strongly inversely correlated with the size of berry (R^2 = 0.83, P < 0.01), with the small berries containing the highest concentrations of anthocyanis on a weight basis (Figure 3.2B). The relationship between these two variables was also observed in the previous studies (Prior et al., 1998; Moyer et al., 2002). Gao & Mazza, (1994) noted that the majority of anthocyanin pigments are primarily located in the skin and outer layer of the pulp, therefore the smallest berries contained the highest surface area to volume ratio, leading to the highest total anthocyanin concentrations.

The profiles of individual anthocyanins from eight different Highbush blueberry cultivars are shown in Figure 3.3. In the visible spectrum (520 nm), fifteen individual anthocyanin peaks were detected in the acidified water blueberry extract samples, corresponding to the glycosylated forms of the five anthocyanidins in their elution order: delphinidin, cyanidin, petunidin, peonidin and malvidin. Specifically, the individual anthocyanin compounds present in these Highbush blueberry varieties included: 1) Delphinidin 3-galactoside 2) Delphinidin 3-glucoside 3) Cyanidin 3-galactoside 4) Delphinidin 3-arabinoside 5) Cyanidin 3-glucoside 6) Petunidin 3-galactoside 7) Cyanidin 3-arabinoside 8) Petunidin 3-glucoside 9) Peonidin 3-galactoside 10) Petunidin 3-arabinoside 11) Peonidin 3-glucoside 12) Malvidin 3-galactoside 13) Peonidin 3- arabinoside 14) Malvidin 3-glucoside and 15) Malvidin 3-arabinoside.

The order of individual anthocyanin elution was deduced based on the retention time of the standard cyanidin-3-glucoside and according to earlier studies on various varieties of Highbush blueberries conducted by Brambilla et al in 2008. It has been commonly reported that the general elution order of glycosides derivatives of anthocyanin is galactoside, glucoside, and arabinoside, respectively; however, the order of the peaks between 11 and 14 reported in the literature is not consistent. Some authors reported that peonidin-3-glucoside (peak 11) coeluted with malvidin-3-galactoside (peak 12) (Lohachoompol et al., 2008; Prior et al., 2001), and peonidin-3-arabinoside (peak 13) coeluted with malvidin-3-glucoside (peak 14) (Lohachoompol et al., 2008). Whilst sometimes, peonidin-3-glucoside and peonidin-3-arabinoside were reported to be eluted before malvidin-3-galactoside and malvidin-3-glucoside respectively (Latti et al., 2009; Brambilla et al., 2008), and sometimes eluting after (Cho et al., 2004).

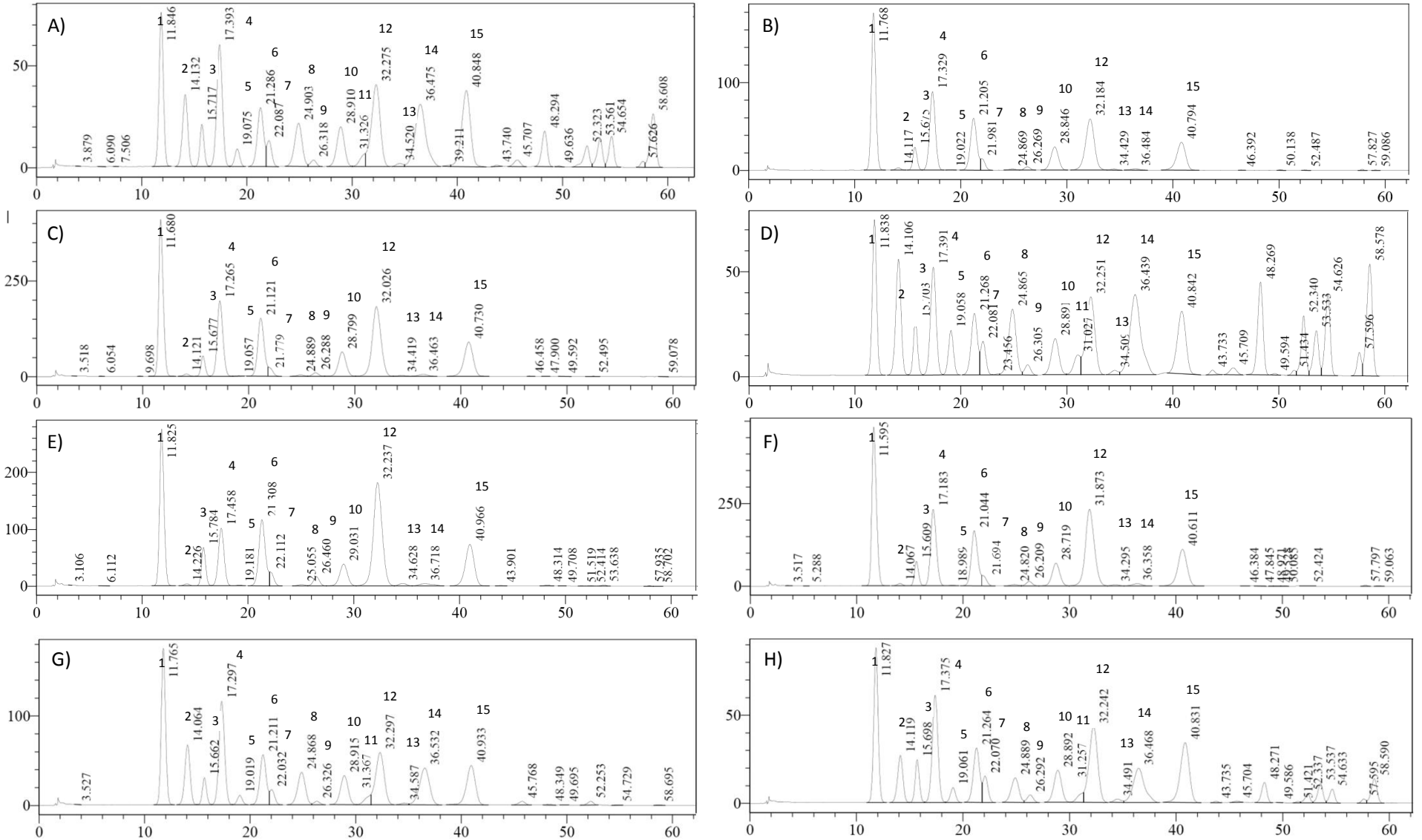


Figure 3.3: Representative chromatograms of eight cultivars of blueberries (water plus 5% formic acid extract) at 520 nm; A) Bluecrop B) Brigitta C) Burlington D) Dixi E) Duke F) Elliott G) Jersey and H) Reka.

Table 3.1: Individual anthocyanin components of the eight Highbush blueberry cultivars extracted with acidified water

Peak no.	Compound	Bluecrop		Brigitta		Burlington		Dixi		Duke		Elliott		Jersey		Reka	
		µg/g	%	µg/g	%	µg/g	%	µg/g	%	µg/g	%	µg/g	%	µg/g	%	µg/g	%
1	Delphinidin-3-galactoside	117	13.2	225	27.8	622	26.2	104	8.4	339	21.4	538	24.4	238	18.4	115	14.5
2	Delphinidin-3-glucoside	71	8.1	3	0.4	10	0.4	96	7.7	4	0.3	9	0.4	111	8.6	44	5.5
3	Cyanidin-3-galactoside	24	2.7	29	3.6	64	2.7	31	2.5	77	4.9	76	3.4	37	2.8	27	3.4
4	Delphinidin-3-arabinoside	125	14.1	148	18.3	386	16.3	96	7.7	171	10.8	333	15.1	207	15.9	113	14.2
5	Cyanidin-3-glucoside	12	1.4	1	0.1	2	0.1	29	2.4	2	0.1	3	0.1	15	1.2	12	1.5
6	Petunidin-3-galactoside	53	5.9	109	13.5	304	12.8	59	4.7	204	12.9	262	11.9	104	8.1	59	7.4
7	Cyanidin-3-arabinoside	16	1.9	13	1.7	34	1.4	21	1.7	31	1.9	38	1.7	24	1.9	20	2.5
8	Petunidin-3-glucoside	47	5.3	2	0.3	8	0.3	71	5.7	4	0.2	7	0.3	81	6.3	31	4.0
9	Peonidin-3-galactoside	4	0.5	5	0.7	16	0.7	8	0.7	25	1.6	19	0.9	6	0.4	7	0.9
10	Petunidin-3-arabinoside	42	4.7	56	6.9	155	6.5	40	3.2	85	5.4	129	5.8	73	5.7	43	5.4
11	Peonidin-3-glucoside	9	1.1	0	0	0	0	16	1.3	0	0	0	0	15	1.2	8	1.0
12	Malvidin-3-galactoside	81	9.1	135	16.7	490	20.6	106	8.5	420	26.4	502	22.7	138	10.7	98	12.5
13	Peonidin-3-arabinoside	2	0.2	2	0.2	5	0.2	3	0.3	9	0.6	7	0.3	3	0.3	4	0.5
14	Malvidin-3-glucoside	83	9.3	3	0.4	13	0.6	130	10.5	12	0.7	16	0.7	109	8.4	57	7.3
15	Malvidin-3-arabinoside	75	8.4	70	8.6	243	10.2	84	6.8	185	11.7	250	11.3	108	8.3	83	10.6
	Total non-acylated anthocyanin (µg/g)	**761**	**85.7**	**803**	**99.0**	**2351**	**99.0**	**894**	**71.8**	**1568**	**98.8**	**2189**	**99.2**	**1270**	**98.0**	**720**	**91.2**
	Total acylated anthocyanin (µg/g)	**128**	**14.3**	**8**	**1.0**	**23**	**1.0**	**353**	**28.2**	**18**	**1.2**	**19**	**0.8**	**26**	**2.0**	**69**	**8.8**

Data expressed as mean of three replicate samples; Peak numbers refer to HPLC chromatogram in figure 3.3; % = percentage of total anthocyanins

Malvidin-3-galactoside and malvidin-3-glucoside were reported to be present at high concentrations in blueberries and always at much higher concentrations than peonidin-3-glucoside and peonidin-3-arabinoside, making us quite confident where the malvidin peaks are. However, the order of the other two little peaks, peonidin-3-glucoside and peonidin-3-arabinoside, cannot be completely finalised and using mass spectroscopy in conjunction with HPLC would be better for accurate identification of these two compounds. However, we did not attempt precise identification by any other technique such as mass spectroscopy or nuclear magnetic resonance.

Although the concentration of total anthocyanins was relatively low when compared to other studies, the pattern of individual anthocyanins was consistent with the thirteen to fifteen commonly reported in previous research using other organic solvents (Ogawa et al., 2008; Borges et al., 2010). Delphinidin, malvidin, and petunidin appeared to be the major contributors to the total anthocyanin concentration of Highbush blueberries, which was in keeping with the finding of Lohachoompol et al., (2008) who analyzed various cultivars of Highbush blueberries in Australia. As shown in Table 3.1, the percentage distribution of monomeric anthocyanins in the eight Highbush blueberry genotypes was delphinidin (24 to 47%), malvidin (26 to 39%), petunidin (14 to 21%), cyanidin (4 to 7%), and peonidin (0.9 to 2.3%). A large variation was also found in the percentage distribution of anthocyanin glycosidic function, with the percentage distribution of galactoside derivatives accounting for 25 to 67%, arabinosides 20 to 36%, and glucosides 1.2 to 28%. Additionally, some cultivars also contained considerable amounts of acylated anthocyanins, the compound which eluted after the major anthocyanins. Three out of eight cultivars observed here including ‘Dixi’, ‘Bluecrop’ and ‘Reka’ exhibited significant concentrations of acylated anthocyanins at approximately 28%, 14% and 9% of total anthocyanins, respectively.

Further analysis using principal component analysis (PCA) was undertaken to identify possible patterns in the anthocyanins composition of the eight blueberry varieties. The PCA was performed on the values of the 15 individual anthocyanins (refer to Table 3.1) for all eight varieties. The score plot for the first two components shown in Figure 3.4 indicated the varietal difference. With respect to principal component 1, the eight blueberry varieties mainly fell into two groups. ‘Burlington’, ‘Elliott’ and ‘Duke’ were on the right hand side of PC1 axis, whereas the other five cultivars including ‘Bluecrop’, ‘Dixi’, ‘Jersey’, ‘Brigitta’ and ‘Reka’ were on the left hand sides of PC1 axis.

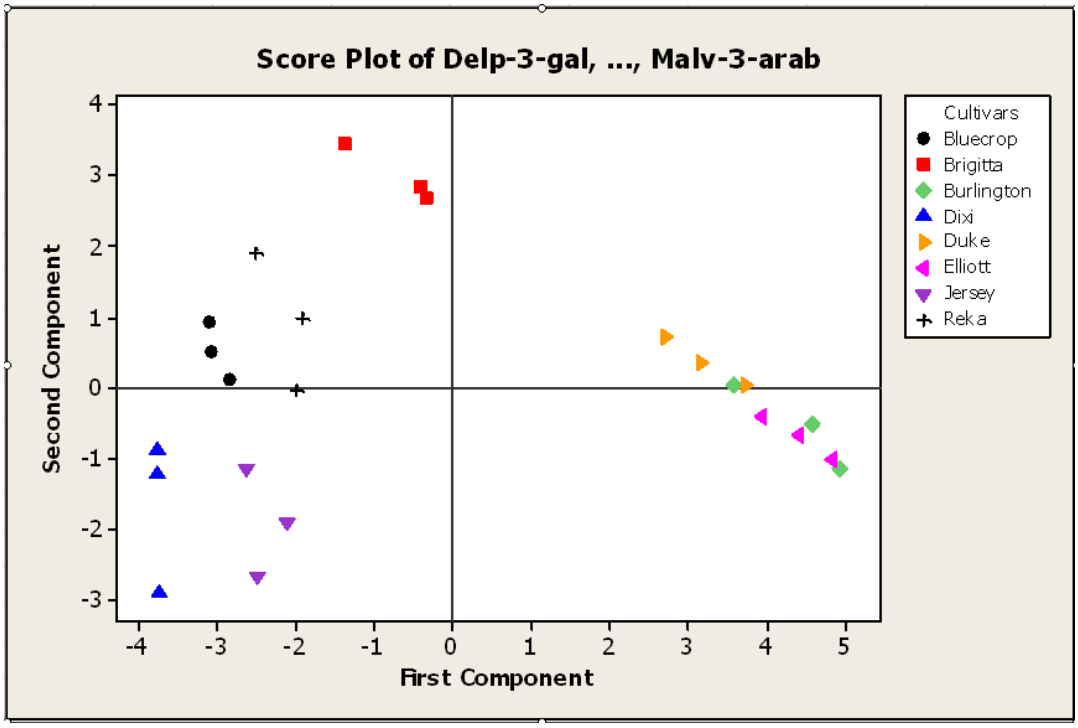


Figure 3.4: Principal component analysis of the anthocyanin composition of the eight Highbush blueberry varieties

A loading plot was then generated to reveal the relationships between variables in the space of the first components, and used to determine which individual anthocyanin component was the major driving force for principal component 1. From the plot (Figure 3.5), it appeared that the main contributor to the differences observed here was the concentration of anthocyanins with the same types of sugar molecules. The left hand side of the PCA score plot contained considerable amounts of the anthocyanidins linked with glucose, as compared with those on the right hand side, which were linked with galactose and arabinose. This result indicated that cultivars 'Burlington', 'Elliott' and 'Duke' contained primarily galactoside and arabinoside derivatives of the anthocyanins, whereas 'Bluecrop', 'Dixi', 'Jersey' and 'Reka' were high in the glucoside derivatives of the anthocyanins. Similar results were also found in other studies (Cho et al., 2004; Brambilla et al., 2008) and the authors suggested that this phenomenon could be related to the specific enzymatic background (glycosyl transferase) that each variety has, which was influenced by common ancestors.

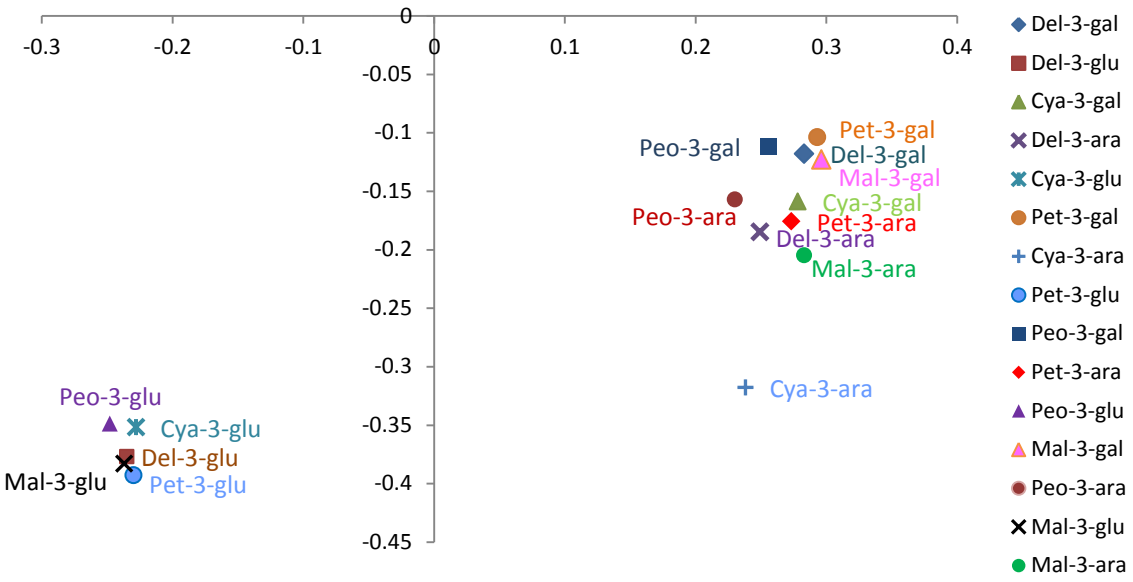


Figure 3.5: Scatter plot for principal components 1 and 2 showing the contribution of each anthocyanin to the observed PCA pattern

In addition to the anthocyanins, we examined the concentration of chlorogenic acid, another polyphenolic compound present at a significant level in blueberries. Similarly to the results of total anthocyanins, 'Elliott' and 'Burlington' had the highest concentrations, with approximately 1.1-1.3 mg/g frozen berries (FB). Conversely, cultivar 'Duke', which had the third highest anthocyanin concentrations, exhibited the lowest concentration of chlorogenic acid (0.5 mg/g FB). 'Bluecrop', which had one of the lowest total anthocyanin concentrations, showed significant chlorogenic acid concentrations (0.9 mg/g FB), which is the third highest value when compared to the other cultivars (Figure 3.6). A number of earlier studies reported a wide range variation in chlorogenic acid concentrations, even within the same variety (Rodriguez-Mateos et al., 2012). However, the values presented here appeared to be comparable or sometimes slightly higher compared to studies using solvent extraction. For example, the concentration of chlorogenic acid reported by Rodriguez-Mateos et al. (2012) for 'Bluecrop' and 'Brigitta' was approximately 0.4 mg/g FB. Other studies reported 'Bluecrop' ranging from 0.3 to 1.0 mg/g FB (Goa & Mazza, 1994; Skrede et al., 2000; Cho et al., 2004; Gavrilova et al., 2011) and for other varieties of Highbush blueberries, 0.3 mg/g FB for 'Duke' cultivar (Gavrilova et al., 2011) and 1.0 mg/g FB for 'Jersey' variety (Taruscio et al., 2004). One possible explanation is that chlorogenic acid has been reported to be more heat sensitive than anthocyanins, and solvent extraction

sometimes requires heat to evaporate such solvents before the extract is injected into the HPLC, whereas the water extraction method employed here did not.

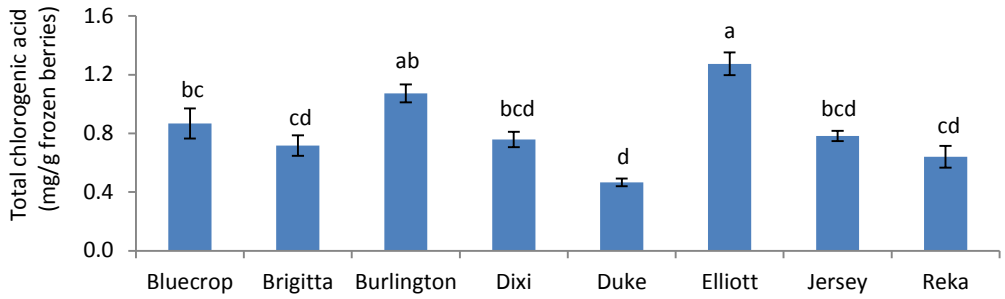


Figure 3.6: Chlorogenic acid concentration of the eight Highbush blueberry varieties (water extracts plus 5% formic acid). Data expressed as mean ± S.E. of triplicate independent experiments (N = 3). Difference superscript letters indicate significant differences between cultivars ($P < 0.05$).

Total phenolic content and antioxidant activity

For the total phenolic content (TPC), each frozen blueberry variety was extracted using three different solvents: mixed solvent (acetone: methanol: water: formic acid, 40:40:20:0.1% v/v), acidified water (5% formic acid) and pure water without acid. The results are shown in Figure 3.7. Across all cultivars, using different solvents caused variation in the TPC, with mixed solvent extracts showing the highest TPC (3.7-4.3 mg GAE/g frozen berries (FB)), followed by acidified water extracts (2.6-3.9 mg/g FB), while water-only extracts showed the lowest results (1.4-2.2 mg/g FB). It should be noted that the presence of acid in the solvent seems to have a significant influence on the extraction of TPC, since adding 5% formic acid gave a significantly higher TPC than using water alone. This is consistent with earlier studies, which have reported that anthocyanins are stable under acidic conditions but are unstable and rapidly break down under neutral conditions (Takikawa et al., 2010). For this reason, acids are usually used in the extraction of samples involving anthocyanins (Ehlenfeldt & Prior, 2001; Brambilla et al., 2008; Ogawa et al., 2008; Borges et al., 2010).

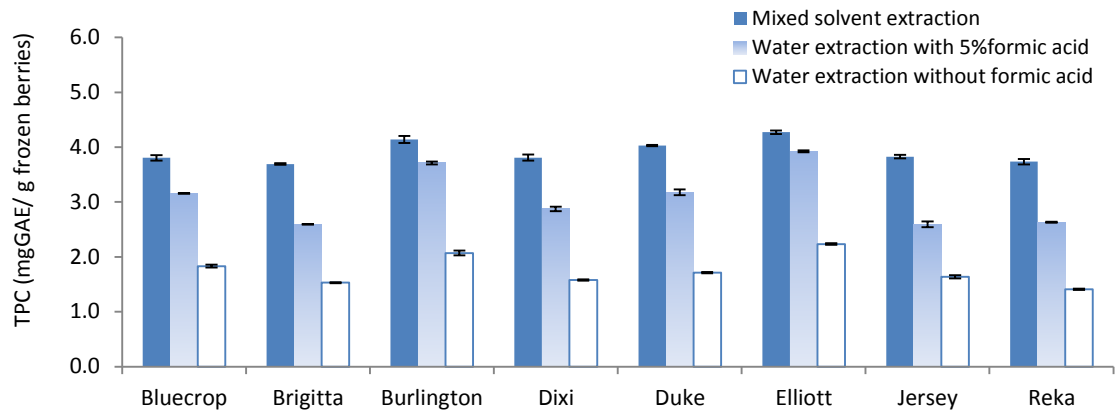


Figure 3.7: Total polyphenolic content (TPC; mg GAE/g frozen berries) in blueberry extracts with mixed solvents (acetone: methanol: water: formic acid, 40:40:20:0.1% v/v), and water with 5% formic acid and water alone. Results are presented as mean ± S.E. of three replicates of the two independent experiments (N = 6).

Substantial differences in the TPC were also seen between the varieties. Across all extractions, 'Elliott' and 'Burlington' exhibited the highest TPC (approximately 3.5 mg GAE/g FB), compared with 'Brigitta' and 'Reka', which contained the lowest values (approximately 2.7 mg GAE/g FB). This finding is consistent with the measurements made by HPLC, which also showed that 'Elliott' and 'Burlington' were among the top two highest concentrations, and 'Brigitta' and 'Reka' were on the low end. Particularly for 'Bluecrop', the TPC of mixed solvent extracts obtained in this study was comparable to those obtained by Howard et al. (2003) (approximately 3.1 mg GAE/g FB), but slightly higher than Prior et al. (1998) (1.9 mg GAE/g FB). Also, our TPC results appeared to be much higher than the value reported by Ehlenfeldt & Prior et al. (2001) (0.5 - 1.8 mg GAE/g FB). As mentioned earlier, several factors, such as different extraction solvents and conditions, berry maturity and agronomic conditions might also be behind these differences. However, our studies are consistent with those of Ehlenfeldt & Prior et al. (2001), in which 'Elliott' exhibited the highest TPC among all eight varieties, followed by 'Burlington' cultivar.

The antioxidant activity was also determined using two commonly used methods: ferric reducing antioxidant power (FRAP), and scavenging of diphenyl-picrylhydrazyl (DPPH)-radicals. The FRAP measures the ability of the extract to reduce Fe (III) iron to Fe (II) iron by donating an electron to Fe (III). The higher the FRAP value, the greater is the antioxidant activity. Another method, DPPH examines the ability of the extracts to donate hydrogen to the DPPH radical, resulting in bleaching of the purple colour of the DPPH solution to a bright yellow. The greater the bleaching action, the greater the hydrogen-donating ability and thus the higher is the radical scavenging activity of the extracts. As shown in Table 3.2, 'Elliott' and 'Burlington' cultivars again possessed the highest reducing ability across all extractions as measured by FRAP (3.1 mg $FeSO_4$/g FB), whereas 'Dixi' and 'Jersey' showed the lowest values (2.5 mg $FeSO_4$/g FB). In the DPPH assay, a single dose of the extracts which was equivalent to 5 mg frozen berries was evaluated. The ability of the extracts to scavenge the DPPH radical was consistent with the activities obtained with FRAP, as 'Elliott' and 'Burlington' exhibited the highest scavenging activity toward DPPH-radical (75% and 66% inhibition, respectively), and 'Jersey' had the lowest inhibitory effects (40% inhibition).

Table 3.2: Least squares means for the effect of different cultivars and extraction methods on the total phenolic content (TPC) and antioxidant activity (FRAP and DPPH assay)

		TPC (mg GAE/g frozen berries)	FRAP (mg $FeSO_4$/g frozen berries)	DPPH (% inhibition)
Cultivar	Bluecrop	3.0^{c}	2.8^{b}	51.8^{c}
	Brigitta	2.7^{e}	2.8^{b}	43.0^{de}
	Burlington	3.4^{b}	3.1^{a}	65.5^{b}
	Dixi	2.9^{d}	2.5^{d}	44.3^{d}
	Duke	3.1^{c}	2.9^{b}	53.1^{c}
	Elliott	3.6^{a}	3.1^{a}	74.6^{a}
	Jersey	2.8^{de}	2.5^{d}	40.4^{e}
	Reka	2.7^{e}	2.6^{c}	42.1^{de}
Extraction	Mixed solvent	3.9^{a}	3.3^{a}	69.4^{a}
	5% formic acid	3.1^{b}	3.2^{b}	46.3^{b}
	Water	1.8^{c}	1.9^{c}	35.9^{c}
P value	Cultivar	<0.01	<0.01	<0.01
	Extract	<0.01	<0.01	<0.01
	Cultivar*Extract	<0.01	<0.01	<0.01

Different superscript letters within the same column indicate significant differences ($P < 0.05$).

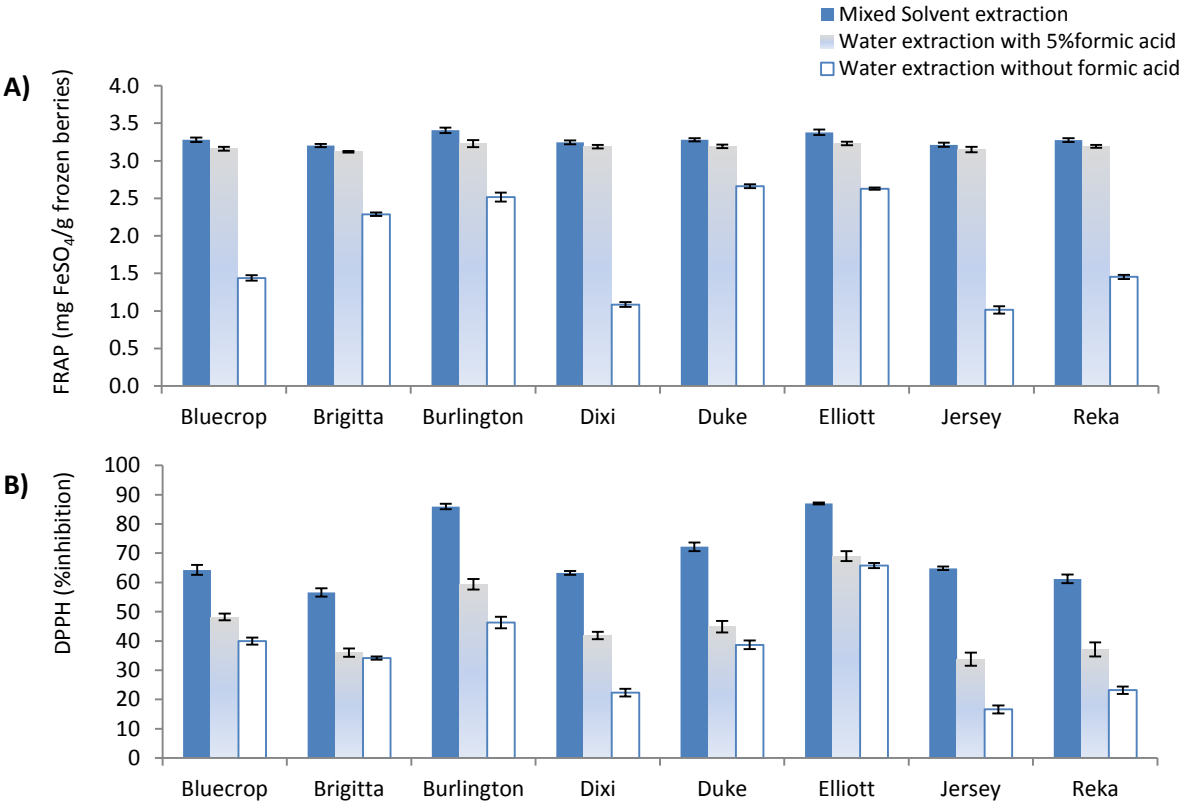


Figure 3.8: Ferric reducing antioxidant power (FRAP; mg $FeSO_4$/g frozen berries) (A), and scavenging of diphenyl-picrylhydrazyl (DPPH)-radical; (% inhibition at 5 mg frozen berries) (B) in blueberry extracts with mixed solvents (acetone: methanol: water: formic acid, 40:40:20:0.1% v/v), water with 5% formic acid and water alone. Results are presented as mean ± S.E. of three replicates of two independent experiments (N = 6).

Solvents used for the extraction also played an important role in antioxidant activity; mixed solvent extracts showed the highest antioxidant capacity in both FRAP and DPPH assays, followed by water extracts plus 5% formic acid, and water only (Figure 3.8). The FRAP values of all varieties in mixed solvent extracts ranged from 3.2 to 3.4 mg $FeSO_4$/g frozen berries, while these values were between 3.1 and 3.2 mg/g in acidified water and ranged from 1.0 to 2.7 mg/g in water only extracts. Across all cultivars, mixed solvent extracts had the highest ability to scavenge DPPH-radical (between 55 and 85% inhibition), whereas water plus 5% formic acid and water only extracts exhibited inhibition in the range of 35% to 70%, and 15% to 65%, respectively. Another possibility for the extraction difference may be related to the fact that in more polar solvents, hydrogen bonding can induce dramatic changes in the H-atom donor activities of phenolic antioxidants which may reduce the antioxidant capacity of phenolics (Pinelo et al., 2004).

When using a two-way ANOVA to examine the effect of cultivars and extraction method as well as their interaction on the TPC and antioxidant activity (Table 3.2), all these values (TPC, FRAP and DPPH) were significantly affected by different cultivars of blueberries (F_{value} = 252, 163, 205, respectively, $P < 0.01$), extraction method (F_{value} = 6664, 5179, 1035, respectively, $P < 0.01$), and the interaction effects of cultivars*extraction (F_{value} = 25, 117, 12, respectively, $P < 0.01$). Furthermore the result also confirmed that across all extractions, cultivar 'Elliott' and 'Burlington' were among the top two highest values. Notably the cultivar 'Bluecrop' which contained a low amount of total anthocyanins exhibited the top four antioxidant activities (both FRAP and DPPH) because this cultivar also contained a considerable amount of chlorogenic acid and TPC. Also, 'Jersey' contained a moderate amount of total anthocyanins but exhibited the lowest antioxidant ability. This result indicated that not only the total anthocyanins but all polyphenols present in blueberries contribute to their antioxidant activities.

In freeze-dried samples, the TPC of all varieties ranged approximately from 4.3 mg to 8.2 mg per gram frozen berries (Figure 3.9A). These values indicate that when compared based on wet weight basis (per gram frozen berries), freeze-dried whole berries tended to give a higher TPC than when frozen berries were extracted with various solvents. This may be because freeze-drying has been recognized to cause the least reduction in several nutrients including total polyphenol content, and grinding tissue into small-sized particles increases solubility and hence may allow higher extraction. Similarly, the antioxidant activity of blueberry powder was significantly ($P < 0.01$) higher than those extracted using mixed solvents-the best reagents for phenolic extraction-confirm that freeze-drying increased the ability to extract polyphenols, both in terms of their quantity and their antioxidant activity. In this study, the eight freeze-dried Highbush blueberries varieties had the reducing ability, which was measured by FRAP, ranging approximately from 12.6 mg to 22.5 mg $FeSO_4$/g frozen berries and exhibited the ability to scavenge DPPH-radical between 40 and 65 % inhibition at a concentration of 5 mg/mL (Figure 3.9B and C). Again, freeze-dried 'Elliott' and 'Burlington' also exhibited the highest TPC and antioxidant ability, while 'Brigitta', 'Jersey' and 'Reka' were on the lowest side, which appeared to be consistent with the results obtained from the frozen berries.

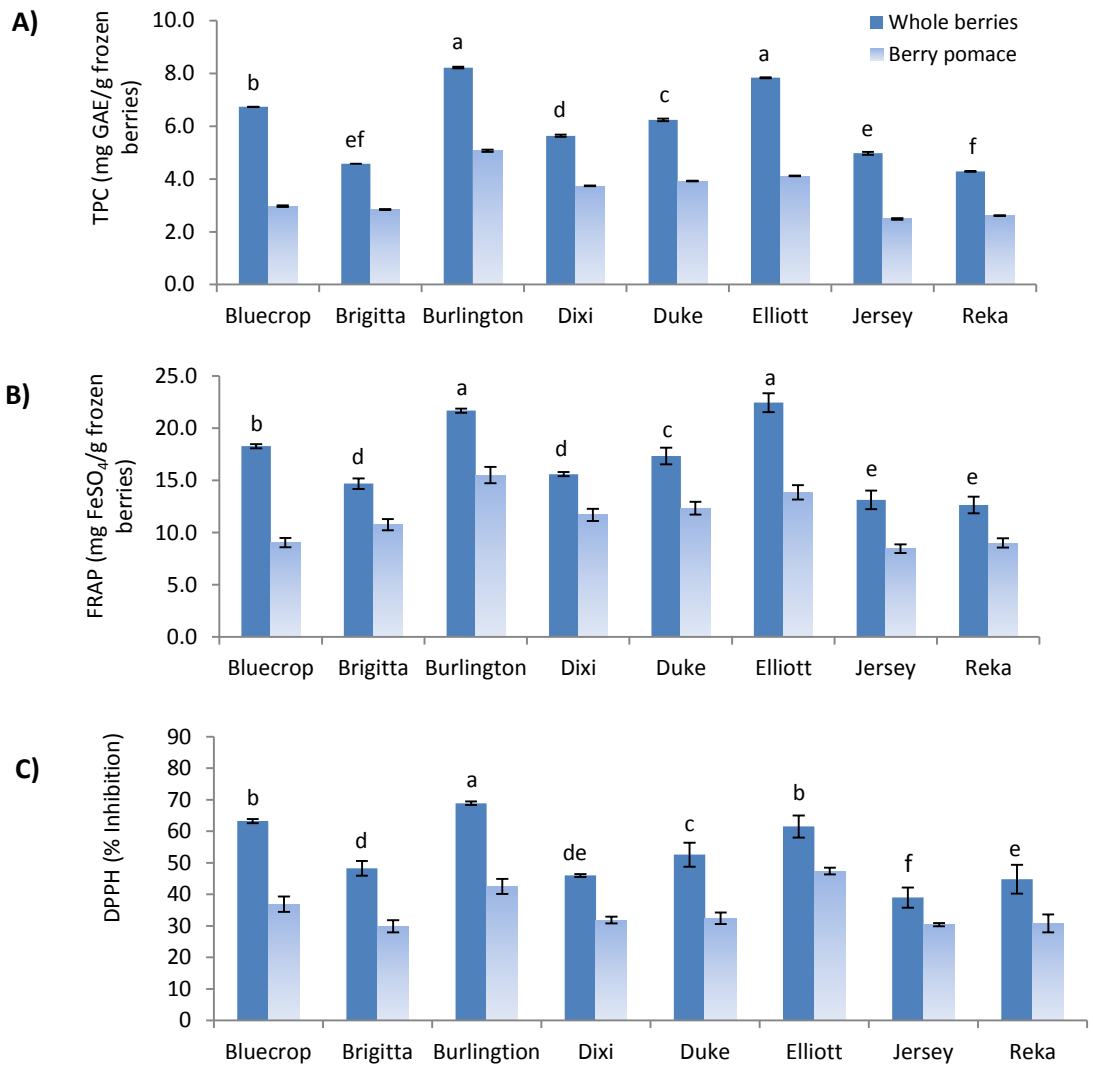


Figure 3.9: Total polyphenolic content (TPC; mg GAE/g frozen berries) (A), ferric reducing antioxidant power (FRAP; mg $FeSO_4$/g frozen berries) (B), and scavenging of diphenyl-picrylhydrazyl (DPPH)-radical; (% inhibition at a concentration of 5 mg/mL) (C) in freeze-dried whole blueberries and berry pomace. Results are presented as mean ± S.E. of three replicates of two independent experiments (N = 6), and a different superscript letter on freeze-dried whole berry samples indicates a statistically significant difference ($P < 0.05$) between blueberry varieties.

In addition, data obtained from the present study indicated the freeze-dried berry pomace, which was left over from the anthocyanin analysis by HPLC, was still coloured and contained moderate amounts of the phenolic compounds and antioxidant activities. Across all cultivars, per gram frozen berries, the TPC of berry pomace ranged from 2.4 to 5.0 mg/g pomace, which was approximately 40% to 60% of the TPC present in whole frozen berries. The pomace also exhibited reducing ability in the range of 8.5 to 15.5 mg $FeSO_4$/g frozen berries and between 30

and 47% scavenging inhibitory ability, which account for approximately 40% to 75% of the total antioxidant capacity presented in whole berries (Figure 3.9). The considerable TPC and antioxidant values found in berry pomace confirmed that extraction using 5% formic acid seemed not efficient enough to extract all TPC/anthocyanins from crude blueberries; consequently, data presented here which are based on acidified water extraction, may not reflect the maximum TPC/anthocyanin concentration as well as antioxidant capacity and were approximately half of the maximum value of each variety.

It is clear that solvent extraction can generate much large concentrations of total phenolics and antioxidant activity in the extracts and provide data on the total concentrations of bioactive compounds, but this is not relevant to the "normal" pattern of dietary intakes. By contrast, the information obtained from acidified water and water extraction may be more likely than those from organic solvents to represent what may be made available to animals in the later *in vivo* trials.

