

**THE EFFECTS OF SUPPLEMENTATION WITH
BLUEBERRIES AND YOGHURT AND THEIR
BIOACTIVE COMPONENTS ON OBESITY
RELATED COMORBIDITIES**

A thesis submitted by

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This thesis is submitted in fulfilment of the requirements for award

Doctor of Philosophy

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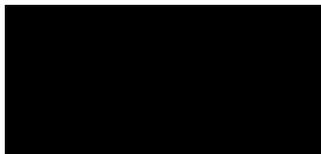
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GENERAL DECLARATION

I, Min Shi, declare that the PhD thesis entitled ‘The effects of supplementation with blueberries and yoghurt and their bioactive components on obesity related comorbidities’ is no more than 100,000 words in length including quotes and exclusive tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work.

Signature:



Date: 23/05/2019

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PUBLISHED MANUSCRIPTS ARISING FROM THIS THESIS

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3. **Min Shi**, Fatah Ahtesh, Michael Mathai, Andrew J. McAinch, Xiao Q. Su*. (2017). Effects of fermentation conditions on the potential antihypertensive peptides released from yoghurt fermented by *Lactobacillus helveticus* and Flavourzyme®. *International Journal of Food Science and Technology*. 52, 137–145.
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CONFERENCE PRESENTATIONS

National and International Conference

1. ANZOS-Breakthrough Discoveries Joint Conference in Melbourne, Australia. 16-18th October 2018. **Min Shi**, Lannie O'Keefe, Anna C Simcocks. Xiao Q Su, Andrew J McAinch. The Impacts of Cyanidin-3-O- β -Glucoside and Peptides Extracted from Yoghurt on Glucose Uptake and Gene Expression in Human Primary Skeletal Muscle Cells from Obese and Diabetic Individuals.

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LIST OF ABBREVIATION

α -MEM	Minimum Essential Medium Eagle, Alpha
AEC	Animal Ethics Committee
ACC	Acetyl-coA Carboxylase
ACE	Angiotensin Converting Enzyme
ACE-I	Angiotensin Converting Enzyme Inhibitor
ACNs	Anthocyanins
AGTR-1	Angiotensin II Receptor Type 1
AICAR	5-Aminoimidazole-4-carboxamide-1-beta-D-ribonucleotide
Akt	Protein Kinase B
AMPK	5' AMP-Activated Protein Kinase
AMP	Adenosine Monophosphate
Ang	Angiotensin
ANOVA	Analysis of variance
AdipoR	Adiponectin Receptors
AT	Adipose Tissue
ATP	Adenosine triphosphate
BB	Blueberry
BCA	Bicinchoninic Acid
BF	Body Fat
BG	Blood Glucose
BMI	Body Mass Index
BSA	Bovine Serum Albumin
BW	Body Weight
C3G	Cyanidi-3-O-beta-D-glucoside
CaCl ₂	Calcium Chloride
CD36	Cluster of Differentiation 36
ChREB	Carbohydrate Responsive Element-binding Protein
CO ₂	Carbon Dioxide
CPT1	Carnitine Palmitoyltransferase I
CRP	C-reactive Protein
CVD	Cardiovascular Disease

D3G	Delphinidin-3-O-glucoside
DBP	Diastolic Blood Pressure
DI	Daily Intake
DMSO	Dimethyl Sulfoxide
DOG	Deoxyglucose
EDL	Extensor Digitorum Longus
EDTA	Ethylenediaminetetraacetic Acid
EI	Energy Intake
ELISA	Enzyme-Linked Immunosorbent Assay
EMLA	Eutectic Mixture of Local Anesthetics
FBS	Foetal Bovine Serum
FI	Food Intake
FoxO1	Forkhead Box Protein O1
FRAP	Ferric-reducing Ability of Plasma
GAPDH	Glyceraldehyde 3-phosphate Dehydrogenase
GLUT1	Glucose Transporter-1
GLUT4	Glucose Transporter-4
G6Pase	Glucose 6-phosphatase
g/kg BW	Gram per Kilogram of Body Weight
HA	Hippuric Acid
HC	Hip Circumference
HCl	Hydrochloric Acid
HDL	High-density Lipoprotein
HDL-C	High-density Lipoprotein Cholesterol
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFD	High Fat Diet
HFHC	High Fat High Fructose
HFHC+B	HFHC Diet Supplemented with Blueberries
HFHC+B+Y	HFHC Diet Supplemented with Blueberries and Yoghurt
HFHC+C	HFHC Diet Supplemented with Cyanidin-3-O- β -glucoside
HFHC+C+P	HFHC Diet Supplemented with Cyanidin-3-O- β -glucoside and Peptides.

HFHC+P	HFHC Diet Supplemented with Peptides
HFHC+Y	HFHC Diet Supplemented with Yoghurt
HHL	Hippuryl-L-histidyl-L-leucine
HL	Histidyl-leucine
HOMA-IR	Homeostasis Model Index of Insulin Resistance
HPLC	High-performance Liquid Chromatography
HPRT-1	Hypoxanthine Phosphoribosyltransferase 1
hsCRP	High Sensitivity C-reactive Protein
kD	Kilo Daltons
kg/m ²	Kilogram per Square of Meter
LAB	Lactic Acid Bacteria
<i>L. bulgaricus</i>	<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i> 1466
LC-MS/MS	Liquid Chromatography-mass Spectrometry/Mass Spectrometry
LDL	Low Density Lipoprotein
LDL-C	Low Density Lipoprotein Cholesterol
<i>L. helveticus</i>	<i>Lactobacillus Helveticus</i> ASCC 881315
IL	Interleukins
IL-1 β	Interleukin-1 beta
IL-6	Interleukin-6
IPGTT	Intraperitoneal Glucose Tolerance Test
IR	Insulin Resistant
IRS-1	Insulin Receptor Substrate 1
IRS-2	Insulin Receptor Substrate 2
IS	Insulin Sensitivity
Lcn2	Lipocalin 2
LFD	Low-fat Diet
LPL	Lipoprotein Lipase
LPS	Lipopolysaccharide
M3G	Malvidin-3-O-glucoside
MAPK	Mitogen-Activated Protein Kinase
MCP-1	Monocyte Chemoattractant Protein
mg/dl	Milligram per Decilitre
mg/g	Milligram per Gram

mg/kg/day	Milligram per kilogram per Day
mg/mL	Milligram per Millilitre
MIG	Monokine Induced by INF- γ
MJ/kg	Megajoules per Kilogram
mL/min	Millilitres per Minute
mL/min/kg	Millilitres per Minute per Kilogram
mmol/L	Millimole per Litre
MRI	Magnetic Resonance Imaging
NaH ₂ PO ₄	Monosodium Phosphate
mRNA	Messenger Ribonucleic Acid
MRS	Glycerol de Man, Rogosa, Sharpe
MgSO ₄	Magnesium Sulfate
NIDDM	Non-insulin-dependent Diabetes Mellitus
NF- κ B	Nuclear Factor κ B
NOS	Nitric Oxide Synthase
O ₂	Oxygen
ORAC	Oxygen Radical Absorbance Capacity
ox-LDL	Oxidized Low-density Lipoprotein
PBS	Phosphate Buffered Saline
KCl	Potassium Chloride
PCR	Polymerase Chain Reaction
PDK	Phosphoinositide-dependent Kinase
PEPCK	Phosphoenolpyruvate Carboxykinase
pg/mL	Picogram per Millilitre
PI	Plasma Insulin
PI3K	Phosphoinositide 3-kinase
PKC ζ	Atypical Protein Kinase C Isoform
PPAR- α	Peroxisome Proliferator-activated Receptor alpha
PPAR- γ	Peroxisome Proliferator-activated Receptor gamma
RAS	Renin Angiotensin System
RBP4	Retinal Binding Protein 4
RCT	Randomized Controlled Trails
RNA	Ribonucleic Acid

ROCK	Rho-associated Kinase
ROS	Reactive Oxygen Species
RP-HPLC	Reversed-phase High-performance Liquid Chromatography
RSM	Reconstituted Skim Milk
SBP	Systolic Blood Pressure
SCD	Standard Chow with 5% Fat
SHR	Spontaneously Hypertensive Rats
SREBF1	Sterol Regulatory Element-binding Transcription Factor 1
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
<i>S. thermophiles</i>	<i>Streptococcus Thermophiles</i> ASCC 1275
T2DM	Type 2 Diabetes Mellitus
TA	Tibialis Anterior
TBS	Tris Based Saline
TBST	Tris Based Saline Tween 20
TC	Total Cholesterol
TEAC	Trolox Equivalent Antioxidant Capacity
TFA	Trifluoroacetic Acid
TG	Triglycerides
TNF- α	Tumour Necrosis Factor- α
$\mu\text{g/ml}$	Microgram per Millilitre
U/kg BW	Unit per Kilogram of Body Weight
VCAM-1	Vascular Cell Adhesion Molecule-1
VCO ₂	Carbon Dioxide Consumption
VO ₂	Oxygen Consumption
vol/vol	Volume for Volume
VPR	Volume Pressure Recording
WC	Weight Circumference
W/H	Waist-to-hip Ratio.
WHO	World Health Organization
x g	Times gravity

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ABSTRACT

Obesity is a leading global health problem contributing to various comorbidities, including cardiovascular disease, type II diabetes mellitus (T2DM) and hypertension. Current conventional medical interventions for the treatment of these diseases are limited with varied efficacy and associated side effects. Thus complementary dietary or alternative therapies have become increasingly popular as alternative treatment methods for obesity and its related comorbidities. There is an immense diversity of plant and animal products, which may be effective agents for attenuating the development of obesity, T2DM and hypertension as they often contain a vast array of bioactive compounds that have been associated with significantly positive health outcomes with minimal side effects. However, the effectiveness of many of these foods and their bioactive molecules in the treatment of human diseases has yet to be fully explored.

The overall focus of this thesis was to determine the effectiveness of supplementation with blueberry, yoghurt and their important respective bioactive components, cyanidin-3-O- β -glucoside (C3G) and peptides, alone or in combination, on the risk factors of obesity and its related comorbidities. To undertake the overall focus of this thesis, the bioactive peptides were extracted from yoghurt and then these peptides as well as C3G were utilised in a human primary skeletal muscle cell culture experiment. This work was further developed with experimentation with these compounds in addition to supplementation with blueberry and yoghurt in isolation and in combination in a high-fat-high-carbohydrate (HFHC) induced obese mouse model.

In order to produce bioactive peptides with angiotensin converting enzyme (ACE) inhibitory activity from yoghurt, the optimal fermentation conditions were determined. The results showed that peptides with ACE inhibitory activity (1.47 ± 0.04 mg/mL of IC_{50} values) were

those obtained from yoghurt fermented by 1% of *L. helveticus* with Flavourzyme® for 12 h. After further separation, one fraction of peptides showed the highest ACE inhibitory activity with an IC₅₀ of 35.75 ± 5.48 µg/mL.

The total content of anthocyanidins and C3G in thirteen brands of blueberry samples were also analysed in order to choose the suitable brand for animal experimentation. Based on these results, Bhatti & Manj blueberries were selected attributed to their higher content of total cyanidin.

In order to determine the effects of C3G and peptides on the regulation of glucose metabolism, different concentrations of C3G and peptides, alone or in combination, on glucose uptake and mRNA expression of key genes were investigated in human primary skeletal muscle cells derived from obese and obese diabetic (obese T2DM) individuals. The results showed that both low and high concentration of peptides and the combination of these peptides with high C3G concentration significantly enhanced glucose uptake in the presence or absence of insulin in the human primary skeletal muscle cells derived from obese individuals. However, high peptide concentration only increased glucose uptake in the absence of insulin in the obese T2DM group. In the obese group, high concentration of peptide alone and its combination with low C3G down-regulated the mRNA expression of angiotensin II receptor, type 1 (AGTR-1), and up-regulated the mRNA expression of insulin receptor substrate 1 (IRS-1), and glucose transporter 4 (GLUT4). In the obese T2DM group, the expression of AGTR-1 was decreased at high peptide concentration and its combinations with C3G.

To further determine whether C3G and peptides, and their original food sources, blueberries and yoghurt, could attenuate obesity and its related comorbidities, a 16-week animal study using HFHC diet induced obese male C57BL/6 mice was conducted. It was found that

blueberry and yoghurt alone, and the combination of peptides and C3G significantly reduced both systolic and diastolic blood pressure. Moreover, yoghurt significantly reduced body weight, percentage body fat and improved intraperitoneal glucose tolerance compared to the HFHC (control) group. Furthermore, peptides and its combination with C3G resulted in a significant reduction in percentage body fat and improvement of intraperitoneal glucose tolerance. However, energy expenditure and locomotor behaviour did not alter in any treatment groups compared to the HFHC group.

The mRNA expression of multiple genes related to glucose metabolism in the skeletal muscle (extensor digitorum longus (EDL) and soleus) of mice supplemented as detailed above was determined by quantitative 'real-time' polymerase chain reaction (PCR). In EDL, yoghurt alone up-regulated the expression of AMPK, IRS-1, PI3K and GLUT4, and down-regulated the expression of AGTR-1. The combination of blueberry and yoghurt down-regulated the mRNA expression of AGTR-1 and FoxO1 in EDL. Furthermore, the combination of C3G and peptides also down-regulated the mRNA expression of AGTR-1 and up-regulated the mRNA expression of GLUT4 in EDL. In soleus, blueberry and yoghurt supplementation alone and their combination down-regulated the mRNA expression of AGTR-1, and up-regulated the mRNA expression of GLUT4.

Therefore, the outcomes of this thesis highlight that yoghurt and its peptides have the potential to reverse or attenuate metabolic disturbances associated with developing obesity, diabetes and hypertension. Blueberries reduced blood pressure while no inhibition on body weight and body fat gain was observed. Furthermore, C3G may not be effective in eliciting beneficial effects on obesity, diabetes and hypertension possibly due to the low dosage utilised. In conclusion, the results presented within the current thesis support that yoghurt as a fermented dairy product may be a beneficial additive to functional foods or

utilized as a dietary component. Blueberry plays a significant role in the treatment of hypertension, which represents a potentially promising dietary intervention worthy of further investigation.

CHAPTER 1: Literature review

1.1 Obesity and its related comorbidities: hypertension and type 2 diabetes mellitus (T2DM)

Body mass index (BMI) – the weight in kilograms divided by the square of the height in meters (kg/m^2) – is a commonly used parameter to classify obesity in adults (Rashad et al. 2005). The World Health Organization (WHO) defines obesity when BMI is equal to or more than 30, and overweight when BMI is equal to or more than 25 (Table 1.1) (NHMRC 2013) while modified criteria have been established for populations with different ethnic background and other conditions. For example, the healthy BMI range tends to be: (1) lower for people of Asian background; (2) higher for those of Polynesian origin; (3) higher for older people; and (4) higher for elite athletes with higher than normal levels of lean body mass (Department of Health 2015).

Table 1.1: BMI classification (NHMRC 2013)

Underweight	< 18.5
Normal range	18.5 – 24.9
Overweight	≥ 25.0
<i>Preobese</i>	25.0 – 29.9
Obese	≥ 30.0
<i>Obese class I</i>	30.0 – 34.9
<i>Obese class II</i>	35.0 – 39.9
<i>Obese class III</i>	≥ 40.0

In 2014, 39% of adults aged 18 years and over (38% of men and 40% of women) were overweight and about 13% of the world’s adult population (11% of men and 15% of women) were obese (WHO 2015) (Figure 1.1). In Australian adults, 28% were obese in 2014 - 2015, and this has increased by 19% compared with data collected in 1995 (AIHW 2018). Available data indicate that the total direct cost for obesity in 2010 was \$14.5 billion in Australia (Council 2014). The worldwide prevalence of obesity has more than doubled from 1980 to 2014, and the trend of childhood obesity becomes prevalence (Chizuru Nishida et al. 2015). In 2013, 42 million children under the age of 5 were overweight or obese (WHO 2015). Once considered a high-income country problem, overweight and obesity are now on the rise in low- and middle-income countries, particularly in urban settings (Bhurosy and Jeewon 2014). In developing countries with emerging economies (classified by the World Bank as lower- and middle-income countries) the rate of increase in childhood overweight and obesity is more than 30% higher than that of developed countries (WHO 2015).

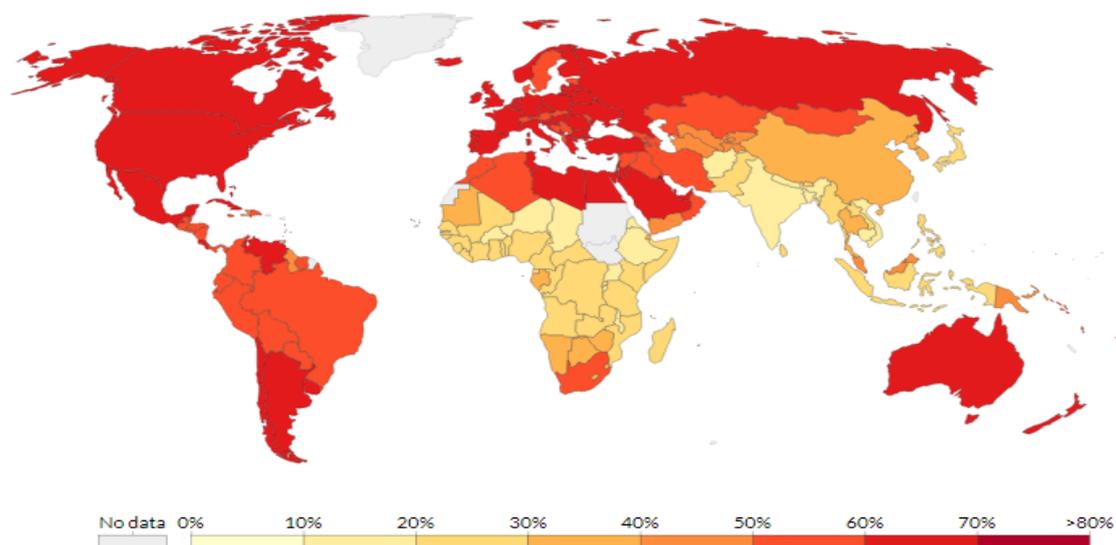


Figure 1.1: Trends of mean body mass index (kg/m²) in 2010-2015.

Percentage of adults aged 18+ years who have a BMI greater than or equal to 25 kg/m² (WHO 2015). Different colour means different percentage of overweight adults.

1.1.1 Risk of obesity

Obesity is a medical condition in which excess body fat has accumulated to the extent that it may have a negative effect on health, causing reduced life expectancy and/or increased health problems (Haslam and James 2005). Obesity is often caused by excessive energy intake coupled with a lack of physical activity, however the complex interplay between genetics and environmental factors means that obesity is difficult to treat (Qi and Cho 2008). Overweight and obesity are linked to more deaths worldwide than underweight. Most of the world's population live in countries where overweight and obesity kill more people than underweight, which includes all high-income and most middle-income countries (Chandrasekaran et al. 2012).

Obesity causes a dysfunction in the metabolic system via a number of mechanisms, including initiation of endothelial dysfunction, increasing free radical production, lipid peroxidation and production of inflammatory cytokines (Chen et al. 2015; Neale et al. 2016). The risks of diabetes, hypertension, cardiovascular disease, liver diseases, metabolic syndrome and dyslipidaemia raise with the increased BMI, thereby reducing life expectancy and greatly increasing the health and societal economic burden; excess bodyweight is now the sixth most important risk factor contributing to the overall burden of disease worldwide (Ezzati et al. 2002; James et al. 2004; Haslam and James 2005; Gill 2016). Furthermore, obesity is associated with a higher risk for various cancers, including breast, colon, prostate, endometrial, liver and renal cancer (Calle and Kaaks 2004; Prieto-Hontoria et al. 2011; Crujeiras et al. 2012).

1.1.2 Obesity as a risk factor for hypertension

Hypertension, or high blood pressure, is a silent, chronic, and progressive disease defined by elevated arterial blood pressure (140/90 mmHg) that is the most common serious chronic

health problem and it is a high-risk factor for arteriosclerosis, stroke, and myocardial infarction and end-stage renal diseases (He, H. L. et al. 2013; Campbell et al. 2014; Morton et al. 2016). In 2010, approximately one billion or 40% of adults globally were treated for hypertension (Campbell et al. 2014). An estimated 10% of healthcare spending (about \$1 trillion) was directly related to hypertension and its complications (Gaziano et al. 2009). Despite extensive public health efforts in implementing a number of behavioural, lifestyle, and pharmacological interventions; hypertension continues to be one of the most common medical diagnoses and one of the most resilient health problems in modern society (Cassidy et al. 2011; Chen, Y. et al. 2014).

Hypertension is closely associated with obesity (Kurukulasuriya et al. 2011; Varughese et al. 2014). The risk of hypertension is up to five times higher among obese people than among those of normal weight, and the variability in response reflects differential genetic susceptibility as well as dietary factors (Haslam and James 2005; Wietlisbach et al. 2013). Data from the Framingham Heart study cohort suggest that ~5% body weight increase from baseline increases hypertension risk by ~30% over a four-year period of observation, while weight loss to about the same degree is associated with a reduction in both systolic and diastolic blood pressure (Stevens et al. 2001; Vasan et al. 2001).

1.1.3 Signalling pathways involved in hypertension

The pathophysiology and etiopathogenesis of obesity related hypertension are complex and appears multifactorial (Landsberg 2000; Kurukulasuriya et al. 2011). Firstly, the kidneys increase sodium reabsorption, the heart increases cardiac output, and arteries respond with vasoconstriction resulting in hypertension (Morse et al. 2005). Secondly, compression exerted by the visceral fat on the renal parenchyma may cause hemodynamic disturbances (Hall et al. 2004). Finally, adipocytes are able to produce aldosterone in response to

angiotensin II (Ang II) and may be considered as a miniature renin-angiotensin-aldosterone system (Briones et al. 2012). Besides, sympathetic nervous system activation, insulin resistance and hyperinsulinemia, endothelial dysfunction, impaired naturesis, obesity-related glomerulopathy, and hyperleptinemia, to name a few are also the contributory factors for obesity hypertension (Landsberg 2000; Zhang and Reisin 2000; Kil and Swanson 2010; Kurukulasuriya et al. 2011).

Among these factors, the renin-angiotensin system (RAS) is thought to be a significant contributor. Normal renal function requires a delicate balance of many components of the angiotensin pathway, since opposing physiological effects can result from altered receptor expression or activation (Morton et al. 2016). The classic RAS pathway begins with circulating renin, which acts on angiotensinogen to produce angiotensin I (Ang I), which in turn is converted into Ang II by angiotensin converting enzyme (ACE) (Atlas 2007). Ang II is considered one primary effector of RAS and induces the release of aldosterone and therefore increases the sodium concentration in kidney and further blood pressure (Humma and Terra 2002; Muro Urista et al. 2011). Ang II exerting its physiologic functions, such as aldosterone release, increased peripheral resistance and growth stimulatory actions, is mediated by Ang II receptor 1 (AGTR-1). Further details of this metabolism are shown in Figure 1.2.

1.1.4 Obesity as a risk factor for T2DM

Obesity increases the risk of developing T2DM, a disease that is characterised by hyperglycaemia with an antecedent phase of insulin resistance (Zierath et al. 1996; Musso et al. 2010). Uncontrolled or poorly managed T2DM can cause changes in the structure and function of major organs and tissues, including blood vessels, heart, nerves, eyes and kidneys which can lead to further serious and life threatening complications such as cardiac

dysfunction, atherosclerosis, and nephropathy (Musso et al. 2010; Pawar and Thompkinson 2014; WHO. 2015). In the early stages of T2DM (or prediabetes), the pancreatic β -cells respond to hyperglycaemia by secreting increased amounts of insulin to facilitate the cellular uptake of the excess plasma glucose. Over time, insulin dependent cells become desensitised to insulin, resulting in insulin resistance and chronic hyperglycaemia if left untreated (Hajiaghaalipour et al. 2015). Furthermore, dyslipidemia and an increase in pro-inflammatory cytokines have been shown to be associated with insulin resistance (Guo, H. et al. 2012b). Oxidative stress is another factor that can cause β -cell dysfunction, impaired glucose tolerance, insulin resistance and eventually T2DM (Evans et al. 2003). Many studies have demonstrated that dietary antioxidants are effective in neutralizing or trapping reactive oxygen species (ROS) and thus antioxidants may be useful anti-diabetic agents (Poudyal et al. 2010; Mussavira et al. 2015; Réus et al. 2016).

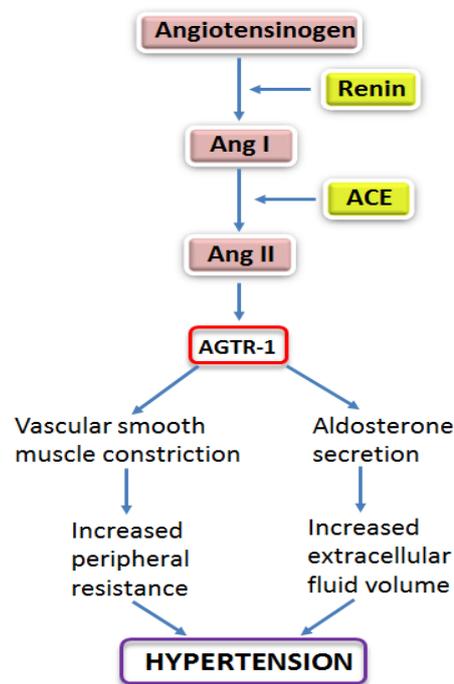


Figure 1.2: Schematic summary of the renin-angiotensin system (RAS) (Humma and Terra 2002; Atlas 2007).

ACE, angiotensin converting enzyme; Ang, angiotensin; AGTR-1, angiotensin II receptor 1.