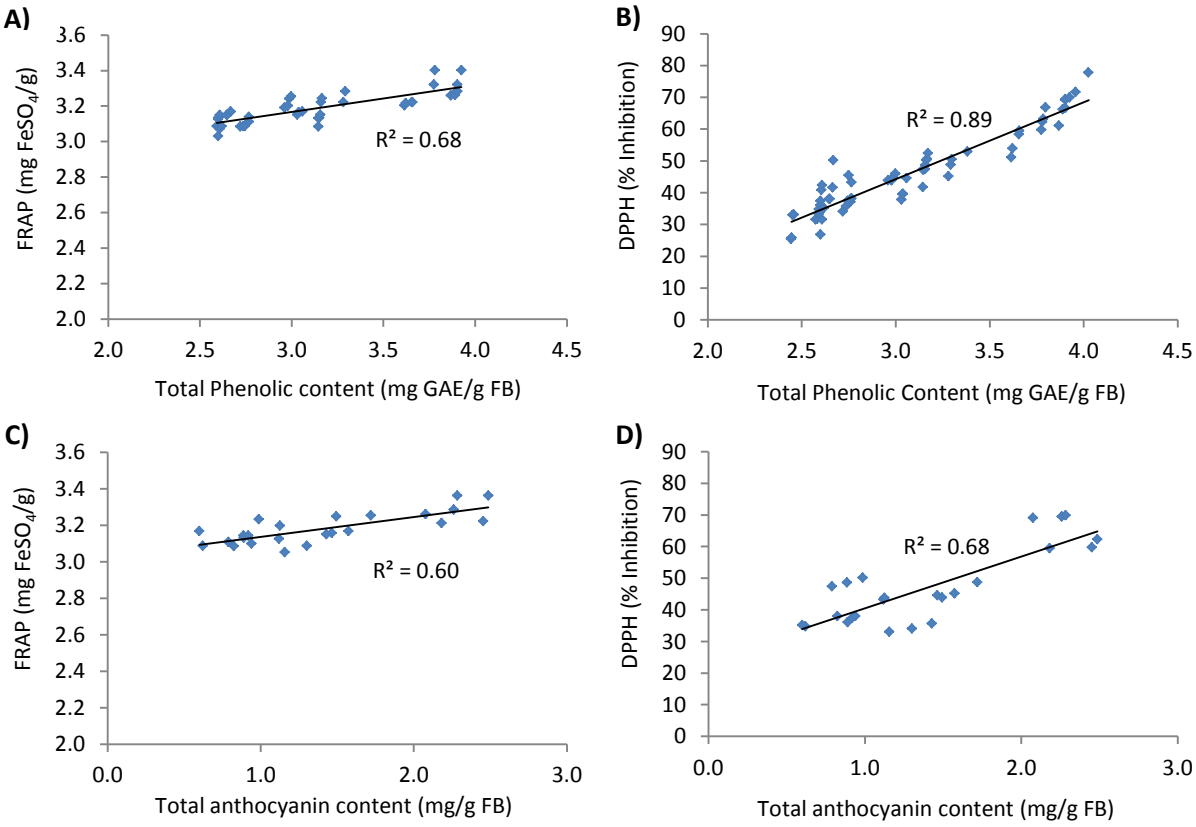


Figure 3.10: Linear correlation between the concentration of total phenolics or total anthocyanins and antioxidant activity in the eight water plus 5% formic acid blueberry extracts: total phenolic content vs ferric reducing antioxidant power (FRAP) (A); total phenolic content vs scavenging of diphenyl-picrylhydrazyl (DPPH)-radical (B); total anthocyanin concentration vs ferric reducing antioxidant power (FRAP) (C); total anthocyanin concentration vs scavenging of diphenyl-picrylhydrazyl (DPPH)-radical (D).

This study agrees with previous reports (Ehlenfeldt & Prior, 2001; Liu et al., 2008; Borneo et al., 2009) in finding a significant linear relationship between total phenolic content and antioxidant activity in blueberries and other plants. As shown in Figure 3.10, there was a high positive correlation between DPPH values and total phenolics ($R^2 = 0.89$, $P < 0.01$), and a moderately high correlation between FRAP and total phenolics ($R^2 = 0.68$, $P < 0.01$) of the water plus 5% formic acid extracts, suggesting that the phenolic compounds are likely to be the major contributors to the antioxidant activity and the TPC could be used as a preliminary indicator for its antioxidant activity. Interestingly, the phenolic concentration in blueberries is more likely to exert a radical scavenging ability rather than the reducing ability.

Anthocyanin concentration has been found to correlate relatively well with antioxidant activity present in fruits (Prior et al., 1998; Connor et al., 2002b; Noda et al., 2002; Kallithraka et al., 2009). Our results also showed significant correlations between the total anthocyanin concentration and antioxidant activities based on the FRAP assay ($R^2 = 0.60$, $P < 0.01$) as well as the DPPH radical inhibition assay ($R^2 = 0.68$, $P < 0.01$). As shown in Figure 3.10, such correlation was normally weaker as compared with the correlation between total phenolic contents and antioxidant values, which confirmed the fact that, beyond anthocyanins, other phenolic compounds in blueberries such as phenolic acids and procyanidins could also contribute to their total antioxidant capacity.

Based on the experimental data, the total phenolic content in blueberries is the major contributing factor to antioxidant capacities, particular on the ability to scavenge the DPPH radicals. Therefore, it was clear that 'Elliot' and 'Burlington' varieties, which had the highest TPC as well as total anthocyanins, possessed the highest antioxidant activities, while 'Brigitta' and 'Reka', which contained the lowest TPC, exhibited the lowest antioxidant capacities.

Additionally, some studies have reported that beyond the total bioactive compounds, some individual anthocyanins are likely to exhibit stronger antioxidant capacities than others due to the number and/or the positions of the hydroxyl group on the B ring of anthocyanidin molecules. The ranking order is delphinidin (3',4',5'-OH) > cyanidin (3',4'-OH), petunidin (4',5'-OH) > malvidin, peonidin, peralgonidin (4'-OH) (Ogawa et al., 2008). Borges et al. (2010) also found that among 15 individual anthocyanins in blueberries, most antioxidant activity was attributed to delphinidin-3-galactoside (peak 1), cyanidin-3-galactoside (peak 3), delphinidin-3-arabinoside (peak 4), petunidin-3-galactoside (peak 6), malvidin-3-galactoside (peak 12) and malvidin-3-

arabinoside (peak 15). Upon examination of the raw data of the present study, 'Elliott' and 'Burlington' not only contained the highest concentrations of these specific peaks, but also showed the highest proportions of these compounds (approximately 90 % of total anthocyanin), followed by 'Duke' (88%). Furthermore, according to earlier evidence, it appeared that the compounds which may possess strong antioxidant activities in blueberries were mostly galactosides and arabinosides rather than the glucosides derivatives of anthocyanins. The result of principal component analysis confirmed that the cultivars which contain mostly galactoside and arabinoside were 'Burlington', 'Elliott' and 'Duke', whereas the other four varieties ('Bluecrop', 'Dixi', 'Jersey' and 'Reka') contained mostly glucoside derivatives. This could be another reason behind the high antioxidant capacity of these cultivars.

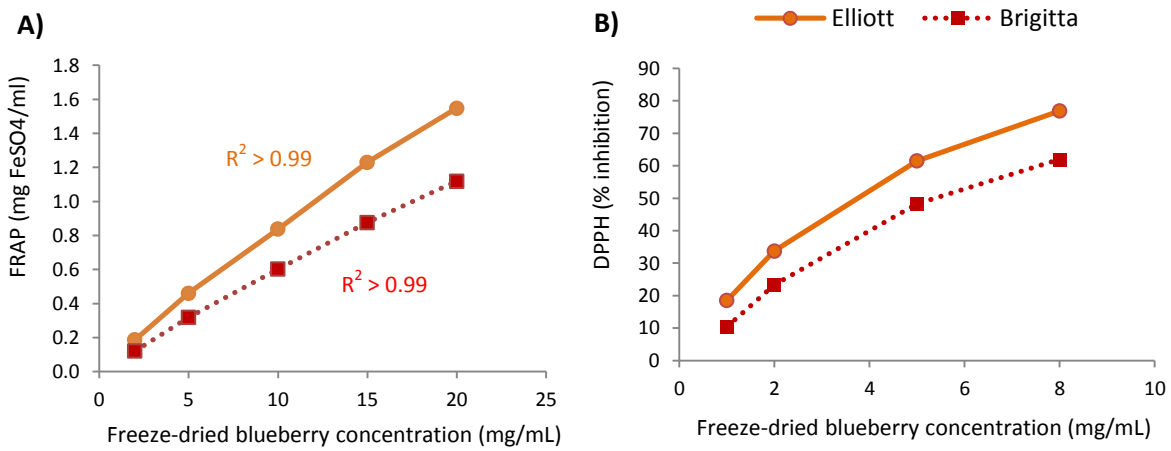


Figure 3.11: Ferric reducing antioxidant power (FRAP; mg $FeSO_4$/mL) (A), and scavenging of diphenyl-picrylhydrazyl (DPPH)-radical; (% inhibition) (B) of 'Elliott' and 'Brigitta' cultivars of freeze-dried whole blueberry sample at different concentrations.

Finally, it is important to note that there was a strong linear response to the concentration of sample for the TPC between 2 and 20 mg/mL with R^2 across all cultivars greater than 0.99 ($P < 0.01$, data not shown). Similarly, a dose-dependent linear curve was observed for the FRAP values (for example; $R^2 > 0.99$ for 'Brigitta' and 'Elliott', $P < 0.01$) as shown in Figure 3.11. These results indicate that the TPC and FRAP values of blueberries were highly dose-dependent and thus TPC and FRAP values can be extrapolated from a single concentration.

Unlike the TPC and FRAP, the scavenging activity toward DPPH-radicals tended to decrease at higher concentrations of freeze-dried sample (Figure 3.11), which is consistent with the fact that the DPPH assay has a narrow dynamic range. For this reason, it has become more common to

report the concentrations of sample required to inhibit DPPH radical formation by 50% (IC_{50}). Although this issue was overlooked at the time of carrying out the assay, it is likely that the data reported in the present study (Figures 3.8B and 3.9C) accurately reflect relative DPPH activities for the eight blueberry cultivars, since most extracts gave % inhibition in a range not far from 50%.

Conclusion :

Our data demonstrated that Highbush blueberries grown in New Zealand possess high amount of total phenolics, total anthocyanins, chlorogenic acid, and exhibit strong ferric-reducing antioxidant power and DPPH-radical scavenging activity. However, the concentration of these bioactive compounds and antioxidant activities vary significantly among blueberry varieties. Generally, cultivars that contain high concentrations of phenolics and anthocyanins were likely to exhibit the highest antioxidant capacity. Additionally, solvents used for the extraction had a significant effect on total phenolics and antioxidant activities, with mixed organic solvent extracts showing the highest total phenolic content and antioxidant capacity, followed by acidified water, and water alone. Across all extractions of the eight varieties tested, 'Elliott' and 'Burlington' exhibit the highest concentration of these bioactive compounds, whereas 'Reka' showed all the lowest values except for the chlorogenic acid concentration. The first obtained principal component distinguished the eight blueberry varieties into two main groups. 'Elliott', 'Burlington' and 'Duke' contained primarily galactosides and arabionosides, while 'Bluecrop', 'Dixi', 'Jersey' and 'Reka' were high in glucoside derivatives of the anthocyanins. A strong inverse correlation was observed between the concentration of total anthocyanins and the size of berries, with the smallest berry size containing the highest concentration of total anthocyanins. This indicates that the anthocyanins were derived mainly from the peel of blueberry fruits. Furthermore, the concentrations of total phenolics were found to be positively correlated to antioxidant capacities across all genotypes, suggesting that phenolic compounds in blueberries are likely to be the major contributors to their antioxidant activity. The present findings provide the fundamental information with regards to predominant bioactive compounds in specific Highbush blueberry grown in New Zealand and our results suggest that these Highbush blueberries are good sources of bioactive compounds as well as antioxidants that may be useful for prevention of oxidative stress-related diseases.

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CHAPTER 4

Effects of blueberry extracts on starch hydrolysis enzyme inhibition and beneficial probiotic bacterial growth *in vitro*

The previous chapter showed that NZ Highbush blueberries contained remarkable amounts of bioactive compounds which may contribute to their health benefits. It has been claimed that antioxidants in blueberries may reduce the risk of oxidative stress-related metabolic syndrome diseases including type II diabetes. Furthermore, recently scientists suggested that beyond their antioxidant activity, the bioactive compounds in blueberries may also possess some particular roles, which may be directly involved in the management of hyperglycemia. For example, blueberries may modulate α-amylase and α-glucosidase, the enzymes directly involved in starch digestion and glucose absorption (Hanhineva et al., 2010). Additionally, they may exert beneficial effects in the gut by modulating the microbiota in the intestinal tract (Delzenne & Cani, 2010) offering another possible mechanism related to obesity and insulin resistance. For these reasons, the present study was carried out to investigate whether these eight varieties of NZ Highbush blueberries can inhibit α-amylase and α-glucosidase activity, or enhance the growth of beneficial probiotic bacteria *in vitro* before progressing to further *in vivo* experiment.

Abstract :

Aqueous extracts of different Highbush blueberries grown in New Zealand were investigated for their potential to contribute to the dietary management of metabolic syndrome through two major mechanisms: inhibition of starch hydrolysis enzymes and improvement of beneficial probiotic bacterial growth. Most blueberry extracts exhibited inhibitory ability against α-amylase and α-glucosidase, with 'Bluecrop' having the highest activity (36.2% and 48.6% inhibition, respectively), followed by 'Jersey' (31.0% and 40.1% inhibition), and 'Burlington' (28.5% and 40.5% inhibition), when the extracts (per well) represented 20 and 25 mg frozen berries, respectively. Enzyme inhibitory activities were not closely correlated with total phenolic content or antioxidant capacities across all genotypes, suggesting that such inhibitory activity was possibly influenced more by some particular phytochemicals in blueberries. Aqueous blueberry extracts appeared to have a beneficial enhancing effect only on the growth of *Lactobacillus acidophilus* but not *Lactobacillus rhamnosus*, with three specific cultivars, 'Burlington', 'Bluecrop' and 'Duke' significantly increasing the growth of *L. acidophilus* by more than 0.5 $\log_{10}$ CFU/mL after 48 h when compared to the control incubations (without blueberry extracts). However, when extracts were characterized by their composition, no specific compounds (chlorogenic acid, anthocyanins, etc.) in blueberries or their antioxidant activities correlated closely with the proliferation of these probiotic bacteria. Across the eight blueberry genotypes tested, 'Bluecrop' and 'Burlington', which exhibited strong enzymatic inhibitory abilities as well as enhanced the growth of beneficial probiotic bacteria, may be potential candidates for early management of metabolic syndrome.

Introduction :

Blueberries are known to be one of the best sources of dietary antioxidants (Borges et al., 2010) and treatment with extracts from blueberries have been reported to reduce a variety of risk factors of metabolic syndrome. Blueberries may protect against the development of glucose intolerance and elevated blood glucose (Vuong et al., 2009; Khanal et al., 2012; Roopchand et al., 2013), suppress appetite (Molan et al., 2008), or normalise lipid parameters (Prior et al., 2010). Several phytochemicals, naturally occurring antioxidants in fruits, are thought to be responsible for their health benefits. However, several reports note that some bioactive compounds in blueberries, such as anthocyanins have a particularly low bioavailability since only small amounts of dietary anthocyanins can be absorbed in the small intestine after consumption,

making the concentration of anthocyanins in the bloodstream typically low (Kay, 2006). For this reason, anthocyanins are unlikely to exert beneficial effects on metabolic parameters by acting as antioxidants *in vivo* (Wallace, 2011), but other mechanisms may be involved. Recent evidence suggested that at low concentrations in the bloodstream, anthocyanins could suppress starch digestion especially by inhibiting the activity of the enzymes α-amylase and α-glucosidase (Hanhineva et al., 2010), or may exert beneficial effects in the gut by modulating microbiota in the intestinal tract (Delzenne & Cani, 2010). These may contribute more directly to the process of glucose homeostasis linked to metabolic syndrome.

In the human body, starch are digested by pancreatic α-amylase in the small intestine into oligosaccharides, which are further hydrolyzed to glucose by intestinal α-glucosidase before being released into the blood stream (Ademiluyi & Oboh, 2013; Adisakwattana et al., 2011). One of the therapeutic approaches for lowering blood glucose is to retard the digestion and absorption of starch after consumption through inhibition of starch hydrolyzing enzymes including α-amylase and α-glucosidase, which may reduce the breakdown of starch into glucose and consequently reduce the postprandial rise in glucose concentration in the blood stream (Kim, 2012). Currently, synthetic drugs such as acarbose, miglitol or voglibose are effectively used to inhibit the action of these starch hydrolyzing enzymes and to lower blood glucose levels in type II diabetic patients (Kim et al., 2005; El-Beshbishy & Bahashwan, 2012); however these drugs have been found to cause various side effects in individuals, for example abdominal distention, flatulence, meteorism and possibly diarrhea (Dong et al., 2012).

Gut microbiota have also been reported to have an important role in metabolic disorders, particularly in relation to energy homeostasis and insulin resistance (Delzenne & Cani, 2010). The large intestine contains a complicated microflora, which is believed to include up to 500-1000 bacterial species with a total population of 10^{14} bacterial cells (Cani et al., 2008). The intestinal microbiota are able to produce a wide range of compounds which have both positive and negative effects on the host. For example, *Lactobacillus* spp. and *Bifidobacterium* spp. have been shown to enhance host immune system, modulate lipid metabolism and participate in activation of provitamins, whereas some species of bacteria like *Clostridium difficile* are associated with negative complications such as development of inflammatory bowel disease (Hidalgo et al., 2012). For this reason, the presence of beneficial or harmful bacteria in the gut significantly contributes to the individual's health status. Importantly, recent evidence indicates that microbiotic composition in the gut has been found to differ between obese and lean individuals

(Bibiloni et al., 2009), suggesting that modification of gut microbiota by incorporating probiotic bacteria may offer a new avenue for the management of metabolic disorders. Two common probiotic species which have been reported in the literature to be involved in energy and glucose homeostasis are *Lactobacillus* spp. and *Bifidobacterium* spp. (Cani & Delzenne, 2009). In particular, a recent study of Laitinen et al. (2009) indicated that incorporation of particular isolates of these probiotic bacteria (*Lactobacillus rhamnosus* GG *and Bifidobacterium lactis* Bb12), into the diet decreased blood glucose concentration, improved glucose tolerance and lowered insulin concentrations in pregnant women, indicating beneficial effects in managing metabolic syndrome.

Bioactive compounds with strong antioxidant activities such as phenolics in various plant extracts have been reported to show a potential to inhibit the action of α-amylase and α-glucosidase enzymes *in vitro* (Kwon et al., 2008; Cheplick et al., 2010; Oboh et al., 2012). On the other hand, these phytochemicals, particularly anthocyanins (isolated from byproducts of wine), have been found to significantly enhance the growth of *Bifidobacterium* spp. and *Lactobacillus-Enterococcus* spp. *in vitro* using mixed faecal batch culture (Hidalgo et al., 2012). Furthermore, another scientist showed that phenolic components extracted from tea significantly modulated the intestinal bacteria population by suppressing the growth of specific pathogenic bacteria such as *Clostridium perfringens*, *Escherichia coli* and *Salmonella typhimurium*, whereas probiotics such *as Lactobacillus* spp. and *Bifidobacterium* spp. were less affected (Lee et al., 2006). This evidence supports the possibility of using phytochemical enriched diets as a complementary anti-metabolic syndrome therapy. Therefore this has led to the increasing search for edible plants rich in bioactive compounds that could be regularly consumed for health benefit purposes without causing side effects.

Blueberries are commonly available in New Zealand. In addition, our first study confirmed that their aqueous extracts not only possess remarkable antioxidant activities, but also contain considerable amounts of bioactive compounds including polyphenolics, anthocyanins, and chlorogenic acid. Therefore, the present study was carried out to investigate the ability of aqueous Highbush blueberry extracts to inhibit α-amylase and α-glucosidase activity, as well as their ability to enhance growth of beneficial probiotic bacteria, namely *Lactobacillus acidophilus* and *Lactobacillus rhamnosus*.

Materials and Methods :

Chemicals

α-glucosidase (EC 3.2.1.20) from Baker's yeast, α-amylase (EC 3.2.1.1) from porcine pancreas, *p*-nitrophenyl α-D-glucopyranoside (*p*NPG), 3,5-dinitrosalicylic acid (DNS), sodium potassium tartrate and soluble potato starch were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). Iodine was kindly provided by the Nutrition laboratory, Institute of Food, Nutrition and Human Health, Massey University, Palmerston North, New Zealand. All chemicals used were of analytical grade and ultrapure water obtained from a Milli-Q system (Millipore, Milford, MA) was used for the preparation of the reagents and blueberry extracts.

Preparation of aqueous blueberry extracts

All eight Highbush blueberry genotypes were extracted using water since this solvent may better represent the normal consumption than using organic solvents. The preparation was done by grinding 50 g of frozen berries with 50 ml of ultrapure Milli-Q water using a small food processor (Breville, MiniWizz Processor WT400, Australia). The mixture was then centrifuged at 10,000 rpm for 10 min (Allegra TM 64 R Centrifuge, Beckman Coulter, CA, USA) and the supernatant collected and used for enzyme inhibitory activity analysis, or the extracts were filtered through membrane syringe filters (0.22 µm pore size) under sterile conditions and used to evaluate the effects of the extracts on growth of the bacteria.

α–Amylase inhibition activity

The effect of the berry extracts on α-amylase inhibitory activity was determined using two different methods. The 3,5-dinitrosalicylic acid (DNS) assay was carried out according to the method described by Gowri et al. (2007) with some modifications. 200 µl of aqueous blueberry extracts were pre-incubated with 200 µl of porcine pancreatic α-amylase (4.0 Unit/mL) at 25^{o}C for 5 min; 400 µl of 0.5% (w/v) soluble starch solution was added, and the mixture was incubated at 25^{o}C. Three min later, the reaction was terminated by adding 400 µl of dinitrosalicylic acid colour reagent (1.0 g of 3,5-dinitrosalicylic acid, 30 g of sodium potassium tartrate and 20 ml of 2N NaOH to a final volume of 100 ml Milli-Q water). The mixtures were then reincubated in a boiling water bath (85-90 ^{o}C) for 10 min in order to develop colour. After cooling to room temperature, 50 µl of the reaction mixture was diluted by adding 175 µl Milli-Q water, and absorbance was measured at 550 nm with a microplate reader (ELx808 BioTek

Instruments Inc, USA). The reading was compared to the control, which contained 200 µl of water instead of blueberry extract (Gowri et al., 2007). An appropriate blank containing blueberry extract and all other components of the assay except the amylase enzyme were used to correct for background absorbance and sugar content. The α-amylase inhibitory activity was expressed as percentage inhibition of enzyme activity and was calculated using the following equation:

$$\text{Inhibition activity (\%)} = \frac{[A_{550}\ \text{control} - (A_{550}\ \text{SEE} - A_{550}\ \text{SEO})]}{A_{550}\ \text{control}} \times 100$$

where A_{550} SEE is defined as the absorbance of sample extract (with enzyme), and A_{550} SEO defined as the absorbance of sample extract (without addition of enzyme).

The second method was starch-iodine testing which was carried out in order to investigate whether the aqueous blueberry extracts had any inhibitory effect on α-amylase. Briefly, 3 ml of aqueous blueberry extracts were pre-incubated with 3 ml of porcine pancreatic α-amylase (4.0 Unit/mL prepared in ice cold water) at 25^{o}C for 5 min. After pre-incubation, 3 ml of 0.5% (w/v) soluble starch solution was added to start the reaction. Then 70 µl of iodine reagent (2% I_2 and 6 % KI in 50% ethanol) was added to develop blue colour. The absorbance was measured at 620 nm against a blank (containing blueberry extract plus the same amount of water in place of enzyme) using a spectrophotometer (Implen NanoPhotometer, Germany) immediately after adding iodine, and at every 2 minutes thereafter to monitor the degradation of blue colour. The control incubation contained 3 ml of water instead of blueberry extract. The absorbance at 620 nm at different time points was plotted and time to 50% starch reduction was calculated by interpolation.

α–Glucosidase inhibition activity

The α-glucosidase inhibitory activity was determined according to the method described by Kim et al. (2005) and Schäfer & Högger (2007) with minor modifications. Briefly, a mixture of 50 µl of aqueous blueberry extracts and 50 µl of yeast α-glucosidase (0.6 Unit/mL) in 100 mM phosphate buffer (pH6.9) solution was incubated at 37^{o}C for 10 min. After pre-incubation, 125 µl of 10 mM *p*-nitrophenyl α-D-glucopyranoside (*p*NPG) solution in 100 mM phosphate buffer (pH6.9) was added and the reaction mixtures were incubated for 30 min at 37^{o}C. The enzymatic reaction was terminated by adding 590 µl of 1M Na_2CO_3 solution to the mixtures. The control sample contained 50 µl of water in place of an aqueous blueberry extract, whereas blueberry

extracts plus phosphate buffer were used as a blank to correct background absorbance. α-glucosidase inhibitory activity was determined by measuring the product (*p*-nitrophenol) released from *p*NPG, measured at 405 nm using a microplate reader (ELx808 BioTek Instruments Inc, USA). The results were expressed as percentage inhibition of enzyme activity and were calculated using the following formula:

$$\text{Inhibition activity (\%)} = \frac{[A_{405}\ \text{control} - A_{405}\ \text{sample extract}] \times 100}{A_{405}\ \text{control}}$$

Determining probiotic bacterial growth using pure cultures of lactic acid bacteria

Pure cultures of two bacteria species, *Lactobacillus acidophilus* and *Lactobacillus rhamnosus*, were obtained from the culture collection held by Environmental Science and Research (ESR), New Zealand. Standard growth curves of these bacteria were first established by optical density readings versus colony counts. All bacteria were grown at 37^{o}C in Man-Rogaso-Sharpe (MRS) broth under anaerobic conditions in a CO_2 incubator for 24 hours. The density of bacterium culture was measured by reading the optical density (OD) of the inoculated broth at 600 nm against the uninoculated (blank) broth, and the OD of culture was consequently adjusted using fresh MRS broth to ensure incubations began with bacteria in log phase of growth and the two sets of the experiment started with an equal amount of bacteria. Then 0.25% (v/v) inocula of these cultures were transferred into 5 ml of fresh MRS broth containing 1, 5 or 10% (v/v) of the blueberry extracts. Controls included a positive control (medium plus bacteria) which contained 1, 5 and 10% of sterile distilled water instead of blueberry extract as well as a negative control containing only medium. All tubes were incubated in CO_2 incubator at 37^{o}C for 8, 24, 48, 72 and 120 hours, and at the end of each incubation period, broths were serially diluted in fresh MRS broth and then a 100 µl aliquot of three appropriate dilutions was spread onto the surface of MRS agar plates in two replicates (Figure 4.1). The plates were then incubated anaerobically at 37^{o}C for 48 hours and the number of viable bacterial colonies was counted using a plate reader. Concentration of each bacterium was expressed as $\log_{10}$ number of bacterial cells per millilitre (CFU/mL).

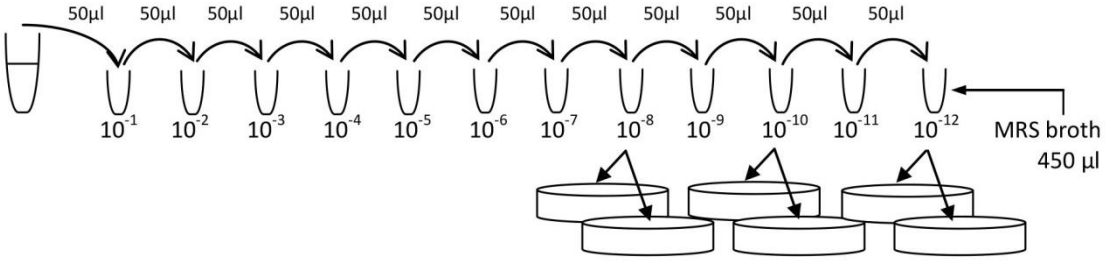

Figure 4.1: Schematic diagram of serial dilutions used for bacterial growth assessment

Statistical analysis

For enzyme inhibitory abilities, the DNS and α-glucosidase assays were performed in triplicate in two independent experiments, whereas one replicate was done for preliminary examination of α-amylase inhibition activity using the starch-iodine test. For probiotic bacterial growth, two independent experiments performed in duplicated were conducted for each bacterium at each concentration. The results were expressed as mean ± S.E. and analyzed using SAS version 9.2 for windows (SAS Institute Inc., Cary, NC, USA). One-way analysis of variance (ANOVA) was used to test for significant differences among means of enzymatic inhibition activities (α-amylase and α-glucosidase). Data for the viable cell counts of probiotic bacteria were compared using repeated measures and two-way analysis of variance (ANOVA) where appropriate. Tukey's post hoc test was used for multiple comparisons and the differences were considered statistically significant at $P < 0.05$. Linear regression analysis (with coefficient of determination, R^2) was performed to evaluate the relationships among variables of interest.

Results and Discussions :

Inhibitory potential of blueberries against α-amylase and α-glucosidase

The α-amylase activity was assayed originally using the DNS method because it is widely used and has been reported in a number of studies with regards to α-amylase inhibition. The principle of the DNS procedure is based on using 3,5-dinitrosalicylic acid (DNS) to react with reducing sugars (the end product hydrolyzed by the activity of α-amylase), to form 3-amino-5-nitrosalicylic acid which absorbs light at 540 nm (Conforti et al., 2005). An increase in colour indicates higher development of reducing sugars, which results from lower inhibitory activity of the sample extracts. This assay was carried out according to the method of Gowri et al. (2007),

who clearly described all details of their procedure to study α-amylase inhibitory activity of bartogenic acid isolated from *Barringtonia racemosa* Roxb. Unfortunately, when we performed the assay in our laboratory, this procedure appeared not to be appropriate for examination of α-amylase inhibitory activity in blueberry fruits since we found that the absorbance of the sample extracts was much higher than the absorbance of the control (without addition of extract). This appears to imply that the blueberry extract had less ability to inhibit the action of the enzyme than pure water used instead of blueberry extract in the control sample, which was unusual. Therefore, since the inhibitory activity of the blueberry extract is calculated by comparing it to the control, the percent inhibition calculation was misleading.

For this reason, a series of experiments were then conducted by varying several experimental conditions in order to standardize the assay. For example, using new pancreatic α-amylase, varying the concentrations of enzyme as well as the extracts, testing the optimal temperature for incubation and increasing the incubation time, etc., however all of these conditions did not solve the problem. The question remained that the problem occurred either from the methodology used or the aqueous extracts themselves did not have any ability to inhibit the enzyme. Consequently, we decided to find another method which is easily performed and also can be used to determine the activity of α-amylase. Iodine solution (the mixture of iodine with potassium iodide) has been used to detect the presence of starch in foods. This solution when reacting with starch will form a very dark purple color, which can be measured using spectrophotometry. The background of this assay is that it measures the amount of starch left over to determine the activity of α-amylase. The higher amount of starch present, the greater ability of the extract to inactivate the enzyme activity. This starch-iodine test was hence used to determine whether these Highbush varieties had inhibitory ability against α-amylase. As shown in Table 4.1, most aqueous extracts tended to show α-amylase inhibition activities as they considerably delayed the hydrolysis of starch into sugar as compared to the control, with 'Jersey' having the highest ability, followed by 'Bluecrop', 'Burlington' and 'Dixi', respectively, while 'Duke' contained the lowest ability which appeared to be comparable to the control.

The result obtained from starch-iodine test supported the notion of an inhibitory effect of the aqueous blueberry extracts against α-amylase *in vitro*. Therefore we hypothesized that there may be something, possibly sugar that can interfere with the DNS test results, making the absorbance of samples higher than usual. Consequently, we undertook the final experiment to confirm this by testing the extract only without addition of any substrate (starch) and enzyme,

and the absorbance reading showed that the blueberry extracts contained some sugar that can react with the DNS and develop colour.

Table 4.1: Inhibitory effect on α-amylase of water extracts from eight Highbush blueberry genotypes using the starch-iodine test

Blueberry genotypes	Time to 50% starch reduction (min)
Bluecrop	6.09
Brigitta	5.31
Burlington	5.56
Dixi	5.36
Duke	3.56
Elliott	4.43
Jersey	6.41
Reka	4.18
Control	**3.36**

Data presented for this assay are from one replication (N = 1).

The final decision was to measure the α-amylase inhibitory ability of blueberry extracts using the DNS assay because this method is accurate and reproducible, but a problem occurred because our samples contained a considerable amount of sugar. The starch-iodine testing, on the other hand, did not contain a termination step and colour continued to develop over time. Since it was confirmed that the sugar in blueberries significantly interfered with the absorbance measurement, the exact amount of sugar in each extract was measured spectrophotometrically and this value was deducted from the total amount of sugar found in each variety of blueberry. Excluding the amount of sugar from the blueberries resulted in an accurate measure of the

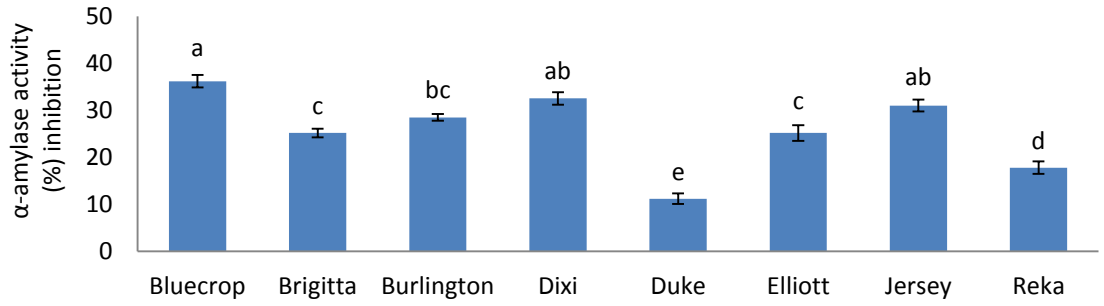

Figure 4.2: Inhibitory effect of water extracts from eight Highbush blueberry genotypes on α-amylase. Extracts used in the assay represented 20 mg frozen berries (per well). Results are presented as mean ± S.E. of three replicates of two independent experiments (N = 6) and different superscript letters indicate statistically significant differences ($P < 0.05$) between blueberry genotypes as determined with one-way ANOVA followed by Tukey's post-hoc analysis

amount of sugar that was truly produced by the activity of α-amylase enzyme. Figure 4.2 shows the inhibitory effects of the blueberry extracts against α-amylase using the DNS assay. Almost in line with iodine test result, 'Bluecrop', 'Dixi' and 'Jersey' were among the top three cultivars which exhibited the highest α-amylase inhibitory effect, ranging from 31.0 to 36.2%, followed by 'Burlington', whereas extracts from 'Duke' showed the lowest value.

The inhibitory activity against α-glucosidase was monitored by using the substrate *p*-nitrophenyl-α-d-glucopyranoside (*p*NPG), which is specifically hydrolyzed by α-glucosidase to release the substance *p*-nitrophenol, a yellow colour product that can be detected at 405 nm (Gowri et al., 2007). In the presence of α-glucosidase inhibitors, less *p*-nitrophenol would be produced and the absorbance value would be decreased. Based on this principle, sugar in the berries did not seem to interfere with the test result, therefore all extracts were tested as usual without taking sugars in berries into account. The *in vitro* inhibitory activity of eight Highbush blueberry cultivars against α-glucosidase is shown in Figure 4.3. Almost all extracts possessed inhibitory activities and 'Bluecrop' showed the highest α-glucosidase inhibiting activity (49%) followed by 'Burlington', 'Jersey' and 'Reka' with more than 40% inhibition. 'Elliott', 'Dixi' and 'Duke' showed moderate inhibitory effects against this enzyme ranging between 20% and 40%, whereas 'Brigitta' showed the lowest α-glucosidase inhibitory value.

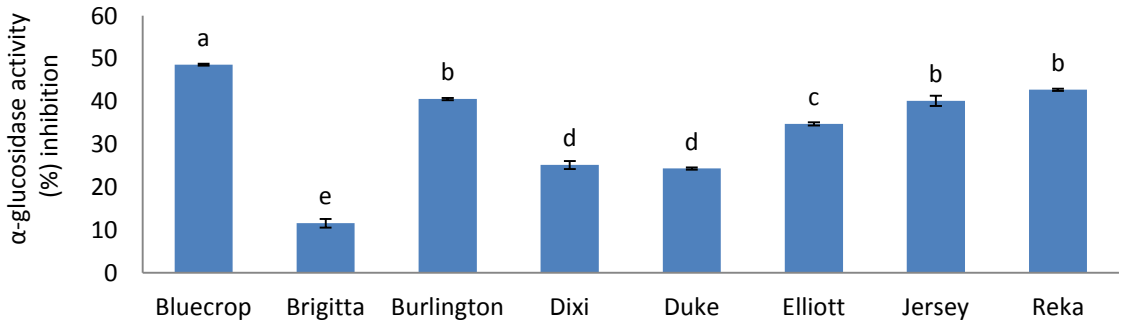

Figure 4.3: Inhibitory effect on α-glucosidase of water extracts from eight Highbush blueberry genotypes. Extracts used in the assay represented 25 mg frozen berries (per well). Results are presented as mean ± S.E. of three replicates of two independent experiments (N = 6) and different superscript letters indicate statistically significant differences ($P < 0.05$) between blueberry genotypes as determined with one-way ANOVA followed by Tukey's post-hoc analysis.

Because these two assays were done at only one concentration of the extracts, we needed to know if there is a linear relationship between the amount of extract used and the extent of inhibition. Therefore, a limited concentration-dependent study on the α-glucosidase inhibiting

activity of all eight blueberry extracts was evaluated at three different levels (25.0, 12.5 and 6.25 mg of frozen berries (FW) per well), and the % inhibition of three selected cultivars ('Bluecrop', 'Elliott' and 'Brigitta') are shown in Figure 4.4. As the amount of berries decreased, the inhibitory ability against α-glucosidase decreased accordingly, indicating dose-dependent relationship but not strictly linear within the range of concentrations studied.

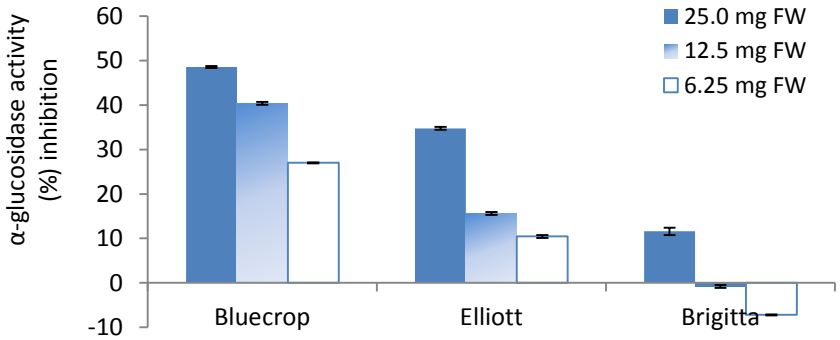

Figure 4.4: Inhibitory effect on α-glucosidase of water extracts of 'Bluecrop', 'Elliott' and 'Brigitta' cultivars at different amounts of extract assayed. Results are presented as mean ± S.E. of three replicates of two independent experiments (N = 6).

It has been reported by some researchers that phenolics or flavonoids possess ability to inhibit these hydrolyzing enzymes. Indeed, a positive correlation has been reported between the α-amylase or α-glucosidase inhibitory activity of the extracts and their phenolic contents or antioxidant capacities (McCue et al., 2005; Kwon et al., 2008; Kim et al., 2010; El-Beshbishy & Bahashwan, 2012; Wang et al., 2012). In contrast to earlier reports, no significant correlation was found between antioxidant values and their enzymatic inhibition activity in this study (Table 4.2). Water extracts of 'Elliott' and 'Burlington', which had the best overall TPC and antioxidant capacity, did not exhibit the highest inhibitory effect on α-amylase and α-glucosidase. Interestingly, 'Bluecrop' and 'Jersey', which contained moderate to low antioxidant activity, possessed the highest inhibitory activity against these starch hydrolysis enzymes. The weak association between these two indicators has also been reported by others in various types of plants. Cheplick et al. (2007) compared α-amylase inhibitory activity of different raspberry genotypes and found that a black raspberry extract exhibited the highest total antioxidant activity but the lowest α-amylase inhibitory ability. Ademiluyi & Oboh (2013) found no association between α-amylase inhibitory activity and the phenolic content of the soybean extracts and suggested that such activity may be associated more with type and quality of the

phenolic compounds. Similarly, Wongsa et al. (2012) also observed no correlation (R = 0.01, *P* > 0.05) between total phenolic content and inhibitory activity against α-amylase for the extracts of all herbs tested in their study.