1.1.5 Signalling pathways involved in glucose metabolism in skeletal muscle

1.1.5.1 Insulin

Insulin is a primary mediator for the normal regulation of glucose transport and metabolism in skeletal muscle (Henriksen and Prasannarong 2013). Insulin induces glucose uptake in skeletal muscles by mediating the translocation of GLUT4 from an intracellular location to the plasma membrane (Bryant et al. 2002). Two intracellular signalling mechanisms are involved to stimulate glucose uptake and GLUT4 translocation in skeletal muscle by insulin (Ryder et al. 2001). One is an insulin-dependent pathway, in which insulin receptor substrate (IRS), phosphatidylinositol 3-kinase (PI3K) and protein kinase B (Akt) are involved (Mokashi et al. 2017). It is now well known that IRS-1 tyrosine phosphorylation generally increases the association of IRS-1 with PI3K, resulting in increased PI3K activity (Scazzocchio et al. 2015). The activation of PI3K subsequently stimulates a serine kinase cascade (including PDK-1 and Akt) to promote translocation of the insulin-responsive glucose transporter GLUT4 from an intracellular compartment to the plasma membrane where GLUT4 facilitates glucose entry into skeletal muscle (Scazzocchio et al. 2015). Therefore, the activation of IRS-1/PI3K/Akt signalling pathway is considered the main player of the metabolic action of insulin on glucose uptake. FoxO1, a member of the O-class of forkhead/winged helix transcription factors (FoxO), has now emerged as a key effector of PI3K/AKT signalling pathway (Guo et al. 1999). The phosphorylation of FoxO1 at three Akt phosphorylation sites, T24, S256, and S319, is induced by insulin or other stimulators of the PI3K/Akt pathway, thus preventing the activation of preproglucagon synthesis (Mancuso et al. 2017).

1.1.5.2 5' AMP-dependent protein kinase (AMPK)

The other key signalling mechanism involved in stimulating GLUT4 translocation is insulin-independent, namely 5' AMP-dependent protein kinase (AMPK) pathway. AMPK is an evolutionarily conserved serine/threonine protein kinase central to the regulation of energy balance at both the cellular and whole-body levels due to its numerous roles in the regulation of glucose, lipid, and protein metabolism (Witczak et al. 2008).

Glucose metabolism comprises the processes involved in the transport of glucose from the blood into cells, and the breakdown and resynthesis of simple sugars, oligosaccharides and polysaccharides (Insel et al. 2006). The activation of AMPK in skeletal muscle has been linked with both an increase in glucose transport and an increase in the synthesis of glycogen (Figure 1.3).

Accumulating evidence suggests that the activation of AMPK inhibits lipid synthesis and can improve insulin action (Scazzocchio et al. 2015). Furthermore the dysregulation of AMPK plays an important role in the development of insulin resistance and T2DM (Ruderman et al. 2013). Important factors for the activation of AMPK are peroxisome proliferator-activated receptors (PPARs), members of the nuclear receptor family that function as ligand-inducible transcription factors in the regulation of genes involved in lipid and glucose metabolism (Chigurupati et al. 2015). Three subtypes, PPAR α , PPAR β , and PPAR γ have been identified with distinct tissue distribution and biological activities (Ferre 2004). Among them, PPAR β is expressed in a wide variety of tissues, with high levels in skeletal muscle (Ehrenborg and Krook 2009; Tan et al. 2017). It has reported that PPAR β agonists increase glucose uptake involved in insulin independent pathway and lead to the phosphorylation of AMPK and p38 mitogen-activated protein kinase in cultured primary human skeletal muscle cells and C₂C₁₂

cells (Luquet et al. 2003; Takahashi et al. 2006; Gan et al. 2011). Therefore, AMPK activation can be an efficient tool in the prevention/therapy of insulin resistance and T2DM.

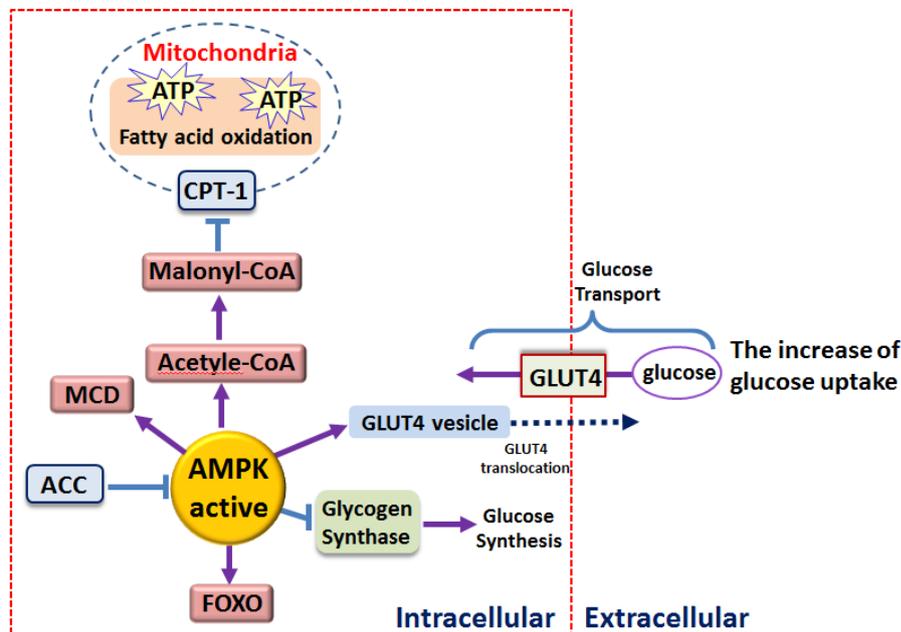


Figure 1.3: The Regulatory Role of AMPK in Glucose Metabolism in Skeletal Muscle (Insel et al. 2006).

ACC, acetyl-CoA carboxylase; AMPK, 5' AMP-activated protein kinase; ATP, adenosine triphosphate; CPT-1, carnitine palmitoyl transferase-1; FoxO, forkhead transcription factor; GLUT4, glucose transporter 4; MCD, malonyl-CoA decarboxylase. Arrow → means regulation. —| means inhibition.

1.1.5.3 Angiotensin converting enzyme (ACE) inhibitor

The RAS is currently being considered as another potential target for the treatment of obesity, insulin resistance, hypertension and diabetes (Passos-Silva et al. 2013). ACE inhibition enhances insulin-induced glucose uptake and further increases GLUT4 expression in skeletal muscle (Shiuchi et al. 2002; Echeverria-Rodriguez et al. 2014). Kishi et al. (1998) proposed that an ACE inhibitor, bradykin, indirectly stimulates GLUT4 translocation and increases glucose uptake through an insulin-independent pathway in both 3T3-L1 adipocytes and L6

myotubes (Kishi et al. 1998). Shiuchi et al. (2002) reported that activation of the bradykinin-NO system by ACE inhibitors enhances the translocation of GLUT4 in skeletal muscle in KK-Ay type 2 diabetic mice, resulting in improvement of insulin resistance (Shiuchi et al. 2002). The ACE inhibitor, temocapril, has also been demonstrated to play a potential role in the increase in glucose uptake in peripheral tissues, leading to the decrease of Ang II, which inhibits insulin signalling in aortic smooth muscle cells with inhibition of PI3K and IRS-1 activation (Folli et al. 1997). Also the treatment of captopril, another ACE inhibitor, induced the up-regulation of myosin heavy chain and the hypertrophic myotubes in differentiating C₂C₁₂ myoblasts, in contrast, overexpression of ACE induced the down-regulation of myosin heavy chain (Mori and Tokuyama 2007). Moreover, another member of the RAS, Ang 1–7, has been demonstrated to improve insulin sensitivity by enhancing insulin-stimulated glucose uptake and the reversal of insulin resistance by restoring the decreased activation of the insulin signalling pathway, including IRS-1, PI3K, Akt and FoxO1 (Furuhashi et al. 2004; Giani et al. 2009; Liu et al. 2012). These results indicate that ACE inhibitor or Ang 1–7 positively regulates insulin signalling in skeletal muscle.

1.1.5.4 Forkhead box O 1 (FoxO1)

Forkhead box O (FoxO) family of transcription factors play a significant role in insulin signalling in all glucose-regulating organs, and becomes a common unifying link of insulin signalling among all glucose-regulating organs (Kousteni 2012). Among all transcription factors involved in energy regulation, the FoxO proteins, and in particular FoxO1, are the main transcriptional modulators of insulin actions (Nakae et al. 2008). FoxO1 is highly expressed in insulin-responsive tissues including pancreas, liver, skeletal muscle and adipose tissue (Xu et al. 2017). In all these tissues FoxO1 orchestrates the transcriptional cascades regulating glucose metabolism. Indeed, FoxO1 is a major target of insulin which inhibits its

transcriptional activity via nuclear exclusion. In skeletal muscle FoxO1 maintains energy homeostasis during fasting and provides energy supply through breakdown of carbohydrates, a process that leads to atrophy and underlies glycaemic control in insulin resistance (Kousteni 2012). Insulin suppresses FoxO1 activity through activation of the PI3K/AKT signalling pathway (Nakae et al. 2008).

FoxO1 is involved in the proliferation of myoblasts, the fusion of mononucleated monocytes into myotubes, in myogenic lineage specification and in the breakdown of muscle fibres (Tsuchiya and Ogawa 2017). In proliferating myoblasts FoxO1 remains inactive, presumably through a PI3K/AKT- independent mechanism of nuclear exclusion. In addition, another potential cause of FoxO1 inactivation is due to direct phosphorylation by the Rho-associated kinase (ROCK) a downstream effector of the small GTPase Rho which is required to maintain extranuclear localization of FoxO1 and at the same time suppresses myoblast differentiation (Nishiyama et al. 2004). However, once in the nucleus FoxO1 promotes myotube formation (Bois and Grosveld, 2003) by upregulating the expression of the genes involved in cell fusion (Furuyama et al. 2003).

In general FoxO1 expression or activation is induced during fasting as a means of maintaining energy homeostasis through utilization of lipids rather than carbohydrates as the energy source in the muscle. In starvation, FoxO1 stimulates pathway related to energy supply through breaking down of muscle protein, an action that leads to muscle loss and atrophy and underlies glycaemic control in insulin resistance (Kousteni 2012).

1.2 Human primary skeletal muscle myotubes

A principal benefit of human samples such as differentiated myotubes, is the maintenance of many features of the donor phenotype, including metabolic properties and disorders observed

in the patients (Gaster et al. 2002; Chen et al. 2005; Chanon et al. 2017). Similar to that observed in skeletal muscle *in vivo*, the human primary skeletal muscle myotubes regulate glucose metabolism through the expression of glucose transporters and the cellular insulin signalling machinery in both healthy individuals and patients with obesity and T2DM (Henstridge et al. 2009). Thus, measurement of glucose uptake and understanding of related molecular mechanisms in primary skeletal muscle are of relevance to characterizing the muscle phenotype of a donor, or investigating the effect of an intervention (drug, nutrition, or physical activity) on the insulin sensitivity in the muscle cell.

In skeletal muscle, insulin binds to the insulin receptor followed by the activation of insulin receptor substrates (Carnagarin et al. 2015). The activated insulin receptor substrates, like IRS-1 subsequently activate downstream insulin signalling proteins, such as PI3K and Akt (Carnagarin et al. 2015). These proteins then stimulate GLUT4 translocation to the plasma membrane, and increase glucose uptake in the tissue. In this signalling pathway, the activated FoxO1, as a negative regulator of insulin sensitivity inhibits the activation of Akt and leads to the decrease of glucose uptake and the development of insulin resistance (Kousteni 2012). On the other hand, activated AMPK promotes GLUT4 translocation, increases glucose uptake and decreases blood glucose level (Eid et al. 2010). In addition, Ang II promotes ROS generation in skeletal muscle and impairs insulin-mediated IRS-1 tyrosine phosphorylation, Akt activation, GLUT4 plasma membrane translocation, and skeletal muscle glucose uptake (Csibi et al. 2010). All of these processes are significantly attenuated by ACE inhibitors or antioxidants (Figure 1.4).

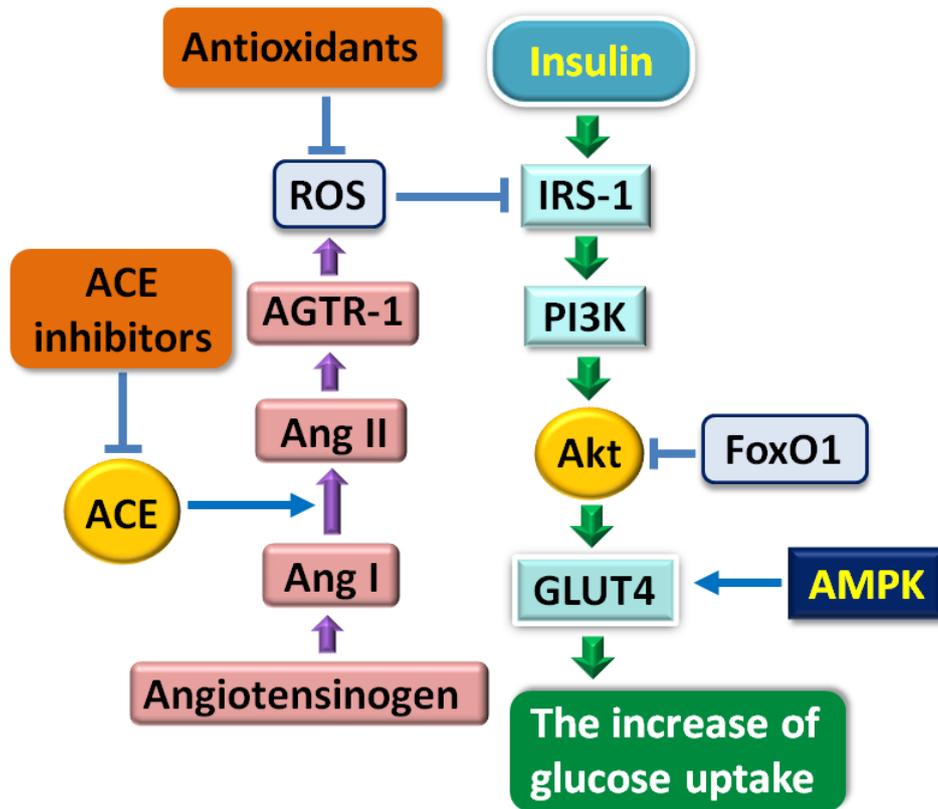


Figure 1.4: Schematic overview of the regulation of major metabolic responses in skeletal muscle by insulin (Csibi et al. 2010).

ACE, angiotensin-converting enzyme; AGTR-1, angiotensin II receptor type 1; AMPK, AMP-activated protein kinase; Akt, protein kinase B; Ang I, Angiotensin I; Ang II, Angiotensin II; GLUT4, glucose transporter 4; FoxO1, forkhead transcription factor 1; IRS-1, insulin receptor substrate 1; PI3K, phosphatidylinositol 3-kinase; ROS, Reactive oxygen species. Arrow → means regulation. —| means inhibition.

1.3 High-fat-high-carbohydrate diet induced obese mouse model

Animal models have been extensively used to study the pathophysiology of various medical conditions and underlying mechanisms. Several factors are important when using a model of diet-induced obesity, such as strains, gender, age, environment factors and so on. C57BL/6 mice are commonly used for obesity research, due to their own characteristics, it is easy to

increase body fat mass, hyperglycemia and hyperinsulinemia with high-fat diet, compared with other mouse models, such as A/J mouse, C57BL/KsJ mouse, BAL/cByJ mouse and db/db mouse (Surwit et al. 1995; Nishikawa et al. 2007; Wang and Liao 2012). Furthermore, many studies showed that the C57BL/6 mice fed high-fat diet become obese within weeks and with prolonged feeding they developed insulin resistance and glucose intolerance, hypertension and low grade (metabolic, meta-) inflammation in metabolic tissues (Jayaprakasam et al. 2006; Winer and Winer 2012; Mykkanen et al. 2014; Elks et al. 2015; Kim and Park 2015). However, the use of high fat diet alone in mice has been reported that the animals only mimic certain aspects of metabolic syndrome but not the entire repertoire (Messier et al. 2007; Panchal and Brown 2011; Wooten et al. 2016). In contrast, a combination of high fat and high carbohydrate diet in animals leads to the development of all typical metabolic complications present in human metabolic syndrome such as increased body weight, increased triglycerides and cholesterol plasma concentrations, abdominal fat deposition, chronic kidney disease and non-alcoholic fatty liver disease (Kohli et al. 2010; Wada et al. 2010; Couturier et al. 2011; Dissard et al. 2013).

Fructose intake has increased worldwide, due to an increased consumption of soft drinks and other beverages, as well as the addition of fructose to breakfast cereals, baked foods, and prepared desserts (Basciano et al. 2005; Le and Tappy 2006). It has been confirmed that fructose, compared with other sugar, disturbs functions of multiple tissues and organs, thereby developing hyperinsulinemia, insulin resistance hypertension and chronic inflammation (Tran et al. 2009; Zhang et al. 2017). Therefore, mouse models with long-term consumption of high-fat and high-fructose diet (HFHC) is probably the most appropriate model to study human metabolic syndrome, especially for the studies of obesity and its related hyperglycemia, hyperinsulinemia, hypertension, inflammation and disorder of liver and kidney function (Nagata et al. 2004; Wooten et al. 2016).

A summary of HFHC induced metabolic syndrome in C57BL/6 mice is provided in Table 1.2. As expected, HFHC has successfully induced metabolic syndrome in C57BL/6 mice including a great gain of body weight, insulin resistance, hypertension, dyslipidemia, and fatty liver as well as up-regulation of inflammatory cytokines (Wada et al. 2010; Wang and Liao 2012). Tsuchiya et al. (2013) showed that C57BL/6 mice fed on HFHC (30% energy from fat plus 35% fructose) for 4 weeks have displayed significantly higher hepatic free fatty acid (FFA) content, fasting serum insulin level and insulin resistance based on homeostasis model assessment of insulin resistance index (Tsuchiya et al. 2013). Similarly, adult male C57BL/6 mice fed HFHC containing 58% kcal from fat and water with 55% fructose and 45% sucrose (wt/ vol) developed obesity and its associated insulin resistance, hepatic comorbidity and non-alcoholic fatty liver disease in the background of increased hepatic ROS and proinflammatory macrophages (Kohli et al. 2010).

Table 1.2: Summary of studies investigating the effect of high-fat and high-fructose diets on obesity and related comorbidities

Diet	Animal model	Duration	Effects	Reference
High-fructose diet (contained 67% carbohydrate (98% of which was fructose), 13% fat, and 20% protein)	Male C57BL/6N	8-week	Compared with diet without fructose: ↑Epididymal fat weight, total cholesterol,	(Nagata et al. 2004)
High-fat diet (60% kcal from fat, mostly from lard) + 15% fructose in drinking water	Male C57BL/6	12-week	Compared with control diet: ↑ BW, BG, Compared with high-fat diet without fructose: ↑ BW ↓ BG	(Messier et al. 2007)
High-fat diet (60% of fat calories from lard and soybean oil) + 20% wt/ vol fructose solution	Male C57BL/6J	14-week	Compared with control diet: ↑ BW, PI, plasma TG, total plasma cholesterol, total liver lipid, liver cholesterol, liver TG Compared with high-fat diet without fructose: ns, BW, PI, total plasma cholesterol, total liver lipid ↓ BG ↑ Plasma TG, liver cholesterol, liver TG	(Wooten et al. 2016)
High-fat and high-fructose diet (45% energy from fat + 30% fructose)	Male C57BL/6J	8-week	Compared with control diet: ↑ BW, energy intake, BF, lean mass, plasma level of TG, cholesterol, HDL, glucose	(Dissard et al. 2013)
High-fat and high-fructose diet (30% energy from fat + 35% fructose)	Male C57BL/6J	8-week	Compared with control diet: ↑ BW, liver free fatty acid, fasting BG levels, fasting serum insulin levels, and HOMA-IR index. ns, liver TG, liver TC.	(Tsuchiya et al. 2013)

High-fat diet (caloric basis 60% fat, 20% carbohydrate, and 20% protein)	Male C57BL/6J	8-week	Compared with control diet: ↑ BW, BG, PIs, blood pressure change measured by the tail cuff method	(Eskens et al. 2014)
High-fat diet (58 kcal % fat) + drinking water with 55% fructose and 45% sucrose (wt/ vol)	Male C57BL/6	12-week	Compared with control diet: ↑ BW gain, BF, fasting BG levels, fasting plasma insulin levels	(Kohli et al. 2010)
High-fat and high-fructose diet (45% kcal from fat, 10% fructose in drinking water)	Male C57BL/6J	12-week	Compared with control diet: ↑ BW, glucose tolerance tests, insulin tolerance tests, fasting BG level, fasting plasma insulin concentration, IR index	(Liu et al. 2016)
High-fat and high-fructose diet (42% calories from fat + 30% fructose in drinking water)	Male C57BL/6J	10-week	Compared with control diet: ↑ PI, plasma cholesterol, plasma IL-6, plasma Lcn2, plasma LPS binding protein, liver TNF- α	(De Sousa Rodrigues et al. 2017)
high-fat and high fructose diet (60% fat + 30% fructose water)	Male C57BL/6J	8-week	Compared with control diet: ↑ BW, BW gain, blood pressure; Serum measurements of fasting glucose, insulin, HOMA-IR index, triglycerides, TC, free fatty acid, and leptin levels; Glucose, insulin, and pyruvate tolerance	(Wada et al. 2010)

ns: no statistical significance ($P > 0.05$) between the treatment and control groups. BF, body fat (%); BG, blood glucose (mmol/L); BW, body weight (g); HDL, high-density lipoprotein; HOMA, homeostasis model index of insulin resistance; IR, insulin resistant; IL-6, interleukin-6 (ng/L); Lcn2, lipocalin 2; LPS, lipopolysaccharides; MCP-1, monocyte chemoattractant protein-1 (ng/L); PI, plasma insulin (pmol/L); TC, total cholesterol (mg/dL); TG, triglycerides (mg/dL); TNF- α , tumor necrosis factor (ng/L); wt/ vol, weight/volume.

1.4 Yoghurt and its peptides

Yoghurt, a semisolid fermented milk product, is produced by lactic acid bacteria (LAB), *Lactobacillus bulgaricus* and *Streptococcus thermophiles*, using milk as a media. Because of yoghurt's specific manufacturing procedure through fermentation and the use of LAB, it shows different nutritional properties and contains higher amounts of several micronutrients, including calcium, riboflavin, magnesium, vitamins B-6, B-12 and D, potassium, zinc and specific fatty acids than other dairy products, such as milk (Tremblay and Panahi 2017). There are a number of studies reporting the positive effects of yoghurt consumption on human health, including inhibiting risk factors for cardiovascular disease (Wu and Sun 2017), lowering risk of type 2 diabetes (Margolis et al. 2011; Gijsbers et al. 2016) and chronic kidney disease (Yacoub et al. 2016), and enhancing development of immunity (Nishida et al. 2016).

The yoghurt fermentation process involves the growth of LAB that converts lactose to glucose and galactose, and milk protein to amino acids and small peptides, which are easily digested and absorbed by the human body (Guan et al. 2017). Among these metabolites, various bioactive peptides released from yoghurt have been considered as functional and alternative therapeutics agents for the numerous potential health benefits due to their antibacterial activity and anticancer activity (Sah et al. 2016), antioxidant properties (Sah et al. 2015). Consequently, peptides provide additional benefits for consumers with preventing allergy-related immune disorders (Song et al. 2016), simulating gastrointestinal digestion (Jin et al. 2016) and suppressing the activity of angiotensin I-converting enzyme (ACE) (Fatah et al. 2016a). However, properties of bioactive peptides in yoghurt mainly depend on proteolytic activities of the cultures used. For example, *Lactobacillus helveticus* (*L. helveticus*) strains are considered as the important cultures to produce biopeptides in dairy

fermentation process, because they have been shown to have high proteolytic activities and their various therapeutic health benefits are recognised (Aihara et al. 2005; Wang et al. 2015; Damodharan et al. 2016).

1.4.1 Properties of *Lactobacillus helveticus* (*L. helveticus*)

L. helveticus is a multifunctional thermophilic LAB with increasing importance in the food industry (Beganovic et al. 2013). It is the dominant LAB species in the starter cultures used for the production of fermented milk beverages and some types of hard cheeses, such as Swiss-type and long-ripened Italian cheeses, based on a range of enzymes, which upon cell lysis can be released into the cheese matrix and impact degradation of proteins, peptides and lipids (Griffiths and Tellez 2013; Spus et al. 2017; Zago et al. 2017). Recently, *L. helveticus* is considered a health-promoting culture in functional food due to their function of acidifying milk and producing bioactive peptides and aromatic compounds from caseins that are reported to be antihypertensive and antimicrobial, to reduce the risk of colon cancer or even to act as immunomodulators in fermented milk and cheese (Vinderola et al. 2007; Genay et al. 2009; Beganovic et al. 2013; Giraffa 2014). In addition, some of *L. helveticus* strains were described as the most proteolytic species of LAB, due to the capability of degradation of casein (Genay et al. 2009; Broadbent et al. 2011). This strong proteolytic system that is capable of producing short peptides and liberating amino acids from the casein matrix is composed of cell envelope proteinases that hydrolyse caseins into oligopeptides. Transport systems that allow uptake of oligopeptides, and various intracellular peptidases with differing and partly overlapping specificities lead to a pool of free amino acids (Sadat-Mekmene et al. 2011a).

1.4.2 Bioactive peptides for the inhibition of angiotensin converting enzyme (ACE) on hypertension

Many dairy products fermented by several *L. helveticus* strains, showed positive antihypertensive activity accompanied by the identification of the ACE inhibitory peptides (Table 1.3). Different types of fermented milk products have been studied for ACE inhibition properties, which include different varieties of fermented milk (Gobbetti et al. 2000; Ashar and Chand 2004; Kilpi et al. 2007; Chen, Y. et al. 2014), fermented sour milk (Nakamura et al. 1995; Sipola et al. 2001; Tuomilehto et al. 2004; Pan and Guo 2010) and fermented skim milk (Qian et al. 2011; Ramchandran and Shah 2011; Beganovic et al. 2013; Nishimura et al. 2013; Fatah et al. 2016b). However Beganovic et al. (2013) reported that milk as a medium does not contain appropriate quantity of free amino acids and peptides with low molecular weight for growth of *L. helveticus* and the production of ACE-I peptides. Yoghurt becomes an alternative medium to provide optimal nutrients after milk fermentation by LAB (Beganovic et al. 2013).