Table 4.2: Correlation coefficients (R^2) between the concentration of total phenolics, anthocyanins, antioxidant activity (FRAP and DPPH) and enzymatic inhibition activity (α-amylase and α-glucosidase) across all eight genotypes.

	Correlation coefficients (R^2)	
	α-amylase inhibition	α-glucosidase inhibition
Total phenolics	0.04	0.08
Total anthocyanins	0.00	0.02
- Galactosylated ACNs	0.08	0.00
- Arabinosylated ACNs	0.01	0.03
- Galactosylated + Arabinosylated ACNs	0.06	0.00
- Glucosylated ACNs	0.34	0.11
- Acylated ACNs	0.20	0.01
- Glucosylated + Acylated ACNs	0.33	0.03
Chlorogenic acid	0.23	0.11
Ferric reducing antioxidant power (FRAP)	0.05	0.00
Scavenging DPPH radical ability	0.01	0.00

As shown in Table 4.2, the lack of correlations between TPC or antioxidant activity and inhibitory effect against starch hydrolyzing enzymes may indicate that neither their TPC nor antioxidant activity are good predictors for enzymatic inhibition in blueberry fruits. Beyond the total antioxidant activity, the inhibitory ability against the enzymes could possibly be associated with some structure-specific properties of particular polyphenols rather than overall phenolic or flavonoid concentrations (Correia et al., 2004; Cheplick et al., 2007). Blueberries are known to contain high concentrations of anthocyanins, and considerable amounts of procyanidin and phenolic acids such as chlorogenic and caffeic acids (Taruscio et al., 2004), and some of these specific compounds have been reported to have α-amylase and/or α-glucosidase inhibitory properties. McDougall et al. (2005) studied anti-hyperglycemic properties of several berry fruits *in vitro* and reported that anthocyanins are potent inhibitors of α-glucosidase activity, while soluble tannins contribute more to α-amylase inhibitory ability. More specifically, it has been suggested by Matsui et al. (2001) that the acylated side-chain structure of anthocyanins appear to be important for the inhibitory activity against α-glucosidase. In addition, caffeic acid

concentration was found to be correlated well (R = 0.68, $P < 0.05$) with inhibitory activity against α-amylase in some families of herbs (Wongsa et al., 2012). However, in a study using various cultivars of strawberries, Pinto et al. (2008) observed no correlation between chlorogenic acid and inhibitory activity against α-glucosidase (R = -0.14, $P > 0.05$). It is becoming clearer that inhibition of α-amylase and α-glucosidase may be caused by different polyphenolic compounds and recent work has focused on the particular bioactive compounds present in plants. Upon examination of raw data with regards to individual anthocyanins determined in the previous chapter (Chapter 3), it is important to highlight that 'Bluecrop' and 'Jersey' which are the most efficient cultivars to inhibit the starch hydrolyzing enzymes, are high in glucoside derivatives of anthocyanins; especially, 'Bluecrop' also contains considerable amounts of acylated anthocyanins. Additionally, correlation between individual anthocyanins (Table 4.2) shows that the specific compound in blueberries which contributes to the inhibitory activity of these starch hydrolysis enzymes may be glucosylated derivatives of anthocyanins. Particularly α-amylase, up to 34% of inhibitory ability of this enzyme could be explained by the concentration of glucosylated anthocyanins found in the fruits; however, the R^2 value was not significant due to the small number of varieties tested ($R^2 = 0.34$; $P = 0.13$). These reasons may partially explain why 'Bluecrop' and 'Jersey', which did not exhibit the highest antioxidant activity, showed the highest efficiency to inhibit both α-amylase and α-glucosidase enzymes.

Probiotic bacteria growth using pure cultures of lactic acid bacteria

An *in vitro* experiment was carried out to investigate the impact of aqueous blueberry extracts on the growth of two species of probiotic bacteria including *Lactobacillus acidophilus and Lactotobacillus rhamnosus*. Overall, a repeated measure analysis over the 5 days indicated that there were no significant differences between cultivars ($P = 0.14$), concentrations ($P = 0.12$), and interaction between cultivars and concentration ($P = 0.46$) on the growth of *L. acidophilus* and *L. rhamnosus*. However, significant differences were observed between incubation time ($P < 0.01$), with the number of bacteria significantly increasing with time until a peak was reached at 48 hours, and then reducing in number when the incubation period was extended up to 72 and 120 hours.

The repeated measure analysis indicated that the appropriate time of culture to observe the maximum size of bacteria population is at 48 h. Consequently, two-way ANOVA was further conducted at the time of maximum growth (48 h) to investigate whether there were any

differences between cultivars and concentrations of the extracts with respect to enhancing the growth of bacteria at this optimal time point.

Table 4.3: Least squares means for the effect of different cultivars and concentrations of blueberry on growth of lactic acid bacteria (*L. acidophilus and L. rhamnosus*) at 48 hours

		Log number of *L.acidophilus* (CFU/mL)	Log number of *L.rhamnosus* (CFU/mL)
Cultivar	Bluecrop	12.94^{ab}	12.45^{c}
	Brigitta	12.62^{bc}	12.45^{c}
	Burlington	13.14^{a}	12.66^{bc}
	Dixi	12.60^{bc}	12.62^{bc}
	Duke	12.92^{ab}	12.92^{a}
	Elliott	12.59^{bc}	12.64^{bc}
	Jersey	12.58^{bc}	12.57^{c}
	Reka	12.69^{abc}	12.83^{ab}
	Control	**12.36^{c}**	**12.56^{c}**
Concentration	1%	12.75	12.72^{a}
	5%	12.82	12.67^{a}
	10%	12.57	12.51^{b}
P value	Cultivar	0.04	< 0.01
	Concentration	NS	< 0.01
	Cultivar*Concentration	NS	< 0.01

Different superscript letters within the same column indicate significant differences ($P < 0.05$).
NS: not significant

When considered at the time of maximum growth (Table 4.3), there were significant differences ($P < 0.05$) between blueberry genotypes on the survivability of *L. acidophilus* and *L. rhamnosus*. The statistical analysis shows that 'Burlington', 'Bluecrop' and 'Duke' significantly enhanced the growth of *L. acidophilus* ($P < 0.05$), whereas 'Duke' and 'Reka' significantly enhanced the growth of *L. rhamnosus* compared to the control. The cultivars 'Brigitta', 'Dixi', 'Elliott' and 'Jersey' appeared to have no ability to increase the population size of these bacteria. However, microbiological experimental error is normally accepted to be 0.5 $\log_{10}$ CFU/mL (Pujol et al., 2012), therefore reliable differences between two colony counts should be greater than 0.5 $\log_{10}$ unit. Using this criterion, aqueous blueberry extracts appeared to have beneficial effects only for the growth of *L. acidophilus*.

Different concentrations of blueberries added to the medium had no effect on the growth of *L. acidophilus*, but seemed to have an effect on the growth of *L. rhamnosus*, with addition of 1 or 5% extracts significantly increasing the number of *L. rhamnosus* when compared to the 10% extract. It is interesting to note that there was no concentration-dependent increase in the numbers of both *L. acidophilus* and *L. rhamnosus* observed in the present study because lower concentrations (1 and 5%) of berry extracts seemed to enhance the survival of these lactic acid bacteria more than a high concentration (10%). It is possible that though the blueberry extracts contain a number of beneficial bioactive substances, adding a higher concentration may replace a significant amount of the medium causing a reduction in essential nutrients for the growth of the bacteria. In future experiments, it would be of interest to repeat this assay using concentrated extracts so that they could be tested using a very small volume and only small amount of broth would be displaced, and hence interfering less with the essential nutrients. However, the difference detected between various concentrations on the growth of *L. rhamnosus* has no biological importance as the difference was less than 0.5 $\log_{10}$ unit.

Table 4.4: Correlation coefficients (R^2) between the concentration of total phenolics, anthocyanins, antioxidant activity (FRAP and DPPH) and the ability to enhance the growth of lactic acid bacteria (*L. acidophilus* and *L. rhamnosus*) across all eight genotypes.

	Correlation coefficients (R^2)					
	L. acidophilus			*L. rhamnosus*		
	1% extract	5% extract	10% extract	1% extract	5% extract	10% extract
Total phenolics	0.27	0.10	0.02	0.17	0.16	0.14
Total anthocyanins	0.14	0.13	0.15	0.23	0.17	0.00
- Galactosylated ACNs	0.22	0.18	0.01	0.29	0.16	0.01
- Arabinosylated ACNs	0.29	0.15	0.17	0.26	0.12	0.05
- Galactosylated + Arabinosylated ACNs	0.24	0.18	0.11	0.28	0.15	0.02
- Glucosylated ACNs	0.23	0.12	0.02	0.18	0.01	0.00
- Acylated ACNs	0.26	0.45	0.09	0.11	0.03	0.00
- Glucosylated + Acylated ACNs	0.29	0.18	0.12	0.17	0.00	0.00
Chlorogenic acid	0.22	0.15	0.00	0.28	0.14	0.11
Ferric reducing antioxidant power (FRAP)	0.42	0.19	0.01	0.20	0.11	0.03
Scavenging DPPH radical ability	0.23	0.05	0.03	0.14	0.07	0.01

The mechanism by which bioactive compounds from fruits may enhance the growth of lactic acid bacteria is still not known. Suggested mechanisms are that the effect may be due to the ability of these phytochemicals to act as antioxidant agents, to modulate oxidative stress in the medium produced by the metabolic activities and consequently provide better environment for the growth of bacteria (Molan et al., 2009; Vodnar et al., 2012). However, these possibilities are not supported in the present study as we did not observe a significant correlation between FRAP or scavenging DPPH radical ability of the extracts and the population size of either probiotic bacterium. Furthermore, there were also no significant correlations between total phenolics or any specific substances measured and the growth of these bacteria (Table 4.4). However, a limitation of our study was a relatively small sample size used. As mentioned earlier, the measurements were conducted using aqueous extracts; however, anthocyanin analysis, was carried out with an independent set of extracts prepared using 5% formic acid to prevent anthocyanin degradation. Since the measurements were done on different extracts, there were no specific links between each set of data. Therefore, we could not perform a correlation between measurements from every single data point and we could only take a mean of each measurement and plot the correlation by variety (8 data points), thus making it difficult to see a significant association. If possible, further experiments should prepare a sample which allows all variables to be measured within the same extract in order to have more replicates for correlation determination.

Several possible factors can affect the growth rate of lactic acid bacteria including temperature, water activity, oxygen concentration, pH and medium composition (Zannini et al., 2005). It was reported earlier that the acid concentration in Highbush blueberries varies among varieties, and the cultivar 'Jersey' had a significantly higher concentration of acid than 'Bluecrop' and 'Duke' (Kampuse et al., 2009). Moreover, another study of Corcoran et al. (2005) found that the enhanced survival of the probiotic lactobaccilli occurred only in the presence of sugars that the bacteria could metabolise. The authors indicated that glucose and fructose, which *L. rhamnosus* could metabolise, enhanced the growth of probiotics in a simulated gastric juice model but lactose did not stimulate survival. Goderska et al. (2008) found that glucose and sucrose were easily utilized by *L. acidophilus* and addition of these sugars into the medium resulted in the highest total number of bacteria. We hypothesize that variation in acid concentration and/or types of sugar in each blueberry variety observed could lead to a variation in pH and sugar composition of the medium, and hence could affect the growth of these two probiotic bacteria.

Addition of sugars especially glucose to the medium has a significant effect on the growth of bacterial cells (Corcoran et al., 2005). Glucose as low as 1.0 mM results in the survival of *L. rhamnosus* to 4.0 $\log_{10}$ CFU/mL above the survival in the absence of glucose. However, a high glucose concentration (5 and 19.4 mM) can lead to only small increases in survival of bacteria (Corcoran et al., 2005). According to the information from the USDA food composition database, raw blueberry fruits contain approximately 10% sugar. Glucose and fructose are generally considered as the main sugars in mature blueberries and they are present in approximately equal proportions in Highbush and Lowbush spp. (Ayaz et al., 2001). Adding 1, 5 and 10% of blueberry extracts (1:1 dilution) in the context of our experiments are equal to approximately 1.4, 7 and 14 mM of glucose, respectively, supplemented into the medium, which could explain the very modest growth increases seen. The sugar present in blueberries resulted in another limitation to our study and it is necessary to take this limitation into account when carrying out any further experiments associated with sugar-containing fruit on the viability of bacterial cells. This could be achieved by having a negative control that contained medium plus sugar at the concentration equivalent to that present in the fruits for comparison. Having such a control would allow an accurate measurement of the growth of the bacteria that excludes the effect caused by the sugar contained in that fruits.

Although some aqueous blueberry extracts showed potential to promote the growth of pure cultures of lactic acid bacteria, specifically *L. acidophilus in vitro*, it remains uncertain whether the bioactive compounds in blueberries contribute to this beneficial effect or other unidentified factors could also be involved. However, it is believed that anthocyanins may be a potential source of nutrients for bacteria in the gut. A study mimicking the conditions of the distal human large intestine found that anthocyanins could be transformed by intestinal microbiota into small phenolic compounds such as gallic acid, syringic acid, *p*-coumaric acid, and pyrogallol, some of which have been found to be more effective than the native molecules for enhancing the growth of *Lactobacillus* spp. (Hidalgo et al., 2012). Therefore, consumption of anthocyanin-rich blueberries for altering the growth and composition of microbial community could be useful for the maintenance of gastrointestinal health. However, further *in vivo* research using animals or human volunteers is necessary to confirm these beneficial effects of blueberries.

Conclusion :

The management of metabolic syndrome is crucial for the early prevention of several chronic diseases including type II diabetes mellitus. This study indicated that aqueous extracts from New Zealand Highbush blueberries exhibited the potential to affect some markers of metabolic syndrome *in vitro* by inhibiting the action of α-amylase and α-glucosidase enzymes, which would likely offer an attractive preventative approach to the problem of postprandial hyperglycemia, and acceleration of the growth of a probiotic microorganism. In particular, two specific blueberry varieties, 'Burlington' and 'Bluecrop', showed significant inhibition against α-amylase and α-glucosidase, as well as the highest ability to enhance the growth of probiotics, especially *L. acidophilus*. These varieties were chosen for further confirmation of their ability *in vivo.* The present study did not find consistent links between antioxidant activities or any specific bioactive compound and inhibitory effects against α-amylase or α-glucosidase, or the growth of probiotic bacteria. Only a trend has been observed between the concentration of anthocyanins with glucose attached and inhibitory effect against α-amylase, but the R-square value was not statistically significant due to the small sample size. Nevertheless, beyond their anthocyanin concentration, there may be other bioactive compounds in blueberries which also contribute to these beneficial effects which were not measured in the present study (such as flavonols, procyanidins); therefore, there is a need for further investigation.

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CHAPTER 5

Effect of Highbush blueberry intake on markers of metabolic syndrome and oxidative stress in rats fed a high-fructose diet

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Our *in vitro* data showed that Highbush blueberries have a high concentration of phenolics and anthocyanins and have high levels of total antioxidant capacity as measured by the FRAP and DPPH assays. The aqueous extract from the tested blueberries also showed *in vitro* inhibitory potential against enzymes (α-glucosidase and α-amylase) involved in hyperglycemia, and the ability to enhance the growth of selected beneficial bacteria. These properties could lead to diminished glucose absorption, anti-hyperglycemia, and possible protection against insulin resistance. Since the *in vitro* findings provided the biochemical rationale, the present study was designed to further examine whether phytochemicals in Highbush blueberries were effective in normalizing parameters associated with metabolic syndrome *in vivo* using a fructose-induced metabolic syndrome Sprague Dawley rat model. The 'Burlington' cultivar was selected for testing initially because it exhibited strong inhibitory abilities against starch hydrolysis enzymes, as well as showed the highest total anthocyanin concentration, antioxidant capacity and probiotic bacterial growth, based on results obtained from *in vitro* experiments.

Abstract :

The aim of the present study was to investigate the effect of Highbush blueberry powder consumption on parameters associated with metabolic syndrome in a model of high fructose-fed Sprague Dawley (SD) rats. Animals (n=10/group) were fed with (1) control diet (60% starch), (2) high fructose diet (60% fructose), (3) high fructose diet + 1% 'Burlington' powder or (4) high fructose diet + 4% 'Burlington' powder for 8 weeks, and blood and tissues were collected for analysis. Body weight gain, fat mass, fasting glucose, insulin, lipids, leptin and adiponectin concentrations, and antioxidant status were found to be similar between the control- and the fructose-fed rats. In addition, no significant changes in these metabolic parameters were observed in rats on a fructose diet supplemented with blueberries, but only non-fat mass and total mass decreased in rats fed with fructose plus 1% blueberry diet. The results suggested that consumption of a 60% fructose diet for 8 weeks was not effective in inducing signs of metabolic syndrome in SD rats and thus the effect of blueberries on these metabolic biomarkers could not be proven in the present study.

Introduction :

High carbohydrate consumption has been documented to generate many features of metabolic syndrome (syndrome X), which includes insulin resistance, glucose intolerance, abdominal obesity, dyslipidemia and predisposition to the development of type II diabetes in Sprague Dawley rats (Bocarsly et al., 2010) and Wistar rats (de Moura et al., 2009). Different theories have been proposed to explain the mechanism behind the diet-induced pathophysiology of insulin resistance and obesity linked to MS. One of the major mechanisms is the impairment of insulin-stimulated glucose metabolism leading to hyperglycemia-induced insulin resistance (Groop et al., 1999). Some research also suggests that an increase in oxidative stress or insufficient antioxidant defence in individuals could be associated with hyperglycemia and hyperinsulinemia (Ceriello & Motz, 2004). In addition, a possible relationship between specific gut microbiota and low-grade inflammation linked to insulin resistance has been reported in the literature (Delzenne & Cani, 2010). As an alternative approach, diets containing bioactive compounds with an ability to inhibit postprandial hyperglycemia, and/or possessing antioxidant or prebiotic activity have received attention for preventing or diminishing insulin resistance and possibly decrease the risk of the metabolic syndrome induced by a high-carbohydrate diet.

Vaccinium corymbosum, also known as Highbush (cultivated) blueberries, are commercially produced in New Zealand. Blueberries have attracted interest not only because of the high concentration of phenolic phytochemicals, in particular anthocyanins, but also because blueberries have been reported to be used as a traditional medicine for diabetic treatment by Quebec (Canada) traditional practitioners for many years (Martineau et al., 2006). Studies from our laboratory have confirmed that various cultivars of Highbush blueberries exhibit high antioxidant capacity and their aqueous extracts are potent scavengers of DPPH radicals and have a strong Fe (III) reducing capability *in vitro.* Beyond their antioxidant capacity, our *in vitro* findings further supported the ability of Highbush blueberries to inhibit alpha-amylase and alpha-glucosidase, the enzymes involved in breaking down starch, which could lead to diminished glucose absorption and hyperglycemia. These extracts also possess the ability to enhance the growth of beneficial probiotic bacteria, another mechanism possibly associated with insulin resistance. However, the evidence in the literature appears to indicate that the bioavailability of anthocyanins is relatively low and hence they are poorly absorbed after consumption (Kay, 2006). For these reasons, the question remains whether increasing consumption of anthocyanin-rich fruits like blueberries which possess antioxidant activity and anti-prediabetic effects *in vitro* have any significant impact on parameters associated with metabolic syndrome physiologically.

In a rodent model for metabolic syndrome study, a diet containing 60% fructose (w/w) has been widely used in a number of earlier studies for inducing metabolic disorders in animals (Liu et al., 2007; Hininger-Favier et al., 2009; El Mesallamy et al., 2010; Kushwah et al., 2010; Sivakumar et al., 2010). Therefore, the present study was designed primarily to examine the effect of consumption of a selected Highbush blueberry ('Burlington') on serum antioxidant status and potential changes in metabolic parameters in rats with high-fructose diet-induced metabolic syndrome.

Materials and Methods :

Preparation of freeze-dried blueberry

The freeze-dried blueberry powder was made from ripe berries of the Highbush blueberry (*Vaccinium corymbosum*) cultivar ('Burlington'), which were obtained from 'Mamaku Blue', Rotorua, New Zealand. The whole blueberries were crushed using a food processor (Breville, MiniWizz Processor WT400, Australia), pre-frozen overnight and then freeze-dried to a

powder using the same method previously described in Chapter 3. Once dried, they were stored at -20^{o}C until used. Total anthocyanin concentration was determined by HPLC using a modification of the method of Wang et al. (2000) as previously described in Chapter 3.

Experimental diets

The formulation of the powder diets (table 5.1) was slightly modified from the studies of Suwannaphet et al. (2010) and Khanal et al. (2010). The control powder diet (CONT) contained 60% carbohydrate mainly from starch, 15% casein, 5% fat, 5% cellulose, 5% minerals and 1% vitamin mix, meeting the nutrient requirements of growing rats as defined by the AIN-93G diet. The fructose diets (HFR) contained 60% fructose instead of starch, whereas the remaining ingredients were held constant. The freeze-dried blueberry powder was mixed with fructose diets at a dose of 1% or 4% by weight. Rats were given freshly-thawed food once daily to avoid degradation of anthocyanins.

Table 5.1 : Composition of the experimental diets.

Ingredients (g/kg diet)	Control	High fructose	High fructose + 1% freeze-dried blueberry	High fructose + 4% freeze-dried blueberry
A. Ingredient composition				
Sodium caseinate	150	150	150	150
Cysteine	2.7	2.7	2.7	2.7
Glycine	3.3	3.3	3.3	3.3
Methionine	1.5	1.5	1.5	1.5
Glutamine	7	7	7	7
Calcium carbonate	12.5	12.5	12.5	12.5
Cellulose	50	50	50	50
Vitamin mix	10	10	10	10
Mineral mix	50	50	50	50
Soybean oil	50	50	50	50
Corn starch	500	-	-	-
Sucrose	100	-	-	-
Fructose	-	600	600	600
Maltodextrin	63	63	53	23
Freeze-dried blueberry powder	-	-	10	40
Total (g)	1000	1000	1000	1000
B. Nutrient composition*				
Crude protein, %	16.0	15.1	15.7	15.5
Fat, %	5.3	5.1	5.2	5.3
Carbohydrate, %	67.8	71.8	70.8	70.8
Energy, kJ/g	17.2	17.3	17.4	17.5

* Nutrient composition was analysed by the Nutrition Laboratoy, Massey University; protein, fat and carbohydrate by proximate analysis, and gross energy by bomb calorimeter.

Animals and Treatments

The animal protocol was approved by the Massey University Animal Ethics Committee (protocol approval #11/39). Forty male Sprague Dawley rats at 6 weeks of age were obtained from the Small Animal Production Unit (SAPU) and were housed individually in cages in a temperature controlled room (22±1 ^{o}C) and a 12-hour light/dark cycle. Animals were accorded a 7-day adaptation period to the normal powder diet and environmental conditions. They were then randomly divided into four groups (n = 10/group) according to their initial body weights and were treated as follows: (1) continued feeding with normal powder diet (control); (2) switched to high-fructose diet (HFR); (3) switched to high-fructose diet containing 1% freeze-dried blueberry powder (HFR1B); and (4) switched to high-fructose diet containing 4% freeze-dried blueberry powder (HFR4B). All animals were maintained in their respective groups for 8 weeks and they were given *ad libitum* access to food and water at all times.

Dietary consumption of animals was monitored daily and body weights were recorded weekly. Every two weeks throughout the study, rats were food-restricted overnight and blood glucose was measured from a drop of blood taken by tail puncture using a OneTouch Ultra glucometer (Life Scan, Inc., Milpitas, CA, USA). One week before the termination of the experiment (week 7), an oral glucose tolerance test (OGTT) was carried out. Rats were fasted overnight before the OGTT was performed by gavaging the conscious rats with a 40% glucose solution at a dose of 2 g of glucose/kg body weight. Blood samples were collected at 0, 30, 60, 90 and 120 min after glucose administration, and glucose concentrations were measured using a OneTouch Ultra glucometer. The calibration of this instrument was verified prior to the test using test strips and artificial blood samples provided by the manufacturer.

Dual-energy x-ray absorptiometry (DEXA) scans

At the end of the study (week 8), animals were DEXA scanned under anaesthesia. For the measurement, each rat was weighed and anaesthetised with an appropriate dose of anaesthetic (0.07 ml/ 100 g body weight). The anaesthetic cocktail, which consisted of a mixture of 0.2 ml of Acepromazine (ACP), 0.5 ml of Ketamine, 0.1ml of Xylazine and 0.2 ml of sterile water in 1 ml total volume, was administered via intraperitoneal injection using a 25G x 5/8” needle and 1 ml syringe. The animals attained a suitable level of anaesthesia approximately 5–10 min after injection and remained under anaesthesia for 2 h.

Body composition was measured with a Hologic Discovery A bone densitometer (Bedford, MA, USA). A daily quality control scan was taken to ensure the precision met the required coefficient of variation. The coefficient of variation for the quality control data was 0.98-1.01. Rats were positioned supine with right angles between the spine and femur and between the femur and tibia, and underwent a high-resolution scan of the whole body.

Euthanasia and tissue collection

Following the DEXA scan, a single blood sample from the overnight fasted-rats was drawn directly from the heart under deep anaesthesia, leading to euthanization through exsanguination, and the rat was dissected. The organs including the liver, kidneys, and caecums were removed and white adipose tissues (retroperitoneal, mesenteric and lower epididymal fat) and interscapular brown adipose tissues (iBAT) were harvested and weighed immediately. Blood samples were allowed to clot at room temperature for 30 min and centrifuged at 3500 rpm for 15 min. Serum was stored at -80^{o}C and biomarkers related to metabolic syndrome including glucose, insulin, leptin, adiponectin, lipid profiles and total antioxidant activity were later analysed.

Blood measurements

The concentration of glucose was determined by the hexokinase method on Vitalab Flexor analyzer using a commercially available kit (Cat. #11447513 216, Roche New Zealand), and insulin levels were assayed using a radioimmunoassay kit (Cat. # RI-13K, Merck Millipore, New Zealand). Total cholesterol, HDL-cholesterol and triglycerides were assayed by enzymatic methods with commercial kits (Cat. #12016630 122, #04713109 190 and #12016648 122, respectively) purchased from Roche, Auckland, New Zealand. Serum leptin was measured by double antiobody radioimmunoassay (Cat. # RL-83K, Merck Millipore, New Zealand), and serum adiponectin was determined using coated microspheres Luminex xMAP technology (Cat. # RADPK-81K-ADPN, Merck Millipore, New Zealand). Total antioxidant capacity was assessed by the FRAP assay according to the procedures described by Molan et al. (2008).

Statistical analysis

Data are expressed as mean ± S.E. The effect on each parameter between groups was statistically analyzed by one-way analysis of variance (ANOVA) followed by the Tukey's test for mean comparison. Statistical analysis on weekly body weight gain, biweekly blood glucose concentration and glucose tolerance curve between groups were compared by repeated

measures ANOVA. All statistical treatments were analyzed with SAS version 9.2 for windows and *P* values less than 0.05 were considered to be statistically significant. The sample size (10 rats per group) was calculated based on an expected difference in blood glucose levels of 4.1 mmol/L at a power of 90% and a significance level of 5%.

Results :

As shown in Figure 5.1, average food and energy consumed did not differ significantly among the four groups, ranging from the lowest found in the HFR+1%BB group (26.5 g/day) to the highest in the CONT group (28 g/day), indicating that neither fructose diet nor administration of blueberry powder affected food intake of SD rats. The CONT, HFR, and HFR supplemented with blueberry diets were formulated to be isocaloric, with each providing 17.2-17.5 kJ of energy/g of diet (Table 5.1). The freeze-dried blueberry ('Burlington') powder contained total anthocyanins at a concentration of 15.8 mg/g freeze-dried weight. Therefore, rats in the HFR+1%BB group consumed an average of 26.5 g of diet/day, which provided 4.2 mg of anthocyanins/day, whereas rats in the HFR+4%BB group consumed an average of 27 g of diet/day, which provided 17.0 mg of anthocyanins/day.

	Control	High fructose	HFR + 1% BB	HFR + 4% BB
Food consumption (g/d)	28.2±0.4	27.0±0.5	26.5±0.7	27.2±0.7
Energy intake (kJ/d)	485.7±6.4	466.3±9.3	460.6±11.8	476.7±11.9

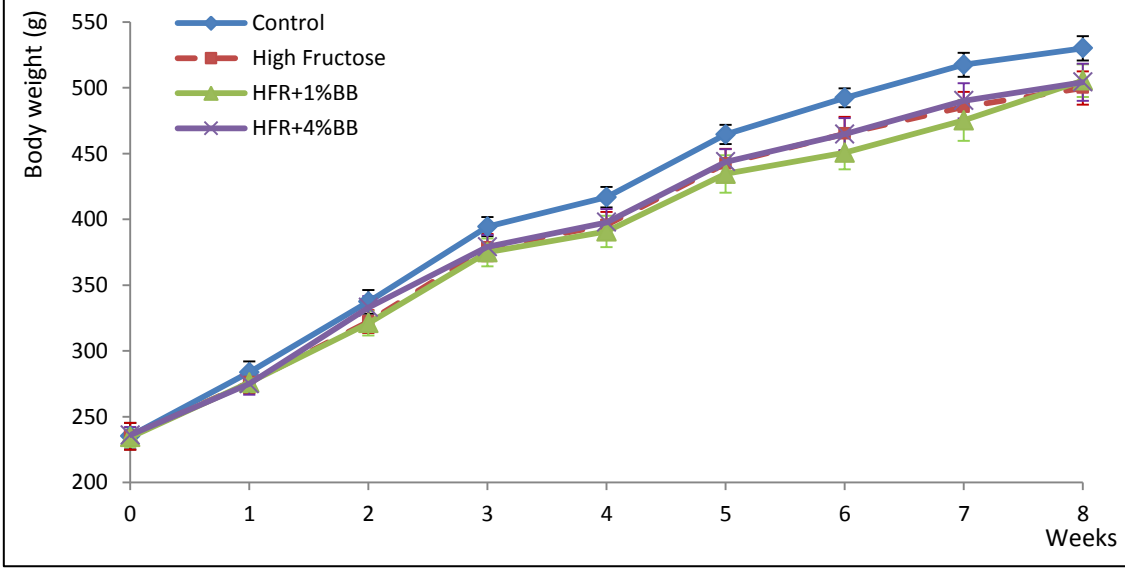

Figure 5.1: Average diet consumption, average total energy intake, and weekly body weight change in rats fed a control diet, a high-fructose diet, or a high-fructose diet supplemented with 1% or 4% freeze-dried blueberry for 8 weeks. Results are expressed as mean ± S.E. of N = 10 rats per group and N = 7 rats for HFR+1%BB group from week 7 onwards (three rats in the HFR+1%BB group died after gavaging at week 7).

At the end of the experiment, unexpectedly we observed no indication that high fructose diet consumption for 8 weeks led to the development of metabolic disorders in SD rats since all metabolic parameters measured in the present study were found to be similar between the control and high-fructose groups. Indeed, control rats appeared to have slightly higher levels than the fructose-fed rats in some particular biomarkers including body weight gain, adipose tissue weights, glucose, insulin and leptin concentrations, as well as glucose tolerance. Although there were no significant differences in average daily food and energy intake between groups, rats fed a control diet had slightly higher diet and energy consumption when compared to the other three groups, hence this group of rats showed a slightly higher increase in body weight starting from week 3 until the end of the study (Figure 5.1). Higher consumption found in the control group may partially explain why this group of rat exhibited higher levels of various signs of MS over the high fructose-fed group.

Table 5.2: Effect of experimental diets on whole body composition, adipose tissue weights and organ weights in rats at the end of study (week 8).

Parameter measured	Control (n=10)	High Fructose (n=10)	HFR+1%BB (n=7)	HFR+4%BB (n=10)
Whole body composition				
Fat mass (g)	87.9±7.7	86.9±8.0	80.0±6.7	87.9±7.5
Non-fat mass (g)	431.9±6.4	401.4±7.5	388.9±13.7*	403.0±12.4
Total mass (g)	519.8±8.9	488.3±12.3	468.8±15.8*	491.0±13.1
Percentage of fat	16.8±1.3	17.6±1.3	17.0±1.2	17.9±1.4
Fat pad (g/kg BW)				
Retroperitoneal	20.9±2.8	20.0±2.5	21.7±2.7	19.7±1.7
Mesenteric	8.9±0.8	8.1±0.8	8.2±1.1	10.1±1.1
Epididymal (lower part)	0.6±0.1	0.6±0.04	0.5±0.03	0.6±0.04
iBAT	1.4±0.2	1.3±0.1	1.3±0.1	1.2±0.1
Organs (g/kg BW)				
Liver	27.7±1.0	29.3±0.9	31.0±1.7	29.6±0.7
Kidneys	7.1±0.3	7.8±0.2	7.9±0.4	7.8±0.2
Caecum	4.2±0.2	4.8±0.3	4.3±0.3	4.5±0.3

Results are expressed as mean ± S.E.
*$P < 0.05$ by one-way ANOVA compared to CONT group at the same time.
Three rats in the HFR+1%BB group died after gavaging at week 7.

When blueberries were added into the high-fructose diet, overall results showed little evidence of a beneficial effect of blueberries on the prevention of MS. In term of signs of obesity, no differences among treatments were observed in body weight gain (Figure 5.1), fat mass, percentage of fat and all adipose tissue weights (Table 5.2) after 8 weeks on their respective

diets. However, non-fat mass and total mass as measured using DEXA were found to be significantly lower ($P < 0.05$) in the rats fed HFR supplemented with 1%BB as compared to the control (Table 5.2). Again, the reason may, in part, due to lower feed intake as well as energy obtained by the HFR+1%BB group.

Table 5.3: Effect of experimental diets on blood measurements of the overnight fasting rats at the end of the experiment (week 8).

Parameter measured	Control (n=10)	High Fructose (n=10)	HFR+1%BB (n=7)	HFR+4%BB (n=10)
Glycemic parameters				
Glucose (mmol/L)	14.6±1.4	12.3±0.8	13.7±1.2	13.8±1.2
Insulin (ng/mL)	0.4±0.2	0.2±0.1	0.2±0.1	0.6±0.3
Lipid profiles				
Triglycerides (mmol/L)	1.0±0.1	1.1±0.1	1.2±0.2	1.2±0.1
Total cholesterol (mmol/L)	2.1±0.1	2.1±0.2	2.1±0.2	2.1±0.1
HDL-cholesterol (mmol/L)	1.2±0.1	1.2±0.1	1.2±0.1	1.1±0.1
Adipocytokines				
Leptin (ng/mL)	5.9±0.6	5.4±0.5	5.6±0.8	6.2±0.6
Adiponectin (µg/mL)	35.5±2.6	33.0±1.7	35.2±4.1	38.5±5.1
Antioxidant status				
FRAP (mg $FeSO_4$/mL)	0.3±0.01	0.3±0.01	0.3±0.01	0.3±0.01

Results are expressed as mean ± S.E.
Three rats in the HFR+1%BB group died after gavaging at week 7.

Similarly to obesity parameters, blueberries supplemented to the HFR diet had no effect on various blood biomarkers associated with insulin resistance. Fasting lipid profiles (total cholesterol, triglycerides and HDL-cholesterol), glucose, insulin, leptin and adiponectin concentrations, as well as total antioxidant status in serum were also similar in all four groups (Table 5.3). However, supplementation of blueberries at dosage of 1% into fructose diet appeared to show a trend to improve glucose tolerance as the animals in this group showed decreased blood glucose levels at 30 and 60 min after glucose load compared to the CONT and HFR groups, although the values did not reach significance (Figure 5.2). Notably, although the different diets did not affect the change in body weight gain and blood glucose level of all groups, a repeated measure analysis over the 8 weeks indicated that these two parameters significantly increased with time ($P < 0.01$), with animals at older age showing heavier weight gain and higher blood glucose concentrations than the younger animals (Table 5.4).

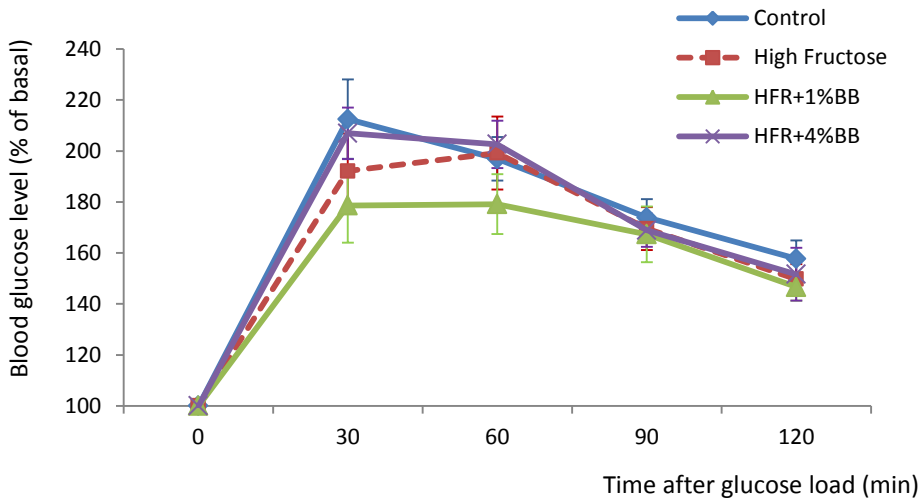

Figure 5.2: Oral glucose tolerance test responses in rats fed a control diet, a high-fructose diet, or a high-fructose diet supplemented with 1% or 4% freeze-dried blueberry for 8 weeks. Results are expressed as mean ± S.E. of N = 10 rats per group and N = 7 rats for HFR+1%BB group (three rats in the HFR+1%BB group died after gavaging at week 7).

Table 5.4: Effect of experimental diets on blood glucose concentration taken via tail puncture at week 2, 4, 6 and 8.

Time period	Blood glucose level (mmol/L)			
	Control	High Fructose	HFR+1%BB	HFR+4%BB
Week 2	2.9±0.2	3.1±0.3	3.2±0.3	2.8±0.1
Week 4	5.1±0.2	4.9±0.2	5.1±0.3	4.7±0.2
Week 6	6.9±0.4	6.5±0.2	6.4±0.3	6.6±0.4
Week 8	13.3±1.1	11.9±0.7	12.5±1.8	13.2±1.0

Results are expressed as mean ± S.E. of N = 10 rats per group and N = 7 rats for HFR+1%BB group at week 8. Three rats in the HFR+1%BB group died after gavaging at week 7

Discussion :

High fructose feeding in animals has been commonly used as a model for studying the metabolic parameters associated with the metabolic syndrome, a complex disorder closely linked to an increased risk for various non-communicable diseases such as CVD and diabetes (Khanal et al., 2010). An increased dietary intake of fructose has been widely documented to cause several serious adverse metabolic effects in rodents. For example, D'Angelo et al. (2005) reported significant increases in fasting plasma glucose, plasma insulin and plasma triglycerides in Sprague Dawley (SD) rats after being fed with a high fructose diet (66% of total calories) for 8 weeks. Al-Awwadi et al. (2005) demonstrated that a diet containing 66% fructose given to SD

rats for 6 weeks induced an increase in the HOMA index of insulin resistance, triglycerides, phospholipid and non-HDL-cholesterol as compared to chow-control fed SD rats. In addition, Suwannaphet et al. (2010) found that insulin resistance can develop within 8 weeks when animal are fed a high fructose diet containing 63% fructose relative to those fed with mainly corn starch. Another study using Wistar rats by Reddy et al. (2009) found elevated levels of blood glucose, insulin and triglyceride, impaired glucose tolerance and impaired insulin sensitivity in animals fed with 66% fructose for 60 days. Hininger-Favier et al. (2009) indicated that signs of insulin resistance including hyperglycemia, hypertriglyceridemia and hyperinsulinemia can develop within 6 weeks in Wistar rats receiving the high-fructose (60% w/w) diet but not in those of the standard chow group. A study investigated an effect of high fructose feeding in form of drinking water (15% fructose solution), similarly, the fructose-fed Wistar rats showed significant increase in plasma levels of insulin, the HOMA index of insulin resistance, and systolic blood pressure after 4 weeks of consumption (Jin et al., 2010).