Table 1.3: Selected fermented milk products, their starter and peptides with ACE inhibitory activity

Starters	Milk products	Precursor protein	Peptide sequence	References
<i>L. helveticus</i> + <i>S. cerevisiae</i>	Sour milk	β -CN*, κ -CN	Ile-Pro-Pro, Val-Pro-Pro	(Nakamura et al. 1995; Takano 1998)
<i>L. helveticus</i> JCM1004 cell-free extract	Skim milk	Skim milk hydrolysate	Ile-Pro-Pro, Val-Pro-Pro	(Rokka et al. 1997)
<i>L. helveticus</i> CP790 proteinase	Casein hydrolysate	β -CN	Lys-Val-Leu-Pro-Val-Pro-Gln	(Maeno et al. 1996)
<i>L. helveticus</i> CPN4	Whey hydrolysate	Whey proteins	Tyr-Pro	(Yamamoto et al. 1999)
<i>L. delbrueckii</i> ssp. <i>bulgaricus</i> SS1 + <i>Lactococcus lactis</i> ssp. <i>cremoris</i> FT4	Milk	β -CN, κ -CN	Many fragments	(Gobbetti et al. 2000)
<i>Lactobacillus delbrueckii</i> ssp. <i>bulgaricus</i> + <i>Streptococcus salivarius</i> ssp.	Yoghurt	β -CN	Ser-Lys-Val-Tyr-Pro-Phe-Pro-Gly-Pro-Ile	(Ashar and Chand 2004)
<i>Lactococcus lactis</i>	Bovine, sheep, goat, pig, buffalo or human milk	α_{s1} -CN, α_{s2} -CN, κ -CN	Many fragments	(Minervini et al. 2003)
<i>Lb. helveticus</i> NCC 2765	Jejunal chyme	β -CN	Tyr-Pro-Phe-Pro-Glu-Pro-Ile-Pro-Asn	(Meisel and Frister 1989)
<i>L. helveticus</i> CNRZ32	Milk	β -CN, α_{s1} -CN	Many fragments	(Kilpi et al. 2007)
Many <i>L. helveticus</i> strains	Milk	Milk hydrolysate	Many fragments, containing high concentration of Val-Pro-Pro and Ile-Pro-Pro	(Chen, Y. et al. 2014)
<i>L. helveticus</i> strains (881315, 881188, 880474, and 880953)	Skim milk	Skim milk hydrolysate	Many fragments	(Fatah et al. 2016b)
<i>Lb. helveticus</i> + <i>S. cerevisiae</i>	Yoghurt	β -CN	Val-Pro-Pro and Ile-Pro-Pro	(Kajimoto et al. 2002)
<i>Lb. delbrueckii</i> + <i>Streptococcus thermophilus</i> + <i>Lb paracasei</i>	Sheep milk Yoghurt	β -CN	Many fragments	(Papadimitriou et al. 2007)

CN* = Casein; ACE** = Angiotensin converting enzyme

Function of ACE inhibition from peptides is associated with the original protein source and based on their inherent amino acid composition and active sequences (Brandelli et al. 2015). The size of active sequences may vary from 2 to 20 amino acid residues (Korhonen 2009). A lot of research has been carried out on casein-derived ACE-I peptides (Giraffa 2014). Caseins are insoluble proteins and easy to be hydrolysed by enzymes and bacteria (Muro Urista et al. 2011). Their open and flexible structure makes it easy for digestion and hydrolysis to release a considerable quantity of active peptides with different amino acid sequences (Silva and Malcata 2005). Many inhibitors of ACE have been isolated from the enzymatic digestion of casein, such as many fragments with ACE-I peptides from bovine β -CN and κ -CN (Nakamura et al. 1995; Gobbetti et al. 2000), converting enzyme inhibitor from bovine α_{s1} -CN (Minervini et al. 2003). Most of the characterized ACE-inhibitory peptides in fermented milk products and hydrolysates are usually short peptides with a proline residue at the carboxyl terminal end, such as isoleucine-proline-proline (Ile-Pro-Pro) valine-proline-proline (Val-Pro-Pro) and leucine-proline-proline (Leu-Pro-Pro) (Ishida et al. 2011a). Also, proline is known to be resistant to degradation by digestive enzymes and may pass from the small intestines into the blood circulation in the sequence of short peptides to show effective antihypertensives (Yamamoto et al. 2003; Hannu and Anne 2006). Many bioactive peptides derived from milk proteins have been shown to be hypotensive in both animal studies (Sipola et al. 2001; Tsai et al. 2008; He, R. et al. 2013; Rodriguez-Figueroa et al. 2013; Chen, Y. et al. 2014) and human trials (Ashar and Chand 2004; Tuomilehto et al. 2004; Aihara et al. 2005; Jauhiainen et al. 2005; Jauhiainen et al. 2010b; Lehtinen et al. 2010; Ishida et al. 2011a; Nakamura et al. 2011; Cicero et al. 2013).

A previous study showed that *L. lactis*-fermented milk presented an important systolic blood pressure (SBP)-, diastolic blood pressure (DBP)- and heart rate-lowering effect after 2, 4, 6 and 24h oral administration in spontaneously hypertensive rats (SHR) (Rodriguez-Figueroa et

al. 2013). Furthermore, it is reported that a single oral dose of fermented milk, enriched with Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP), significantly attenuated SBP and DBP of SHR after both a short period treatment (6 or 12 hours) and long-term daily treatment (7 weeks) (Chen, Y. et al. 2014). The above findings are consistent with the results obtained by Tsai et al., (2008), who observed that SBP and DBP were reduced significantly in SHR, after 8 weeks of oral administration of diluted whey (with Tyr-Pro-Tyr-Tyr of 4.9 mg/mL) (Tsai et al. 2008). It was concluded that feeding diets supplemented with fermented milk containing high concentration peptides resulted in reduced blood pressure in SHR.

A limited number of human studies have focused on the hypotensive effect of different milk protein hydrolysates which contain ACE-I peptides (Sekiya et al. 1992; Jauhiainen et al. 2005; Jauhiainen et al. 2010b; Cicero et al. 2013). Sekiya et al. (1992) were the first to report that the consumption of 20 g/day of a tryptic hydrolysate of casein for 4 weeks could reduce both SBP and DBP in hypertensive human volunteers (Sekiya et al. 1992). A previous study showed that *L. helveticus* fermented milk containing bioactive peptides with a high concentration of tripeptides (IPP 7.5 mg/100 g and VPP 10 mg/100 g), in daily use, could also decrease SBP and DBP in hypertensive subjects (Jauhiainen et al. 2005). Furthermore, a meta-analysis demonstrated that the combination of two peptides (IPP and VPP) yielded a statistically significantly greater effect on SBP and DBP than single IPP or VPP, compared with placebo (Cicero et al. 2013). However, some human trials presented inconsistent results. There was only a significant decrease in DBP in the high-normal blood pressure group for 4 weeks of treatment with two tripeptides (VPP and IPP), compared with the placebo group (Aihara et al. 2005). Interestingly, in the mild hypertension group, only SBP, not DBP, decreased significantly, compared with the placebo group (Aihara et al. 2005). These findings indicate that individuals with various phenotype, age and characteristics have different responses to the ACE-I peptides. For example, each additional year of age could reduce the

effect of VPP and IPP in SBP by 0.09 mm Hg, which might be related to isolated systolic hypertension, a condition often encountered in the elderly, who may be poorly responsive to the first-line of treatment for hypertension (Cicero et al. 2013).

1.5 Blueberries on obesity and its related comorbidities

Section 1.5 has been published in 2017 and no alterations to the text has been made with the exception of formatting/layout and referencing style to ensure consistency with the rest of the thesis. See Appendix 1 for details of publication entitled “**Blueberry as a source of bioactive compounds for the treatment of obesity, type 2 diabetes and chronic inflammation**”. Min Shi, Hayley Loftus, Andrew J. McAinch, Xiao Q. Su. *Journal of Functional Foods*, 30, 16-29.

1.5.1 Summary

Recent experimental and clinical studies suggest that consumption of blueberry products has potential health benefits in ameliorating the development of obesity and its related comorbidities, including type 2 diabetes mellitus (T2DM) and chronic inflammation. Blueberry fruits are enriched with numerous bioactive components such as vitamins, phenolic acids and anthocyanins which could contribute to these protective effects. Possible mechanisms by which blueberries exert their beneficial properties include counteracting oxidative stress, regulating glucose metabolism, improving lipid profile, and lowering inflammatory cytokine levels in animal models and preliminary human trials. This review focuses on the potential role of blueberries as a functional food in the prevention and treatment of obesity and its comorbidities. Although the current evidence is promising, further randomized controlled studies in the longer term are needed to evaluate the role of blueberries and blueberry extracts to support human health.

1.5.2 Introduction

Obesity is a medical condition in which excess body fat has accumulated to the extent that it may have a negative effect on health, causing reduced life expectancy and/or increased health problems (Haslam and James 2005). Obesity causes a dysfunction in the metabolic system via a number of mechanisms, including initiation of endothelial dysfunction, increasing free radical production, lipid peroxidation and production of inflammatory cytokines (Chen et al. 2015; Neale et al. 2016). Obesity predisposes to various diseases, especially obstructive sleep apnoea, cardiovascular disease (CVD), T2DM and certain cancers (Haslam and James 2005). Obesity is caused by excessive energy intake coupled with a lack of physical activity, however the complex interplay between genetics and environmental factors means that obesity is difficult to treat.

Obesity increases the risk of developing T2DM, a disease that is characterised by hyperglycaemia with an antecedent phase of insulin resistance (Zierath et al. 1996; Musso et al. 2010). Uncontrolled or poorly managed T2DM can cause changes in the structure and function of major organs and tissues, including blood vessels, heart, nerves, eyes and kidneys which can lead to further serious and life threatening complications such as cardiac dysfunction, atherosclerosis, and nephropathy (Zierath et al. 1996; Musso et al. 2010). In the early stages of T2DM (or prediabetes), the pancreatic β -cells respond to hyperglycaemia by secreting increased amounts of insulin to facilitate the cellular uptake of the excess plasma glucose. Over time, insulin dependent cells become desensitised to insulin, resulting in β -cell dysfunction, insulin resistance and chronic hyperglycaemia if left untreated (Hajiaghaalipour et al. 2015). Furthermore, dyslipidemia and an increase in pro-inflammatory cytokines have been shown to be associated with insulin resistance (Guo, H. et al. 2012b). Oxidative stress is another factor that can cause β -cell dysfunction, impaired glucose tolerance, insulin

resistance and eventually T2DM (Evans et al. 2003). Many studies have demonstrated that dietary antioxidants are effective in neutralizing or trapping reactive oxygen species (ROS) and thus antioxidants may be useful anti-diabetic agents (Laplaud et al. 1997; Martineau et al. 2006; DeFuria et al. 2009; Poudyal et al. 2010).

It is well known that obese and diabetic patients often present with dyslipidemia, characterized by elevated triglycerides (TG), low high density lipoprotein cholesterol (HDL-C) and predominance of small-dense low density lipoprotein (LDL) particles (Chan et al. 2014). Dyslipidaemia in visceral obesity is principally the result of insulin resistance, which perturbs the kinetics of both apolipoprotein B- (apoB) and apolipoprotein A- (apoA) containing lipoproteins (Chan et al. 2002; Martinez-Fernandez et al. 2015). Effective management of dyslipidaemia in obesity and T2DM therefore often requires lipid regulation.

Obesity is related to chronic inflammation due to an increased infiltration of inflammatory cells into tissues such as liver and adipose tissue (Jung and Choi 2014). Excess body fat, especially central adiposity, is correlated with a concomitant and persistent increase in low grade inflammation, which results in increased pro-inflammatory adipokines, cytokines and chemokines such as monocyte chemoattractant protein-1 (MCP-1), interleukin (IL)-6, nuclear factor-kappa B (NF- κ B) and tumour necrosis factor alpha (TNF- α), and reduced production of anti-inflammatory adipokines, including adiponectin (Matsuzawa 2010; Joseph et al. 2014).

Dietary and/or complementary strategies to alleviate the metabolic complications of obesity and its related metabolic conditions have aroused considerable interest and are now under exploration as alternatives to pharmaceutical interventions. This paper will review the possible health benefits of one such dietary component, blueberries and blueberry extracts, emphasizing emerging evidence for its potential to ameliorate the impacts of obesity, T2DM

and chronic inflammation. Moreover, data collected from studies on bioactive compounds of blueberries, in particular phytochemical constituents are included. The mechanisms of action of blueberries, as well as mechanistic and signalling pathways involved in the effects of blueberries on obesity and its related chronic diseases are also discussed. Figure 1.5 shows the proposed effects of blueberries on obesity and its related comorbidities, as well as associated metabolic and molecular pathways.

1.5.3 Bioactive constituents in blueberries

Blueberries are perennial flowering plants with indigo-coloured berries from the family Ericaceae within the genus *Vaccinium* (Luby et al. 1999). Many species of blueberry come predominantly from North America, however they are now produced in almost all countries, including Australia, New Zealand and European countries. Depending on the growing season and harvesting time, several types of blueberries are commonly available, including highbush blueberry plants (*Vaccinium corymbosum* L.), the rabbiteye blueberry (*Vaccinium ashei* Reade), lowbush blueberry plants or wild blueberry (*Vaccinium angustifolium* Aiton), and bilberry (*Vaccinium myrtillus* L.) (Maatta-Riihinen et al. 2004; Michalska and Lysiak 2015). Bilberry is a European wild blueberry that contains a higher content of anthocyanins (ACNs) than cultivated blueberry species (Chu et al. 2011). Blueberries are nutritious fruits as they are rich sources of carbohydrates, vitamins and minerals (Liu, S. X. et al. 2015). Blueberries are also a good source of dietary fibres that constitutes 3% – 3.5% of fruit weight (Michalska and Lysiak 2015). In addition, blueberries have a high content of several phytochemicals, including ascorbic acid and phenolics. Many of the proposed beneficial health effects associated with blueberry consumption are linked to the bioactive properties of the phytochemical constituents. The predominant bioactive components contained in blueberries are ascorbic acid, flavonols (including kaempferol, quercetin and myricetin),

hydroxycinnamic acids (including caffeic acids, ferulic acids and coumaric acids), hydroxybenzoic acids (including gallic acids and procatechuic acids), pterostilbene, resveratrol, and ACNs. The potential benefits of blueberry for human health have received much attention in recent years due to these bioactive components (Chen et al. 2010; Koupy et al. 2015).

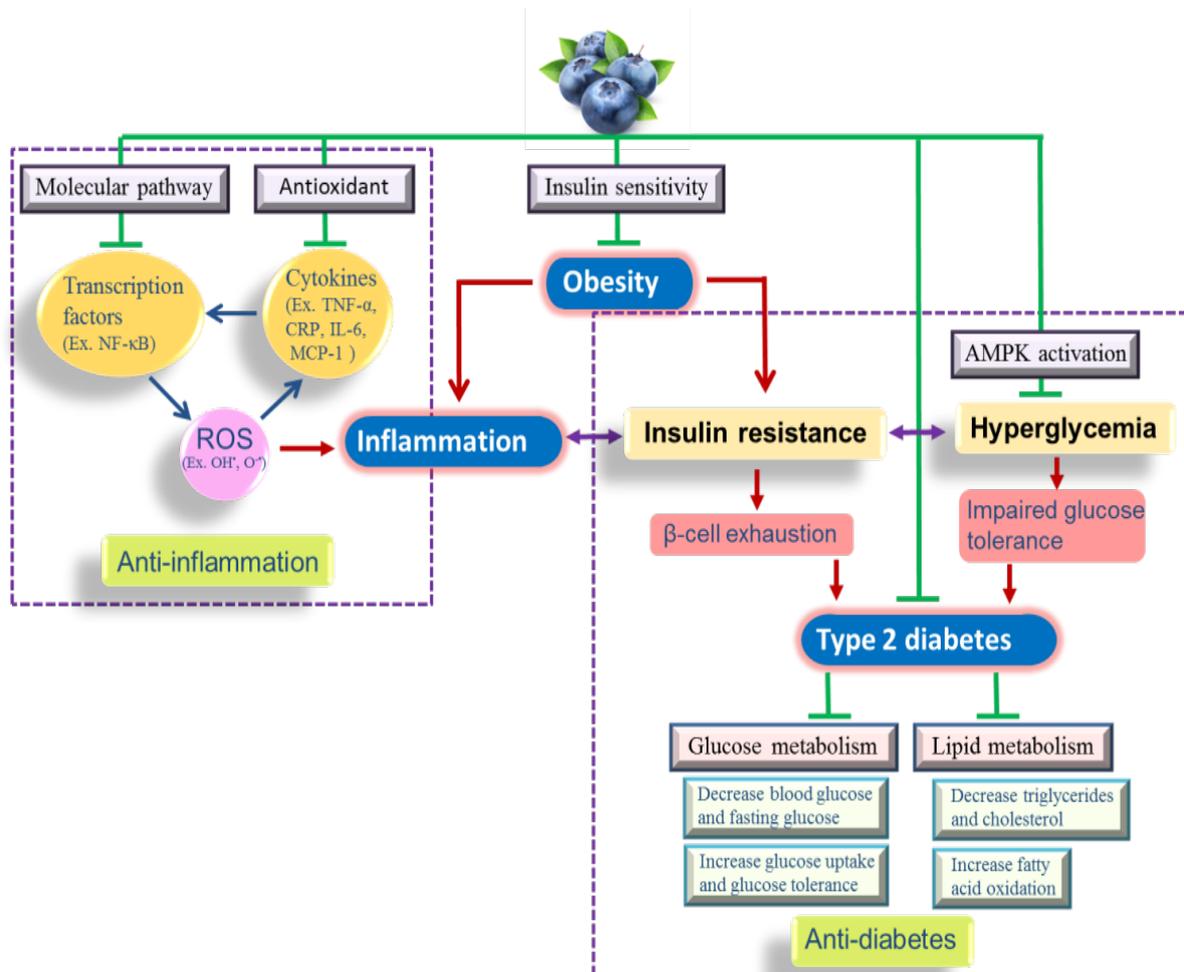


Figure 1.5: Proposed metabolic pathways involved in the effects of blueberry on obesity and related comorbidities.

Blueberry possibly inhibits obesity and type 2 diabetes mellitus via improving hyperglycemia through the activation of 5' adenosine monophosphate-activated protein kinase (AMPK), and the increase of insulin sensitivity. These inhibitions possibly include the reduction of blood glucose, triglyceride and total cholesterol, and increase of glucose uptake, glucose tolerance

and fatty acid oxidation. Blueberry consumption may also have anti-inflammatory potential through the inhibition of reactive oxygen species (ROS) production, for example, hydroxyle radical (OH^{\bullet}) and superoxide radical ($\text{O}^{\bullet-}$); and inflammatory cytokines including tumor necrosis factor ($\text{TNF-}\alpha$), C-reactive protein (CRP), interleukin-6 (IL-6), and monocyte chemoattractant protein-1 (MCP-1) due to the strong antioxidant activity of blueberry and its extracts. Nuclear factor-kappa B (NF- κ B) is a main signally pathway. Arrows \rightarrow , \leftrightarrow mean regulation. $\text{---}|$ means inhibition.

1.5.3.1 Ascorbic acid

Blueberries are rich in ascorbic acid, which is a water-soluble compound that fulfils several roles in living systems, including enhancing immunity and reducing inflammation (Nile and Park 2014; Liu, F. et al. 2015). Ascorbic acid is an antioxidant vitamin and is widely distributed in various blueberry species and varieties. On average 100 g of blueberries provide 10 mg of ascorbic acid, which is equal to one third of the daily recommended dietary intake (Prior et al. 1998; Capra 2006), however varying amounts of ascorbic acid have been reported in different species. The content of ascorbic acid in highbush blueberries (total eight species) ranged from 5 to 15 mg/100 g of fresh fruit, compared with 16.4 mg/100 g in lowbush blueberry (Prior et al. 1998). Fresh bilberry only contains small quantities of ascorbic acid (3 mg/100 g) (Graff and Upton 2001). Rabbiteye blueberries contain different amounts of ascorbic acid due to the variety of species. Six species of rabbiteye blueberry were found to have a lower amount of ascorbic acid (6 to 10 mg/100g) compared to the average content (Prior et al. 1998). However, it has been reported that the concentration of ascorbic acid was high and up to 41 mg/100 g in fresh Ochlockonee fruit, belonging to the rabbiteye species, and 25 mg/100 g in fresh highbush blueberry (Gündüz et al. 2015). There are also other contributors to the potential variation in ascorbic acid in blueberries, such as cultivation, climate, weather conditions and storage time. The concentration of ascorbic acid

decreases when conditions such as oxygen level and temperature are suboptimal during storage. Moreover, after storage for 8-days at 20 °C the content of ascorbic acid in fresh fruit decreases by 27% (Kalt et al. 1999).

1.5.3.2 Phenolics

Phenolic compounds belong to a wide and heterogeneous group of chemical substances that possess one or more aromatic rings with a conjugated aromatic system and one or more hydroxyl groups. Phenolic compounds occur in free or conjugated forms with sugars, acids, and other biomolecules as water-soluble (phenolic acids, flavonoids and quinones) or water-insoluble compounds (condensed tannins) (Skrovankova et al. 2015). The total content of phenolic compounds in blueberries is highly variable, with variation upwards of 10-times higher or lower (e.g. ranges from 48 up to 304 mg/100 g of fresh fruit weight (up to 0.3%) (Ehlenfeldt and Prior 2001; Moyer et al. 2002) depending on the cultivar (Taruscio et al. 2004), growing conditions and maturity (Castrejón et al. 2008), and its estimation may vary depending on the method of analysis (Maatta-Riihinen et al. 2004; de Souza et al. 2014). Phenolic compounds presented in blueberries contain stilbenoids, tannins [hydrolyzable tannins (gallotannins and ellagitannins) and condensed tannins (proanthocyanidins)], and flavonoids, including flavan-3-ols, ACNs, and their polymeric condensation products, flavanones, flavonols (i.e., kaempferol, quercetin, myricetin) and flavones (Taruscio et al. 2004; Seeram 2008; Borges et al. 2010). High amounts of phenolics are found in blueberry and account for 50%–80% of the total polyphenol content, which can reach a concentration of up to 300 mg/100 g fresh weight (Muller et al. 2012; Kuntz et al. 2015).

Tannins are a unique group of phenolic metabolites with molecular weights between 500 and 30,000 Da, which are widely distributed in all berry species and specific berries may contain an abundance of a particular group of tannins (Ferreira et al. 2005; Serrano et al. 2009). It has

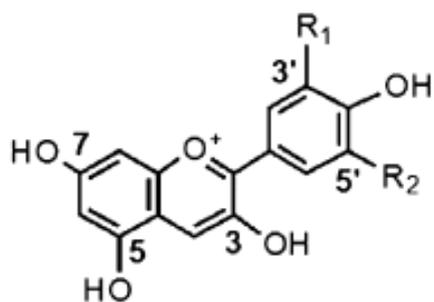
been suggested that tannins may have therapeutic potential in the treatment of diabetes, mainly through two ways; (i) they may lower glucose levels by delaying intestinal glucose absorption and an insulin-like effect on insulin-sensitive tissues, and (ii) they may delay the onset of insulin-dependent T2DM by regulating the antioxidant environment of pancreatic β -cells (Serrano et al. 2009). Previous studies showed that tannins were an effective inhibitor of intestinal α -glucosidase activity (Toda et al. 2001; McDougall et al. 2005), and they also inhibited glucose uptake in intestinal cells (Song et al. 2002). Proanthocyanidins, known as condensed tannins, are the most widely represented products of plant secondary metabolism throughout nature, after lignins (Gu et al. 2003). Blueberries contain predominantly proanthocyanidins, compared with other berries, such as blackberries, black raspberries, red raspberries, and strawberries, which contain predominantly ellagitannins (Seeram 2008). Therefore, the unique biological properties of blueberries may be associated with the specific chemical structures of tannins. The distinct biological effects of blueberries on neuronal function in different regions of the brain and behaviour in aging animals may be due to the effects of individual classes of tannins (Shukitt-Hale et al. 2007).

Flavonoids are a large heterogenic group of benzo- γ -pyron derivatives, which are abundantly present in food products and beverages derived from fruits and vegetables (Heo and Lee 2004). Many physiological benefits of flavonoids have been attributed to their antioxidant and free radical scavenging properties to exert positive health effects on chronic disease, including cancer and neurodegenerative disorders (Lau et al. 2007; Neto 2007; Nile and Park 2014). Blueberries have also been demonstrated to contain high levels of flavanoid compounds, ranking them among the foods showing the highest antioxidant activity (Moyer et al. 2002; Borges et al. 2010; Barberis et al. 2015).

The predominant flavonoids in blueberries are quercetin glycosides (quercetin-3-galactoside, quercetin-3-glucoside, quercetin-3-rutinoside) and myricetin glycosides (myricetin-3-glucoside, myricetin-3-rhamnoside) (Skrovankova et al. 2015). Quercetin, one of the most frequently researched flavonoids, has shown antioxidative and anti-carcinogenic activities to protect against oxidative stress (Heo and Lee 2004). The content of quercetin in blueberry and bilberry were 2.4 and 3 mg/ 100 g fresh fruit, respectively, which were accounted to 50% and 60% of total flavonoids (Hakkinen et al. 1999). Several *in vitro* studies indicated its efficacy in the prevention of different types of cancer induced by potent carcinogens, such as benzo(a)pyrene, azoxymethane, and N-nitrosodiethylamine (Volate et al. 2005; Kamaraj et al. 2007; Seufi et al. 2009) and its anti-cancer capability has also been demonstrated in animal models (Caltagirone et al. 2000; Devipriya et al. 2006). Myricetin is a bioflavonoid abundant in berries and it was reported that the anti-diabetic effectiveness of myricetin is due to its anti-inflammatory activity (Wang et al. 2010; Fu et al. 2013; Wu et al. 2016). The content of total flavonoids in blueberries ranged from 2.5 to 387.48 mg/100 g fresh fruit (Hakkinen et al. 1999; Sellappan et al. 2002), depending on the species and the method used (Taruscio et al. 2004; Borges et al. 2010; Buran et al. 2014). Taruscio et al. (2004) reported the contents of flavonols extracted from eight blueberry species, including three species of highbush blueberry, three species of half-highbush blueberry and two species of bilberry. The HPLC analytical results showed that myricetin and quercetin were the principal flavonols in blueberries (Taruscio et al. 2004). Bilberry contained the highest level of quercetin (16.36 mg/100 g in frozen fruit) followed by half-highbush blueberry (10.25 mg/100 g in frozen fruit) and highbush blueberry (8.64 mg/100 g in frozen fruit) (Taruscio et al. 2004). Bilberry also contained the highest content of myricetin (200 µg/g in frozen fruit) at the level of nearly 10 and 15-fold higher, compared to half-highbush blueberry (1.98 mg/100 g in frozen fruit) and highbush blueberry (1.29 mg/100 g in frozen fruit) (Taruscio et al. 2004).