In contrast to expected outcomes based on earlier studies reported in the literature, inclusion of 60% fructose in the diet in the current study did not produce a significant response in any biomarkers associated with metabolic syndrome in Sprague Dawley rats. One possibility is that several previous studies used a ‘‘chow’’ as a control and made a comparison with a high-fructose (treatment) diet without balancing all the nutrients in the process (Harati et al., 2004; Al-Awwadi et al., 2005; D'Angelo et al., 2005; Hininger-Favier et al., 2009; Kushwah et al., 2010). Chow diets contain natural plant-based ingredients which may vary in nutrient composition according to the time of harvest, whereas purified diets vary by only a single ingredient (i.e. fructose replaces starch) having little variability and hence providing more consistency between batches. Therefore, it is difficult to make a comparison when chow and semi-synthetic diets are used together in an experiment. Furthermore, chow diets may also contain some phytochemicals such as phytoestogens which have been found to have a minimizing effect on some parameters of MS (Gajda, 2008). This could create confounders, and observed beneficial effects found in studies could be the result of the treatment diets or due to other unknown ingredients in the diets. In order to avoid these discrepancies, all diets used in the current study were isocaloric and contained the same ingredients which were closely matched between treatment diets.

Another possible factor leading to our null effect may involve the characteristic of fructose itself; it has been reported in several studies that fructose has a lower glycemic index than other types of sugar or other foods rich in starch and hence produce a smaller glycemic response than that

of glucose and sucrose (Crapo et al., 1982; Kim et al., 1988; Blaak & Saris, 1995). Metabolism of fructose, unlike glucose, is not dependent on insulin so an increase of fructose in the blood circulation does not stimulate insulin secretion from the pancreatic β-cells, therefore fructose ingestion consequently causes only a limited rise in insulinemia (Bray et al., 2004). Moreover, because leptin production is regulated by the insulin response to meals, the lower insulin response caused by fructose could also lead to a smaller production of circulating leptin (Elliott et al., 2002). Interestingly, some nutritionists believe that fructose is a safer form of sugar than sucrose or glucose, particularly for people with type II diabetes, because moderate fructose intake does not adversely affect blood glucose and insulin levels, at least in the short-term (Gaby, 2005; Tappy et al., 2010). For these reasons, when fructose is given to animals, it is difficult to discern clear signs of metabolic syndrome when compared to those on a starch-control meal. Although we did not observe the deteriorative effect of fructose on MS parameters in this study, some previous publications documented its deleterious effect when consumed for a long period of time (Blakely et al., 1981; Lê & Tappy, 2006). It is possible that the present experiment may not have been long enough to significantly induce symptoms of MS using a high fructose diet.

The inability of a high fructose diet to modify biomarkers of metabolic syndrome in animals has also been reported in some previous studies. For instance, Stark et al. (2000) fed male SD rats with a high fructose (53%) diet for 3 months and found no differences in various metabolic parameters measured including weight gain, fasting glucose and insulin levels, circulating lipid profiles (cholesterols and triglycerides) and signs of glucose intolerance. A study of Khanal et al. (2010) demonstrated that feeding a fructose diet to SD rats could not increase fasting plasma glucose, abdominal fat, body weight or impaired glucose tolerance in these rodents. In line with two earlier rodent studies, a report in rhesus monkeys indicated that an infusion of glucose markedly increased plasma glucose and insulin concentrations, and progressively increased plasma leptin, whereas fructose infusion only modestly increased plasma glucose but did not increase circulating insulin and leptin concentrations (Havel, 1997). Another study in humans by Teff et al, (2004) found that dietary fructose (consumed with mixed meals) failed to stimulate circulating insulin and leptin concentrations compared to dietary glucose.

Even though the trend towards a beneficial effect of 1% blueberry supplementation on decreasing total mass as measured by DEXA and improving glucose tolerance found remains unexplained in the present study, we did not observe an improved effect with 4% blueberry

supplementation. This result is in line with the study of Prior et al. (2010) in which the effect of blueberries on metabolic parameters was not a dose-dependent relationship. In addition, our results also support the notion that some phytochemicals, in particular anthocyanins are poorly absorbed in the GI tract and hence only small amounts can be detected in the circulation (McGhie & Walton, 2007). There were no changes induced by the phytochemicals in antioxidant status (measured by FRAP assay) in the serum of rats fed with freeze-dried blueberries possibly due to the poor absorption. Indeed, these values were almost similar to those of the control groups (without blueberries). Because their circulating levels are found to be considerably low, bioactive compounds in blueberries are unlikely to make a contribution to their beneficial effect *in vivo* by acting as antioxidant in the blood directly.

Since evidence in the literature supporting the feeding of a high fructose diet to develop a response of metabolic syndrome is conflicting, and no clear development of symptoms of metabolic disorder due to a high fructose diet was found in the present study. Prior to testing the ability of a phytochemical-enriched diet on prevention or delaying onset of metabolic syndrome, a diet-induced MS animal model should be first established and validated to ensure that the treatment results obtained are reliable. For this reason, the next study was conducted in an attempt to find an appropriate diet which could induce symptoms of MS in Sprague Dawley rats in a short period of time, to be then used as a model for investigating the effect of phytochemical-rich fruits on features associated with metabolic syndrome.

Notably, the oral glucose tolerance test (OGTT) is a useful marker for measuring insulin resistance in animals at dynamic state. However, this test is considered invasive, causes much stress and pain to experimental animals and hence three of our rats from the 1% blueberry treatment group died unexpectedly at the step of being gavaged with a glucose solution, representing the three missing samples in this group in all MS parameters measured on week 7 onwards. Gavaging a solution to rats appears to be not appropriate, in particular for those of larger body size, therefore droplet-fed glucose solution was chosen to be used instead of gavaging in the next study in order to minimise stress and reduce pain to the experimental animals.

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CHAPTER 6

Short-term and long-term effects of excessive consumption of saturated fats and/or sucrose on metabolic variables in Sprague Dawley rats: a pilot study

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The first animal study found that feeding Sprague Dawley rats for 8 weeks with a diet containing 60% carbohydrate (fructose) and 5% fat (soybean oil) failed to induce several symptoms associated with metabolic syndrome. This might be due to the type and amount of sugar and fat included in the diets which may not have been extreme enough to induce insulin resistance. According to Stark et al. (2000), healthy growing Sprague Dawley rats may be able to adapt to the intervention diets without developing metabolic syndrome. Since types and amounts of fat and sugar, as well as the length of feeding period, are crucial for establishing diet-induced metabolic syndrome in the Sprague Dawley rat model, the present study was designed to investigate short-term (4 weeks) and long-term (8 weeks) effects of excess consumption of saturated fat (lard) and/or sucrose incorporated into the diet in order to establish an appropriate model for inducing symptoms of metabolic syndrome in Sprague Dawley rats.

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Abstract :

Growing evidence supports the notion that feeding high-fat and/or high-sugar diets to rats leads to a change in markers of metabolic syndrome. However, types and amounts of fat and sugar as well as the length of the experiment for establishing diet-induced metabolic syndrome in the rat model, especially in Sprague Dawley (SD) rats, remain uncertain. The objective of this study was to examine the effects of consuming excess lard and/or sucrose on several biomarkers related to metabolic syndrome including weight gain, abdominal fat mass, body composition, fasting blood glucose, insulin concentration and glucose tolerance in SD rats. Four groups of 9 week-old male SD rats (n=5/group) were *ad libitum* fed either a control diet (CONT; 5% soybean oil, 60% corn starch), a high-sugar diet (HS; 5% lard, 60% sucrose), a high-fat-high-sugar diet (HFHS; 20% lard, 45% sucrose), or a high-fat diet (HF; 35% lard, 30% corn starch). Body weights were measured weekly, while remaining metabolic parameters were measured at week 4 (short-term effect) or week 8 (long-term effect). Consumption of the combination of HFHS significantly increased weight gain and abdominal fat weights ($P < 0.05$), and the animals also began to develop signs of impaired glucose tolerance and insulin resistance (11%, 34% and 58% increases at week 4, and 13%, 50% and 73% increases at week 8 in fasting glucose, insulin concentrations, and HOMA-IR index, respectively compared to the control group). The HF diet mainly affected weight gain and fat deposition in animals, whereas feeding the rats with HS diet seemed to induce glucose intolerance, but not the obesity-related parameters. Animals fed the starch-based control diet showed a tendency towards insulin resistance and glucose intolerance when feeding for long-term period. The lard plus sucrose-based HFHS is the most efficient diet for inducing signs of MS and SD rats fed this diet for 8 weeks successfully develop obesity and insulin resistance which can be used as a model for MS research.

Introduction :

Metabolic syndrome (MS) is characterized by abdominal obesity, dyslipidemia, hypertension, and elevated blood glucose. These symptoms have been recognized to be key risk factors for development of various chronic diseases, such as type II diabetes (Grundy et al., 2004; Streja, 2004). Both genetic and environmental factors contribute to this development, and diet represents an important environmental factor that can influence a metabolic disorder. Specifically, the detrimental effect of saturated fats and simple carbohydrates are of concern because chronic consumption of such macronutrients leads to excess circulating glucose and

free fatty acids (FFA), which may contribute to the development of insulin resistance (Arner, 2002; Tomas et al., 2002).

Animal models, especially rodents, are commonly used to mimic human diseases to improve understanding of the causes and development of disease symptoms. High fat diets used in laboratory animal research vary between 30% and 60% of calories from saturated fat such as lard, beef tallow, or coconut oil. The sugar content in the experimental diets typically comprises 60-65%, and the common sugars used are sucrose and fructose (Gajda et al., 2007; Hariri & Thibault, 2010; Panchal & Brown, 2011).

Growing evidence from animal studies indicates that feeding high fat and/or high sugar diets to rats leads to insulin resistance, impaired glucose metabolism, and hyperlipidemia (Gajda et al., 2007). Jiao et al. (2008) found that Sprague Dawley (SD) rats can develop insulin resistance within 3 to 4 weeks after being fed a high fat diet (23% lard and 21% sucrose). In addition, Suwannaphet et al. (2010) reported that insulin resistance can develop within 8 weeks when SD rats are fed a high sugar diet containing 63% fructose relative to those fed with mainly corn starch.

In contrast, a previous study by Stark et al. (2000) showed no development of any signs of insulin resistance and impaired glucose tolerance in SD rats fed either a high fructose (53%) or high fat (25% soybean oil) diet for 3 months. Khanal et al. (2010) also reported no increase in fasting plasma glucose, abdominal fat accumulation or body weight and impaired glucose tolerance in growing rats fed a high fructose diet (53% w/w). Importantly, our previous animal trial also found that feeding rats for 8 weeks with a diet containing 60% fructose plus 5% soybean oil failed to induce obesity or insulin resistance in SD rats. Since data with regards to diet-induced metabolic syndrome in SD rat model obtained from literature remain inconsistent, it is hypothesized that different types and amounts of diet as well as the period of time the animals are exposed to the experimental diet may significantly affect metabolic parameters in SD rats. Therefore, the objective of the present study was to compare the effects of consuming HF, HS and their combination (HFHS) diet for a short time (4 weeks) and a long time (8 weeks) on various markers related to metabolic syndrome in order to find out the suitable diet and optimal experimental period for establishing diet-inducing metabolic disorders using the SD rat model.

Materials and Methods :

Diets

Four experimental powdered diets: control (CONT), high-sugar (HS), high-fat (HF), and high-fat-high-sugar (HFHS) were prepared to meet the nutrient requirements of laboratory animals (Modified AIN-93G; National Research Council, 1995). All ingredients were purchased from local suppliers within New Zealand; sodium caseinate (Tatua, Waikato), corn starch, soybean oil and sugar (Davis Trading, Palmerston North), lard (Mad Butcher, Palmerston North), purified amino acids (cysteine, glycine, methionine, and glutamine), and calcium carbonate (Merck, Auckland), cellulose (Hawkins Watts, Auckland), and vitamin mix (Unitech Industries, Auckland). The diet composition and the nutritive values are shown in Table 6.1.

Table 6.1 : Composition and nutritive values of the experimental diets.

Ingredients (g/kg diet)	Control (CONT)	High sugar (HS)	High fat (HF)	High fat high sugar (HFHS)
A. Ingredient composition				
Sodium caseinate	200	200	200	200
Cysteine	2.7	2.7	2.7	2.7
Glycine	3.3	3.3	3.3	3.3
Methionine	1.5	1.5	1.5	1.5
Glutamine	7	7	7	7
Calcium carbonate	12.5	12.5	12.5	12.5
Cellulose	50	50	50	50
Vitamin mix	10	10	10	10
Mineral mix	50	50	50	50
Lard	0	48	320	170
Soybean oil	50	30	30	30
Corn starch	513	0	263	0
Sugar	100	585	50	463
Total (g)	1000	1000	1000	1000
B. Nutrient composition*				
Crude protein (g/100g)	19.8	18.8	19.5	19.6
Total fat (g/100g)	5.2	7.8	35.1	19.9
Saturated fat	0.8 (16%)	2.4 (31%)	13.1 (39%)	7.1 (37%)
Monounsaturated fat	1.2 (24%)	2.9 (38%)	15.2 (45%)	8.4 (44%)
Polyunsaturated fat	3.0 (60%)	2.3 (30%)	5.3 (16%)	3.6 (19%)
Carbohydrate (g/100g)	64.1	68.5	37.5	55.3
Starch (g/100g)	51.3	0	26.3	0
Sugar (g/100g)	9.3	49	2.2	38
Energy, kJ/100g	1750	1880	2440	2130
Energy from protein	360 (23%)	360 (22%)	360 (16%)	360 (19%)
Energy from fat	188 (12%)	294 (18%)	1319 (60%)	754 (40%)
Energy from starch	859 (55%)	0 (0%)	440 (20%)	0 (0%)
Energy from sugar	167 (11%)	980 (60%)	84 (4%)	775 (41%)

* Crude protein, fat, carbohydrate and energy were analysed by the Nutrition Laboratory, Massey University. The remaining data was calculated based on diet composition.

Animals and Treatments

Forty male Sprague Dawley rats aged 9 weeks (reared in the Small Animal Production Unit, Massey University, Palmerston North, New Zealand) were individually housed in a temperature controlled room (22 ± 1 ^{o}C) and a 12-hour light/dark cycle. All animals were fed the control (starch-based) diet for 7 days of acclimatisation. Rats were then randomised by body weight into four groups composed of ten rats each and given test diets: (1) CONT diet; (2) HS diet; (3) HF diet; (4) HFHS diet. Diets were replaced daily and supplied *ad libitum* except during the fasting period prior to anaesthesia. Feed and water intakes were measured daily. Body weights were recorded weekly.

Half the rats from each group were euthanased after 4 weeks on the test diet (short-term), while the remainder were euthanased after 8 weeks (long term). Three days before euthanasia, rats were fasted overnight before an oral glucose tolerance test (OGTT) was performed. Fasted animals were droplet-fed a 120% glucose solution (at the dose of 2 g glucose/ kg body weight). Drops of blood were taken via tail vein puncture at times 0, 30, 60, 90 and 120 min after glucose administration and analyzed for glucose using a OneTouch Ultra glucometer (Life Scan, Inc., Milpitas, California 95035, USA).

On the final day, rats were anesthetised, scanned for whole body composition using dual-energy X-ray absorptiometry (DEXA) under anaesthesia as described in Chapter 5, and euthanased for collection of blood and tissue samples including liver, kidneys, pancreas, caecum as well as white adipose tissues (retroperitoneal, mesenteric and epididymal) and interscapular brown adipose tissues (iBAT). For the purposes of the studies described in this thesis, the adipose tissue lining the dorsal peritoneal cavity, excluding the embedded kidneys, were defined as retroperitoneal fat; the adipose tissue connecting the intestines, including the embedded mesenteric lymph nodes, were defined as mesenteric fat; the large anterior portion of the epididymal fat pad was defined as upper epididymal fat; the small posterior portion of the epididymal fat pad attached directly to the testes was defined as lower epididymal fat. All biological tissues were snap-frozen immediately in liquid nitrogen and stored at -80^{o}C until time of assay. All animal experiments were carried out with the approval of the Massey University Animal Ethics Committee (protocol approval #11/84).

Biochemical variables

Blood samples were collected and separated into 2 tubes. For plasma, blood were collected on fluoride-coated tube and immediately centrifuged at 3500 rpm for 15 min for the glucose determination. The rest of samples were allowed to clot at room temperature for approximately 30 min, then centrifuged at the same condition to recover serum, which was used for the assay of insulin.

Plasma glucose was determined using the hexokinase method on Vitalab Flexor analyzer with commercially available kit (Cat. #11447513 216, Roche New Zealand). Insulin concentrations were assayed in serum samples using a radioimmunoassay kit (Cat. # RI-13K, Merck Millipore, New Zealand). The degree of insulin resistance was calculated according to the homeostasis model assessment for insulin resistance (HOMA-IR) using the following formula: [fasting glucose (mmol/L) x fasting insulin (μIU/mL)/22.5] (Bonora et al., 2000).

Statistical analysis

Data are expressed as mean ± S.E. Statistical analysis was performed either by one-way or two-way analysis of variance (ANOVA) where appropriate. The effect of various diets on weekly animal weight gain and incremental blood glucose at each time point after glucose challenge were compared using repeated measures ANOVA. Tukey's test was used for mean comparisons and $P < 0.05$ was considered to be statistically significant. Simple linear regression analysis (with coefficients of determination, R^2) was done to determine the correlation between two variables. All statistical tests were analyzed using the SAS program for Windows version 9.2 (SAS Institute Inc., Cary, NC, USA). Since the present study is a pilot study, the power analysis was not used for sample size calculation.

Results :

Food consumption and body weight

At the end of the acclimatization period (week 0), no significant difference was observed in food consumption or energy intake among the four groups. They were then switched onto their test diets (HF, HFHS, HS, or CONT) for either a short-term (4 weeks) or a long-term (8 weeks) period. Generally, the average daily food consumption was not significantly different

between rats fed the HFHS diet and the HS diet compared to the CONT group, ranging between 25 g and 27 g/day; whereas rats fed the HF diet consumed the lowest amount of food (22 g/day) throughout the study. However, energy intakes in rats fed the HF as well as HFHS diets (ranging between 538 and 555 kJ/day) were higher than in HS-fed rats (approximately 500 kJ/day), and were significantly different ($P < 0.05$) from the CONT rats (approximately 450 kJ/day) (Table 6.2).

Table 6.2: Average dietary consumption, average energy intake and body weight in rats fed a HF, a HFHS, a HS, or a CONT diet during the adaptation period (week 0), and over the short-term (week 1-4) and long-term (week 1-8) periods.

Study period	Diets	Parameters				
		Diet intake (g/d)	Energy intake (kJ/d)	Body weight at the end of each period (g)	Total weight gain (g)	Total weight gain (%)
Adaptation period (week0)	HF	26.9±0.8^{a}	470.8±13.4^{a}	361.4±7.7^{a}		
	HFHS	28.1±0.6^{a}	491.0±10.8^{a}	361.5±9.7^{a}		
	HS	27.2±0.7^{a}	475.8±12.6^{a}	361.0±8.2^{a}		
	CONT	27.0±1.1^{a}	472.5±18.6^{a}	361.9±7.3^{a}		
Short term period (week 1-4)	HF	22.1±0.6^{b}	538.7±13.9^{a}	573.5±11.4^{a}	212.1±12.6^{a}	59.2±4.1^{a}
	HFHS	26.0±0.8^{a}	554.6±16.2^{a}	577.1±13.7^{a}	215.7±8.5^{a}	60.0±2.7^{a}
	HS	26.9±1.0^{a}	505.5±19.1^{ab}	540.9±16.6^{ab}	180.0±12.8^{ab}	49.9±3.5^{ab}
	CONT	26.5±0.8^{a}	464.3±14.5^{b}	513.3 ±9.4^{b}	151.4 ±7.6^{b}	42.1±2.5^{b}
Long term period (week 1-8)	HF	22.2±0.7^{b}	541.9±16.0^{a}	656.5 ±13.0^{a}	305.8 ±18.5^{a}	88.1±7.2^{a}
	HFHS	26.0±1.6^{a}	554.5±34.8^{a}	659.0±32.4^{a}	296.2±21.6^{ab}	82.2±6.5^{a}
	HS	26.6±1.0^{a}	499.9±18.3^{ab}	618.3±17.0^{ab}	261.4±15.1^{ab}	73.7±5.5^{ab}
	CONT	25.2±0.9^{ab}	440.9±16.2^{b}	571.3±13.2^{b}	227.8 ±13.7^{b}	66.5±4.5^{b}

Results are expressed as mean ± S.E. of N = 10 rats per group from week 0-4, and N = 5 rats per group from week 5-8. Mean values not sharing the same superscript letters (a,b) within a column at the same period were significantly different ($P < 0.05$) by one-way ANOVA.

At the beginning of the experiment, animals were randomized to 4 groups such that initial weights were not different between treatments; therefore all groups of animal started with similar mean body weights (361.6 ± 0.6 g). A repeated measure analysis over the 8 weeks

indicated that there were significant differences between diets ($P = 0.03$), times ($P < 0.01$), with interactions between diet and either animal or time ($P < 0.01$). The body weight of the animals increased over the 8 week period of the experiment, with the rats on HFHS diet having the heaviest body weight, followed by the HF- and HS-fed rats, while the CONT-fed group had the lowest weight gain throughout the study (Figure 6.1). The HFHS diet significantly ($P = 0.03$) affected weekly body weight gain in the animals, while the HF diet showed a tendency to increase animal body weight ($P = 0.09$); but the HS diet had no significant effect on the weight change. Furthermore, after only one week of feeding the HFHS diet, the body weight of these rats was significantly ($P < 0.05$) higher than the control group, and they maintained a significantly higher body weight throughout the study compared to the other three groups. The HF-fed rats showed no significant difference from the control at the beginning but significant ($P < 0.05$) increases were observed from week 3 onwards until the end of experiment; whereas, the HS-fed rats showed no significant difference at any time when compared with the control group. This result suggests that the HFHS is the most efficient diet to induce animal body weight gain in a short period of time.

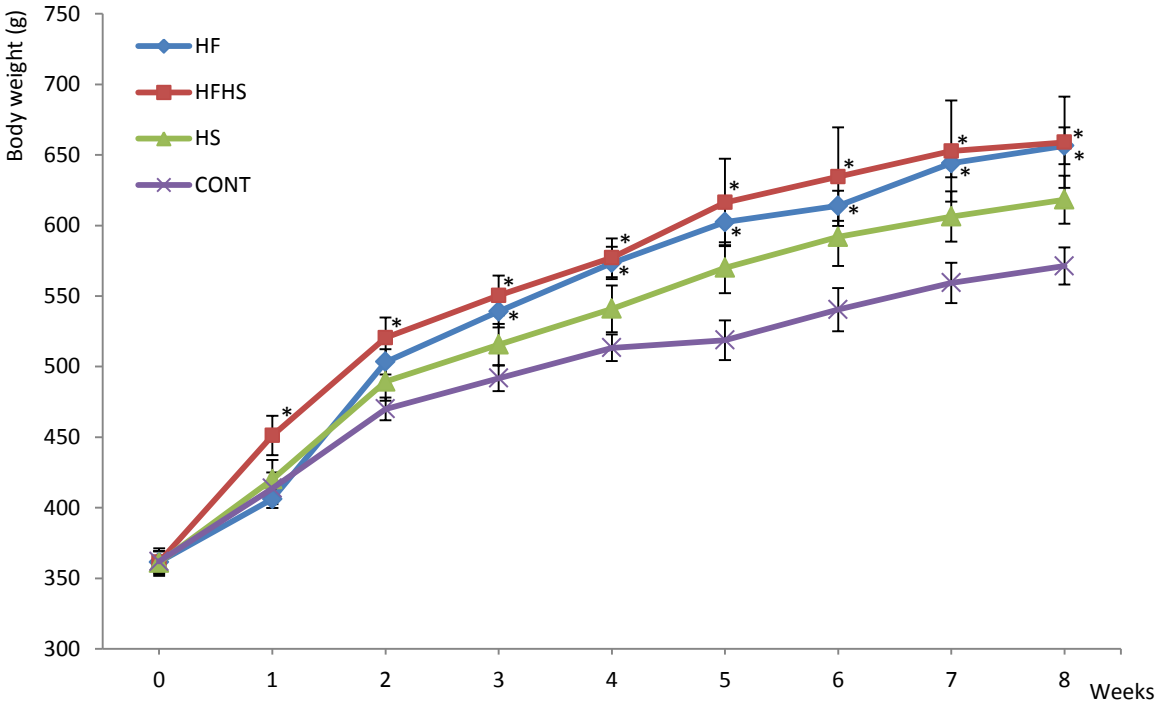


Figure 6.1: Weekly body weight change. Rats were fed with four different diets (HF, HFHS, HS and CONT). All values are expressed as mean ± S.E. of N = 10 rats per group from week 0-4, and N = 5 rats per group from week 5-8. *$P < 0.05$ compared with the normal control group at the same time.

Percentage of fat and body composition by DEXA analysis

At weeks 4 and 8, animals from each group were DEXA scanned for whole body composition. The parameters measured included fat mass, non-fat mass, total mass and percentage of fat. The results of whole body composition obtained from DEXA analysis indicated that significant weight gain in HF and HFHS-fed rats was mainly contributed by accumulation of fat mass rather than non-fat mass. As seen from Table 6.3, the HF and HFHS groups showed a remarkable increase in fat mass and percentage of fat compared to CONT-fed rats. More specifically, the HF and HFHS-fed rats had approximately 53% and 44% increase in fat mass and 38% and 33% increase in percentage of fat respectively compared to the control at week 4. After feeding the animals for 8 weeks, these two groups continued to show a marked increase in fat mass and percentage of fat, but the rate of increase was higher in HFHS-fed than the HF-fed rats. Unlike fat deposition, the rats fed with HF and HFHS diets had only a slight gain in non-fat mass (approximately 2 %) at week 4 and up to about 13 % at week 8 compared to the control group.

Table 6.3: Body composition by DEXA scan in rats at week 4 and week 8.

Parameter measured	Week 4				Week 8			
	HF	HFHS	HS	CONT	HF	HFHS	HS	CONT
Fat mass (g)	124.2 ±15.7	117.1 ±8.9	92.1 ±15.4	81.1 ±8.9	189.2 ±30.0	194.1 ±22.4	161.3 ±15.3	122.4 ±11.8
Non-fat mass (g)	432.1 ±5.7	431.1 ±6.0	413.8 ±12.2	424.8 ±11.5	478.2 ±24.7	474.9 ±29.6	436.5 ±10.4	422.4 ±5.2
Total mass (g)	556.3 ±19.9	548.3 ±11.4	505.9 ±25.4	506.0 ±11.4	667.5 ±19.1	669.0 ±48.3	597.8 ±22.5	544.8 ±12.6
Percentage of fat	22.1 ±2.1	21.3 ±1.3	17.9 ±2.1	16.0 ±1.7	28.2 ±3.9	28.8 ±1.6	26.8 ±1.7	22.3 ±1.7

Results are expressed as mean ± S.E. of N = 5 rats per group.

By performing two way analysis of variance, the whole body composition of animals was found to be significantly affected by study duration ($P < 0.05$), with week 8 being greater than week 4 for total mass, and also for each individual parameter. However, there was no significant interaction between effect of diet x length of study ($P > 0.10$), therefore it is reasonable to consider the significant difference between diets across the experiment (Table 6.3A). The statistical analysis indicates that HF and HFHS diets significantly increased total fat mass ($P = 0.01$) and percentage of fat ($P = 0.03$), but not non-fat mass ($P = 0.09$), which confirmed that fat accumulation is the major contributor to the increased body weight of HF and HFHS rats. In

contrast, the HS-fed rats had similar levels of all parameters (fat mass, non-fat mass, total mass and % fat) compared to those on the control diet. In terms of the whole body composition, the results clearly suggest that HF and HFHS diets were more efficient than the HS diet for inducing weight gain as well as fat deposition in the SD rat model.

Table 6.3A: Least squares means for the effect of different diet on whole body composition

Parameter measured	Diets			
	HF	HFHS	HS	CONT
Fat mass (g)	156.7^{a}	155.6^{a}	126.7^{b}	101.8^{b}
Non-fat mass (g)	455.2^{a}	453.0^{a}	425.2^{a}	423.6^{a}
Total mass (g)	611.6^{a}	608.6^{a}	551.8^{ab}	525.4^{b}
Percentage of fat	25.1^{a}	25.0^{a}	22.3^{ab}	19.2^{b}

Different superscript letters within the same row indicate significant differences ($P < 0.05$).

The acceleration in body weight gain in HF and HFHS rats was associated with fat accumulation. Therefore, at the termination of each study period, euthanased animals were dissected and visceral white adipose tissues including retroperitoneal, mesenteric and epididymal (upper and lower parts) as well as interscapular brown adipose tissue (iBAT) were collected and weighed to further examine whether this fat deposition influenced any specific type of fat pad.

As shown in Table 6.4, the HF and HFHS-fed groups which had heavier body weight and percentage of fat than the HS and CONT groups, showed heavier adipose tissues and some organ weights. In order to allow us to examine the real differences between the treatments, the tissue and organ weights were then calculated as gram per kg of body weight of each rat. After 4 weeks on the test diets, the animals fed with HF and HFHS had considerable gains in total white adipose tissue (WAT) weight (33% and 25%, respectively), but exhibited only slight increases of iBAT weight (10% and 5%, respectively) compared to those on the control diet. In particular, HF and HFHS rats had 23% and 18% increases in retroperitoneal fat weight, 40% and 20% increases in mesenteric fat, 41% and 39% increases in upper epididymal fat, and 23% and 13% increases in lower epididymal fat, respectively. Consistent with whole body composition data, the HS rats had smaller increases in total as well as in each individual white fat pad when compared to the other two treatments (HF and HFHS). This result indicates that the significant increase in fat mass as well as in the percentage of fat which was found in HF and HFHS groups was mostly due to the accumulation of several parts of white adipose tissue, but not brown adipose tissue. Furthermore, after 8 weeks on their respective diets, the HF and HFHS groups continued to show

Table 6.4: Adipose tissue and organ weights in rats at week 4 and week 8.

Parameter measured	Week 4				Week 8			
	HF	HFHS	HS	CONT	HF	HFHS	HS	CONT
Fat pad (g)								
Retroperitoneal	18.0 ±2.3	16.8 ±1.6	15.3 ±1.8	13.3 ±0.6	28.8 ±2.1	32.5 ±3.9	22.2 ±2.7	16.1 ±2.9
Mesenteric	9.0 ±1.4	7.6 ±0.8	6.6 ±1.1	5.9 ±0.6	14.1 ±2.1	15.7 ±2.2	10.8 ±1.6	7.8 ±2.1
Epididymal (upper part)	14.6 ±1.1	14.2 ±1.7	10.2 ±0.9	9.5 ±0.5	19.6 ±1.0	20.8 ±1.8	16.8 ±1.5	11.2 ±1.4
Epididymal (lower part)	0.5 ±0.04	0.4 ±0.1	0.4 ±0.03	0.3 ±0.03	0.5 ±0.03	0.6 ±0.04	0.6 ±0.1	0.4 ±0.1
Total WAT	42.1 ±4.5	39.1 ±3.8	32.3 ±3.5	29.0 ±1.4	63.0 ±4.0	69.5 ±7.5	50.3 ±5.3	35.4 ±6.4
iBAT	0.8 ±0.1	0.7 ±0.1	0.7 ±0.1	1.6 ±0.1	1.0 ±0.1	1.0 ±0.03	0.8 ±0.1	0.7 ±0.03
Fat pad (g/kg BW)								
Retroperitoneal	31.0 ±3.1	29.6 ±2.6	28.6 ±2.5	25.2 ±1.3	43.8 ±3.0	49.0 ±4.6	35.5 ±3.6	27.8 ±4.4
Mesenteric	15.6 ±1.9	13.4 ±1.2	12.2 ±1.5	11.1 ±1.1	21.4 ±2.8	23.7 ±2.7	17.3 ±2.3	13.4 ±3.4
Epididymal (upper part)	25.4 ±1.3	25.0 ±2.8	19.2 ±1.4	18.0 ±0.8	30.0 ±1.8	31.4 ±1.6	27.0 ±1.8	19.4 ±2.0
Epididymal (lower part)	0.8 ±0.1	0.7 ±0.1	0.7 ±0.1	0.6 ±0.04	0.8 ±0.04	0.9 ±0.1	0.9 ±0.1	0.7 ±0.1
Total WAT	72.8 ±5.8	68.7 ±6.0	60.6 ±4.5	54.9 ±2.8	96.0 ±5.6	105.0 ±8.3	80.8 ±6.9	61.3 ±9.8
iBAT	1.3 ±0.1	1.3 ±0.1	1.4 ±0.1	1.2 ±0.1	1.5 ±0.1	1.5 ±0.04	1.3 ±0.2	1.2 ±0.1
Organs (g)								
Liver	14.7 ±1.3	15.4 ±0.7	14.1 ±1.0	13.9 ±0.4	16.6 ±0.5	17.1 ±1.8	17.0 ±0.4	14.2 ±0.6
Kidneys	3.8 ±0.1	3.9 ±0.1	3.6 ±0.1	3.6 ±0.1	3.9 ±0.1	3.8 ±0.2	4.0 ±0.2	3.8 ±0.2
Pancreas	0.9 ±0.1	0.9 ±0.1	1.0 ±0.03	0.8 ±0.04	1.3 ±0.1	1.5 ±0.2	1.5 ±0.1	1.4 ±0.1
Caecum	2.2 ±0.2	2.0 ±0.1	2.2 ±0.1	2.8 ±0.3	2.7 ±0.7	2.9 ±0.5	2.1 ±0.4	2.8 ±0.4
Organs (g/kg BW)								
Liver	25.5 ±1.7	27.1 ±1.1	26.7 ±1.1	26.2 ±0.6	25.3 ±0.6	25.8 ±1.7	27.5 ±0.5	24.8 ±1.1
Kidneys	6.6 ±0.3	6.9 ±0.3	6.7 ±0.2	6.8 ±0.2	5.9 ±0.1	5.7 ±0.1	6.5 ±0.2	6.7 ±0.4
Pancreas	1.6 ±0.1	1.5 ±0.2	1.8 ±0.1	1.6 ±0.1	1.9 ±0.1	2.3 ±0.2	2.4 ±0.1	2.5 ±0.1
Caecum	3.8 ±0.1	3.6 ±0.2	4.2 ±0.4	5.4 ±0.7	4.2 ±1.2	4.3 ±0.6	3.4 ±0.6	5.0 ±0.7

Results are expressed as mean ± S.E. of N = 5 rats per group.

increases in weight of total WAT and all specific white adipose tissues (56% and 71% increased total WAT; 58% and 76% increased retroperitoneal; 59% and 77% increased mesenteric; 54% and 61% increased upper epididymal fat; and 18% and 26% increased lower epididymal fat, respectively). In accordance to the DEXA results, HFHS rats seemed to gain more weight of total WAT and each individual white fat than those on the HF diet at this period (Table 6.4).

Since no interaction effect between diet and time was found for any adipose tissue weight ($P > 0.05$), a two way ANOVA indicated that the weights of total WAT as well as each individual white fat pad, except the lower part of epididymal were significantly affected by diets ($P < 0.05$), whereas there was no significant difference ($P = 0.35$) in iBAT weight between diets (Table 6.4A). Similarly, it was confirmed that the only HF and HFHS groups had significantly heavier total WAT ($P < 0.01$) and also the three major white fat pads ($P < 0.01$ retroperitoneal; $P = 0.02$ mesenteric; and $P < 0.01$ upper epididymal) than the controls. However, there were no significant differences among these biomarkers between HS-fed and control-fed animals; only the upper epididymal weight almost reached the levels of significance.

Table 6.4A: Least squares means for the effect of diet on adipose tissue weights (g/kg BW)

Adipose tissues	Diets			
	HF	HFHS	HS	CONT
Retroperitoneal	37.4^{a}	39.3^{a}	32.1^{ab}	26.5^{b}
Mesenteric	18.5^{a}	18.6^{a}	14.7^{ab}	12.3^{b}
Epididymal (upper part)	27.7^{ab}	28.2^{a}	23.1^{bc}	18.7^{c}
Epididymal (lower part)	0.8^{a}	0.8^{a}	0.8^{a}	0.7^{a}
Total WAT	84.4^{a}	86.8^{a}	70.7^{ab}	58.1^{b}
iBAT	1.4^{a}	1.4^{a}	1.4^{a}	1.2^{a}

Different superscript letters within the same row indicate significant differences ($P < 0.05$)

As expected, there was a greater increase in the weight of white adipose tissue with time, with animals at week 8 showing significantly heavier retroperitoneal ($P < 0.01$), mesenteric ($P < 0.01$), upper epididymal ($P < 0.01$), lower epididymal ($P = 0.04$) and total WAT ($P < 0.01$) than those at week 4. In contrast to white fats, there was no significant change in brown adipose tissue (iBAT) weight over time.

For organ weights, analysis of variance showed that there were no significant differences in the liver, kidneys, pancreas and caecum weights among treatments, indicating that the diets did not influence the weight of any organ. Also, no diet-by-time interaction for organ weights was observed. However, it may be important to note that kidney weight significantly decreased over

time, implying that kidney growth did not keep pace with the growth in body weight. Conversely there was a significant increase over time in the weight of pancreas (Table 6.4B).

Table 6.4B: Least squares means for the effect of time on organ weights (g/kg BW)

Internal organs	Duration of study	
	4 weeks	8 weeks
Liver	26.4[a]	25.8[a]
Kidneys	6.8[a]	6.2[b]
Pancreas	1.6[b]	2.3[a]
Caecum	4.3[a]	4.2[a]

Different superscript letters within the same row indicate significant differences ($P < 0.05$).

As shown in Figure 6.2A-C, highly positive correlations between rat's body weight and total mass ($R^2 = 0.97$) were found, indicating that body weight correlated well with total mass measured by DEXA. A tendency to positive correlation were also observed between body weight of rats and percent body fat ($R^2 = 0.63$) as well as between body weight and total fat pad weight ($R^2 = 0.58$). We also examined the relationship between body weight and each white fat pad weight but found no evidence for preferential partitioning of deposited fat to any one particular white fat

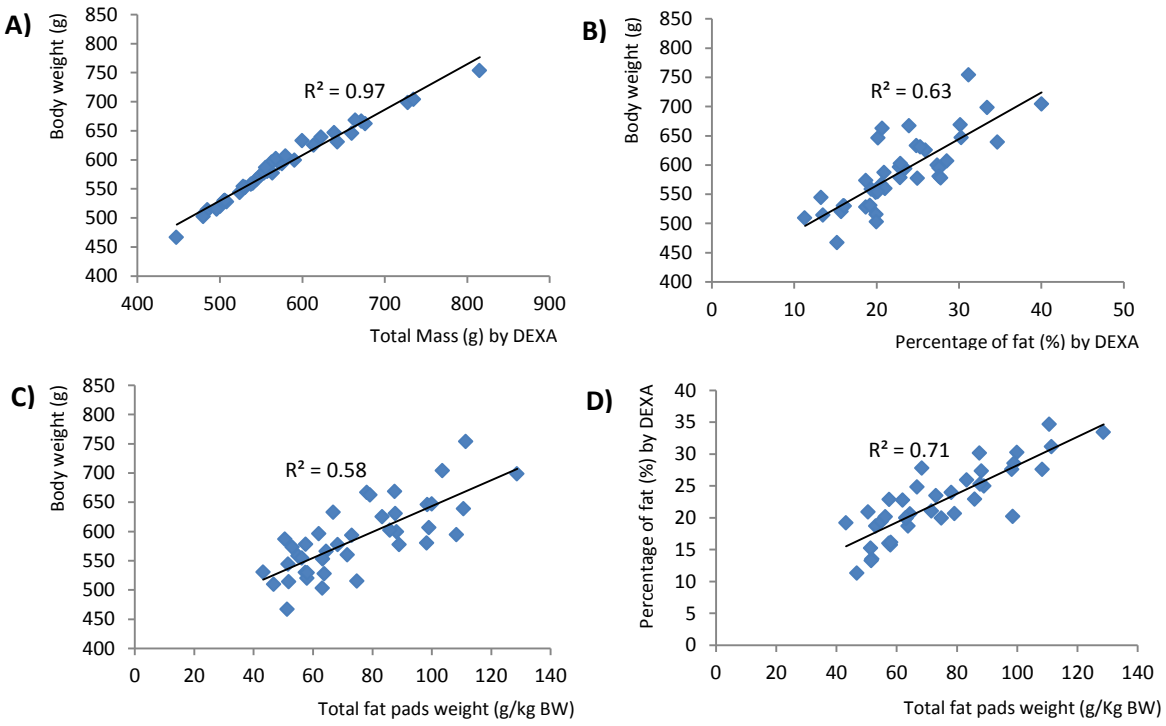


Figure 6.2: Linear correlations between the rat's body weight vs. the total mass measured by DEXA (A); percentage of fat (B); total fat pad weights (C); and between percentage of fat vs. total fat pad weights (D).

(data not shown). These results indicate that the increases of body weight in animals simply related to the fact that these animals proportionally accumulated all parts of white fat rather than put on any particular white fat pad. Notably, measurement of total fat pad weight correlated strongly with DEXA fat mass measurement (R^2 = 0.71), demonstrating that these two methods reflect each other, and verify that abdominal white fat is the key parameter to measure as a proxy for total body fat (Figure 6.2D).

In summary, the same pattern was found in all parameters associated to obesity, in which the HF and HFHS-fed rats had significant higher body weight, fat mass, percentage of fat, and total as well as individual visceral white adipose tissue weights than the control-fed rats, whereas the HS-fed rats were somewhere in between. The results clearly indicated that the HF and HFHS diets are more efficient than the HS diet for inducing obesity in the SD rat model.

Fasting glucose, insulin concentrations, and insulin resistance (HOMA-IR) index

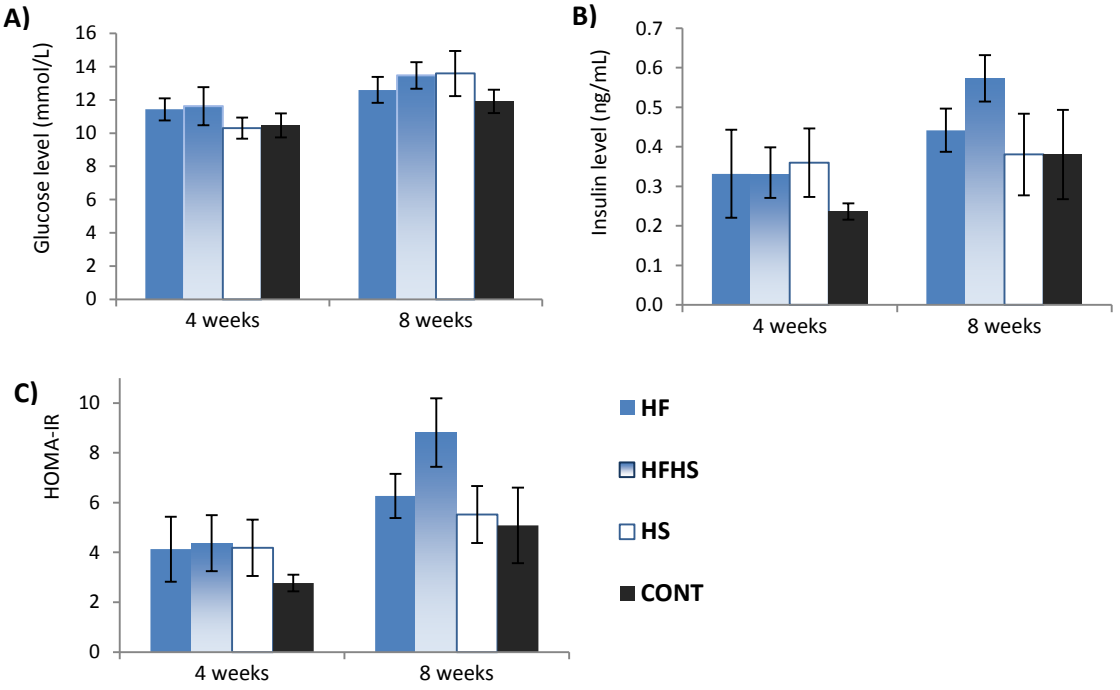


Figure 6.3: Effect of different diets on fasting plasma glucose levels (A); serum insulin concentrations (B) and homeostasis model assessment of insulin resistance (HOMA-IR) index (C) in rats at week 4 and week 8. Results are expressed as mean ± S.E. of N = 5 rats per group.

Major parameters relevant to metabolic syndrome were measured at week 4 and week 8 to investigate short-term and long-term effects of the test diets on blood glucose and insulin concentrations. As shown in Table 6.5, analysis of variance demonstrated that the diets did not

significantly influence fasting glucose (P = 0.50), insulin concentrations (P = 0.41) or HOMA-IR score (P = 0.16) within 8 weeks of this study period. However, these three indices significantly increased over time (P < 0.05), confirming that symptoms of metabolic syndrome linked to type II diabetes are more likely to occur in middle-aged or older animals rather than younger ones. Again, no interaction effects between diet and time in these parameters were observed (P > 0.50). Although, there were no differences among tested diets, excessive consumption of fat and/or sugar showed a trend to increased levels of fasting glucose and insulin for both time points. For example, rats fed with HF or HFHS for 4 weeks had a 9% and 11% increase in plasma glucose concentration, and 35% and 34% increase in serum insulin concentration, respectively when compared to the control group at the same time. Importantly, the HF and HFHS induced an increase in the degree of insulin resistance (HOMA-IR) by 49% and 58% compared to the control diet. Similar to the obesity parameters, these glycaemic indices were also found to be more pronounced at week 8 in the HFHS group, which had up to a 13% increase in glucose, 50% increase in insulin levels, and 73% increased HOMA-IR score. However, these three parameters tended to be less pronounced when feeding the rats with HF diet up to 8 weeks (6% increased glucose, 16% increased insulin, and 23% increased HOMA-IR, compared to the control group at week 8).

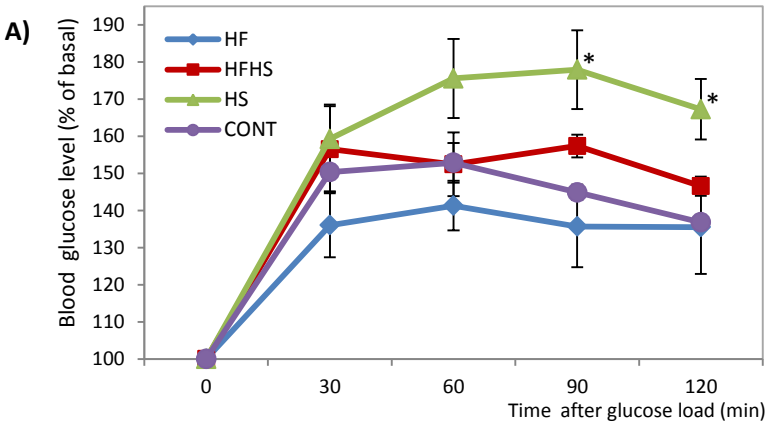
Table 6.5: Least squares means for the effect of diet and duration of study on fasting plasma glucose and insulin concentrations

Parameter measured	Diets				Duration of study	
	HF	HFHS	HS	CONT	4 weeks	8 weeks
Glucose (mmol/L)	12.0^{a}	12.6^{a}	12.0^{a}	11.2^{a}	11.0^{b}	12.9^{a}
Insulin (ng/mL)	0.4^{a}	0.5^{a}	0.4^{a}	0.3^{a}	0.3^{b}	0.4^{a}
HOMA-IR	5.2^{a}	6.6^{a}	4.9^{a}	3.9^{a}	3.9^{b}	6.4^{a}

Different superscript letters within the same row indicate significant differences (P < 0.05).

The oral glucose tolerance test (OGTT)

Figure 6.4 shows the changes in blood glucose concentrations of rats following an oral glucose challenge carried out in week 4 and week 8 of the experiment. After 4 weeks on test diets (Figure 6.4A), the HS and HFHS rats had a progressive increase in their blood glucose, especially in the HS group which became significantly higher (P < 0.05) than those of the control group at 90 and 120 min after the glucose loading. This result indicates that animals fed with HS or HFHS diets for 4 weeks seemed to develop signs of impaired glucose tolerance: a condition



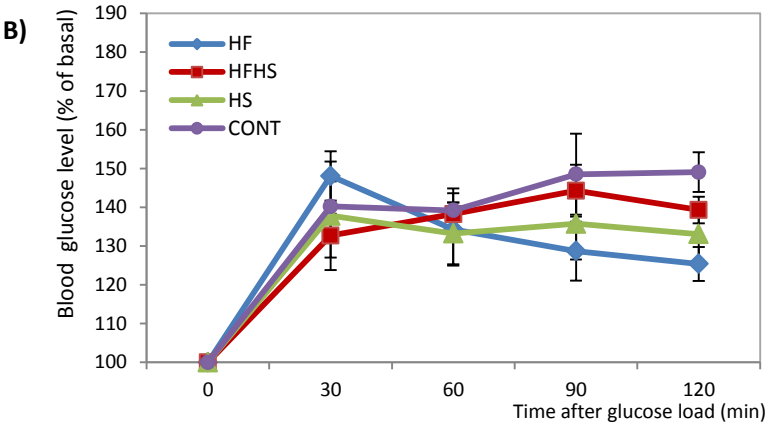


Figure 6.4: Oral glucose tolerance test responses in rats fed a HF diet, a HFHS diet, a HS diet, and a CONT diet for 4 weeks (A) and 8 weeks (B). Results are expressed as mean ± S.E. of N = 5 rats per group. *P < 0.05 compared with the normal control group at the same time.

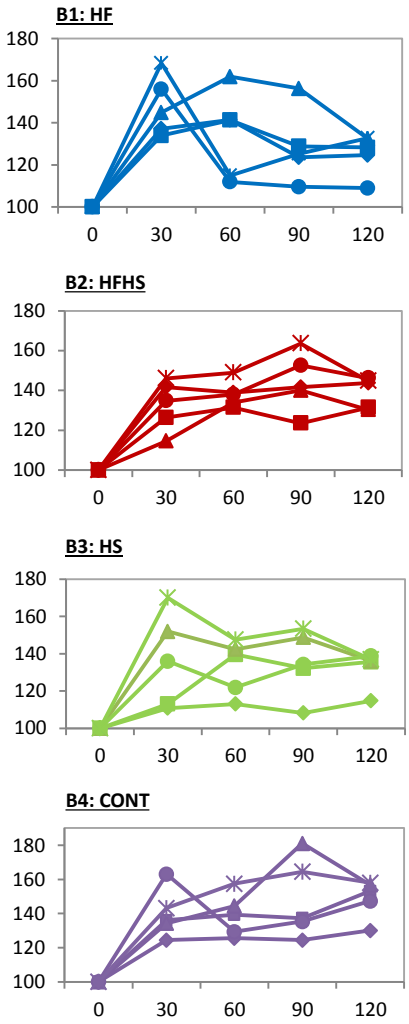


Figure 6.4(B1:B4): Oral glucose tolerance test responses at week 8 in individual rats (n = 5/group) fed a HF diet (B1), a HFHS diet (B2), a HS diet (B3), and a CONT diet (B4).

in which the body cannot dispose of glucose efficiently after a glucose load. However, there was no significant difference in blood glucose concentration between either HS or HFHS and CONT group at any time point at week 8 (Figure 6.4B). The lack of a treatment effect is because, by 8 weeks, the CONT diet appeared to be inducing signs of impaired glucose tolerance as well, which is in line with the increase of degree of insulin resistance (HOMA-IR) index found in this group at the end of week 8. When considering data for individual rats in each group (Figure 6.4:B1-B4), all animals in HFHS groups developed signs of glucose intolerance, whereas approximately 80% of animals in the HS groups showed impaired glucose tolerance. Unlike these two groups, animals fed the HF diet did not develop marked evidence of glucose intolerance over either short or long periods of time. Therefore, in term of glycemic parameters generally, HFHS is considered to be the most efficient diet for inducing metabolic syndrome because it successfully induced the rise in fasting glucose and insulin concentrations, insulin resistance as well as impaired glucose tolerance in the SD rat model.

Discussion :

The present study was designed to examine the effectiveness of diet in inducing metabolic syndrome in Sprague Dawley (SD) rats in an attempt to establish an appropriate model in this strain. It has been well documented that excess consumption of fat and/or sugar in animal models can lead to the development of various signs of metabolic syndrome including body weight gain, abdominal fat pad weight gain, glucose intolerance, hyperglycemia and hyperinsulinemia, and consequently a reduction of insulin sensitivity (Pagliassotti et al., 1996; Ghibaudi et al., 2002; Brenner et al., 2003; Johnson et al., 2007).

Some of our results are consistent with previous findings. As expected HF and HFHS diets induced an increase in body weight, body fat, abdominal fat pads weights. Although animals fed a HF or HFHS diet had lower food intake, they consumed more calories than animals fed control diets, which was the likely reason for the accumulation of fat mass and body weight gain. In addition, we observed that chronic consumption of sugar alone was not effective for inducing obesity in animals, as has been suggested in the literature (Chicco et al., 2003; Ryu & Cha, 2003; Sumiyoshi et al., 2006; Khanal et al., 2010). Furthermore, this study did not find a significant effect of high consumption of fat and sugar on the weight of any organs, except the weight of pancreas which was found to increase over time. Diabetes (both type 1 and 2) has been found to be associated with reduced pancreatic size (presumably linked to reduced insulin secretion,

Alzaid et al., 1993; Altobelli et al., 1998) but there is paucity of literature in animal or human trials on the relation of metabolic syndrome or prediabetes with pancreatic size alteration, and there is a need for further investigation.

Fasting blood glucose and insulin concentration are normally used to calculate insulin resistance, a condition associated with the early stage of diabetes in which the body produces insulin but the cells of the body fail to respond properly. Most earlier studies clearly report the adverse effect of consumption of high fat and/or high sugar diet on hyperglycemia and hyperinsulinemia. For example, Han et al. (1997) fed Wistar rats 50% fat diets for 32 weeks and observed increased blood glucose concentrations from 16 weeks onwards. Suwannaphet et al. (2010) suggested that feeding 60% fructose diet to SD rats for 8 weeks effectively increased fasting plasma glucose and insulin concentrations. La Fleur et al. (2011) clearly showed the effect of feeding HFHS diets to male Wistar rats for 4 weeks in the development of hyperglycemia and hyperinsulinemia. Similarly, the results of this study also confirm that excessive intake of fat and/or sugar markedly elevate fasting glucose and insulin concentrations, although the elevation did not reach statistical significance by the end of the 8 week period of our experiment, which may be due to the small sample size used per group (N = 5).

However, we did observe a tendency for development of signs of hyperglycemia and hyperinsulinemia in tested animals. Nevertheless, consideration of only the elevation of fasting glucose and/or insulin concentrations in blood may lead to misinterpretation because at some stages, the concentration of glucose and insulin in the fasting period can be increased simply because glucose/insulin production from organs is faster than glucose/insulin clearance from blood (Bock et al., 2006). Accordingly, HOMA-IR, an indicator calculated using both glucose and insulin concentrations is used for an evaluation of insulin resistance (IR). This indicator is reasonably valid and has been accepted worldwide for determining the degree of IR because it correlates well with results obtained from euglycemic hyperinsulinemic clamp, a ‘gold standard’ for measuring IR (Matthews et al., 1985).

The results of this study show that HFHS and HF diets were effective for inducing IR, with HFHS being more effective than HF diet, and high consumption of sugar alone being the least efficient. This may be due to the fact that elevated concentations of circulating free fatty acids in animals fed with HF/HFHS diets enhance the regulation of insulin secretion from pancreas, consequently, the animals develop sign of insulin resistance (Tomas et al., 2002). This finding is in line with other work in the B/6J mouse model by Surwit et al. (1995), who demonstrated that fat was the

primary stimulus for the elevation of fasting glucose and insulin, whereas sugar had no effect for these two keys parameters in the absence of fat.

Abnormal glucose tolerance is also used in various clinical settings to determine insulin resistance/sensitivity because this method reflects the clearance of glucose load by insulin (Stumvoll et al., 2000). Additionally, OGTT are commonly used together with fasting glucose in order to determine the pre-diabetic condition in subjects. The present study found that HFHS and HS diets, but not the HF diet, moderately impaired glucose tolerance, especially when feeding the animals for a short-term (4 weeks) period. Interestingly, we did not find this impaired effect at 8 weeks. We speculate that the starch-based control diet may also gradually cause impaired glucose tolerance, making the difference between the fat/sugar-based treatments and starch-based control not able to be differentiated statistically. HOMA-IR results obtained from this study confirm that starch-fed rats developed insulin resistance at week 8 as their HOMA-IR indexes were found to be increased to nearly the same concentration as the HS group. A number of previous studies have reported that starch appears to also influence postprandial glucose and insulin responses, but generally produce a flatter curve than simple carbohydrates (Crapo et al., 1976; Reaven, 1979). Specifically, according to Crapo et al. (1976), raw starch ingestion resulted in a 44 % lower glucose response and a 35-65 % lower insulin response than glucose or sucrose ingestion. In a later study, a wide range of starchy foods were compared for their glucose and insulin response curves and the data demonstrated that dextrose and potato give similar plasma glucose as well as insulin responses, and these responses were greater than that of bread, rice and corn (Crapo et al., 1981). Interestingly, starch from potato has been found to have almost an identical response compared to glucose or sucrose (Bantle et al., 1983; Blaak & Saris, 1995). Recent data suggest that not all types of starch give similar glycemic and insulinemic responses, and the differences are due to several factors, including amylose-amylopectin ratio, cooking condition and particle size of starches (Ray & Singhania, 2011).

The duration required for a high-fat diet to cause impaired glucose tolerance in animals has been shown to vary from 10 to 78 weeks (Ahrén & Scheurink, 1998; Sumiyoshi et al., 2006; Prior et al., 2008; Braz De Oliveira et al., 2010; Zhang et al., 2010). The present study did not observe an effect of HF intake on glucose intolerance. The 8-week feeding period may have been too short to induce the glucose impairment effect; animals fed this diet would likely develop signs of

glucose intolerance eventually, but a HF diet does not appear to be as efficient as HFHS in inducing pre-diabetes and/or insulin resistance.

The inability of high fat and/or high sugar diets to modify biomarkers of metabolic syndrome in SD rats has also been reported in some previous studies. Stark et al. (2000) fed male SD rats with a high fat (25% soybean oil and 35% corn starch) or a high fructose (53% fructose and 10% sucrose) diet for 3 months compared to a control (53% cornstarch, 10% sucrose and 7% soybean oil). The researchers found no differences between test diets in all metabolic parameters measured including animal weight gain, fasting glucose and insulin concentrations, circulating lipid profiles (cholesterols and triglycerides) and signs of glucose intolerance. In contrast, Khanal et al. (2010) demonstrated that feeding a fructose diet to SD rats was only partially effective in inducing some biomarkers of metabolic syndrome, such as increased fasting plasma insulin, cholesterol and triacylglycerols, but not fasting plasma glucose, area under the curve after OGTT, abdominal fat or body weight. Several possibilities explaining the inability of high-fat and/or high-fructose diets to induce glucose impairment have been proposed by the authors. For instance, Sprague Dawley is an outbred strain which has been found to have greater genetic variability among colonies, and SD rats used in the experiment could be resistant to a diet producing insulin resistance. In addition, fat and sugar content used in their research may not have been extreme enough to induce insulin resistance.

The types of fat as well as sugar might vary in their ability to induce metabolic syndrome. It has been shown in a number of previous studies that of animals fed similar amounts of fat, those fed diets containing mono- or polyunsaturated fat had improved lipid profiles and did not gain as much weight as those fed diets with more saturated fat; and the change from a diet high in saturated fat to one enriched in unsaturated fat tended to reverse metabolic damage (Ikemoto et al., 1996; Wang et al., 2002; Arapostathi et al., 2011). Indeed, we observed an inability of fructose to induce any sign of metabolic syndrome in SD rats, as reported in Chapter 5. For this reason, soybean oil, which is high in mono- and polyunsaturated fat does not seem appropriate and fructose may be less suitable than sucrose with regard to a diet-induced metabolic disorder research model.

There are some limitations to the present study. The small sample size reduced the statistical power, though we did observe a significant difference between treatment and control diets in parameters related to obesity and also found a tendency for the development of insulin resistance indicators. In addition, the oral glucose tolerance test is performed on non-sedated

animals with blood samples collected every 30 minutes. This introduces stress in experimental animals and may increase the variability in the blood glucose data (Nowotny et al., 2010). To overcome this problem, a longer time interval between blood sample collections of 45 mins or 1 hour may enable animals to become more relaxed and relieve their stress. Finally, it is also important to note that the SD rats used in this study are an outbred stock which has been found to have genetic variability. As outlined by the data sheet of the Jackson Laboratory (2012), using inbred strains mouse enables more reproducible outcomes. Specifically, C57BL/6J is considered to be an efficient and the most intensively inbred mouse strain used (Winzell & Ahrén, 2004). However, using outbred stocks more accurately mimics the genetic heterogeneity in human populations and eliminates the need to repeat the study on multiple inbred lines. In addition, mice are too small for repeated blood collection and DEXA scanning. Therefore, although the C57BL/6J mouse may be the most widely used in metabolic syndrome research, the use of the mouse model severely limits the parameters that can be tested and the reproducibility of the measurements. As a result, the SD rats may constitute another appropriate alternative for a diet-driven model of MS.

In conclusion, the results of this study demonstrate that excessive intake of the combination of high fat sourced from lard and high sugar sourced from sucrose in SD rats effectively induced the development of abdominal obesity and showed promise to develop insulin resistance and glucose intolerance, with longer HFHS intake inducing more severe signs of metabolic syndrome. Chronic consumption of high fat alone increased weight gain and obesity rather than insulin resistance or glucose intolerance, whereas high sugar alone tended to induce glucose intolerance, but not weight gain and fat deposition. From the above findings it can be concluded that SD rats fed a high-fat-high-sugar (20% lard and 45% sucrose) diet for 8 weeks successfully developed signs of metabolic syndrome and can potentially be used as a model for metabolic syndrome research associated with obesity as well as insulin resistance.

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CHAPTER 7

Effect of Highbush blueberry intake on markers of metabolic syndrome in rats fed a high-fat-high-sugar diet

The results from the second animal study showed that feeding a high-fat-high-sugar diet (20% lard and 45% sucrose) to Sprague Dawley (SD) rats for 8 weeks leads to obesity, and increases the trend to develop insulin resistance. We therefore used this animal model for further testing the potential anti-metabolic syndrome effects of Highbush blueberries grown in New Zealand. Although evidence from *in vitro* and animal models supports the beneficial role of blueberries on metabolic syndrome, most studies were done using Lowbush cultivars, which are not common in New Zealand. Our *in vitro* data showed the beneficial effect of Highbush varieties on inhibition against starch hydrolysis enzymes and improvement of probiotic bacterial growth, indicating a possible protective effect against the development of metabolic syndrome *in vivo*. For these reasons, the present study was designed mainly to evaluate the actual physiological effect (both preventive and therapeutic) of NZ Highbush blueberries on alleviation of symptoms associated with metabolic syndrome in SD rats fed with a high-fat-high-sugar diet.

Abstract :

Chronic consumption of high-fat and/or high-sugar diets may lead to the development of metabolic disorders. We tested the hypothesis that supplementation of whole blueberry powder into the diet may protect against obesity and insulin resistance and the objectives were (1) to investigate whether blueberries alleviate symptoms of metabolic syndrome in rats fed with a high-fat-high-sugar diet, and (2) to compare the efficacy of two specific blueberry cultivars ('Burlington' enriched in galactosylated, and 'Bluecrop' enriched in glucosylated anthocyanins) in preventing these metabolic changes. In experiment A, four groups of SD rats (n=7/group) were fed with either a control diet (60% starch), or a high-fat-high-sugar (HFHS) diet, or a HFHS diet supplemented with 1% (w/w) of freeze-dried 'Burlington' or 'Bluecrop' berries for 8 weeks. In experiment B, two groups of rats (n=7/group) were fed with a HFHS diet for 4 weeks to induce symptoms of MS and then switched onto HFHS diet with either 1% 'Burlington' or 'Bluecrop' berries added to the diet, for another 4 weeks, whereas the last two groups were fed with CONT for 4 weeks and then switched to either CONT+'Burlington' or CONT+'Bluecrop' for another 4 weeks for comparison. At the end of the experiment, animals were challenged with an oral glucose tolerance test, and parameters associated with metabolic syndrome were measured. Compared to the starch-CONT, HFHS-fed rats developed signs of impaired glucose tolerance, and had increased body weight gain, fat mass, fasting plasma glucose and insulin concentrations. However, inclusion of blueberries into the diets tended to improved glucose tolerance as indicated by a reduction in peak blood glucose level in BB-treated rats during the OGTT compared to non-BB fed counterparts. Furthermore, blueberry feeding lowered fasting insulin level and degree of insulin resistance (HOMA-IR) induced by the HFHS diet by 33% and 36%, respectively in experiment A, and 20% and 30% in experiment B, but this anti-hyperinsulinemic effect was muted when blueberry was added into the CONT diet. Blueberry supplementation either into HFHS or CONT diets had no significant effect to normalise hyperglycemia and did not prevent weight gain and fat accumulation. Notably, the ability of 'Burlington' and 'Bluecrop' to modulate these metabolic parameters was not significantly different, suggesting the nature of their anthocyanins was not important. Results obtained suggest that addition of blueberries to the HFHS diet tended to decrease hyperinsulinemia and improve symptoms of insulin resistance, but did not alter obesity parameters.

Introduction :

Metabolic syndrome (also known as syndrome X) is a combination of symptoms that are associated with an increased risk of developing cardiovascular disease and type II diabetes. Based on the guidelines from the National Cholesterol Education Program Adult Treatment Panel (ATPIII), it is diagnosed when three of the following characteristics are present; abdominal obesity, elevated serum triglycerides, low serum HDL-cholesterol, raised blood pressure, and elevated fasting glucose (Grundy et al., 2004; Streja, 2004). According to the statistics of the International Diabetes Federation (2006), it is estimated that around a quarter of the adult population globally have metabolic syndrome. This number of people with metabolic syndrome is increasing rapidly in every country due to imbalance in dietary intake, physical inactivity, and excess body weight as well as to genetic and physiological factors. Therefore, the emphasis on approaches to managing obesity and/or lowering hyperglycemia should help to decrease the prevalence of metabolic syndrome, with subsequent lowering of the risk of development of various related diseases. Beyond physical activity and genetic variables, dietary modification is a more amenable target for change than genetic or physiological factors. In particular, increasing the intake of foods enriched in phytochemicals which possess anti-metabolic syndrome characteristics may provide an alternative way of decreasing the risk of metabolic disorders. This approach has gained much interest and there is an increase in the search for naturally derived foods that can be consumed regularly on a daily basis.

Highbush blueberries (*Vaccinium corymbosum*) are commonly cultivated and widely consumed in New Zealand; in addition various members of the *Vaccinium* genus have been used as a traditional medicine by Quebec traditional practitioners for diabetic control for many decades (Martineau et al., 2006). Previous evidence has also demonstrated that blueberries may provide a beneficial effect improving a variety of metabolic symptoms, for instance, anti-hyperglycemia, anti-insulinemia, anti-obesity or induction of satiety (McDougall et al., 2005; Martineau et al., 2006; Molan et al., 2008; DeFuria et al., 2009; Grace et al., 2009; Prior et al., 2010; Roopchand et al., 2013). These findings have been interpreted as indicating that anthocyanins, the predominent phytonutrient in blueberries, may contribute to these beneficial effects. In addition to the total anthocyanins, recent studies reported that some individual anthocyanin components are likely to possess more anti-diabetic activity than others. For example, Jayaprakasam et al. (2005) found that cyanidin 3-glucoside and delphinidin 3-glucoside were particularly effective at increasing insulin secretion. In addition, Grace et al. (2009) studied the hypoglycaemic effects of

pure anthocyanins and showed that malvidin-3-glucoside had the ability to lower blood glucose levels in animal models.

Our *in vitro* data demonstrated that Highbush blueberries grown in New Zealand contain significant concentrations of total anthocyanins (Chapter 3). In addition, it has been found in a subsequent study (Chapter 4) that these Highbush cultivars have the ability to inhibit the activity of alpha-amylase and alpha-glucosidase, the enzymes involved in the metabolism of starch, and also exhibited potential to enhance the growth of *Lactobacillus* spp., a group of beneficial probiotic bacteria that has been shown to be associated with glucose homeostasis. In particular, we have observed a trend among individual anthocyanins, that glucosylated anthocyanins are better correlated with the α-amylase inhibitory effect (R^2 = 0.34; *P* = 0.13), and therefore may be the effective compounds inducing the anti-hyperglycemic effects by these fruits when consumed. Therefore, the present study was designed (1) to investigate whether NZ Highbush blueberries can alleviate symptoms of metabolic syndrome in SD rats fed with a high-fat-high-sugar diet, and (2) to compare the efficacy of two specific blueberry cultivars ('Burlington' which is enriched in galactosylated, and 'Bluecrop' which is enriched in glucosylated anthocyanins) in modulating these metabolic parameters.

Materials and Methods :

Experimental diets

The control (CONT) diet contained 60% carbohydrate, 20% casein, 5% fat from soybean oil, 5% cellulose, 5% minerals and 1% vitamin mix, which were prepared to meet the nutrient requirements of growing rats (Modified AIN-93G). The high fat-high sugar diets contained the same amount of casein, cellulose, minerals and vitamins as the control diet, but the source and percentage of fat and carbohydrate (20% fat from lard and 45% carbohydrate from sucrose) were varied. The two types of freeze-dried blueberry powder were prepared from Highbush blueberry (*Vaccinium corymbosum*) cultivars ('Burlington' and 'Bluecrop') as previously described in Chapter 3, and were mixed with the test diets at the dose of 1% (w/w) once a week and then stored at -20^{o}C. Freshly-thawed diets were replaced every day in order to minimize oxidation of the fats and deterioration of the anthocyanins. All diets were powdered and supplied *ad libitum* to animals at all times except during the fasting period. The composition and nutritive values of the diets are given in Table 7.1.

Table 7.1: Composition of the experimental diets.

Ingredients (g/kg diet)	CONT	CONT + 1% Burlington	CONT + 1% Bluecrop	HFHS	HFHS + 1% Burlington	HFHS + 1% Bluecrop
A. Ingredient composition						
Sodium caseinate	200	200	200	200	200	200
Cysteine	2.7	2.7	2.7	2.7	2.7	2.7
Glycine	3.3	3.3	3.3	3.3	3.3	3.3
Methionine	1.5	1.5	1.5	1.5	1.5	1.5
Glutamine	7	7	7	7	7	7
Calcium carbonate	12.5	12.5	12.5	12.5	12.5	12.5
Cellulose	50	50	50	50	50	50
Vitamin mix	10	10	10	10	10	10
Mineral mix	50	50	50	50	50	50
Lard	0	0	0	170	170	170
Soybean oil	50	50	50	30	30	30
Corn starch	513	503	503	10	0	0
Sugar	100	100	100	453	453	453
Freeze-dried blueberry powder	0	10	10	0	10	10
Total (g)	**1000**	**1000**	**1000**	**1000**	**1000**	**1000**
B. Nutrient composition*						
Crude protein, %	20.4	20.1	20.4	19.8	19.3	19.1
Fat, %	6.1	7.0	6.2	21.4	22.4	21.2
Carbohydrate, %	62.8	61.9	62.2	53.6	52.9	54.5
Energy, kJ/g	17.7	17.7	17.6	21.5	21.5	21.2
Sugar, %	8.3	9.2	9.4	40	41	42

* Nutrient composition was analysed by the Nutrition Laboratory, Massey University; protein, fat and carbohydrate by proximate analysis; gross energy by bomb calorimeter and sugar by phenol-sulphuric acid method.

Anthocyanin analysis of blueberry powder

Freeze-dried blueberry powder was dissolved in ultrapure milli-Q water at the concentration of 1 g/50 mL extract and the anthocyanin component was determined by HPLC using a modification of the method of Wang et al. (2000) as described in Chapter 3. Quantification of anthocyanins was calculated based on peak areas at 520 nm and using cyanidin 3-O-glucoside chloride as a standard.

Animals and Treatments

The experimental animal protocol was approved by the ethics committee on the use of live animals for research, testing and teaching, Massey University, Palmerston North (protocol approval #12/11). Fifty-six male Sprague Dawley rats, around 8 weeks old (250 ± 20 g) obtained from the Massy University Small Animal Production Unit (SAPU), were individually housed in a temperature controlled room (22 ± 1^{o}C) and a 12-hour light/dark cycle. After a 7-day adaptation period on the powder CONT diet, the rats were randomly divided into two experiments; experiment A was carried out for investigation of the preventive effect, whereas the experiment B was conducted to assess the therapeutic effect of Highbush blueberry on the MS parameters.

Experiment (A): Twenty-eight rats were divided into four groups composed of seven rats each based on their initial body weight and were fed with the experimental diets for 8 weeks as follows: (1) feeding with CONT diet; (2) feeding with HFHS diet; (3) feeding with HFHS diet supplemented with 1% freeze-dried blueberry powder from 'Burlington' cultivar; and (4) feeding with HFHS diet supplemented with 1% freeze-dried blueberry powder from 'Bluecrop' cultivar.

Experiment (B): The rest of the animals (28 rats) were also divided into four equal groups based on baseline body weight data. The first two groups (14 animals) were fed with CONT diet for 4 weeks, and then half of them (7 animals) were switched onto CONT+ 1% freeze-dried 'Burlington', whereas the another half (7 animals) were switched to CONT+ 1% freeze-dried 'Bluecrop' for another 4 weeks. The second two groups (7 rats each) were fed with HFHS diet for 4 weeks, and then further treated with either HFHS diet plus 1% freeze-dried 'Burlington', or HFHS diet plus 1% freeze-dried 'Bluecrop' from week 5 to week 8.

Rats were given *ad libitum* access to food and water throughout the study and maintained in their respective groups (Figure 7.1) for a total 8-week period. The food intake of each rat was measured daily and their body weight was recorded weekly.

After 4 weeks of being fed with the test diet (only for experiment A), rats were fasted overnight before an oral glucose tolerance test (OGTT) was performed. At the end of week 8 of each experiment, an OGTT was carried out on the group of rats over a period of three consecutive days before euthanasia. On the euthanasia day, rats were anaesthetized, and then scanned using Dual-energy X-ray absorptiometry (DEXA). Once finished, blood samples from the rats were drawn directly from the heart under anaesthesia. This procedure served to euthanize the rats through exsanguination under anaesthesia followed by dissection. The caeca were collected

for gut microflora determination and other tissues including the liver, kidneys, pancreas, white adipose tissues (retroperitoneal, mesenteric and epididymal fat) as described in Chapter 6 and interscapular brown adipose tissues (iBAT) were carefully removed and weighed immediately before being snap-frozen in liquid nitrogen and then stored at -80^{o}C until analysis. Selected biomarkers related to metabolic syndrome including glucose, insulin, and adiponectin levels, and lipid contents (triglycerides, total cholesterol and HDL-cholesterol) were determined, and the numbers of caecal lactobacilli and bifidobacteria were estimated using real-time PCR assay.

<table>
<tr><td rowspan="2">Exp</td><td rowspan="2">Groups</td><td colspan="9">Study period</td><td rowspan="2"></td></tr>
<tr><td>W1</td><td>W2</td><td>W3</td><td>W4</td><td></td><td>W5</td><td>W6</td><td>W7</td><td>W8</td></tr>
<tr><td rowspan="4">A</td><td>CONT</td><td colspan="4">CONT diet</td><td rowspan="4">OGTT</td><td colspan="4">CONT diet</td><td rowspan="4">OGTT+EUTHANASIA</td></tr>
<tr><td>HFHS</td><td colspan="4">HFHS diet</td><td colspan="4">HFHS diet</td></tr>
<tr><td>HFHS + 1% Burlington</td><td colspan="4">HFHS diet + 1% freeze-dried Burlington</td><td colspan="4">HFHS diet + 1% freeze-dried Burlington</td></tr>
<tr><td>HFHS + 1% Bluecrop</td><td colspan="4">HFHS diet + 1% freeze-dried Bluecrop</td><td colspan="4">HFHS diet + 1% freeze-dried Bluecrop</td></tr>
<tr><td colspan="11"></td></tr>
<tr><td rowspan="4">B</td><td>CONT+ 1% Burlington</td><td colspan="4">CONT diet</td><td rowspan="4"></td><td colspan="4">CONT diet + 1% freeze-dried Burlington</td><td rowspan="4">OGTT+EUTHANASIA</td></tr>
<tr><td>CONT+ 1% Bluecrop</td><td colspan="4">CONT diet</td><td colspan="4">CONT diet + 1% freeze-dried Bluecrop</td></tr>
<tr><td>HFHS + 1% Burlington</td><td colspan="4">HFHS diet</td><td colspan="4">HFHS diet + 1% freeze-dried Burlington</td></tr>
<tr><td>HFHS + 1% Bluecrop</td><td colspan="4">HFHS diet</td><td colspan="4">HFHS diet + 1% freeze-dried Bluecrop</td></tr>
</table>

Figure 7.1: Schematic diagram of the experiments.

Glucose Tolerance Test: Rats were fasted overnight before a glucose tolerance test was performed. Each rat was droplet-fed a glucose solution using a 1 ml syringe at the dose of 2 g of glucose/kg of body weight over a period of 1 – 3 minutes. The rat's tail was placed in warm water (35 - 40 degrees) for approximately 1 minute to dilate the veins, and the rat was then restrained in a plastic holder. A drop of blood was taken via tail vein puncture using a 23G x 3/4" needle at times 0, 45, 120, 180 and 240 min after glucose supplementation and the blood analyzed for glucose using a OneTouch Ultra® glucometer (Life Scan Inc., Milpitas, California, USA). This procedure took place in a laboratory which was temperature controlled and rats were visually monitored for stress. After the last blood sample was collected, the rats were returned to their respective cages and provided with food and water.

Blood collection and analysis: Blood samples were collected after an overnight fast. Half of samples were put into fluoride-containing tubes and immediately placed on ice. Plasma was obtained by centrifugation at 3500 rpm for 15 min for the assay of glucose concentration. The rest of blood samples were collected in tubes without anticoagulant and allowed to clot at room temperature for approximately 30 min, then centrifuged at the same conditions to recover serum, which was used for the assay of insulin, lipid profiles and adiponectin levels. Samples for all assays were kept frozen at -80 ^{o}C until analysis. The plasma glucose concentration was analysed by the hexokinase method on a Vitalab Flexor analyzer using a commercially available kit (Cat. #11447513 216, Roche New Zealand), and insulin levels in serum were measured with a radioimmunoassay kit (Cat. # RI-13K, Merck Millipore, New Zealand). The degree of insulin resistance was calculated according to the homeostasis model assessment for insulin resistance (HOMA-IR) using the following formula: [fasting glucose (mmol/L) x fasting insulin (µIU/mL)/22.5] (Bonora et al., 2000). Serum total cholesterol, triglycerides and cholesterol in the HDL fraction were assayed by enzymatic methods with commercially available kits (Cat. #12016630 122, #12016648 122 and #04713109 190 respectively, Roche New Zealand). Serum total adiponectin was assayed using coated microspheres Luminex xMAP technology with commercially available kits, according to the manufacturer's instructions (Cat. # RADPK-81K-ADPN, Merck Millipore, New Zealand).

Dual-energy x-ray absorptiometry (DEXA) scans: For the DEXA measurement, each rat was weighed and anaesthetised with an appropriate dose of anaesthetic (0.07-0.08 ml/ 100 g body weight). The anaesthetic drug, which consisted of a mixture of 0.2 ml of Acepromazine (ACP), 0.5 ml of Ketamine, 0.1ml of Xylazine and 0.2 ml of sterile water in 1 ml total volume, was administered via intraperitoneal injection using a 25G x 5/8" needle and 1 ml syringe. The animals attained a suitable level of anaesthesia approximately 5–10 min after injection and remained under anaesthesia for 2 h. Body composition was measured with a Hologic Discovery A bone densitometer (Bedford, MA, USA). A daily quality control scan was taken to ensure the precision met the required coefficient of variation. The coefficient of variation for the quality control data was 0.98-1.01. Rats were positioned supine with right angles between the spine and femur and between the femur and tibia, and underwent a high-resolution scan of the whole body.