Anthocyanins (ACNs), pigments that contribute to the intense colours in blueberry, have been shown to exhibit numerous bioactive properties, such as anti-inflammatory, antioxidant and anti-cancer activities (Faria et al. 2010; Zepeda et al. 2012; Vendrame et al. 2013). The most common anthocyanidin aglycones are peonidins, pelargonidins, malvidins, delphinidins, cyanidins and petunidins (Li et al. 2011). These then combine with organic acids and sugars to generate various ACNs (Figure 1.6) (Rodriguez-Mateos et al. 2014). Muller et al. (2012) found that malvidin and delphinidin are the main components and constitute almost 72% of all identified anthocyanins (Muller et al. 2012). However, other studies reported less concentrations of malvidin (22% – 33%) and delphinidin (27% – 40%) in five genotypes of blueberries (Cho et al. 2004). There are up to 27 different ACNs found in blueberries (Prior et al. 1998). The content and type of ACNs depend on the species, fruit size, ripening stage, as well as on climatic, pre-harvest environmental conditions and storage (Scibisz and Mitek 2007; Muller et al. 2012). The concentration of ACNs is up to 800 mg/100 g fresh weight in highbush species and more than 1000 mg/100 g fresh fruit in lowbush species (Cho et al. 2004; Hosseinian and Beta 2007). The high content of ACNs in different *Vaccinium* species is a main contributor to their antioxidant activity and is responsible for about 84% of total antioxidant capacity (Borges et al. 2010). Whereas ascorbic acid was only found to contribute to 10% of the antioxidant capacity despite being present in a significant amount (Barberis et al. 2015).

Although structural and categorical diversity can be noticed among bioactive constituents in blueberries, other factors influence this diversity including, but are not limited to, species and genetic makeup of blueberries, agricultural practices, growing condition, season of harvest, irrigation, and storage of the fruits (Scibisz and Mitek 2007; Castrejón et al. 2008).



Anthocyanidin	R₁	R₂
Pelargonidin	H	H
Cyanidin	OH	H
Delphinidin	OH	OH
Peonidin	OCH ₃	H
Petunidin	OCH ₃	OH
Malvidin	OCH ₃	OCH ₃

Figure 1.6: Structures of the major anthocyanidins in blueberry.

1.5.3.3 Phenolic acid

Phenolic acid, in general, describes phenols that possess one carboxylic acid functionality (Robbins 2003). Phenolic acids account for approximately one-third of the dietary phenols present in plants (Zadernowski et al. 2005). Researchers have become increasingly interested in phenolic acids and their derivatives due to their high nutritional and antioxidant properties in foods (Chalas et al. 2001; Zadernowski et al. 2005). Clifford (1999) estimated that the average amount of phenolic acids consumed is between 25 mg and 1 g daily (Clifford 1999). In blueberries, only a minor fraction of phenolic acid exists as free forms, with the majority of phenolic acid existing in conjugated forms, which are linked with esters, amides and glycosides (Robbins 2003). Vanillic acid, hydroxycinnamic acids, ferulic acid, caffeic acid, chlorogenic acid, p-coumaric acid, gallic acid and salicylic acid are the principal phenolic acids in blueberry (Zadernowski et al. 2005). Among them, chlorogenic acid is the most abundant in blueberry species (Kang et al. 2015), however its content was highly variable between species with highbush and lowbush blueberry varieties ranging from 34.3 to 113.8

mg/100 g fresh weight (Rodriguez-Mateos et al. 2012). This high concentration of chlorogenic acid present in blueberries is likely to contribute to the anti-inflammatory effects of blueberries (Santos et al. 2006). A previous study showed that seven phenolic acid mixture including hydroxycinnamic acid, hippuric acid, 3-(3-hydroxyphenyl)propionic acid, 3-(4-hydroxyphenyl) propionic acid, hydroxyphenylacetic acid, hydroxybenzoic acid and ferulic acid from blueberry inhibited lipopolysaccharide (LPS)-induced production of pro-inflammatory cytokine, IL-6 and TNF- α by the reduction of mitogen-activated protein kinase, Jun amino-terminal kinases (JNK), p38 and Erk1/2 phosphorylation in murine macrophage cell line RAW 264.7 (Xie et al. 2011).

1.5.4 Effects on body weight and fat mass

The anti-obesity effects of blueberries and blueberry extracts have been investigated in both clinical studies and also several animal models, such as Obese Zucker rats, KKAY mice, C57BL/6J mouse and Sprague-Dawley rats (Seymour et al. 2009; Vuong et al. 2009; Prior et al. 2010; Seymour et al. 2011). Tables 2.4 and 2.5 summarise the impacts of consumption of blueberries and blueberry extracts on obesity in animal models and human trials.

Table 1.4: Summary of the effects of blueberry and its extract on obesity and comorbidities in animal studies

Type of blueberry	Amount (per day)	Animals	Days	Obesity			T2DM		Lipid profiling	Inflammatory cytokines (Serum marker)	Reference
				Food intake (FI); Metabolic variables	Body weight (BW) or fat (BF)	Tissue weight (g) or fat (%)	Glucose	Insulin			
Lowbush BB	8% (w/w)	Obese Zucker rat	56						↓serum TC, TG	↑adiponectin ↓IL-6, TNF- α , CRP-1	(Vendrame et al. 2013)
Lowbush BB powder	8% (w/w)	Zucker Fatty rats	56	Nil FI					Nil plasma HDL-C; ↓plasma TG, TC		(Vendrame et al. 2014)
		Zucker Lean rats		Nil FI					Nil serum TG, TC, HDL-C		
Highbush BB powder, freeze-dried	2% (w/w)	Zucker Fatty rats	90	Nil FI	Nil BW	↓weight of liver, AT fat	Nil fasting glucose; ↓BG	↓PI	Nil fatty acids; ↓serum TG, free fatty acids	Nil IL-6, TNF- α	(Seymour et al. 2011)
		Zucker Lean rats	90	Nil FI	↑BW; Nil BF		Nil fasting glucose, BG	Nil fasting insulin	Nil serum TG, fatty acids, TC		
BB powder	4% (w/w)	C57BL/6 J mice	56	Nil energy intake, heat produce	Nil BW	Nil AT fat		Nil PI; ↓IR			(DeFuria et al. 2009)
Bilberry, freeze-dried	5% (w/w)	C57BL/6 J mice	84	Nil FI, energy intake	Nil BW gain		Nil BG		Nil serum free fatty acids		(Mykkanen et al. 2012)
Bilberry	5% or 10%	C57BL/6 J mice	90	Nil FI, energy	Nil BF; ↓BW,		Nil fasting	Nil IR	Nil serum TC	Nil adiponectin,	(Mykkanen et al. 2014)

	(w/w)			intake, heat produce			glucose;			leptin ↓resistin, TNF- α, IL-1β, MCP- 1	
BB powder, freeze-dried	10% (w/w)	C57BL/6 J mice	84		↑BW gain, BF	↑AT weight	Nil BG	Nil PI	Nil serum TC, liver TC, TG; ↑serum TG	↓leptin	(Prior et al. 2009)
Purified ANCs from BB	10% (w/w)				↓BW gain, BF			↓PI	Nil liver TG; ↓serum TG, liver TC; ↑serum TC	Nil IL-6, TNF-a; ↓leptin, MCP-1	
BB powder, freeze-dried	10% (w/w)	C57BL/6 J mice	92		↑BW, BF	Nil weights of liver; ↓weights of kidney, heart; ↑AT fat	Nil BG				(Prior et al. 2008)
Purified ANCs from BB	3.75 mg		70	Nil BW; ↓BF	Nil weights of kidney, liver, heart						
BB juice	ad libitum	C57BL/6 mice	84		↓BW	Nil weights of heart, liver, kidney, AT	Nil BG		Nil liver lipids, cholesterol; ↓TC, liver TG	↓leptin; Nil adiponectin	(Wu et al. 2013)
BB juice	ad libitum	C57BL/6 J mice	72	Nil energy intake; ↓FI	Nil BF; ↓BW	Nil weights of heart, liver, kidney, AT; ↓total fat, AT fat	Nil fasting glucose	Nil PI	Nil serum cholesterol, TG	↓ leptin	(Prior et al. 2010)
Purified ANCs (0.2 or 1.0 mg/mL)	ad libitum			Nil FI; ↓energy intake	↓BW, BF	Nil weights of heart, liver, kidney; ↓ total fat,	↓fasting glucose	Nil PI	Nil serum cholesterol, TG		

						AT fat					
BB juice	(80 ml/kg)	Obese KKAY mice	28	↓FI	↓BW gain	Nil weights of kidney; ↓weight of liver, AT fat	↓BG; ↑glucose tolerance	Nil PI		Nil leptin, adiponectin	(Vuong et al. 2009)
BB polyphenol	ad libitum	Obese C57BL/6 mice	91	Nil FI	↓BW gain		↑glucose tolerance ↓fasting glucose	Nil PI	Nil serum TG; ↓serum cholesterol	Nil IL-6, IL-1 β , TNF-a, adiponectin, RBP4	(Roopchand et al. 2013)
BB, ANCs, phenolic-fractions	500 mg/kg	Diabetic C57BL/6 J mice	42				↓BG				(Grace et al. 2009)
ACNs (10 g/kg in diet)	ad libitum	Diabetic KKAY mice		Nil FI, energy intake	Nil BW	Nil weights of AT; ↓weights of liver	↓BG	Nil PI; ↓IR	↓total lipid, TC, TGA of liver, serum TG, serum TC	Nil adiponectin	(Takikawa et al. 2010)
BB peel extracts	15.8% (w/w)	Sprague-Dawley rats	35	Nil FI	↓BW, BW gain	↓weight of AT			↓serum TC, TG; ↑serum HDL-C		(Song et al. 2013)
BB pomace by-products	8% or 10% (w/w)	Golden Syrian hamsters	21	↑FI	Nil BW, BW gain	Nil weights of liver, AT			Nil hepatic total lipid, free cholesterol, TG ↓hepatic TC		(Kim et al. 2010)
C3G	0.2% (w/w)	Diabetic db/db mice	35	Nil FI	Nil BW	Nil weights of kidney, heart, AT fat; ↓weights of	↓BG, fasting glucose	Nil PI; ↓IR	↓hepatic TG	↑adiponectin ↓IL-6, TNF-a, CRP-1	(Guo, H. et al. 2012b)

						liver; ↑AT fat					
		C57BL/6 J obese mice			Nil BW	Nil weights of kidney, heart, AT fat; ↓weights of liver; ↑AT fat	↓fasting glucose, BG	↓PI, IR	↓hepatic TG	Nil IL-6; ↑adiponectin; ↓MCP-1, TNF- α	
C3G	2% (w/w)	Diabetic KKAy mice	35	Nil FI	Nil BW, AT weight		↓BG	Nil PI; ↓IR	Nil serum TG, TC		(Sasaki et al. 2007)
C3G (2 g/kg in diet)	ad libitum	Diabetic db/db mice	56							↑adiponectin	(Liu et al. 2014)

Nil: no statistically significant differences between the blueberry and control groups.

ACNs, anthocyanins; AT, adipose tissue; BB, blueberry; BF, body fat (%); BG, blood glucose (mmol/L); BW, body weight (g); C3G, cyanidin-3-glucoside; CRP, high levels of C-reactive protein (ng/L); FI, food intake (g); HDL-C, high-density lipoprotein cholesterol (mg/dL); IR, insulin resistant; IL-1 β , interleukin-1 beta (ng/L); IL-6, interleukin-6 (ng/L); MCP-1, monocyte chemoattractant protein-1 (ng/L); PI, plasma insulin (μ mol/L); RBP4, retinol binding protein 4; TC, total cholesterol (mg/dL); TG, triglycerides (mg/dL); TNF- α , tumor necrosis factor (ng/L).