Real time PCR: Total DNA from the caecum contents was extracted using ISOLATE Fecal DNA kit (Bioline, NSW, Australia) in accordance with the manufacturer's instruction. Aliquots of the DNA

extracted from each caecal sample were measured for DNA concentration using a spectrophotometer (Nanophotometer 2000 IMPLEN, Munchen, Germany) and then stored at -20^{o}C until analysed. Real-time PCR amplification reactions were performed with the LightCycler^{TM} 480 (Roche Diagnostics Ltd., Indianapolis, USA) using SYBR Green I Mastermix (Roche Ltd.) with two primer sets (Geneworks, Adelaide, Australia) of *Lactobacillus* and *Bifidobacterium* spp. as follows: Lab-0159-F (5'-GGA AAC AGR TGC TAA TAC CG-3'), UnivL-0515-R (5'-ATC GTA TTA CCG CGG CTG CTG GCA-3'), Bif1-F (5'-TCG CGT CYG GTG TGA AAG-3') and Bif2-R (5'-CCA CAT CCA GCR TCC AC-3'), respectively (Collier et al., 2003; Rinttila et al., 2004). PCR reaction was performed in a 20 µl volume in 96-well plates in triplicate and the composition of each reaction mix contained 1 µl of each primer, template DNA diluted with Nuclear free water in a total volume of 8 µl, and 10 µl of SYBR Green I Master (Roche), which includes *Taq* polymerase, reaction buffer, a deoxynucleotide triphosphate mixture, SYBR Green I dye and hot start antibody. The PCR cycle condition consisted of an initial denaturation and anti-*Taq* DNA polymerase antibody-inactivation step (5 min 95^{o}C), an amplification step (45 cycles of 15 s at 95^{o}C, 20 s at 63^{o}C and 10 s at 72^{o}C), and a melting-curve determination step (65^{o}C to 95^{o}C with 30 s hold). Measurement of SYBR green fluorescence was performed at the end of each amplification step and the threshold cycle (C_T) was defined as the cycle at which a significant increase in the magnitude of the signal generated by the PCR reaction was first detected. A standard curve for each bacterium culture in the range of 10-fold serial dilution was established by plotting a linear relation of bacteria concentration against C_T value, and this curve was subsequently used to quantify the precise number of specific bacteria in unknown samples using the LightCycler^{TM} software on the basis of the C_T value detected in each sample.

Statistical analysis

Data are expressed as mean ± S.E. and analysis was done with SAS version 9.2 for Windows. The effect of treatments on each parameter between two independent groups was compared using Student's t-test. One-way analysis of variance (ANOVA) was used to determine significant difference among three or more groups followed by Tukey's post-hoc test for means comparison. For the data of the number of *Bifidobacterium* spp. (in the treatment of HFHS+Blueberries), which was found to be non-normally distributed, non-parametric Kruskal-Wallis followed by Mann-Whitney tests was applied. The incremental blood glucose at each time point after glucose challenge was compared using repeated measures ANOVA. P values less than 0.05 were considered to be statistically significant. Statistical power analysis was performed

using data from the previous pilot study, and the sample size (7 rats per group) was calculated based on an expected difference in serum insulin (week 8) of 0.19 ng/mL at a power of 80% and a significance level of 5%.

Results :

Diet and energy consumption

The mean dietary consumption was not significantly different among groups in experiment A, ranging from the lowest in the HFHS group (23 g/day) to the highest in the CONT group (26 g/day). In experiment B, average food intake was found to be significantly higher ($P < 0.05$) in CONT+'Burlington' and CONT+'Bluecrop' groups as compared to their HFHS-fed counterparts (Table 7.2). However, the average daily energy consumption among groups of animals was not statistically different (between 470 and 500 kJ/day).

Table 7.2: Average dietary and energy consumption in rats.

Parameter measured	Average feed intake (g/d)	Average energy intake (kJ/d)	Total anthocyanin intake (mg/d)
Experiment A :			
CONT	25.8±1.0	455.8±17.4	-
HFHS	23.2±0.7	499.8±14.4	-
HFHS + 1% Burlington	24.5±0.6	527.5±12.7	4.2±0.1[a]
HFHS + 1% Bluecrop	24.0±1.3	509.9±26.4	2.7±0.1[b]
Experiment B :			
CONT + 1% Burlington	26.7±1.1[a]	473.0±18.5	4.6±0.2[a]
CONT + 1% Bluecrop	28.5±1.5[a]	503.7±27.0	3.2±0.2[b]
HFHS + 1% Burlington	23.1±0.9[b]	496.2±19.9	4.0±0.2[a]
HFHS + 1% Bluecrop	22.7±0.6[b]	484.5±11.9	2.6±0.1[b]

Results are expressed as mean ± S.E. of N = 7 rats per group. Different superscript letters within the same column in each experiment indicate significant differences ($P < 0.05$).

Freeze-dried 'Burlington' powder contained 17.2 mg total anthocyanins/g FD weight, which is composed of 10.8 mg galactoside (63%), 0.2 mg glucoside (1%), and 6.1 mg arabinoside (35%) derivatives of ACNs, and 0.1 mg acylated anthocyanins (1%). Freeze-dried 'Bluecrop' contained anthocyanins (11.2 mg total anthocyanins/g FD weight (galactosides 3.4 mg (30%), glucosides 2.5 mg (22%), arabinosides 3.7 mg (33%) and acylated anthocyanins 3.7 mg (33%)). Therefore, rats in the 'Burlington' groups consumed diets in the range between 23 and 27 g/day, which provided approximately 4 mg of total anthocyanins/day. 'Bluecrop'-fed rats consumed diet

between 23 and 29 g daily, which provided anthocyanins/day in the range of 2.6 to 3.2 mg. Due to higher total anthocyanin concentration found in freeze-dried 'Burlington', the rats fed with CONT+'Burlington' or HFHS+'Burlington' received significantly more anthocyanins ($P < 0.05$) than their counterparts which consumed the 'Bluecrop' variety.

HFHS diets induced signs of obesity and insulin resistance

As shown in Table 7.3, the HFHS diet induced an increase in obesity parameters, especially increasing the overall percentage of body fat and the abdominal fat pads when compared to CONT diet-fed rats, but no significant difference was found in the rats' body weight gain ($P = 0.11$). Furthermore, within the 8-week period this diet also showed a trend to induce signs of insulin resistance and glucose intolerance. Fasting blood glucose and insulin concentrations were higher in the HFHS-fed rats than in the control. In addition, the insulin resistance index (HOMA-IR) in the HFHS group rose to almost triple the level compared to the control group. After oral glucose loading, postprandial blood glucose concentration in HFHS group was increased dramatically compared to the control animals (Figure 7.2), particularly at time = 120 min which was found to be significantly different between the two groups.

Table 7.3: Effect of starch-based (CONT) diet and high-fat-high-sugar (HFHS) diet on selected metabolic biomarkers.

Parameter measured	CONT	HFHS	P-value
At week 4:			
Body weight (g)	499.9±17.2	515.6±13.6	0.49
At week 8:			
Body weight (g)	555.7±18.7	605.2±22.0	0.11
% fat	17.0±1.6	27.5±1.6	< 0.01
Total WAT (g)	33.3±4.0	58.0±5.1	< 0.01
Total WAT (g/kg BW)	59.5±6.4	95.2±5.9	< 0.01
Glucose (mmol/L)	10.1±0.4	12.2±1.0	0.08
Insulin (ng/mL)	0.2±0.03	0.2±0.1	0.06
HOMA-IR	2.5±0.3	7.2±2.4	0.08

Results are expressed as mean ± S.E. of N = 7 rats per group
Total WAT comprised of retroperitoneal, mesenteric and epididymal fat pads

Body weight gain was measured and the oral glucose tolerance test was performed at week 4. These measurements were done in order to investigate whether the rats developed signs of obesity and glucose intolerance at this stage and used as background information before

blueberry supplementation was given to animals (for studying the therapeutic effect in the experiment B). A slight gain in body weight was observed in animals fed with the HFHS diet compared to the CONT group. More importantly, the oral glucose tolerance test data indicated that animals could develop signs of impaired glucose tolerance as early as 4 weeks when fed with the HFHS diet. This was because their blood glucose concentration was considerably higher than that of the control group at every time point measured, including baseline, and a significant difference was found at the time of 45 min after glucose loading.

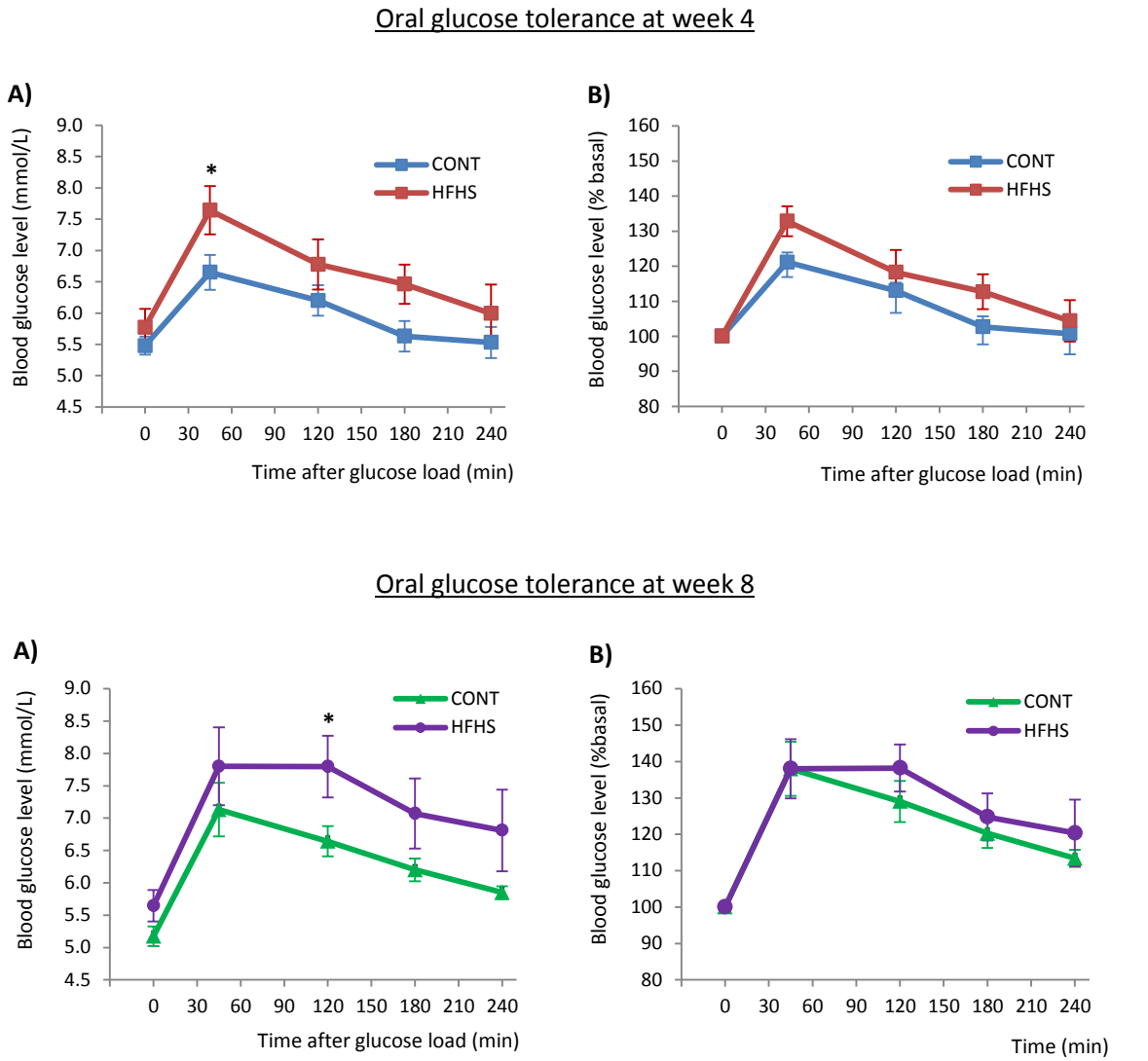

Figure 7.2: Oral glucose tolerance test responses in rats fed either CONT or HFHS diet for short term (4weeks) or long term (8 weeks) (A); and normalised to time 0 (B). Results are expressed as mean ± S.E. of N = 7 rats per group. *$P < 0.05$ compared with the CONT diet at the same time.

Comparison of two different varieties of blueberries ('Burlington' vs. 'Bluecrop') on metabolic variables

The effect of two specific blueberry cultivars ('Burlington' enriched in galactosylated and 'Bluecrop' enriched in glucosylated anthocyanins) on metabolic changes was compared using a t-test among three independent pairs as followed: 1) when supplemented into the HFHS diet for 8 weeks (Experiment A), 2) when supplemented into the HFHS diet for 4 weeks (Experiment B), and 3) when supplemented into the CONT diet for 4 weeks (Experiment B). As shown in Table 7.4, no significant differences were observed between 'Burlington' and 'Bluecrop' on modulation of parameters related to obesity, insulin resistance or glucose tolerance in any of the group. This result indicated that the ability of galactosylated anthocyanins was not different from the ability of glucosylated anthocyanins, suggesting that the nature of anthocyanin derivatives was not important in modulating these metabolic biomarkers.

Since no significant differences were found in any parameter measured between 'Burlington' and 'Bluecrop', the data of these two varieties were pooled and the effect of consumption of freeze-dried blueberry powder in general on each metabolic biomarker was then further examined.

Effect of freeze-dried blueberry powder consumption on metabolic variables associated with insulin resistance, glucose tolerance and obesity

Experiment A was carried out to investigate whether blueberry powder consumption protects against obesity and insulin resistance in animals fed a HFHS diet. At the end of 8-week HFHS+BB feeding, analysis of blood parameters showed that there was no difference between the HFHS+BB and the HFHS diet groups in term of fasting plasma glucose. However, we observed a trend to prevent an increase in fasting insulin concentration and insulin resistance index with blueberry supplementation. This was because fasting insulin levels and HOMA-IR index in rats receiving blueberry powder were found to be 33 % and 36 % lower than in rats on the HFHS diet (Table 7.5). In addition, consuming freeze-dried blueberry powder appeared to improve glucose tolerance in the animals. As seen in Figure 7.3, the incremental glucose concentration of HFHS supplemented with BB was lower than the HFHS group at 120, 180 and 240 min after an oral glucose loading; however these values did not reach statistical significance.

Table 7.4 : Comparison of the effect of different cultivars of blueberry ('Burlington' and 'Bluecrop') supplemented into the CONT diet for 4 weeks, and supplemented into the HFHS diet for 4 and 8 weeks on selected metabolic biomarkers.

Parameter measured	Experiment A			Experiment B					
	HFHS+Burl	HFHS+Blue	P-value	HFHS+Burl	HFHS+Blue	P-value	CONT+Burl	CONT+Blue	P-value
	Long term duration (wk8)			Short term duration (wk4)			Short term duration (wk4)		
Body weight (g)	657.0±16.3	627.9±33.7	0.45	605.3±21.8	603.8±21.8	0.96	570.7±22.1	568.3±19.5	0.94
% fat	28.7±1.0	27.5±2.1	0.60	25.5±2.4	28.3±1.8	0.36	22.6±0.4	22.4±2.6	0.96
Total WAT (g)	69.9±2.8	62.7±6.7	0.34	52.5±6.4	49.6±4.8	0.72	38.7±2.3	40.0±5.9	0.84
Glucose (mmol/L)	12.0±0.6	11.9±1.0	0.94	11.6±0.6	11.2±0.7	0.70	11.1±1.0	10.5±0.4	0.57
Insulin (ng/mL)	0.4±0.04	0.3±0.1	0.21	0.4±0.1	0.4±0.04	0.59	0.4±0.10	0.3±0.04	0.58
Oral Glucose Tolerance Test (mmol/L) at week 8									
time 0 min	5.5±0.6	5.4±0.8	0.90	5.4±0.5	5.4±0.3	0.92	5.1±0.4	5.1±0.5	0.99
time 45 min	7.8±0.6	7.9±1.4	0.83	7.0±0.7	7.4±0.8	0.29	6.5±0.9	6.6±0.8	0.90
time 120 min	7.4±0.8	7.2±1.6	0.81	6.9±0.9	6.8±0.8	0.84	6.3±0.8	6.4±0.4	0.72
time 180 min	6.7±0.8	6.7±1.5	0.90	6.1±0.9	6.0±0.7	0.87	5.9±0.6	5.9±0.5	0.90
time 240 min	6.4±0.6	5.9±1.0	0.34	5.9±0.7	5.9±0.8	0.87	5.7±0.6	5.7±1.0	0.96
Oral Glucose Tolerance Test (mmol/L) at week 8 (normalised to time 0)									
time 0 min	5.6±0.0	5.6±0.0	1.00	5.6±0.0	5.6±0.0	1.00	5.6±0.0	5.6±0.0	1.00
time 45 min	7.9±0.2	8.1±0.4	0.65	7.1±0.2	7.6±0.3	0.23	7.2±0.4	7.3±0.5	0.85
time 120 min	7.5±0.2	7.4±0.6	0.90	7.0±0.2	7.0±0.4	0.94	6.9±0.3	7.1±0.4	0.70
time 180 min	6.8±0.1	6.8±0.5	1.00	6.2±0.2	6.2±0.3	0.99	6.5±0.3	6.5±0.3	0.86
time 240 min	6.5±0.2	6.1±0.3	0.27	6.0±0.1	6.0±0.3	0.93	6.2±0.2	6.3±0.4	0.92

Results are expressed as mean ± S.E. of N = 7 rats per group

Total WAT comprised of retroperitoneal, mesenteric and epididymal fat pads

Unlike insulin and glucose tolerance indices, feeding of freeze-dried blueberry powder did not seem to have any beneficial effect in terms of preventing obesity. Unexpectedly, inclusion of the whole blueberry powder in the HFHS diet slightly increased weight gain, the percentage of fat, and the total white adipose tissue weight in animals compared to the HFHS-fed group (Table 7.5). This may partly be due to higher average food and energy intake in the HFHS+BB group as compared to the HFHS-fed group; which in itself may suggest that the diet was simply more palatable in the presence of blueberries.

Table 7.5: Effect of freeze-dried blueberry supplementation added into either HFHS or CONT diets on selected metabolic biomarkers.

Parameter measured	**HFHS 8 wk**	**HFHS+BB 8 wk**	**HFHS 4 wk ↓ HFHS+BB 4 wk**	**CONT 8 wk**	**CONT 4 wk ↓ CONT+BB 4 wk**
Feed intake (g/d)	23.2±0.7	24.3±0.7	22.9±0.5	25.8±1.0	27.6±0.9
Energy intake (kJ/d)	499.8±14.4	518.7±14.3	490.4±11.2	455.8±17.4	488.3±16.3
Body weight (g)	605.2±22.0	642.4±18.4	604.6±14.8	555.7±18.7	569.5±14.2
% fat	27.5±1.6	28.1±1.2	26.9±1.5	17.0±1.6	22.5±1.3*
Total WAT (g)	58.0±5.1	66.3±3.6	51.0±3.9	33.3±4.0	39.4±3.0
Total WAT (g/kg BW)	95.2±5.9	102.6±3.3	83.5±4.9	59.5±6.4	68.4±4.1
Glucose (mmol/L)	12.2±1.0	11.9±0.5	11.4±0.5	10.1±0.4	10.8±0.5
Insulin (ng/mL)	0.5±0.1	0.3±0.03	0.4±0.05	0.2±0.03	0.3±0.05
HOMA-IR	7.2±2.4	4.6±0.6	5.0±0.7	2.5±0.3	4.2±0.8

Results are expressed as mean ± S.E. of N = 7 rats in CONT and HFHS groups, and N = 14 rats in CONT+BB and HFHS+BB groups. *$P < 0.05$ compared with the CONT diet
Total WAT comprised of retroperitoneal, mesenteric and epididymal fat pads

In experiment B, all rats initially consumed the HFHS for 4 weeks to induce the signs of metabolic syndrome before being assigned to the HFHS+BB diets. The main objective of this experiment was to investigate the therapeutic effect of blueberry powder supplementation on alleviation of selected metabolic parameters. In general, the treatment effect of BB consumption was in line with the preventive effect found in experiment A. Whole freeze-dried blueberry supplementation tended to normalize fasting insulin concentration and insulin resistance index induced by the HFHS diet. As shown in Table 7.5, fasting insulin concentration and HOMA-IR score of the HFHS+BB were decreased by 20 % and 30 %, respectively compared to the HFHS-

treated group. More importantly, following an oral glucose load, the incremental blood glucose levels in HFHS+BB-fed rats fell to a value significantly lower than that of the HFHS-fed counterparts ($P < 0.05$) at 120, 180 and 240 min (Figure 7.3A), which was found to decrease to near those observed in the CONT-fed animals. The findings indicate that blueberry supplementation had a significant effect on improving glucose tolerance in animals. Similarly to experiment A, consumption of whole blueberry powder did not show any beneficial effect either in reducing body weight gain or in decreasing fat accumulation. Notably, this group of rats (HFHS+BB 4 wks) had slightly lower energy intake compared to the HFHS group and consequently showed lower levels of body weight, percentage of fat and total WAT weight than the HFHS-fed counterpart. The data obtained may, in part, confirm the notion that beyond the composition of the diet, the amount of diet and energy consumed greatly affect obesity parameters in animals.

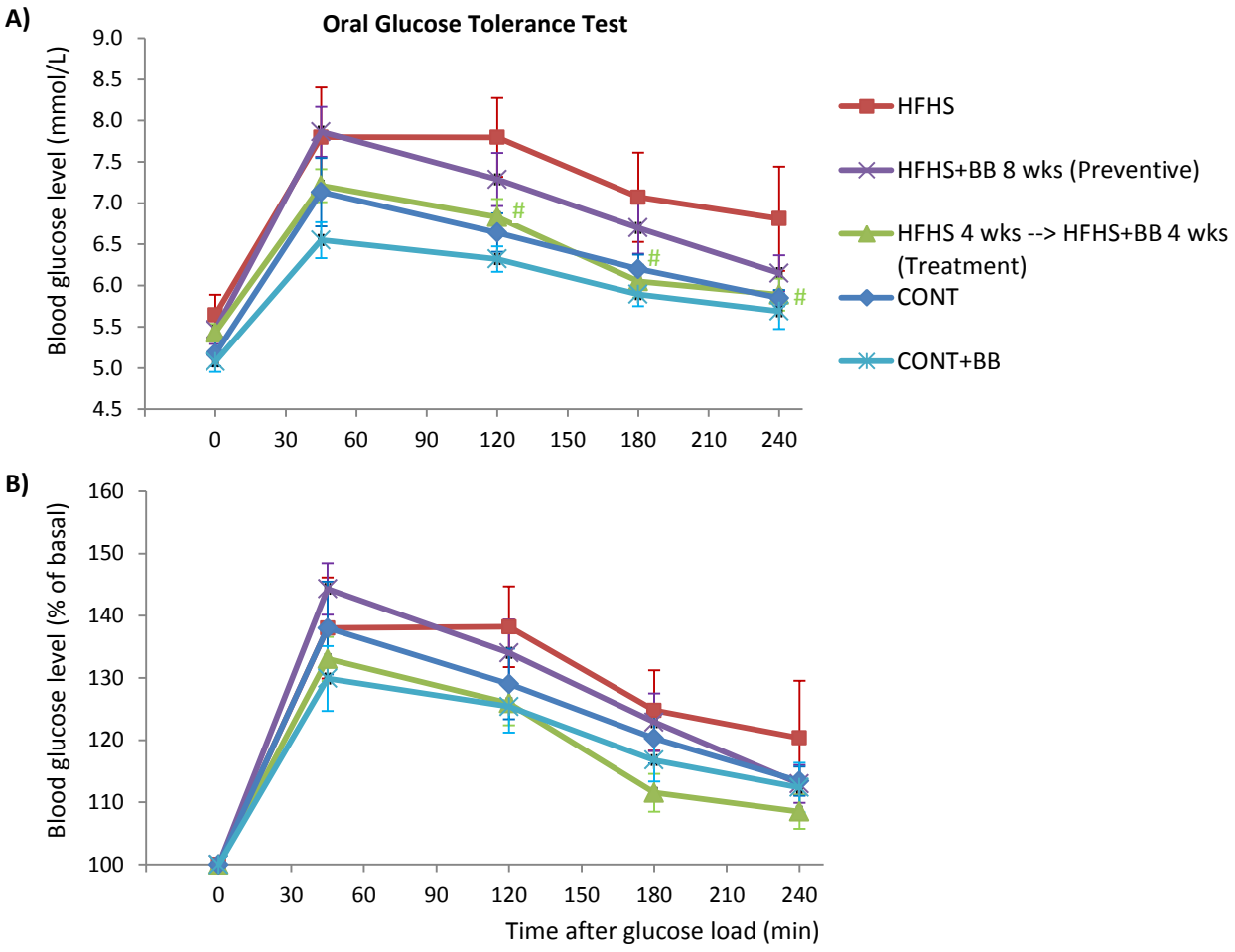

Figure 7.3: Oral glucose tolerance test responses in rats fed different diets (A); and normalised to time 0 (B). Results are expressed as mean ± S.E. of N = 7 rats in CONT and HFHS groups, and N = 14 rats in CONT+BB and HFHS+BB groups. #$P < 0.05$ compared with the HFHS diet.

In addition to supplementing the HFHS diet with BB, supplementing the CONT diet with BB was carried out for the same period of time (4 weeks) for comparison. In this case, the only beneficial effect after blueberry treatment was that it showed a trend to slightly lower the increments by which the glucose concentrations changed during the OGTT (Figure 7.3A and B). However, the effect of blueberries on biomarkers associated with insulin resistance was somewhat unexpected. Fasting glucose and insulin concentrations as well as HOMA-IR index of CONT+BB-fed rats were found to be higher than the CONT-fed counterpart. Again, consumption of whole freeze-dried blueberries did not reduce body weight gain or total WAT weights; in contrast it significantly increased the percentage of fat in animals (Table 7.5).

Effect of freeze-dried blueberry powder on intestinal microbiota

Table 7.6 shows the effect on the population size of *Lactobacillus* and *Bifidobacterium* spp. in the caeca of rats after administration of whole freeze-dried blueberry powder into the HFHS diet for 8 weeks in experiment A (preventive effect) or 4 weeks in experiment B (treatment effect). Real time PCR results showed that blueberry powder tended to increase the growth of both *Lactobacillus* spp. (P = 0.20) and *Bifidobacterium* spp. (P = 0.15; Kruskal-Wallis test). For both short term and long term feeding, *Lactobacillus* spp. increased by approximately 0.3 $\log_{10}$ CFU/g caecal contents, while *Bifidobacterium* spp. increased by between 0.5 and 0.6 $\log_{10}$ CFU/g caecal contents after blueberry consumption, compared to the HFHS-fed counterparts.

Table 7.6: Effect of freeze-dried blueberry supplementation added into HFHS diets on the growth of probiotic bacteria

Bacteria (log 10 CFU/g caecal contents)	**HFHS**	**HFHS + Blueberries for long term period (8 weeks)**	**HFHS + Blueberries for short term period (4 weeks)**
Lactobacillus spp.	10.0±0.2	10.3±0.1	10.3±0.1
Bifidobacterium spp.	11.4±0.4	11.9±0.1	12.0±0.1

Results are expressed as mean ± S.E. of N = 7 rats in CONT and HFHS groups, and N = 14 rats in CONT+BB and HFHS+BB groups.

Similarly to the HFHS diet, supplementation of the freeze-dried whole blueberries into the CONT diet appeared to slightly increase the numbers of *Lactobacillus* spp. in the rats' caecal content (approximately 0.25 $\log_{10}$ CFU/g caecal contents). However, an addition of freeze-dried blueberries resulted in a significant increase in the numbers of *Bifidobacterium* spp. (increased approximately 0.5 $\log_{10}$ CFU/g caecal contents; P = 0.01), when compared to unsupplemented CONT group (Table 7.7).

Table 7.7: Effect of freeze-dried blueberry supplementation added into CONT diets on the growth of probiotic bacteria

Bacteria (log 10 CFU/g caecal contents)	**CONT**	**CONT + Blueberries**	**P-value**
Lactobacillus spp.	10.3±0.1	10.5±0.1	0.11
Bifidobacterium spp.	11.6±0.2	12.1±0.1*	0.01

Results are expressed as mean ± S.E. of N = 7 rats per groups. *$P < 0.05$ compared with the CONT diet

Discussion :

Overconsumption of unsaturated fat and simple sugar is well documented as one of the major causes of development of metabolic disorders including insulin resistance, hyperglycemia, hyperinsulinemia and dyslipidemia, which are closely linked to the development of various chronic diseases such as diabetes and cardiovascular disease (Wilcox, 2005; Cornier et al., 2008). There is a growing number of reports in the literature describing an increase in body weight, blood glucose and insulin concentrations, or other metabolic biomarkers with the excessive consumption of high fat and/or high sugar diets in both animal and human studies (Daly, 2003; Gajda et al., 2007). Our results are consistent with previous reports which found that consumption of a high-fat-high-sugar diet markedly induces an increase in body weight in SD rats, and in particular an increase in the percentage of fat and abdominal fat pad weight. Moreover, this diet also induced an increase in fasting hyperglycemia associated with hyperinsulinemia, and consequently induced the development of insulin resistance. A significant increase in blood glucose level after glucose loading during OGTT was also found in HFHS-fed rats, indicating that the ability of insulin to stimulate glucose uptake was impaired and hence compensated by an increase in insulin secretion from the beta cells in the pancreas.

Blueberries are polyphenolic-rich fruits that exhibit a high antioxidant capacity and have a unique composition of anthocyanin compounds. Earlier studies have documented beneficial effects of blueberry supplementation on various metabolic parameters *in vitro* (Martineau et al., 2006), in animals models (DeFuria et al., 2009; Grace et al., 2009), and in human participants (Basu et al., 2010; Stull et al., 2010). The doses of blueberry powder provided to animals in the present study were calculated based on the assumption that a 70 kg human could consume approximately 330 mg of total anthocyanins per day (Clifford, 2000), which is equivalent to around 1.2 mg of total anthocyanins/250 g body weight in the rat/day. Depending upon the previous data obtained in Chapter 3 and the conversion from frozen weight to freeze-dried

weight, the 'Burlington' and 'Bluecrop' cultivars contained an average of 6.0 mg total anthocyanins/g freeze-dried berries. Therefore, to provide 1.2 mg of total anthocyanins to rats, they needed to consume at least 0.2 g of freeze-dried blueberry per day. Based on the previous data, SD rats consume approximately 20 g of diet per day, therefore 0.2 g of freeze-dried blueberry should be supplemented into 20 g of diet, which is equivalent to 1 g/100 g diet or at the dose of 1%.

In the present study, our data showed that inclusion of whole freeze-dried blueberry powder into the HFHS diet could normalise insulin concentrations and effectively suppress glucose intolerance induced by the HFHS diet, indicating a beneficial role for both prevention and treatment of diet-induced insulin resistance by inclusion of blueberries in the diet. Besides the effects on insulin resistance, blueberry treatment tended to influence growth of both *Lactobacillus* and *Bifidobacterium* spp. It is believed that these two bacterial strains exert a wide range of beneficial health effects including inhibition of pathogen growth, increasing synthesis of vitamins and other beneficial bioactive compo3unds from food components etc. More importantly, these bacteria are believed to be responsible for the inflammatory process linked to insulin resistance (Delzenne & Cani, 2010). However, the effect of blueberry consumption on fasting blood glucose reduction was not significant. Feeding rats with freeze-dried blueberries for a short period of time (4 weeks) induced a larger response reducing blood glucose than the long term feeding (8-weeks). However, levels of fasting glucose can be influenced by many factors and the slight effect of lowering fasting glucose concentration which occurred in this group of animals may be partially explained by a higher insulin secretion.

Unlike the parameters associated with insulin resistance, whole freeze-dried blueberry powder was not effective in preventing or normalising obesity parameters including body weight, percentage of fat and abdominal fat pad weight. Our results with regards to the anti-obesity effects of blueberries were inconsistent with some earlier studies, which demonstrated a significant protective effect of blueberry feeding on various parameters associated with obesity in animal models (Vuong et al., 2009). The contradictory findings might be due to different types of blueberries used in the experiments or different concentrations of blueberries provided to animals etc. However, our findings are in agreement with the study of Prior et al. (2008) who showed that C57BL/6J mice fed with a high-fat diet (45% calories from fat; HF45) plus whole freeze-dried blueberry for 92 days gained significantly more body weight, body fat (% body weight), and epididymal fat weight than those in the HF45-fed controls, therefore demonstrating

no protective effect by the blueberries. Furthermore, DeFuria et al. (2009) also found no protective effect of whole blueberry powder consumption against high fat diet (HFD)-induced weight gain and adiposity, as the body weight and the epididymal adipose tissue mass of mice fed the HFD and the HFD+BB did not differ and were greater than that of mice fed the LFD. We observed that rats fed a HFHS diet supplemented with blueberries gained more fat than rats on unsupplemented HFHS diet. However, the mechanism for this is not clear. According to the nutrient composition of the experimental diets (Table 7.1), addition of 1% freeze-dried whole blueberries would have increased the amount of sugar approximately 1 g/100 g diet. Therefore, if animals consumed approximately 25 g of diet daily, they would receive about 0.25 g of sugar more than their control counterparts per day. This substitution, however, is not large enough to account for the increased obesity in animals fed with blueberries.

It is believed that bioactive compounds in blueberries, in particular anthocyanins play an important role in modulating factors associated with metabolic syndrome (Grace et al., 2009; Prior et al., 2010). Some previous studies highlighted the beneficial effect of some specific anthocyanins on metabolic syndrome both *in vitro* and *in vivo*. For instance, Jayaprakasam et al. (2005) found that cyanidin 3-glucoside and delphinidin 3-glucoside were effective compounds in relation to increased insulin secretion. Adisakwattana et al. (2009) reported the inhibition of α-glucosidase activities by cyanidin 3-galactoside *in vitro*, whereas Grace et al. (2009) studied hypoglycaemic activity of different anthocyanins in animal models and found that malvidin-3-glucoside gavaging with Labrasol had the ability to lower blood glucose levels. To our knowledge, this is the first study to compare the effects between the galactoside derivatives and the glucoside derivatives of anthocyanins (by using two specific blueberry cultivars 'Burlington' and 'Bluecrop", which have different profiles of individual anthocyanins) on parameters associated with metabolic syndrome in animals. However, we found no significant differences between 'Burlington', which is high in galactoside derivatives of anthocyanins, and 'Bluecrop', which is high in glucoside derivatives of anthocyanins, on modulating any metabolic parameter assessed here. Only a slightly higher number of probiotic bacteria and a lower level of fasting insulin were found in 'Bluecrop'-fed rats compared to the 'Burlington'-treated groups (data shown in the Appendix II). Since the composition of individual anthocyanins in 'Burlington' cultivar is different from the anthocyanin composition in 'Bluecrop' cultivars, it appears that glucoside derivatives of anthocyanins are likely to possess more anti-insulin resistance properties than galactoside derivatives of anthocyanins; however, the effect was not strong enough to detect a statistically significant difference.

It is not possible from the data in our study to identify which mechanisms were responsible for the anti-hyperinsulinemia and anti-glucose tolerance effects of the blueberry powder; however, the findings presented here demonstrated that blueberry supplementation could improve the insulin sensitivity as indicated by a more rapid clearance of the incremental glucose during the OGTT and decreased circulating plasma insulin levels found in the blueberry-fed animals. Insulin sensitivity is a condition describing how sensitively the body cells react to the effect of insulin. Individuals with low insulin sensitivity, also refered to as insulin resistance, will require larger amounts of insulin in order to keep their blood glucose stable. The body, therefore, tries to compensate by producing more insulin over time and once the body no longer produces as much insulin as needed, diabetes will develop (NIDDK, 2008). An earlier study by Martineau et al. (2006) described the ability of an extract from Lowbush blueberry fruits to increase pancreatic beta cell proliferation in a cell culture-based assay, suggesting a potential ability to restrain beta-cell damage which could hence be associated with improvement in insulin sensitivity. DeFuria et al. (2009) also reported that whole blueberry powder (1:1 Rabbiteye and Highbush blueberry) could attenuate insulin resistance in an animal model. The authors stated that blueberry supplementation at the dose of 4% for 8 weeks improved insulin sensitivity, as indicated by lower levels of glucose AUC (area under the curve) during an intraperitoneal insulin tolerance test (ITT) in mice fed the HFD+BB (AUC: 549.6 ± 19.9 mmol/L·min) compared with those fed the HFD (AUC: 787.8 ± 35.2 mmol/L·min). In addition, Stull et al., (2010) who first reported the effect of supplementation with blueberries on whole body insulin sensitivity in humans, consistently demonstrated that insulin sensitivity as measured by hyperinsulinemic euglycemic clamps was more enhanced in the blueberry group (1.7 ± 0.5 mg·kg FFM^{-1}·min^{-1}) after 6-week consumption as compared to the placebo group (0.4 ± 0.4 mg·kg FFM^{-1}·min-^{1}) without significantly altering energy intake and adiposity in individuals.

Although it is widely believed that obesity, one of the key physiological features of metabolic syndrome, is closely associated with the development of metabolic disorders and the increased risk of type II diabetes, it has been shown previously that this association may not be true in all cases. A number of epidemiological studies demonstrated that metabolic syndrome and type II diabetes can develop in non-obese people or among individuals with a healthy weight (Reaven, 1993: Yoon et al., 2006). In particular, the South Asian population have been found to develop metabolic abnormalities at a lower body mass index and waist circumference than other groups of population (Enas et al., 2007). Therefore, it is possible that, despite a lack of evidence for blueberry supplementation suppressing energy intake or decreasing weight gain and abdominal

obesity, bioactive components in blueberries may affect some of the factors associated with metabolic syndrome, possibly through mechanisms associated with insulin.

Another mechanism by which blueberries could reduce the risk of development of metabolic syndrome may involve inhibitory activity of blueberry extracts against pancreatic α-amylase and intestinal α-glucosidase enzymes. The data described in the current chapter found a remarkable decrease in incremental glucose after glucose loading (OGTT) (Figure 7.3) as well as a trend towards a reduction in the level of serum insulin in the HFHS+BB-fed groups compared to their non-BB-fed counterparts (Table 7.5). It is possible that the bioactive compounds in blueberries could delay the digestion of carbohydrate and/or suppress glucose absorption, which led to a reduction of postprandial hyperglycemia and possibly contributed to lower levels of insulin being released from the pancreas. The results obtained from the present animal study are consistent with the ability of blueberries to inhibit starch hydrolysis enzymes, as found in our previous *in vitro* studies.

More recent observations have suggested that polyphenolic compounds including anthocyanins can modulate intestinal microbiota (Dolara et al., 2005; Hidalgo et al., 2012), which is another possible mechanism by which insulin resistance can be altered. It has been well documented that inflammation in the insulin responsive tissues is one of the major contributors to insulin resistance and related metabolic diseases (Hirabara et al., 2012). Previous data suggests that lipopolysaccharide (LPS), a membrane component of Gram negative bacteria, acts as a trigger for the development of inflammation (Cani & Delzenne, 2009). The leakage of gut lumen contents results in an increase of LPS concentration in the portal blood (D'Aversa et al., 2013). Previous findings support the idea that modulation of gut microbiota, particularly *Bifidobacterium* spp., by treatment with prebiotics restored normal intestinal epithelial integrity and hence decreased plasma LPS levels in obese *ob/ob* mice, and this change in LPS levels in the blood was associated with improved glucose tolerance and a normalised inflammatory status (Cani et al., 2007; Cani et al., 2009). Our results showed an increase in the number of *Bifidobacterium* spp. in caecal contents and there was a trend to enhance the growth of *Lactobacillus* spp. in caecal contents after consumption of powdered freeze-dried whole blueberries. These positive changes in the microflora may contribute to a reduction of the impact of high-fat-high-sugar diet-induced inflammation and insulin resistance. Our data are in agreement with other published studies. In a study by Molan et al. (2009), oral administration of the water extracts from two Rabbiteye blueberry cultivars, namely 'Maru' and 'Centurion', for 6 days significantly increased the number

of lactobacilli and bifidobacteria in the fecal samples of SD rats. Furthermore, Vendrame et al. (2011) documented that daily consumption of a powdered wild blueberry drink for six weeks significantly increased the population size of *Bifidobacterium* spp. in the gut of healthy men.

When either 'Burlington' or 'Bluecrop' powder was added into the CONT diet, increases in body weight gain, fat mass, fasting concentrations of glucose and insulin were found in rats fed with CONT+BB diet compared to their counterparts given CONT diet without blueberries. The explanation could be the difference of daily energy consumption between these two groups. We observed that CONT+BB-fed rats consumed more diet and therefore had a higher energy intake compared to the CONT-fed group, which may have resulted in the observed obesity and symptoms of hyperglycemia; similarly, the blueberries possibly made the CONT diet more palatable. Another possibility for a lack of benefit may be due simply to variability arising from genetic variation in these outbred animals. It has been widely accepted that metabolic responsiveness depends not only on dietary and environmental factors, but is also influenced by the genetic background of an individual. It is possible that rats in CONT+BB group were more susceptible to starch-induced metabolic impairment, consequently may have produced higher basal fasting glucose and insulin concentrations than the CONT-fed rats, and blueberry supplementation for only 4 weeks was too short to normalise these effects.

In the present study, blueberries have been shown to enhance insulin sensitivity only in the HFHS-fed groups but not in the CONT-fed group. Several previous studies also found a different responsiveness between lean and obese phenotypes. For instance, Livingstone et al. (2000) found an effect of metformin and rosiglitazone (insulin-sensitizing drugs) on the improvement of insulin sensitivity in obese but not lean Zucker rats. Also, pioglitazone, an antihyperglycemic agent, has been shown to normalise hyperglycemia only in Wistar fatty rats, but not in lean rats in a previous study by Kobayashi et al. (1992). In addition, in a study by Jeyakumar et al. (2005), supplementation of dietary vitamin A at a high dose for 2 months resulted in a significant decrease in the adiposity index and retroperitoneal fat weight in obese rats of the WNIN/Ob strain, whereas a marginal reduction was found in lean animals. The latter authors explained that the observed differences between lean and obese individuals with regard to various metabolic parameters could be, in part, due to the differences in their genetic composition.

Furthermore, our study consistently showed that addition of blueberries into either HFHS or CONT diet increased adipose tissue accumulation. Adiponectin is a hormone that helps to modulate a number of metabolic processes, including glucose homeostasis and fatty acid catabolism. High levels of the hormone are associated with a reduced risk of metabolic conditions (Snehalatha et al., 2003). Since the levels of circulating adiponectin are normally found to be inversely correlated with the accumulation of adiposity, greater deposition of fat in the blueberry-fed groups may partially explain the lower levels of adiponectin found in the HFHS+BB and CONT+BB-fed animals when compared to their HFHS or CONT counterparts (data shown in the appendix).

In conclusion, the results of this study suggest that consumption of whole freeze-dried blueberry powder from two selected cultivars of Highbush blueberries ('Burlington' and 'Bluecrop') was effective in normalising hyperinsulinemia and improving insulin resistance in animals at risk of metabolic syndrome. However, no significant differences were found between specific individual anthocyanins (galactoside vs. glucoside derivatives) on modulation of any parameter associated with metabolic syndrome, suggesting the nature of their anthocyanins was not important *in vivo*. Furthermore, the blueberry powder administered into both CONT and HFHS diets was able to favourably modulate the composition of the intestinal microbiota, by increasing the population size of particular bacteria strains from the genus Lactobacillus and Bifidobacterium, which are among the most health promoting bacterial groups. However, the precise mechanisms which underlie the effects are not fully understood. Further studies to investigate the underlying mechanisms and pinpoint the specific polyphenolic compounds in blueberries that may be responsible for such effect are required.

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CHAPTER 8

General discussion and conclusions

The primary aim of this research was to evaluate the potential ability of selected NZ Highbush blueberries to ameliorate metabolic disorders associated with type II diabetes, and to provide further knowledge with regards to the role of individual anthocyanins present in blueberries on modulation of metabolic syndrome parameters. In order to achieve these objectives, various NZ Highbush blueberry varieties were screened *in vitro* for total polyphenolic content, individual anthocyanins and antioxidant activity, and for their ability to inhibit starch hydrolysis enzymes and increase the growth of probiotic bacteria associated with insulin resistance. In addition, ability of two selected blueberry cultivars, which contain different compositions of individual anthocyanins (rich in glucosides vs. galactosides), were compared *in vivo* to examine their actual physiological responses as well as to investigate whether any of these compounds contribute more to the beneficial effects on MS. The results obtained from the present study may be useful for a recommendation to consumers and/or to provide better understanding in the role of anthocyanins in blueberries on metabolic parameters.

8.1 Bioactive compounds and antioxidant activity (AOA)

Evidence indicates that the beneficial effects of a plant-based diet are mainly derived from their bioactive compounds, and possibly from the AOA present in the plants. Therefore, examining the polyphenolic profile and AOA of Highbush blueberries is crucial to serve as background information. Data reported in Chapter 3 demonstrated that Highbush blueberries grown in New Zealand have a high total phenolic content (TPC) and antioxidant capacity as the TPC (extracted by mixed solvent) obtained in this study were slightly higher than those presented by other authors for Highbush cultivars (Prior et al., 1998; Ehlenfeldt & Prior, 2001; Moyer et al., 2002; Giovanelli et al., 2009; Rodrigues et al., 2011). In addition, the AOA of these Highbush blueberries are relatively similar to those reported by Koca et al. (2009) and Rodrigues et al. (2011), although our values were found to be lower than those reported by Bunea et al.

(2011). However, it is important to note that the TPC and AOA could differ between studies due to various factors involved such as the cultivation of the berries and climate conditions, the degree of ripeness, the storage condition, the methods employed for analysis and/or the different solvents used for sample preparation.

The present study agrees with earlier reports finding considerable variation in blueberry polyphenol concentrations and AOA between cultivars (Prior et al., 1998; Howard et al., 2003; Molan et al., 2010; Bunea et al., 2011) as well as variation caused by the solvent systems tested (Barnes et al., 2009; Flores et al., 2013). In the present study, the most efficient solvent for phenolic extraction and AOA evaluation from blueberries was mixed solvent (acetone: methanol: water: formic acid, 40:40:20:0.1% v/v), whereas the least effective solvent was pure water. Organic solvents can penetrate cell membranes readily, whereas water can only extract materials that are already accessible to water (Naczk & Shahidi, 2004). Another possibility is the polarity of solvents, where hydrogen bonding in highly polar solvents may interfere with the H-atom donor activity of phenolics which could lead to a reduction in antioxidant activity measured (Pinelo et al., 2004). In addition, the form of berries could affect the TPC and AOA values. Freeze-drying and grinding the tissue allowed a higher extraction of phenolics and antioxidant activity when calculated gram per fresh weight than could be obtained from frozen and homogenized berries, which reinforces the notion that freeze-drying is an efficient method to preserve the quality of the fruits (Wojdylo et al., 2009), and hence freeze-drying was selected as the method of choice for preparing extracts for physiological studies in animals.

The correlation between antioxidant activity and total phenolic content was also studied to test whether TPC contribute to AOA. A significant correlation was found between TPC and AOA as measured by both FRAP ($R^2 = 0.68$, $P < 0.01$) and DPPH ($R^2 = 0.89$, $P < 0.01$), indicating that TPC was a major component of total antioxidants present in Highbush blueberries. In addition, the relationship between total anthocyanins and AOA suggested that between 50% and 60% of the AOA can be attributed to total anthocyanins. The data found in this study corroborated the results obtained by others (Moyer et al., 2002; Zheng & Wang, 2003; Rodrigues et al., 2011), who demonstrated that anthocyanins account for a large proportion of the polyphenolic compounds present in blueberries, and are responsible for a large part of the AOA in the fruits. Since anthocyanins are the predominant bioactive in blueberries, these compounds were our focus in this thesis.

For anthocyanin quantification, acidified water was used for the extraction since this solvent is more likely to represent what is made available in normal consumption than the organic solvents. A total of fifteen individual anthocyanins were found in Highbush blueberries extracted with acidified water, which was in keeping with others who analyzed the fruits using organic solvents. However, colour was indeed observed in the pomace after extraction, which was then found to contain significant amounts of TPC and AOA. The results obtained indicated that anthocyanin compounds were not well extracted by acidified water, and approximately only half of them could be extracted from the berries with a single extraction of acidified water. Since the total anthocyanins in the present study were extracted using acidified water, the concentration was considerably lower than as reported in other studies for Highbush blueberry cultivars (Goa & Mazza et al., 1994; Ehlenfeldt & Proir, 2001; Connor et al., 2002; Cho et al., 2004).

It was clearly shown that among all eight cultivars observed, 'Burlington' and 'Elliott' exhibited the highest AOA (as measured by both FRAP and DPPH) since these two varieties contained the highest TPC and total anthocyanin concentrations. It is also important to note that the high AOA of these two cultivars may, in part, be explained by high amounts of delphinidin-3-galactosides and delphinidin-3-arabinosides present in the fruits. It has also been previously reported that different structures of individual anthocyanins affect antioxidant capacity; aglycones with increasing numbers of hydroxyl groups on the B ring structure (Figure 2.5 in Chapter 2; delphinidin > cyanidin, petunidin > malvidin, peonidin) increase the antioxidant capacity (Ogawa et al., 2008), and anthocyanidins containing galatosides and arabinosides appear to exhibit higher AOA than those containing glucosides (Borge et al., 2010).

8.2 Starch hydrolysis enzymes inhibition and beneficial probiotic bacterial growth

Beyond their AOA, it was of interest to determine whether Highbush blueberries exert an effect on glucose/energy metabolism, the cornerstone of insulin resistance pathophysiology linked to MS. Therefore, the ability of all eight cultivars to inhibit starch hydrolysis enzymes and increase the growth of specific beneficial bacteria was investigated *in vitro*.

It has been suggested in a number of studies that α-amylase or α-glucosidase inhibitory activity of the plant extracts mainly arises from their phenolic compounds and antioxidant activities

(McCue et al., 2005; Kwon et al., 2008; Kim et al., 2010; El-Beshbishy & Bahashwan, 2012; Wang et al., 2012b). Therefore, it was expected that fruits containing high TPC and AOA would exhibit high inhibiting ability against starch hydrolysis enzymes. However, this trend was not observed in the present study. The different findings between the previous and the present studies could be ascribed mainly to the use of different plants, as such a correlation has not been demonstrated in other research involving blueberries so far. Data presented in Chapter 4 found only a weak correlation between TPC or AOA and α-amylase or α-glucosidase inhibitory activities. These observations suggested that in blueberry fruits, the TPC or AOA are not the major contributor to their ability to inhibit the starch hydrolysis enzymes and it is possible that some other specific bioactives are making a greater contribution, which cannot be clearly identified in the present study, and need further clarification. This weak correlation was also found in some types of plants, for example in raspberries (Cheplick et al., 2007), soybean (Ademiluyi & Oboh, 2013) and herbs (Wongsa et al., 2012) and is an important reminder that strong correlations do not always indicate a causal connection.

Evidence seemed to suggest that anthocyanins may be the compounds inhibiting α-glucosidase and procyanidins effectively inhibiting α-amylase. Since anthocyanins are the predominant bioactives in blueberries, blueberry fruits are likely to possess the ability to inhibit α-glucosidase rather than α-amylase, as has been suggested previously in the literature. However, the percentage inhibition of blueberries on α-glucosidase observed in the present study was not much different from the percentage inhibition against α-amylase. One possible explanation for this discrepancy is that most previous studies used blueberries extracted with mild acidified solvents, whereas in the present study only water was used for this assay. It is known that anthocyanins are not stable in neutral conditions (McGhie & Walton, 2007), which may lead to significant loss in α-glucosidase inhibitory effects of the aqueous extracts. Conversely, procyanidins, reputed to be the effective compound against α-amylase, appear to be more stable than anthocyanins under neutral conditions (Bermúdez-Soto et al., 2007). This may partially explain why higher inhibition capacity was not observed against α-glucosidase as compared to α-amylase as in previous studies. In addition, it is difficult to compare the inhibitory values to earlier publications due to the use of different cultivars and assay conditions. Nevertheless, the percentage inhibition on α-glucosidase of 'Bluecrop' and 'Duke' shown in this study were ranked in the same order as the study of Wang et al. (2012a) but our values were found to be lower, presumably due to the different solvent used for the extraction. In future

studies, it would be more meaningful to compare these values with known concentrations of a commercial standard such as acarbose.

Aqueous extracts of Highbush blueberries showed a trend to enhance the growth of a probiotic species, *L. acidophilus*. This trend was also found in the study by Molan et al. (2009) and Sutherland et al. (2009), who showed higher proliferation of *L. rhamnosus* after supplementing culture medium with blueberries, while Biswas et al. (2012) found the growth-stimulating effect of blueberry juice on the survival of *L. bulgaricus*. However, to the best of our knowledge, there is no information so far available on the effect of Highbush blueberries on the growth of *L. acidophilus in vitro*, and hence no comparison is possible. It was also demonstrated by Sutherland et al. (2009) that aqueous extracts were more beneficial for the growth of probiotics than solvent extracts since chemicals in organic solvents may be toxic to living cells and as a result their growth was inhibited. This is one of the reasons why water extraction rather than solvent extraction was used.

However, in this study, we cannot identify which compound in blueberries is responsible for some beneficial effects since no significant correlations were found between the growth of bacteria and any measured variable, including TPC, AOA, or anthocyanin concentration. It is not possible to ignore a contribution from sugar in blueberries on the growth of these bacteria, as reported by other authors (Corcoran et al., 2005). In order to examine the actual effect of polyphenols in the blueberries, sugar should be eliminated from the extracts before carrying out the analyses in future. However, we chose to study the extracts from whole berries because that is the most available and consumed form, and it reflects the real consumption pattern of consumers.

8.3 Metabolism of fructose, unlike glucose

Data from Chapter 5 revealed that consumption of a large quantity of fructose for 8 weeks did not induce a metabolic disorder in SD rats. This observation is contrary to expectations and in contrast with reports by other researchers (Al-Awwadi et al., 2005; D'Angelo et al., 2005; Reddy et al., 2009; Suwannaphet et al., 2010). After consumption, glucose enters peripheral tissues via the GLUT-4 transporter that is dependent on the regulation of insulin. In contrast, fructose mainly transports to the liver via the portal vein, and enters hepatic cells by GLUT-5,

which is insulin-independent (Elliott et al., 2002). Therefore, once fructose is given *in vivo*, it produces a smaller rise in circulating glucose and insulin than when a similar amount of glucose is given (Bantle, 2009). This may be one explanation why we did not observe a detrimental effect of fructose on hyperinsulinemia and insulin resistance**.** Additionally, corn starch diet feeding for 8 weeks also produced slightly increased circulating glucose and insulin concentrations (Chapter 6), making it is more difficult to see significant differences between the starch-control and the high fructose fed groups.

In addition, once inside the cells, fructose molecules are metabolized by fructokinase into fructose-1-phosphate, which is then split by aldolase B to form trioses that are the cornerstone for the synthesis of phospholipids and triglycerides. Also, a high intake of fructose may also lead to the synthesis of long-chain fatty acids (Bray et al., 2004). This effect may be involved in the development of hyperlipidemia linked to insulin resistance. Indeed, a human study has demonstrated that the ingestion of fructose resulted in a significantly increased rate of lipogenesis (Schwarz et al., 1993). For these reasons, it could be suggested that regular consumption of fructose may eventually lead to the development of metabolic syndrome like glucose does, but possibly via different metabolic pathways.

8.4 Diet-induced metabolic syndrome SD rat model

Careful choice needs to be made when planning a diet-induced metabolic syndrome study, since the metabolic pathway is very complex and multiple genetic and environmental factors can significantly contribute to the development of metabolic disorders in animals.

An initial and important decision has to be made regarding the type of diet. The results of the pilot study reported in Chapter 6 are in line with other reports (Ghibaudi et al., 2002; Brenner et al., 2003; Johnson et al., 2007) in which chronic consumption of saturated fat and/or sugar resulted in the development of metabolic syndrome in animals. Our findings demonstrated that a diet containing lard, or sucrose, or the combination of lard/sucrose at a dose between 60% and 65% (w/w) of total diet induced specific metabolic changes in SD rats within our expected time frame (8 weeks). Specifically, the high-fat diet significantly increased weight gain and fat accumulation, whereas the high-sucrose diet did not induce excessive body weight but tended to affect hyperglycemia and insulin resistance. Saturated fat and sucrose appears to have

synergistic effects since their combination enhanced development of both obesity and insulin resistance, and the effect was more pronounced compared with the intake of high-lard or high-sucrose alone. Certainly, longer exposure to these diets resulted in more severe signs of metabolic syndrome. To date, no universal cut-off values have been established for the insulin resistance index (HOMA-IR) in rats since there are large variations in insulin values between laboratories due to lack of standardized insulin measurements (Cacho et al., 2008). Therefore we cannot confirm whether the rats in our studies remained normal or were heading towards insulin resistance. However, the assay for glucose seems more reproducible. Some earlier studies used the range between 100 and 130 mg/dL (5.5 and 7.0 mmol/L) as the normal blood glucose levels for rats (Kohn & Clifford 2002), and SD rats fed with high-lard and/or high-sucrose diets in our studies appeared to have higher levels of plasma glucose (approximately 70 % increase) than those normal proposed values.

The second criterion to be considered is the species of animal to be used. Since genetic background can influence metabolic syndrome development, the strain of rodents used in the experiments needs to be considered. Some inbred mouse strains, for example SJL/J and SWR/J, have been found to be not responsive to high-fat diet-induced obesity (West et al., 1992), whereas the high-fat diet-fed C57BL/6J mouse model is widely used in several studies involving obesity and/or insulin resistance (Winzell & Ahrén, 2004; Prior et al., 2008; Grace et al., 2009) and seem to be a model of choice. The present study was carried out on Sprague Dawley (SD) rats because of reproducibility of measurements within the same animals. Our data suggest that SD rats are well suited to studies involving the induction of MS with high-saturated fat (lard) and/or high-sucrose diets, but for a high-fructose diet study, it seems that SD rats were not a good choice. Khanal et al. (2010) reported a similar finding when their SD rats were fed with high fructose diets and have proposed that Wistar rats may be a better choice for high-fructose diet-induced MS in rats since this phenotype appears to be more sensitive to fructose in the diet.

It is noteworthy that the age of the animal may also be important for the development of metabolic disorders. In our study, growing rats aged between 6-8 weeks were used since this age range of animals was frequently used in a number of other diet-induced metabolic syndrome experiments. However, we unexpectedly found that not every individual animal developed signs of metabolic syndrome even though they were all exposed to identical experimental conditions. This difference may be due to genetic and physiological variations between animals. Pagliassotti et al. (2000) revealed that older (56 weeks) rats fed with high-

fat/high carbohydrate diets for 5 weeks exhibited considerably higher levels of plasma insulin and free fatty acids in both basal and during euglycemic hyperinsulinemia clamp conditions than young (8 weeks) and weanling (3 weeks) rats, suggesting ageing animals may be more susceptible to metabolic syndrome. Furthermore, it has been shown previously that animals starting at 6 weeks of age were able to adapt to long-term feeding (3 months) of high-fat or high-fructose diets without developing metabolic disorders (Stark et al., 2000). Since metabolic syndrome appeared to be age-associated, ageing animals may serve as a better choice for this model. A drawback, however, is the relative cost; aged rats are much more expensive to rear or procure than weanling or young rats.

Animal gender may be another concern; Horton et al. (1997) have noted that female rats did not develop insulin resistance in response to 8-weeks high-sucrose feeding, in contrast to males which can develop sucrose-induced insulin resistance within 2 weeks. The presence of female sex hormones, estrogen and/progesterone, may affect the metabolic response. Therefore the male gender may be more appropriate, and hence was used in all of our experiments.

8.5 Overall physiological effects of Highbush blueberries

Since aqueous extracts of Highbush blueberries have shown promise *in vitro* on some aspects associated with metabolic syndrome, *in vivo* animal studies were subsequently carried out in order to examine their actual physiological effects, and find out the relevant MS biomarkers to be proposed for a human intervention study in the future.

Data reported in Chapter 5 reveal that the beneficial effect of bioactive compounds in the blueberries on MS was not likely to be a result of a direct antioxidant effect in the blood circulation because there was no increase in antioxidant status (measured by FRAP assay) observed in serum of rats-fed with freeze-dried blueberries. This is not surprising because the absorption of polyphenols, especially anthocyanins, is particularly low (McGhie & Walton, 2007).

However, Highbush blueberries showed a trend to improve insulin sensitivity as indicated by a reduction in HOMA-IR index and the improvement of glucose tolerance (OGTT), although the specific mechanism remains unclear. The HOMA-IR represents body insulin resistance at the basal state and is calculated based on fasting serum glucose and insulin levels. Our data in

Chapter 7 revealed that the effect of blueberries on the HOMA-IR score were due to a reduced hyperinsulinemia rather than a reduced hyperglycemia. Since fasting glucose levels mostly depend on glucose production by the liver, individuals with fasting hyperglycemia reflect hepatic insulin resistance (Boyda et al., 2012). By contrast, hyperinsulinemia appears to be a result of impairment of pancreatic beta-cell function, leading to ineffective insulin released (Demir et al., 2008). Based on these data, bioactive compounds in blueberries may improve the sensitivity to insulin by reducing the beta-cell damage or improving beta-cell function.

Consumption of blueberries also improved glucose tolerance in animals. The OGTT reflects sensitivity of insulin at non-steady state to stimulate glucose uptake into peripheral tissues. Therefore, another possible mechanism is that blueberry polyphenols may facilitate the GLUT 4 system, stimulate insulin receptor phosphorylation, or regulate expression of selected enzymes participating in glucose translocation and insulin signalling pathways in peripheral target cells such as insulin receptor substrates (IRS), phosphotidylinositol 3-kinase (PI3K), and AMP-activated protein kinase (AMPK). Future studies at the molecular level will assist to elucidate the actual mechanisms.

In addition, our results found a trend towards an increase in the population size of *Bifidobacterium* spp. and *Lactobacillus* spp., the two common probiotic species that have been shown to be involved in glucose and energy homeostasis (Cani & Delzenne, 2009), in the rat caecum after consumption of blueberry powder. Accumulated evidence has proposed that bioactive compounds in blueberries, especially anthocyanins, may undergo considerable changes by these beneficial microbiota in the intestine before exerting their biological effects, since these compounds have been found to be absorbed very poorly (McGhie & Walton, 2007). Therefore, it is possible that it may not be the berries' polyphenolic compounds themselves that actually promote metabolic benefits, but rather the products of their metabolism, and this is another area that needs further investigation.

Another finding in Chapter 7 revealed no significant difference in any observed biomarkers for metabolic syndrome between 'Burlington' and 'Bluecrop' cultivar. Based upon phytochemical data comparison between the two varieties, 'Burlington' contained significantly higher amounts of total anthocyanins with higher percentage of galactoside/glucoside derivatives compared to 'Bluecrop'. These results suggest that galactosylated anthocyanins were not different from glucosylated anthocyanins physiologically in the modulation of the metabolic parameters

observed here. It may be possible that benefits from blueberry consumption could also be attributed to other bioactive compounds in blueberries that we did not measure in the present study such as procyanidins or flavonols. In addition, it is important to note that blueberry powder also contains some dietary fibre (approximately 18% in freeze-dried wild blueberry powder; Mazza et al., 2002), which may also contribute to the metabolic effect.

Although blueberries show promise at improving insulin sensitivity, they did not affect obesity-related parameters. This finding implies that the pathway of insulin resistance and obesity development may not necessarily occur concurrently and hence support the previous report which found that not all people with metabolic syndrome and/or insulin resistance are obese (Enas et al., 2007). However, an interesting question remaining is why whole freeze-dried blueberry powder failed to exert a significant effect on any biomarkers related to obesity, in contrast to blueberries in the form of juice and the purified anthocyanins which have been found to protect against weight gain and fat accumulation (Prior et al., 2010). Anthocyanins obtained from blueberry juice and purified anthocyanins may be absorbed earlier in the stomach or small intestine, whereas the cell walls of blueberries may delay the absorption of anthocyanins in the freeze-dried whole blueberry powder, allowing them to reach the lower gastrointestinal tract and hence could interact with other phenolics and their metabolites (Prior et al., 2010). Notably, we did not vary the dose of blueberries in the present study; however, some previous studies highlight that a higher dose of berries may not always provide better protection against MS (Khanal et al., 2010; Prior et al., 2010), suggesting that a high dose may be harmful or could suppress protective mechanisms. In the future, the form and dose of blueberries that are beneficial to consumers for reducing obesity still need to be clarified.

8.6 Large variations in metabolic parameters

As observed in the present thesis, large variations in metabolic parameters were found in the animals although they were all exposed to the same diet and identical experimental conditions. For example, in rats fed with high-sucrose diets for 8 weeks, the range of fasting insulin concentrations was observed from the lowest at 0.16 ng/mL to the highest at 0.77 ng/mL (Figure 6.3 in Chapter 6), and fasting triglyceride concentrations of rats fed with high-fat diets (at week 4) varied between 0.5 and 1.6 mmol/L (data not shown). Furthermore, the OGTT data reported in Chapter 6 (Figure 6.4) showed that approximately 80% of rats in the high-sucrose

groups exhibited impaired glucose tolerance, whereas in the high-fat groups, around 40% of them showed no signs of deterioration. This finding suggests that not every individual reacts to the same treatment in the same manner. Similarly, blueberries appeared to show a beneficial impact only in some animals. Based on data of individual animals after being fed with HFHS+'Burlington' for 8 weeks, 3 out of 7 rats decreased their fasting glucose concentrations, and 6 out of 7 rats reduced their fasting insulin levels relative to the mean of the HFHS-control group, whereas the remaining rats were unaffected. It is also interesting to note that within the HFHS+'Burlington' group, the animal that had the highest weight was not the one exhibiting the highest levels of glucose and/or insulin (data not shown). This may be another indication that obesity and insulin resistance are not necessarily linked.

Although it is well accepted that genetic factors can affect the development of metabolic disorders, particularly in the outbred strain which was used in our study, we did not expect to encounter such huge variability in metabolic changes among individuals. More surprisingly, these variations can also be found in genetically identical mice. As demonstrated in the study of Burcelin et al. (2002), C57BL/6J inbred mice fed with a high-fat diet for 9 months showed wide scatter in their body weight gain, with approximately 50% of these mice being defined as obese, 30% intermediate and the remaining 20% were lean. These observations support the complexity of metabolic syndrome which can be strongly influenced by numerous variables, including physiology, genetics and environment, and suggest that in term of metabolic syndrome research, the sample size used in these kinds of research needs to be large enough to overcome the variability in order to detect a significant difference among treatments. Alternatively, prolonged cross-over trials may be more desirable, where animals become their own controls.

Conclusions :

The present study addressed the major objective whether Highbush blueberries, which generally contain lower concentrations of polyphenols, but are more widely consumed than Lowbush blueberries, would provide any beneficial effects against metabolic syndrome.

The key findings obtained demonstrated that selected NZ Highbush blueberries contained relatively high amount of polyphenolics and antioxidant activities *in vitro*. However these values varied considerably depending on varieties. Furthermore, aqueous extracts of Highbush blueberries which exhibited the lowest TPC and AOA still showed some promise in controlling metabolic alterations through the inhibition of starch digestive enzymes (α-amylase and α-glucosidase) and affecting the growth of selected lactic acid probiotic bacteria. The metabolic benefits presented *in vitro* were consistent with the results demonstrated subsequently *in vivo*, since signs of an improvement of glucose tolerance, a reduction in the degree of insulin resistance (HOMA-IR), and an increase in the number of beneficial intestinal microbiota (*Lactobacillus* spp. and *Bifidobacterium* spp.) were also seen in animals receiving high-fat-high-sugar diets containing blueberries.

The results obtained from this thesis will help to improve the understanding of the health benefits of selected Highbush blueberry cultivars and shed light on a possible use of NZ blueberries for protection against insulin resistance

Recommendations for future research :

1. Future experiments on a larger animal population group of SD rats comparing various forms of Highbush blueberries (i.e. juice, frozen, freeze-dried) with the purified bioactive compounds extracted from blueberries are required to gain deeper understanding of health benefits of blueberries on metabolic syndrome, and to identify the active compound(s) in blueberries, if any, responsible for these benefits.

2. Effect of blueberry bioactive compounds appears to not be a dose-dependent response; therefore, the optimum physiological dosage that provides benefit against insulin resistance needs to be established and determined in human participants.

3. Since genetic variability plays a significant role in metabolic parameters in individuals, comparing between pre- and post-treatment within subjects could help to avoid the problem of variability among subjects and serve as another approach when assessing parameters associated with metabolic syndrome.

4. Molecular studies are an important further step needed to explore the possible mechanisms underlying the anti-insulin resistance effect of blueberries. Observations from the present study suggested that the pathways involve the function of pancreatic beta-cell, the translocation of glucose transporter 4 and the insulin signaling cascades could serve as possible candidates.

5. Finally, it is important to remember that blueberry bioactive compounds may be metabolized by intestinal microflora, therefore monitoring these metabolites *in vivo* may also be required.

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Prior, R. L., Wu, X., Gu, L., Hager, T. J., Hager, A., & Howard, L. R. (2008). Whole berries versus berry anthocyanins: interactions with dietary fat levels in the C57BL/6J mouse model of obesity. *Journal of Agricultural and Food Chemistry, 56*(3), 647-653.

Prior, R. L., Wilkes, S. E., Rogers, T. R., Khanal, R. C., Wu, X., & Howard, L. R. (2010). Purified blueberry anthocyanins and blueberry juice alter development of obesity in mice fed an obesogenic high-fat diet. *Journal of Agricultural and Food Chemistry, 58*(7), 3970-3976.

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APPENDICES

Metabolic Syndrome Animal Models (Preventive effect)

Animal	Diet	Supplement	Experimental Procedures (Preventive effect)	Time Period	Biomarkers	Result
Prior, R. L., Wu, X., Gu, L., Hager, T. J., Hager, A., & Howard, L. R. (2008). Whole berries versus berry anthocyanins: interactions with dietary fat levels in the C57BL/6J mouse model of obesity. *Journal of Agricultural and Food Chemistry, 56*(3), 647-653.						
Male C57BL/6J mice (age 21 days)	Low fat diet (10% energy from fat) High fat diet (45% or 60% energy from fat)	Freeze-dried whole BB and STW powder (100 g/~1 kg diet) Purified anthocyanins From blueberry and strawberry	Exp1: Divided into 6 groups (12 animals /group) 1. Control low fat (10% fat) 2. LF + 10% freeze dried blueberry powder 3. LF + 10% freeze dried strawberry powder 4. Control high fat (45% fat) 5. HF + 10% freeze dried blueberry powder 6. HF + 10% freeze dried strawberry powder Exp2: Divided into 6 groups (9 animals /group) 1. Control low fat (10% fat) 2. Control high fat diet (45% fat) 3. HF diet (45% fat) + 10% freeze dried blueberry powder 4. High fat diet (60% fat) 5. HF diet (60% fat) + purified anthocyanins from blueberry provided in drinking water 6. HF diet (60% fat) + purified anthocyanins from strawberry provided in drinking water	92 days 70 days	- Body weight - % Body fat and epididymal fat weight - Body protein - Glucose tolerance test - Plasma glucose	HF45+BB: ↑ Body weight gain, body fat, epi fat weight compared to HF45-C HF45+STW: Body weight ~ HF45-C BB and STW feeding did not alter glucose tolerance. HF60+purified ACN from BB: ↓ body weight gain, body fat compared to HF60-C Authors suggested that whole BB did not prevent and may have increased obesity, however, feeding purified anthocyanin from BB reduced obesity.

Animal	Diet	Supplement	Experimental Procedures (Preventive effect)	Time Period	Biomarkers	Result
DeFuria, J., Bennett, G., Strissel, K. J., Perfield Ii, J. W., Milbury, P. E., Greenberg, A. S., et al. (2009). Dietary blueberry attenuates whole-body insulin resistance in high fat-fed mice by reducing adipocyte death and its inflammatory sequelae. *Journal of Nutrition, 139*(8), 1510-1516.						
Male C57BL/6J mice	Low fat diet (10% energy from fat) High fat diet (60% energy from fat)	Freeze-dried whole BB powder (40.9 g/1 kg diet) (Powder consist of 1:1 blend of Tifblue and Rubel)	After several days of acclimation, mice were assigned into 3 groups (n=8) 1. A low fat diet (10% energy) 2. A high fat diet (60% energy) 3. HFD with 4% (w/w) freeze-dried BB powder	8 weeks	- Energy intake and indirect calorimetry - Insulin tolerance test - Glucose concentration - Plasma insulin levels - Adipocyte death - Inflammatory gene expression	BB (2.7% total energy): - Did not affect alterations in energy intake, metabolic rate and body weight - ↓ Insulin resistant compared to the HFD - Reduction in adipocyte death - Protect against HFD-induced ATMϕ inflammary gene expression in *e*AT
Kim, H., Bartley, G. E., Rimando, A. M., & Yokoyama, W. (2010). Hepatic gene expression related to lower plasma cholesterol in hamsters fed high-fat diets supplemented with blueberry peels and peel extract. *Journal of Agricultural and Food Chemistry, 58*(7), 3984-3991.						
Male Golden Syrian hamsters (80 g)	High fat (HF) diet consisted of 18% protein, 45& carb, 37% fat supplemented with 0.15% cholesterol	Blueberry (BB) pomace byproducts - Dried whole blueberry peels - Dried ethanol extract of peels - Residue from ethanol extracted peel	Animals were acclimatized and given free access to water and rodent chow for 1 week prior to the initiation of the experimental diets. 1. HF+ 8% whole BB peels (BBPWHL) 2. HF+ 6% BB peel ethanol extract (BBPX) 3. HF+ 2% BB residue from ethanol extract (BBPEXT) 4. HF + 5% microcrystalline cellulose MCC)-Control	3 weeks	- Body weight - Plasma and hepatic lipid content - Hepatic gene expression	BB pomace : - ↓Plasma VLDL, T-CHOL, but for LDL (only BBPX) - ↓Hepatic cholesterol (only BBPWHL & BBPX) - Did not affect BW, hepatic total lipid, TG - Hepatic CYP7A1 expression was up-regulated by all BB diets, and the expression of CYP51 was up-regulated by BBPX and BBPEXT

Animal	Diet	Supplement	Experimental Procedures (Preventive effect)	Time Period	Biomarkers	Result
Prior, R. L., E. Wilkes, S., R. Rogers, T., Khanal, R. C., Wu, X., & Howard, L. R. (2010). Purified blueberry anthocyanins and blueberry juice alter development of obesity in mice fed an obesogenic high-fat diet. *Journal of Agricultural and Food Chemistry, 58*(7), 3970-3976.						
Male C57BL/6J mice	Low fat diet (10% energy from fat) High fat diet (45% energy from fat)	Blueberry juice and purified blueberry ACNs (0.2 and 1.0 mg/ml) Whole blueberry powder (11.5%)	There were 9 animals/treatment The treatment included 1. Low fat diet (10% kcal from fat) 2. LF + blueberry juice in place of drinking water 3. LF + purified blueberry ACNs in drinking water (0.2 mg/ml) 4. LF + purified blueberry ACNs in drinking water (1.0 mg/ml) 5. High fat diet (45% kcal from fat) 6. HF + blueberry juice in place of drinking water 7. HF + ACNs in drinking water (0.2 mg/ml) 8. HF + ACNs in drinking water (0.2 mg/ml) 9. HF + sucrose in drinking water (88 mg/ml) 10. HF + 11.5% whole blueberry powder	72 days	- Whole body composition - Serum cytokines (leptin, ACTH, osteocalcin, RANKL) - Serum insulin - Serum glucose, triglycerides, cholesterol - Insulin resistance and β-cell function - Hepatic triglyceride and cholesterol	ACNs (0.2 mg/ml) : - ↓adipose tissue weight - ↓fasting serum glucose - ↑β-cell function score (HOMA-BCF) - ↓serum leptin Blueberry juice : - ↓serum leptin, but not alter serum glucose The whole BB powder : - not alter parameters measured relative to development of obesity except for a lowered serum leptin level Likewise, the higher dose of ACNs (1.0 mg/ml) - not effective in decreasing the development of obesity

Animal	Diet	Supplement	Experimental Procedures (Preventive effect)	Time Period	Biomarkers	Result
Khanal, R. C., Howard, L. R., Wilkes, S. E., Rogers, T. J., & Prior, R. L. (2010). Urinary excretion of (epi) catechins in rats fed different berries or berry products. *Journal of Agricultural and Food Chemistry, 58*(21), 11257-11264.						
Male Sprague Dawley rats (175±8.4 g)	AIN-93 based control High-fructose diet (53-65% of the diet)	Commercial concentrated cranberry powder (CCP) (3.3, 6.6 and 33 g/kg diet) Freeze-dried whole cranberry (CB) blueberry (BB) black raspberry (BRB) (50g/kg of diet)	Exp1: (n=10) 1. Control (AIN-93 based) 2. High fructose diet 3. HF + low CCP 4. HF + medium CCP 5. HF + high CCP Exp2: 1. Control (AIN-93 based) 2. High fructose diet 3. HF + CB 4. HF + BB 5. HF + BRB	50 days	- Catechin/ Epicatechin intake and excretion	Catechin/ Epi-C intake Exp1: Intakes were consistant with their conc in the diet with high CCP > medium CCP > lowCCP Exp2: CB > BB >BRB group Catechin/ Epi-C excretion - Excretion of epi-C ranged from 30-47% of ingested amount, whereas catechin range from 9-31% EXP1: Urinary excretion of epi-c was dose dependent and increased with the amount of epi-C present in the diet, in contrast, methylated catechins in rats fed high CCP were either not detected in the urine or present in only small amount EXP2: Catechins were excreted in small amounts in animals fed CB and BB at 9 and 28%, respectively. However, epi-C were excreted in higher amounts in BB-fed group than CB group. Data suggesting that the bioavailability of epi-C > catechins and that epi-C may be more available from BB compared to CB

Animal	Diet	Supplement	Experimental Procedures (Preventive effect)	Time Period	Biomarkers	Result
Al-Awwadi, N. A., Araiz, C., Bornet, A., Delbosc, S., Cristol, J. P., Linck, N., et al. (2005). Extracts enriched in different polyphenolic families normalise increase cardiac NADPH oxidase expression while having differential effects on insulin resistance, hypertension, and cardiac hypertrophy in high-fructose-fed rats. *Journal of Agricultural and Food Chemistry, 53*(1), 151-157.						
Male Sprague Dawley rats (185-200 g)	Fructose enriched diet (66% fructose, 22% protein, and 12% fat)	- A red grape skin polyphenolic extract enriched in anthocyanins - A grape seed extract enriched in procyanidins and galloylated procyanidins - The commercial preparation Vitaflavan, rich in catechin oligomers (All treatment were administered at the same dose of 21.42 mg/kg of polyphenols)	After an adaptation period of 1 week, the rats were divided into 5 groups of homogeneous weight of nine animals each. 1. Control group (C): Fed with standard chow 2. Fructose enriched diet group (F) Fed with 66% fructose diet 3. Anthocyan-treated fructose-fed group (FANT) 4. Procyanidin and galloylated procyanidins-treated fructose-fed group (FPRO) 5. Vitaflavan-treated fructose-fed group (FVITA) The rats were daily treated by gavage with 10 ml/kg of water only (C and F groups) or the same amount of solution containing anthocyanins (ANT), Procyanidins (PRO), and Vitaflavan (VITA)	6 weeks	- Body weight - Blood pressure - Plasma insulin - Plasma glucose - Total cholesterol, triglyceride, HDL, and phospholipid - Determination of superoxide anion production	- The ANT prevented hypertension, cardiac hypertrophy and production of ROS - The PRO prevented insulin resistance, hypertriglyceridemia and overproduction of ROS - Vitaflavan prevented hypertension, cardiac hypertrophy and overproduction of ROS Mean plasma glucose, insulin and HOMA-IR: - All values obtained from ANT, PRO and VIT-treated rats were intermediated between and not sig different from C and F groups Mean plasma lipids: - TG: ↓ by PRO - HDL: ↑ by ANT & PRO - Phosholipids and LDL: not changed

Animal	Diet	Supplement	Experimental Procedures (Preventive effect)	Time Period	Biomarkers	Result
Suwannaphet, W., Meeprom, A., Yibchok-Anun, S., & Adisakwattana, S. (2010). Preventive effect of grape seed extract against high-fructose diet-induced insulin resistance and oxidative stress in rats. *Food and Chemical Toxicology, 48*(7), 1853-1857.						
Male Sprague Dawley rats (180-200 g)	High-fructose (HF) diet (g/kg diet): Casein 200 g Fructose 630 g Soybean oil 70 g Mineral mix 35 g Vitamin mix 10 g Cellulose powder 50 g L-cystine 3 g Choline bitartrate 2.5 g	Freeze-dried grape seed water extract (0.5 and 1.0g per 100 g diet)	The rats were fed standard laboratory chow with water ad libitum and fasted overnight before the experiments. Then, they were randomly assigned to four groups of six animals. 1. Group 1: Normal diet 2. Group 2: High fructose diet 3. Group 3: HF diet + 0.5% grape seed extract 4. Group 4: HF diet + 1.0% grape seed extract	8 weeks	- Body weight - Plasma glucose - Plasma insulin - Oral glucose tolerance test - Hepatic markers of oxidative stress	1% grape seed extract : - ↓fasting plasma glucose, insulin and HOMA-IR - Improved glucose intolerance - Increased activity of hepatic superoxide dismutase, catalase, - ↓lipid peroxidation - However, did not change in hepatic glutathione peroxidase
Yang, X., Yang, L., & Zheng, H. (2010). Hypolipidemic and antioxidant effects of mulberry (*Morus alba* L.) fruit in hyperlipidaemia rats. *Food and Chemical Toxicology, 48*(8-9), 2374-2379.						
Male Wistar rats (193±12g) (2-mth-old)	High fat diet: 10% lard, 1% cholesterol, 0.5% sodium cholate, 88.5% commercial diet	Mulberry fruit freeze-dried powder (5 and 10%)	Before exp, rats were fed a commercial diet for a week while adapting, then were randomly divided into 6 groups of 8 rats each. 1. Normal diet (ND)-commercial diet 2. ND + 5% MFP (NDM I) 3. ND + 10% MFP (NDM II) 4. High fat diet (HF) 5. HF + 5% MFP (HFM I) 6. HF + 10% MFP (HFM II)	4 weeks	- Body weight - Serum and liver lipid levels - Lipid peroxidation and antioxidant enzymes	MFP (10%): - Sig ↓ serum TC, TG and LDL - Sig ↓ by 17% for liver TC and 54.3% for Liver TG - Sig ↑serum HDL MFP (5%): - Did not affect lipid parameters exp serum TG MFP (5% & 10%): - Sig ↓ serum and liver TBARS - Sig ↑ the RBC and liver SOD and blood GSH-Px level (all compared to HF-group)

Animal	Diet	Supplement	Experimental Procedures (Preventive effect)	Time Period	Biomarkers	Result
Jang, E. M., Choi, M. S., Jung, U. J., Kim, M. J., Kim, H. J., Jeon, S. M., et al. (2008). Beneficial effects of curcumin on hyperlipidemia and insulin resistance in high-fat-fed hamsters. *Metabolism: Clinical and Experimental, 57*(11), 1576-1583.						
Male Golden-Syrian hamsters (4 weeks old)	High fat high cholesterol diet (10% coconut oil, 0.2% cholesterol wt/wt)	Curcumin 0.05 g/100 g diet	All hamsters were fed a pelletized commercial chow diet for 1 week and then divided into 2 groups (n=8) 1. Group I (control): High fat diet 2. Group II : High fat diet + curcumin	10 weeks	- Body weight - Plasma Leptin level - Plasma and hepatic lipids and apolipoproteins - Hepatic lipid-regulating enzyme activities - Lipid peroxide levels - Plasma glucose, Insulin levels and insulin resistance index	Curcumin : - Did not altered plasma glucose, but sig ↓plasma insulin and HOMA-IR - Sig ↓plasma leptin FFA, TG ↑ HDL, Apo A-I
Sivakumar, A. S., Viswanathan, P., & Anuradha, C. V. (2010). Dose-dependent effect of galangin on fructose-mediated insulin resistance and oxidative events in rat kidney. *Redox Report, 15*(5), 224-232.						
Male albino Wistar rats	High-fructose diet (60g/100g diet):	Galangin (50, 100 and 200 µg/kg body weight)	Rats were divided into 6 groups containing 6 animals each. 1. Group 1: A starch-based control diet 2. Group 2: A high fructose diet 3. Group 3: A high fructose diet + galangin 50 µg/kg body weight 4. Group 4: A high fructose diet + galangin 100 µg/kg body weight 5. Group 5: A high fructose diet + galangin 200 µg/kg body weight 6. Group 6: A starch-based control diet + galangin 200 µg/kg body weight	60 days	- Blood glucose - Insulin levels - Insulin resistance - Oral glucose tolerance test - Kidney oxidative stress	Galangin : (Dose-dependent) - Normalized blood glucose and insulin levels - Exaggerated the response to oral glucose challenge - Maintain antioxidant balance - The minimum effective dose was 100 µg/kg BW

Animal	Diet	Supplement	Experimental Procedures (Preventive effect)	Time Period	Biomarkers	Result
Reddy, S. S., Ramatholisamma, P., Karuna, R., & Saralakumari, D. (2009). Preventive effect of *Tinospora cordifolia* against high-fructose diet-induced insulin resistance and oxidative stress in male Wistar rats. *Food and Chemical Toxicology, 47*(9), 2224-2229.						
Male Albino Wistar rats (140-160 g) (age 6 weeks)	High fructose diet (66% fructose)	Aqueous extract of *Tinospora cordifolia stem* (TCAE) (400 mg/kg/day)	Animals were acclimatized for 7 days and then randomly assigned into 4 groups (8 rats/group) 1. Group C: Normal control rats received tap water and control diet 2. Group C+TC: TCAE treated normal rats received TCAE and control diet 3. Group F: High fructose fed rats received tap water and fructose diet 4. Group F+ TC: TCAE treated fructose fed rats received TCAE and fructose diet Vehicle (tap water) and TCAE (dissolved in tap water) were administered orally by gastric intubation.	60 days	- Body weight - Fasting plasma glucose - Insulin and triglycerides levels - Oral glucose tolerance test - Hepatic oxidative stress markers and antioxidants	TCAE treatment: - Prevented the rise in glucose levels by 21.3% insulin by 51.5% triglyceride by 54.12% glu-insulin index by 59.8% of the fructose-fed rats - Lowering lipid peroxidation, protein carbonyls - Showed higher GSH levels and activities of enzymatic antioxidants
El Mesallamy, H. O., El-Demerdash, E., Hammad, L. N., & El Magdoub, H. M. (2010). Effect of taurine supplementation on hyperhomocysteinemia and markers of oxidative stress in high fructose diet induced insulin resistance. *Diabetology and Metabolic Syndrome, 2*(46), 1-11.						
Male Wistar rats (170-190 g)	Fructose diet: (60% fructose)	Taurine (300 mg/kg/day)	Animals were acclimatized for 2 weeks, and then randomly divided into 4 groups (n=8) 1. Normal control group: received control diet 2. Taurine treated normal group: received taurine via i.p.route and control diet 3. Fructose fed group: received high fructose diet 4. Taurine treated fructose-fed group: received taurine via i.p.route and HF diet	35 days	- Oral glucose tolerance test - Body weight - Serum glucose, insulin - Lipid profile - Total antioxidant capacity - Paraoxonase activity and nitric oxide metabolites - Plasma Hcy	Taurine: - Ameliorated the rise in HOMA by 56%, triglycerides by 22.5%, Chol by 11%,LDL by 21.4% - The AUC of OGTT was sig ↓ by 4.5% - Sig ↓ HOMA results - 34.7% ↑ in Hcy levels (compare to fructose-fed group)

Animal	Diet	Supplement	Experimental Procedures (Preventive effect)	Time Period	Biomarkers	Result
Kushwah, A. S., Patil, B. M., & Thrppeswamy, B. S. (2010). Effect of *Phyllanthus fraternus* on fructose induced insulin resistance in rats. *International Journal of Pharmacology, 6*(5), 624-630.						
Male Sprague-Dawley rats (200-210 g)	Fructose diet (g/kg diet): Casein 207 g DL-methionine 3g Fructose 600 g Lard 50 g Cellulose 79.81 g Mineral-mix 50 g Zinc carbonate 0.04g Vitamin mix 10g	Aqueous extract of *Phyllanthus niruri* (PF) (250 mg/kg/day)	Prior to dietary manipulation, all rats were acclimatized for 7 days and then divided into 4 groups of seven each. 1. Group C: (Received standard chow diet) 2. Group CT: (Received standard chow diet plus PF dose 250 mg/kg) once daily 3. Group F: (Received fructose rich diet) 4. Group FT: (Received fructose rich diet plus PF dose 250 mg/kg) once daily	3 weeks	- Body weight and blood pressure - Glucose levels - Plasma insulin - Total cholesterol, triglyceride and HDL - Oral glucose tolerance test and Insulin sensitivity index	PF (250 mg/kg/day): - Sig prevented the increase in plasma glucose, insulin levels and AUC of OGTT - Sig ↓ HOMA-IR values - Sig prevented the increase in triglyceride but did not affect total-Chol levels as compared to fructose-fed rats
Hininger-Favier, I., Benaraba, R., Coves, S., Anderson, R. A., & Roussel, A. M. (2009). Green tea extract decreases oxidative stress and improves insulin sensitivity in an animal model of insulin resistance, the fructose-fed rat. *Journal of the American College of Nutrition, 28*(4), 355-361.						
Male Wistar rats (6 weeks old) (150 g weight)	Fructose diet (g/kg diet): Casein 200 g Fructose 600 g Corn oil 50 g Alphacel 50 g DL methionine 3g Choline bitartrate 2g Mineral mix 35 g Vitamin mix 10 g	The green tea solid (1 or 2 g/kg diet)	All rats were adapted and fed a standard chow diet for 1 week. They then were randomly divided into 4 groups of 10 rats. 1. FD group received the FD ad libitum 2. FD plus 1 group received FD + 1 g green tea solids/kg diet 3. FD plus 2 group received FD + 2 g green tea solids/kg diet 4. Control group received standard chow diet	6 weeks	- Body weight - Insulin, Glucose and triglyceride levels - Plasma malonedialdehyde (MDA) - Plasma sulfhydryl - DNA damage determination (Comet)	Green tea solid: - Did not change food intake - Tended to lower body weight - Sig ↓ plasma glucose, triglyceride and insulin concentrations - Lower plasma lipid peroxidation, SH-group oxidation and DNA oxidative damage

Animal	Diet	Supplement	Experimental Procedures (Preventive effect)	Time Period	Biomarkers	Result
Jin, X., Amitani, K., Zamami, Y., Takatori, S., Hobara, N., Kawamura, N., et al. (2010). Ameliorative effect of *Eucommia ulmoides* Oliv. leaves extract (ELE) on insulin resistance and abnormal perivascular innervation in fructose-drinking rats. *Journal of Ethnopharmacology, 128*(3), 672-678.						
Male Wistar rats (6-wk old)	15% fructose solution in drinking water	The Eucommia ulmoides Oliv. leaves extract (ELE) (500 and 1000 mg/kg/day)	Rats were divided into 4 groups (n=6/group) 1. Control rats : Received tap water 2. Fructose drinking rats (FDR): Received 15% fructose solution 3. FDR+ ELE 500 15% fructose solution + orally administrated ELE solution at dose of 500 mg/kg/day 4. FDR + ELE 1000 15% fructose solution + orally administrated ELE solution at dose of 1000 mg/kg/day	4 weeks	- Systolic blood pressure and heart rate - Body weight - Blood glucose - Serum insulin - Immunostaining density of CGRP-like immunoreactive (CGRP-LI) and TH-LI nerve fivers	ELE (500 and 1000 mg/kg/day): - Sig ↓plasma insulin, HOMA-IR - Did not affect blood glucose levels - Sig ↑CGRP-LI nerve fiber density and sig ↓TH-LI nerve density
Harati, M., & Ani, M. (2004). Vanadyl sulfate ameliorates insulin resistance and restores plasma dehydroepiandrosterone-sulfate levels in fructose-fed, insulin-resistant rats. *Clinical Biochemistry, 37*(8), 694-697.						
Male Wistar rats (8-wk old) (200-220g)	Fructose-enriched diet (66% fructose)	vanadyl sulphate (0.2 mg/ml dissolved in tap water)	Rat were divided into 3 groups 1. Control rats (n=8) Fed with standard lab chow + tap water 2. Fructose fed rats (n=10) Fed with Fructose-enriched diet + tap water 3. Fructose-fed, vanadyl-sulfate-treated rats (n=10) Fed with Fructose-enriched diet + tap water supplemented with 0.2 mg/ml vanadyl sulphate	7 days	- Plasma glucose and triglyceride levels - Plasma insulin levels - Plasma DHEAS levels	Vanadyl treatment : - Did not affect fasting plasma glucose levels - Prevented the increase in plasma insulin and TG - Prevented the decrease in plasma DHEAS (Compared with the F-F group)

Animal	Diet	Supplement	Experimental Procedures (Preventive effect)	Time Period	Biomarkers	Result
Braz De Oliveira, P. R., Da Costa, C. A., De Bem, G. F., Reis Marins De Cavalho, L. C., Vieira De Souza, M. A., De Lemos Neto, M., et al. (2010). Effects of an extract obtained from fruits of *Euterpe oleracea* Mart. in the components of metabolic syndrome induced in C57BL/6J mice fed a high-fat diet. *Journal of Cardiovascular Pharmacology, 56*(6), 619-626.						
C57BL/6J mice	High fat diet (60% fat) Control diet (10% fat)	Euterpe oleracea Mart (aĉaí) seed extract (ASE) (300 mg/kg/day)	1. Group 1: Control diet 2. Group 2: Control diet + ASE 300 mg/kg/day 3. Group 3: High fat diet 4. Group 4: High fat diet + ASE 300 mg/kg/day	12 weeks	- Body weight - Plasma total cholesterol and triglyceride - Plasma glucose and insulin levels - Oral glucose tolerance test - Oxidative damage	ASE (300 mg/kg/day) - ↓body weight, plasma TG, T-CHOL, glucose levels, insulin resistance, glucose intolerance and malondialdehyde levels
Shirosaki, M., Koyama, T., & Yazawa, K. (2008). Anti-hyperglycemic activity of kiwifruit leaf (*Actinidia deliciosa*) in mice. *Bioscience, Biotechnology and Biochemistry, 72*(4), 1099-1102.						
Std:ddY mice (6-7 weeks old)	Soluble starch, Maltose, Glucose (1000 mg/kg BW) Sucrose (1000 mg/kg BW)	The methanol extract of kiwifruit leaf (1000 mg/kg BW)	The animals had free access to water and standard feed for 1 week to accustom them to their surroundings. Then, mice (n=6/group) were deprived of food for 24 h and consequently administrated with 1. soluble starch with or without kiwifruit leaf 2. maltose with or without kiwifruit leaf 3. glucose with or without kiwifruit leaf 4. sucrose with or without kiwifruit leaf	-	- Blood samples were taken from the lateral tail vein at 0,30,60,90 and 120 min after the administration of carbohydrate	- In control group, the blood glucose reached a max at 30 min after administration. -When kiwi orally admin with starch or sucrose, the bl. glucose at 30 min was sig suppressed, In contrast, no suppressive effect in maltose or glucose-group. - Authors suggested that Kiwi did not inhibit glucose absorption but probably inhibited the carb-hydrolyzing enz.

Metabolic Syndrome Animal Models (Treatment effect)

Animal	Experimental Procedures (Treatment effect)		Biomarkers	Results
	Induce insulin resistance period	**Treatment period**		
Montagut, G., Blade, C., Blay, M., Fernandez-Larrea, J., Pujadas, G., Salvado, M. J., et al. (2010). Effects of a grapeseed procyanidin extract (GSPE) on insulin resistance. *Journal of Nutritional Biochemistry, 21*(10), 961-967.				
Wistar female rats (weight 160-175 g)	After 1 week in quarantine, The animals were divided into 2 groups: 1. Control group (12 animals) Fed with a standard diet 2. Cafeteria group (36 animals) Fed with a cafeteria diet (bacon, sweets, biscuits with cheese, muffins, carrots, milk with sugar) Time period: 13 weeks	→ The control group continually fed with a standard diet → The cafeteria group was divided into 3 subgroups (12 animals/group) 1. Cafeteria group Treated with vehicle (sweetened condensed milk) 2. Cafeteria + 25 Treated with 25 of GSPE/kg BW/day 3. Cafeteria + 50 Treated with 50 of GSPE/kg BW/day Rats were fed either the vehicle or GSPE dissolved in the vehicle by controlled oral intake with a syringe. Time period: After 10 days, 6 animals from each group were sacrificed (Effect of short treatment). After 30 days, other 6 animals from each group were sacrificed (Effect of long treatment).	- Glucose tolerance test - Blood glucose levels - Serum insulin - Insulin resistance - mRNA levels of adipocyte markers	- GSPE did not change body weight and plasma leptin - The 25 mg of GSPE/kg BW/d dose being the most effective - The lower dose (25 mg) admin. for 30d ↓ Adipose tissue ↓ plasma insulin - However, only 30 day-treated rats at the dose of 25 mg maintained their fasting glycemia and showed a healthier HOMA-IR index.

Animal	Experimental Procedures (Treatment effect)		Biomarkers	Results
	Induce insulin resistance period	**Treatment period**		
Grace, M. H., Ribnicky, D. M., Kuhn, P., Poulev, A., Logendra, S., Yousef, G. G., et al. (2009). Hypoglycemic activity of a novel anthocyanin-rich formulation from Lowbush blueberry, *Vaccinium angustifolium* Aiton. *Phytomedicine, 16*(5), 406-415.				
Five-week-old male C57BL/6J mice (10-20 g)	• Mice were acclimatized for 1 week • Then, animal were randomly divided into 2 groups 1. Low fat diet (10% Kcal from fat) 2. Very high fat diet (60% Kcal from fat) Time period: 12 weeks	Animals fed with LFD were fasted for 4 h and measured blood glucose levels. Animals fed with VHFD were fasted for 4 h and Then, gavaged with 1. Plant extract- phenolic-rich (500 mg/kg) 2. Plant extract- anthocyanin-enriched fraction (500 mg/kg) 3. Pure anthocyanin (300 mg/kg) (delphinidin-3-glucoside and malvidin-3 glucoside) 4. Negative control: Vehicle (Labrasol 66% w/v) 5. Positive control: Metformin (300 mg/kg)	- Blood glucose levels (Blood glucose reading were made at 0,3 and 6 h)	- Treatment by gavage (500 mg/kg BW) with plant extract (phenolic and ANT-enrich) formulate with Labrasol ↓elevated bl.glucose by 33 and 51%, respectively. - The hypoglycemic activities of these plant extracts were comparable to that of the anti-diabetic drug metformin (27% at 300 mg/kg). - However, the extracts were not significantly hypoglycemic when administered without Labrasol. - Treatment by gavage (300 mg/kg BW) with pure ANT formulate with Labrasol, showed that malvidin-3 glucoside was sig. hypoglycemic while delphinidin-3-glucoside was not.

Animal	Experimental Procedures (Treatment effect)		Biomarkers	Results
	Induce insulin resistance period	**Treatment period**		
Poudyal, H., Panchal, S., & Brown, L. (2010). Comparison of purple carrot juice and beta-carotene in a high-carbohydrate, high-fat diet-fed rat model of the metabolic syndrome. *British Journal of Nutrition, 104*(9), 1322-1332.				
Wistar rats (8-9 wks) (337±5 g)	Rats were divided into 2 groups 1. Maize starch (Control group) 2. High carbohydrate, High fat diet Time period: 8 weeks In addition, the drinking water of all HCHF diet-fed rats contain 25% fructose At week 8, 12 rats from HCHF group were killed to assess the pathophysiological state before supplementation	Divided into 6 groups (12 rats /group) 1. Maize starch (control) 2. Maize starch + beta carotene 3. Maize starch + purple carrot juice 4. High carb, High fat 5. High carb, High fat + beta carotene 6. High carb, High fat + purple carrot juice Supplement doses: Purple carrot: 50 ml/kg diet Beta-carotene: 400 mg/kg diet Time period: 8 weeks	- Systolic blood pressure - Blood glucose - Oral glucose tolerance test - Plasma chol, TAG, NEFA, malondialdehyde and CRP concentration	Purple carrot juice: - ↓% BW, abdominal fat pads, plasma T-CHOL, TAG, NEFA and CRP concentrations - Improved oral glucose tolerance - Attenuated the changes in uric acid and malondialdehyde β-carotene: - Improved OGT and ↓CRP concentrations, but ↑ TAG and NEFA
Kim, H. Y., Okubo, T., Juneja, L. R., & Yokozawa, T. (2010). The protective role of amla (*Emblica officinalis* Gaertn.) against fructose-induced metabolic syndrome in a rat model. *British Journal of Nutrition, 103*, 502-512.				
Wistar male rats (Weight 217±6 g)	After adaptation for 7 days, Rats were fed a standard chow or a high-fructose (65%) diet Time period: 1 week	Then, randomized into 4 groups (8 rats /group) 1. Normal diet: continually fed with standard chow 2. High fructose diet 3. High fructose diet + 10 mg/kg BW/d EtOAc extracted of amla 4. High fructose diet + 20 mg/kg BW/d EtOAc extracted of amla Consumption of diet level: 16 g/day/rat Time period: 2 weeks	- Serum glucose, TAG, Cholesterol, Lipoprotein fraction - Serum glycated protein and TBARS levels - Hepatic TAG and cholesterol - Blood pressure	EtOAc ext. of amla (20 mg/kg) : - Slightly ↓ in BW - Attenuated serum glucose, T-CHOL, TAG VLDL, and LDL - Normalized blood pressure - Sig inhibited the increased serum and hepatic TBARS - Did not affect protein level of PPARα & SREBP-2

Animal	Experimental Procedures (Treatment effect)		Biomarkers	Results
	Induce insulin resistance period	**Treatment period**		
Jalal, R., Bagheri, S. M., Moghimi, A., & Rasuli, M. B. (2007). Hypoglycemic effect of aqueous shallot and garlic extracts in rats with fructose-induced insulin resistance. *Journal of Clinical Biochemistry and Nutrition, 41*(3), 218-223.				
Albino Wistar rats (180-240 g)	After 2 weeks of acclimation period, the animals were divided into 2 groups. 1. Control group (n=10): Standard rat chow and tap water 2. Insulin resistance group(n=24): 10% w/v fructose dissolved in drinking water Time period: 8 weeks	The control received 500 ml of 0.9% saline Further divided into three subgroups (n=8) Group1: IR Control-Daily injected with 500ml of 0.9% saline Group2: Daily treated by injection of shallot extract (500 mg/kg body weight) Group3: Daily treated by injection of garlic extract (500 mg/kg body weight) Time period: 8 weeks	- Blood glucose, triglyceride, and cholesterol - Serum insulin levels - Intraperitoneal glucose tolerance test	Shallot extract: - Sig ↓fasting blood glucose - ↑ intraperitoneal glucose tolerance and diminish fasting insulin resistance index (FIRI) Garlic extracts: - Sig ↓fasting blood glucose but did not affect FIRI
Shih, C. C., Lin, C. H., & Lin, W. L. (2008). Effects of *Momordica charantia* on insulin resistance and visceral obesity in mice on high-fat diet. *Diabetes Research and Clinical Practice, 81*(2), 134-143.				
Male C57BL/6J mice (5 week of age)	After 1 week acclimation period, mice were divided into 2 groups. 1. Control group: Low fat diet (20% protein, 70% carb, 10% fat) 2. Experimental group: 45% high fat diet (20% protein, 35% carb, 45% fat) Time period: 8 weeks	The control were treated with vehicle (n=9) The exp group was subdivided into 6 groups (n=9). 1. High fat control: Treated with vehicle 2. HF + 0.5 g/kg/day P fraction extracts of bitter melon 3. HF + 1.0 g/kg/day P fraction extracts of bitter melon 4. HF + 0.2 g/kg/day G fraction extracts of bitter melon 5. HF + 1.0 g/kg/day G fraction extracts of bitter melon 6. HF + 10.0 mg/kg/day resiglitazone Time period: 4 weeks	- Body weight - Plasma lipid - Plasma glucose and insulin - Leptin concentrations - Epididymal WAT PPARγ - Lipoprotein lipase - Hepatic PPARα mRNA levels	Bitter melon: - Showed a significant reduction in plasma glucose - No dose-dependent effect in T-CHOL, TG and FFA - ↓the weights of epididymal white adipose tissue and visceral fat - ↓adipose leptin and resistin mRNA levels

Animal	Experimental Procedures (Treatment effect)		Biomarkers	Results
	Induce insulin resistance period	**Treatment period**		
Liu, I. M., Tzeng, T. F., Liou, S. S., & Lan, T. W. (2007). Myricetin, a naturally occurring flavonol, ameliorates insulin resistance induced by a high-fructose diet in rats. *Life Sciences, 81*, 1479-1488.				
Male Wistar rats (age 8 weeks)	Rats were divided into 2 groups 1. Standard chow (Control group) ⋯► • Normal control: Vehicle (70% ethanol: saline 1:19)(n=7) • Fructose diet control: Vehicle (70% ethanol: saline 1:19) 2. Fructose chow (60% fructose) (Induce insulin resistance) ⋯► • Treatment : Myricetin at the dose 0.1 mg/kg/injection • Treatment : Myricetin at the dose 0.5 mg/kg/injection • Treatment : Myricetin at the dose 1.0 mg/kg/injection (7 rats/groups) Myricetin or vehicle was injected into the lateral tail vein 3 times daily (6.00, 14.00 and 22.00 h) Time period: 6 weeks	Time period: 2 weeks	- Oral glucose tolerance test - Plasma glucose, cholesterol & triglycerides - Glycogen synthesis in hepatocytes - Insulin receptor activation	Myricetin : - Sig ↓plasma glucose, TG and HOMA-IR - ↑whole-body insulin sensitivity index during the OGTT
Zhang, H. M., Chen, S. W., Zhang, L. S., & Feng, X. F. (2008). The effects of soy isoflavone on insulin sensitivity and adipocytokines in insulin resistant rats administered with high-fat diet. *Natural Product Research, 22*(18), 1637-1649.				
Male Sprague-Dawley rats (15-180 g)	After 1 week of adaptation period, Rat were randomly assigned 1. A basal diet control group (n=10) ⋯► The basal diet rats continually fed with a basal diet 2. High fat diet fed group (n=70) ⋯► The IR model rats were randomly assigned into 4 groups 1. IR model control group (intra-gastric administration of sterilized water) 2. Low dosage of soy isoflavone (50 mg/kg/d intra-gastric administration of SIF) 3. Medium dosage of soy isoflavone (150 mg/kg/d intra-gastric administration of SIF) 4. High dosage of soy isoflavone (450 mg/kg/d intra-gastric administration of SIF) Time period: 2 months	Time period: 30 days	- Serum adiponectin, leptin, resistin - Blood glucose level - Plasma insulin - Serum TNF-α - mRNA expression	Soy isoflavone (450 mg/kg/d): - ↓body weights and depositions of visceral adipose tissue - Improved insulin resistance by ↓ plasma insulin and HOMA-IR - Did not affect fasting blood glucose - A negative correlation between adiponectin and HOMA-IR (R= -0.881) was found

Animal	Experimental Procedures (Treatment effect)		Biomarkers	Results
	Induce insulin resistance period	**Treatment period**		
Palanisamy, N., Viswanathan, P., & Anuradha, C. V. (2008). Effect of genistein, a soy isoflavone, on whole body insulin sensitivity and renal damage induced by a high-fructose diet. *Renal Failure, 30*(6), 645-654.				
Adult male Wistar rats (150-160 g)	The rats were fed with A control diet or a high fructose diet (60g fructose/100 g diet in place of corn starch) <u>Time period</u>: 15 days	The animal were divided into 4 groups of six rats each 1. Control animals received the control diet containing corn starch as the carb source. 2. Fructose-fed animals received the high fructose diet 3. Fructose-fed animals received genistein (1 mg/kg/day in 0.5 ml dimethyl sulfoxide) 4. Control animals received genistein (1 mg/kg/day in 0.5 ml dimethyl sulfoxide) Genistein was given to the rats daily from the 16th day until the 60th day. <u>Time period</u>: 45 days	- Body weight - Plasma glucose and insulin - Oral glucose tolerance test - Plasma and urinary levels of urea and creatinine - TBARS & GSH - Histopathology examination	Genistein treatment: - ↓plasma glucose, insulin and HOMA values - For OGTT, fasting glucose were sig ↓ in FD+genistein group than FD group. - ↓plasma urea, uric acid, creatinine - ↓lipid peroxide (TBARS) and ↑ glutathione(GSH) compared to fructose-fed rats
Zhang, W., Liu, C. Q., Wang, P. W., Sun, S. Y., Su, W. J., Zhang, H. J., et al. (2010). Puerarin improves insulin resistance and modulates adipokine expression in rats fed a high-fat diet. *European Journal of Pharmacology, 649*, 398-402.				
Male Sprague-Dawley rats (200 g)	After acclimatization for 1 week, SD rats were fed with std chow or a high fat diet (43.6% fat, 24.5% carb and 14.7% protein) <u>Time period</u>: 6 weeks	Then, rats were randomly allocated into 4 groups 1. Normal control diet: continued with standard chow 2. High fat control diet (HFD) 3. HFD + 100 mg/kg/day puerarin 4. HFD + 200 mg/kg/day puerarin Puerarin was intraperitoneally administrated at 6 weeks of HFD consumption until 14 weeks. <u>Time period</u>: 8 weeks	- Oral glucose tolerance test - Intraperitoneal insulin tolerance test - Serum leptin, adiponectin and resistin levels - Real-time RT-PCR	Puerarin treatment: - Reduced BW gain - Improved glucose/ insulin tolerance (Bl.glucose after glucose and insulin injection were markedly ↓ in HFD+puerarin gr. compared to HFD gr.) - Decreased serum leptin and resistin, but not that of adiponectin

Table 1: Effect of freeze-dried blueberry supplementation added into either HFHS or CONT diets on diet and energy consumption, whole body composition, blood parameters and number of probiotic bacteria in rats'caecal contents.

Parameter measured	HFHS 8 wk	HFHS+Burl 8 wk	HFHS+Blue 8 wk	HFHS 4 wk ↓ HFHS+Burl 4 wk	HFHS 4 wk ↓ HFHS+Blue 4 wk	CONT 8 wk	CONT 4 wk ↓ CONT+Burl 4 wk	CONT 4 wk ↓ CONT+Blue 4 wk
Average feed intake (g/d)	23.2±0.7	24.5±0.6	24.0±1.3	23.1±0.9	22.7±0.6	25.8±1.0	26.7±1.1	28.5±1.5
Average energy intake (kJ/d)	499.8±14.4	527.5±12.7	509.9±26.4	496.2±19.9	484.5±11.9	455.8±17.4	473.0±18.5	503.7±27.0
Body weight at wk 8 (g)	605.2±22.0	657.0±16.3	627.9±33.7	605.3±21.8	603.8±21.8	555.7±18.7	570.7±22.1	568.3±19.5
Body composition by DEXA								
Fat mass (g)	165.7±13.2	187.1±7.7	174.0±22.8	149.7±19.0	168.9±14.1	93.2±9.9	126.4±5.7	126.9±18.4
Non-fat mass (g)	434.2±14.0	464.9±14.0	446.8±15.2	430.2±13.4	424.5±14.5	449.9±13.5	434.0±16.0	428.0±10.1
Total mass (g)	599.9±20.9	652.0±16.1	620.8±34.9	579.9±22.8	593.4±21.3	543.1±17.7	560.5±21.2	554.9±19.1
Percentage of fat	27.5±1.6	28.7±1.0	27.5±2.1	25.5±2.4	28.3±1.8	17.0±1.6	22.6±0.4	22.4±2.6
Blood measurements								
Glucose (mmol/L)	12.2±1.0	12.0±0.6	11.9±1.0	11.6±0.6	11.2±0.7	10.1±0.4	11.1±1.0	10.5±0.4
Insulin (ng/ml)	0.5±0.1	0.4±0.04	0.3±0.05	0.4±0.1	0.4±0.04	0.2±0.03	0.4±0.1	0.3±0.04
Triglycerides (mmol/L)	1.0±0.1	1.1±0.2	0.9±0.2	0.8±0.1	0.8±0.2	0.7±0.1	1.0±0.1	1.1±0.2
Cholesterol (mmol/L)	1.7±0.2	1.8±0.1	1.6±0.2	1.5±0.2	1.5±0.1	1.5±0.1	1.8±0.1	1.7±0.2
HDL-cholesterol (mmol/L)	1.0±0.1	1.1±0.1	1.0±0.1	1.0±0.1	0.9±0.1	1.1±0.1	1.1±0.1	1.0±0.1
Adiponectin (µg/ml)	42.3±2.9	39.9±4.7	35.2±4.5	35.7±4.0	35.8±4.5	35.1±4.3	31.7±2.5	35.5±1.3
Log number of probiotic bacteria (CFU/g caecal content)								
Lactobacillus	10.0±0.2	10.1±0.2	10.5±0.1	10.1±0.2	10.5±0.1	10.3±0.1	10.5±0.03	10.6±0.1
Bifidobacteria	11.4±0.4	11.9±0.1	11.9±0.1	11.9±0.2	12.0±0.1	11.6±0.2	12.0±0.1	12.1±0.1

Results are expressed as mean ± S.E. of N = 7 rats per groups.

Table 2: Effect of freeze-dried blueberry supplementation added into either HFHS or CONT diets on adipose tissue and organ weights.

Parameter measured	HFHS 8 wk	HFHS+Burl 8 wk	HFHS+Blue 8 wk	HFHS 4 wk ↓ HFHS+Burl 4 wk	HFHS 4 wk ↓ HFHS+Blue 4 wk	CONT 8 wk	CONT 4 wk ↓ CONT+Burl 4 wk	CONT 4 wk ↓ CONT+Blue 4 wk
Fat pad weights (g)								
Retroperitoneal	24.5±2.2	31.6±1.8	26.7±2.9	24.0±3.5	21.9±2.6	14.9±1.5	17.3±1.3	18.0±2.5
Mesenteric	14.0±1.4	16.4±1.1	15.1±1.9	11.9±1.6	10.0±1.3	6.9±1.2	8.1±0.5	7.7±1.1
Epididymal (upper)	19.0±1.8	21.4±0.7	20.3±2.2	16.0±1.4	17.1±2.2	11.1±1.5	12.8±1.0	13.8±2.4
Epididymal (lower)	0.6±0.04	0.6±0.04	0.6±0.1	0.5±0.04	0.6±0.1	0.5±0.03	0.4±0.1	0.5±0.1
Total WAT	58.0±5.1	69.9±2.8	62.7±6.7	52.5±6.4	49.6±4.8	33.3±4.0	38.7±2.3	40.0±5.9
iBAT	1.2±0.1	1.3±0.1	1.0±0.1	1.2±0.1	1.0±0.1	0.9±0.1	0.9±0.1	1.0±0.1
Fat pad weight (g/kg BW)								
Retroperitoneal	40.3±2.8	48.3±3.1	42.0±2.8	39.2±4.9	36.2±4.1	26.6±2.4	30.4±1.9	31.1±3.4
Mesenteric	22.9±1.8	24.8±1.2	23.6±1.7	19.4±2.2	16.3±1.5	12.2±1.9	14.1±0.4	13.4±1.6
Epididymal (upper part)	31.0±2.2	32.7±1.0	31.9±1.9	26.3±1.8	27.8±2.7	19.9±2.4	22.5±1.4	23.8±3.4
Epididymal (lower part)	0.9±0.1	0.8±0.1	1.0±0.1	0.9±0.1	1.0±0.1	0.8±0.1	0.8±0.1	0.9±0.1
Total WAT	95.2±5.9	106.6±3.8	98.6±5.3	85.7±8.7	81.3±5.3	59.5±6.4	67.8±2.7	69.1±8.2
iBAT	2.0±0.1	1.9±0.1	1.6±0.1	2.0±0.2	1.7±0.1	1.6±0.2	1.6±0.1	1.7±0.1
Organ weights (g)								
Liver	15.4±0.9	16.2±0.6	15.2±1.2	14.5±0.8	14.4±0.9	13.7±0.6	14.2±0.9	13.9±0.9
Kidney	3.8±0.1	3.9±0.1	3.9±0.2	3.8±0.1	3.9±0.1	3.8±0.2	3.7±0.2	3.6±0.1
Pancreas	1.3±0.1	1.3±0.1	1.5±0.1	1.0±0.1	1.3±0.1	1.5±0.1	1.2±0.1	1.3±0.1
Caecum	2.4±0.2	2.3±0.1	2.1±0.3	2.5±0.2	2.4±0.3	3.0±0.4	3.0±0.3	2.7±0.2
Organ weights (g/kg BW)								
Liver	25.3±0.8	24.6±0.5	24.1±0.7	24.0±0.7	23.7±0.7	24.6±0.3	24.7±1.0	24.4±0.8
Kidney	6.3±0.1	5.9±0.2	6.3±0.2	6.4±0.2	6.4±0.3	6.9±0.2	6.4±0.1	6.4±0.2
Pancreas	2.1±0.1	2.0±0.1	2.3±0.1	1.7±0.1	2.1±0.2	2.7±0.1	2.1±0.1	2.3±0.2
Caecum	4.0±0.4	3.5±0.2	3.4±0.6	4.2±0.3	4.1±0.6	5.4±0.8	5.2±0.7	4.7±0.3

Results are expressed as mean ± S.E. of N = 7 rats per groups.

List of Publications

Peer-reviewed scientific paper

Pranprawit, A., Wolber, F. M., Heyes, J. A., Molan, A. L., & Kruger, M. C. (2013). Short-term and long-term effects of excessive consumption of saturated fats and/or sucrose on metabolic variables in Sprague Dawley rats: a pilot study. *Journal of the Science of Food and Agriculture, 93*(13), 3191-3197.

Pranprawit, A., Heyes, J. A., Molan, A. L., & Kruger, M. C. (2014). Potential inhibitory effects of blueberry extracts against key enzymes relevant for hyperglycemia. *Journal of Food Biochemistry*. (Submitted 25th March, 2014).

Conference presentations

Pranprawit, A., Molan, A. L., Heyes, J. A., & Kruger, M. C. (2010). Antioxidant capacity, phenolic contents and anthocyanin concentrations of various cultivars of New Zealand highbush blueberries. (abstract) *Proceedings of the Nutrition Society of New Zealand, 34*, 142-142.
Winner of "the best student poster" prize at the Nutrition Society of New Zealand Annual Scientific Conference, Wellington, New Zealand.

Pranprawit, A., Wolber, F.M., Molan, A. L., Heyes, J. A., & Kruger, M. C. (2012). Short term and long term consumption of high-fat, high-sugar and high-fat-high-sugar diets differently affect markers of metabolic syndrome in Sprague Dawley rats.
Poster presentation at the New Zealand Institute of Food Science & Technology (NZIFST) Conference, Hamilton, New Zealand.

Pranprawit, A., Wolber, F.M., Molan, A. L., Heyes, J. A., & Kruger, M. C. (2012). Blueberry exhibits potential to improve insulin resistance in high-fat-high-sugar diet-induced rat model for metabolic syndrome.
Oral presentation at the Firth Internaltion Symposium on Human Health Effects of Fruits and Vegetables, Karnataka, India.

Pranprawit, A., Wolber, F.M., Molan, A. L., Heyes, J. A., & Kruger, M. C. (2013). Effect of blueberries against high-fat-high-sugar diet-induced metabolic syndrome in rats.
Oral presentation at the NZIAHS INZSPB Plant Science Conference, Palmerston North, New Zealand.

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Araya Pranprawit

Name/Title of Principal Supervisor: Prof Marlena Kruger

Name of Published Research Output and full reference:

Pranprawit, A., Wolber, F. M., Heyes, J. A., Molan, A. L., & Kruger, M. C. (2013). Short-term and long-term effects of excessive consumption of saturated fats and/or sucrose on metabolic variables in Sprague Dawley rats: A pilot study. Journal of the Science of Food and Agriculture, 93(13), 3191-3197.

In which Chapter is the Published Work: Chapter 6

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate: 80%

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Araya Pranprawit
Digitally signed by Araya Pranprawit
DN: cn=Araya Pranprawit, o=Massey University, ou, email=hui_ap@yahoo.com, c=TH
Date: 2013.10.30 10:19:56 +13'00'

Candidate's Signature

30/10/2013
Date

Marlena Kruger
Digitally signed by Marlena Kruger
DN: cn=Marlena Kruger, o=Massey University, ou=Professor, email=m.c.kruger@massey.ac.nz, c=NZ
Date: 2013.10.30 10:52:47 +13'00'

Principal Supervisor's signature

30/10/13
Date