Table 1.5: Summary of the effects of blueberry on obesity and its comorbidities on human trials

Type of blueberry	Amount (per day)	Population	Days	Obesity			T2DM		Lipid Profiling (in serum)	Serum marker	Inflammatory cytokines (In plasma)	Reference
				Daily intake (DI); Energy intake (EI)	Circumference of weight, hip (WC, HC); Waist-to-hip ratio (W/H)	Body weight, fat (BW, BF); Body mass index (BMI)	Glucose	Insulin				
Lowbush BB powder, (freeze-dried)	100 g	Healthy	7						Nil TG	↓TEAC; ↑ORAC		(Mazza et al. 2002)
Bilberry fruit (frozen)	100 g	Metabolic syndrome	40		↓WC	↓BW		↑PI		↓adiponectin	↓VCAM-1, TNF- α ,	(Lehtonen et al. 2011)
Bilberry fruit (fresh)	400 g	Metabolic syndrome	126	Nil DI, EI	Nil WC	Nil BW, BF, BMI	Nil fasting BG		Nil TG, TC, HDL-C, LDL-C, free fatty acids	Nil leptin, adiponectin	↓hsCRP, IL-6, IL-12, LPS	(Kolehmainen et al. 2012)
Bilberry juice	330 mL	Cardiovascular disease	28			Nil BW, BMI			Nil TG, TC	Nil ORAC, FRAP	Nil MCP-1, IL-1 β ; ↓CRP, IL-6, IL-15, MIG; ↑TNF- α	(Karlsen et al. 2010)
BB beverage	480 mL (twice)	Obese	56	Nil DI	Nil	Nil BW, BMI	Nil BG	Nil IR	Nil TG, TC, HDL-C, LDL-C; ↑ox-LDL	Nil adiponectin	Nil CRP, IL-6	(Basu et al. 2010)
BB	22.5 g	Obese,	42	Nil EI		Nil BW,	Nil BG	Nil PI;	Nil TG,		Nil TNF- α ,	(Stull et al.

bioactives	(twice)	nondiabetic, IR				BF, BMI		↑IS	TC, HDL-C, LDL-C		CRP, MCP-1	2010)
Bilberry extract	0.47 g (36% ACNs)	T2D	14				↓BG	↓PI		Nil TEAC, FRAP	Nil MCP-1	(Hoggard et al. 2013)
Blueberin (250 g BB + 50 mg myricetin)	300 mg (three times)	T2DM	28				↓fasting BG				↓CRP	(Abidov et al. 2006)
ACNs	160 mg (twice)	dyslipidemic	84	Nil EI	Nil WC, HC, W/H	Nil BW, BMI	Nil BG		Nil TG, TC; ↑HDL-C; ↓LDL-C	Nil adiponectin		(Qin et al. 2009)
ACNs	160 mg (twice)	Hypercholesterolemia	168	Nil DI			Nil BG	Nil PI	Nil TG, TC; ↑HDL-C; ↓LDL-C		Nil TNF- α , ↓hsCRP, IL-1 β , VCAM-1	(Zhu et al. 2013)

Nil: no statistically significant differences between the blueberry and control groups. ACNs, anthocyanins; AT, adipose tissue; BB, blueberry; BF, body fat (%); BG, blood glucose (mmol/L); BMI, body mass index (kg/m²); BW, body weight (g); CRP, high levels of C-reactive protein (ng/L); CVD, cardiovascular disease; DI, daily intake (g); EI, energy intake (kcal/d); FRAP, ferric-reducing ability of plasma (μ mol/L); HC, hip circumference (cm); HDL-C, high-density lipoprotein cholesterol (mg/dL); hsCRP, high sensitivity C-reactive protein (ng/L); IL-1 β , interleukin-1 beta (ng/L); IL-6, interleukin-6 (ng/L); IR, insulin resistant; IS, insulin sensitivity; LDL-C, low-density lipoprotein cholesterol (mg/dL); LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein-1 (ng/L); MIG, monokine induced by INF- γ ; ORAC, oxygen radical absorbance capacity (μ mol of Trolox equiv/L); ox-LDL, oxidized low-density lipoprotein (U/L); PI, plasma insulin (μ mol/L); T2DM, type 2 diabetes mellitus; TC, total cholesterol (mg/dL); TEAC, trolox equivalent antioxidant capacity (μ mol of Trolox equiv/L); TG, triglycerides (mg/dL); TNF- α , tumor necrosis factor (ng/L); VCAM-1, vascular cell adhesion molecule-1; WC, weight circumference (cm); W/H, waist-to-hip ratio.

1.5.4.1 Whole fruit or juice

It has been reported that body weight, liver weight, and total fat weight were significantly reduced in Obese Zucker rats fed a low-fat diet (LFD) combined with 2% (w/w) whole highbush blueberry powder (Seymour et al. 2009; Seymour et al. 2011). These results are consistent with the study of Prior et al. (2010) who reported that supplementation with blueberry juice (0.2 mg/mL) prevented weight gain in C57BL/6J mice that were fed a HFD (45% of kcal from fat). Furthermore, Voung et al. (2009) showed that incorporating blueberry juice in drinking water significantly reduced weight gain in obese KKAy mice. These positive results possibly related to the improved glucose tolerance and enhanced insulin sensitivity seen in these animals (Vuong et al. 2009). Contrary to these results, blueberry supplementation did not affect the body weight of C57BL/6J mice fed a HFD (60% of energy) with 4% (w/w) whole blueberry powder for 8 weeks (DeFuria et al. 2009) or of Sprague-Dawley rats supplemented with 10% freeze-dried whole blueberry for 3 weeks (Seymour et al. 2009). Another study found no significant differences in weight gain after the 12 weeks of feeding C57BL/6J mice with 5% bilberry compared with mice fed a HFD (45% kcal fat) (Mykkanen et al. 2012). Conversely, Prior et al. (2008) demonstrated that diets supplemented with 10% whole blueberry powder increased adiposity and body weight in C57BL/6J mice fed a HFD. However, blueberry-fed mice in this study consumed approximately 12% more energy/day than the control HFD group, which may have contributed to these outcomes (Prior et al. 2008).

Most of the clinical studies have shown that dietary supplementation with whole blueberry or blueberry juice failed to reduce body weight and waist circumference (Qin et al. 2009; Basu et al. 2010; Stull et al. 2010). This has been demonstrated in a randomised controlled trial with 48 obese participants (4 males and 44 females) in which participants consumed a freeze-

dried blueberry beverage (50 g freeze-dried blueberries equivalent to 350 g of fresh blueberries) or water for 8 weeks. There were no significant differences observed in waist circumference, body weight or dietary intakes between the treatment group and the control group (Basu et al. 2010). Similar results were also observed in non-diabetic obese participants who were supplemented with either 22.5 g blueberry powder or a placebo twice daily for 6 weeks in that there were no significant differences observed between the treatment and control groups in body weight, adiposity and energy or macronutrient consumption (Stull et al. 2010). Overall there is limited evidence to suggest that blueberry supplementation alone affects adiposity in obese or overweight individuals. Future studies are encouraged to focus on calorie restriction and longer intervention periods in conjunction with supplementation, however whether this will result in clinically significant improvements in weight loss compared to calorie restriction alone is uncertain.

1.5.4.2 Extracts of blueberries

Several studies have examined the effects of blueberry extracts, particularly ACNs from fresh blueberry fruit, juice and peel on control of body weight and have indicated that the anti-obesity capability of blueberry extract is quite different to whole fruit or juice (Prior et al. 2008; Prior et al. 2009; Prior et al. 2010). Although the reasons for these disparities are not clear, one possible explanation is that there are different types and amounts of bioactive constituents contained in blueberry and its products, which might change the response to extracts from blueberry, compared with purification or single components. For instance, blueberry juice contains not only ACNs but also other components such as procyanidins, chlorogenic acid, and other water-soluble compounds including sugars (Prior et al. 2010). Another possibility is that there are some specific components, such as uronic acids, neutral sugars, noncellulosic sugars including xylose and arabinose, or other factors as an obstruction

in whole blueberry to counteract the potential benefit of blueberry consumption (Vicente et al. 2007). Wu et al. (2013) showed that ACNs from blueberry juice decreased body weight up to 7.3% in dietary-induced models of obesity. Dietary-induced weight gain, perirenal adipose tissue and epididymal weights were significantly lowered in male Sprague-Dawley rats fed a HFD supplemented with blueberry peel extracts for 5 weeks compared to an equivalent control group. It has been reported that blueberry peel extracts may potentially affect obesity by a reduction of adipogenesis and inhibition of fat accumulation through the PI3K/Akt/GSK3 β pathway in 3T3-L1 preadipocytes (Song et al. 2013).

Further studies are required to assess the effect of ACNs consumption at various doses to establish the specific concentration of ACNs required for ameliorating the development of obesity. According to a previous study conducted by Prior et al. (2010), the low concentration of ACNs (0.2 mg/mL) decreased retroperitoneal and epididymal fat (% body weight) by 31% and 25%, respectively in mice fed a LFD, and 26% and 29%, respectively in mice fed a HFD for 72 days. However, retroperitoneal and epididymal fat levels were not decreased in HFD-fed mice treated with higher concentration of ACNs (1.0 mg/mL) but were similar to, or slightly higher than the HFD mice without ACNs (Prior et al. 2010). ACNs intake was measured as 0.6 and 3.4 mg/day for each mouse fed a LFD, and 0.5 and 1.8 mg/day for each mouse fed a HFD, according to liquid intake with the low concentration (0.2 mg/mL) and high concentration (1.0 mg/mL) of ACNs (Prior et al. 2010). This indicated that low concentrations of ACNs are potentially more beneficial compared to higher doses; however the exact reasons for this observation are unknown. Conversely, another independent study has demonstrated that supplementation of a higher concentration (2.8 mg/day/mouse) of purified ACNs for 92 days significantly prevented the development of obesity, but 3.75 mg/day/mouse failed to prevent body weight gain in HFD induced obese mouse model (Prior et al. 2008). Thus it appears to be no clear dose dependent effect and further investigation is

needed to define the effective dose of ACNs or blueberries for body weight control in cases of obesity.

1.5.5 Effect on glucose metabolism and insulin signalling

Animal models (Table 1.4) and clinical studies (Table 1.5) have demonstrated that supplementation or consumption of blueberry or blueberry bioactive compounds cause changes in glucose metabolism and improve insulin sensitivity.

1.5.5.1 Whole fruit or juice

Supplementation of 2% freeze-dried blueberry powder for 13 weeks in Obese Zucker rats have demonstrated significant reductions in glucose, fasting insulin and insulin resistance, as indicated by the Homeostasis Model Index of Insulin Resistance (HOMA-IR) (Seymour et al. 2009; Seymour et al. 2011). Likewise, Vuong et al. (2009) showed that fermented blueberry juice by the *Serratia vaccinii* bacterium significantly reduced blood glucose levels and maintained the glycaemia of pre-diabetic KKAY mice to a normal level. These results indicate that blueberry intake could reduce phenotypes of diabetes in obesity-prone rats by regulating glucose metabolism. Conversely, Prior et al. (2008) reported that long term supplementation with freeze-dried whole blueberry powder did not affect the results of a glucose tolerance test that were administered to C57BL/6J obese mice. These inconsistent results mainly depend on the variation of animal models, the duration of the treatment, and the dose of bioactivity components in blueberry. Furthermore, clinical studies have also reported that blueberry supplementation did not show the impact on fasting serum glucose (Basu et al. 2010; Stull et al. 2010; Kolehmainen et al. 2012). Specifically, Basu et al. (2010) documented that a freeze-dried blueberry beverage (50 g freeze-dried blueberries equivalent to 350 g of fresh blueberries) for 8 weeks to 48 obese participants (4 males and 44 females) was not able to significantly change their serum glucose concentration. Also, glucose and

insulin responses did not differ between the bilberry group (400 g fresh fruit) and the control group, when obese individuals consumed a diet rich in bilberries for 8 weeks (Kolehmainen et al. 2012). Likewise, no changes were observed in serum glucose during the intervention with 22.5 g blueberry bioactive twice daily for 6 weeks, although insulin sensitivity was improved significantly more in the blueberry group compared to the placebo group in participants who were obese, nondiabetic, and insulin resistant (Stull et al. 2010). *In vitro* studies have however consistently shown that blueberry improves glucose uptake. For instance, 6-h incubation of fermented blueberry juice with and without insulin enhanced glucose uptake into the adipocyte and muscle cells and increased the phosphorylation/activation of proteins in the insulin-independent pathway (i.e., AMP-activated protein kinase) but had no effect on phosphorylation of key proteins in the insulin-dependent pathway (i.e., AKT and ERK1/2) (Vuong et al. 2007). These findings showed that the bioactive components in fermented blueberry improved glucose uptake into the cells via an insulin-independent mechanism. These positive cellular mechanistic studies provide evidence on the improvement of insulin sensitivity *in vitro*, however why the variation in the *in vivo* studies remains to be determined.

1.5.5.2 Extracts from blueberries

While the effects of blueberry juice on glucose tolerance *in vivo* is varied, supplementation with ACNs appear to have a more positive effect as it has been previously indicated that fasting serum glucose concentrations were decreased and oral glucose tolerance was increased in mice fed a HFD supplementation with ACNs compared to blueberry juice (Prior et al. 2010). This result is possibly attributed to other constituents in blueberry juice such as procyanidins, chlorogenic acid, and other water-soluble compounds including sugars, which are not present in ACNs. It is possible that this beneficial effect of ACNs on glucose

tolerance may be due to a direct effect on the liver as blueberry ACNs (0.05–10 mg/mL) have been demonstrated to significantly reduce glucose production by 24%–74% in H4IIE hepatocytes (Roopchand et al. 2013). In addition, diabetic C57BL/6J mice supplemented with 500 mg/kg body weight of a phenolic-rich fraction or an anthocyanin-rich fraction showed reductions in blood glucose levels by 33% and 51%, respectively. In these fractions, 287 mg/g ACNs was in a phenolic-rich fraction, while 595 mg/g ACNs (cyanidin-3-glucoside equivalents) was in an anthocyanin-rich fraction, which suggested that higher ACNs concentration in different fractions may contribute to more hypoglycaemic activity of the extracts (Grace et al. 2009).

Bilberry extract also reduces blood glucose level and enhances insulin sensitivity in diabetic KKAY mice (Sasaki et al. 2007). Furthermore, in the same study, the GLUT4 was upregulated and retinol binding protein 4 (RBP4) was downregulated in the white adipose tissue in bilberry extract group (Sasaki et al. 2007). These results indicated that bilberry extract has a potent effect on glucose metabolism through the regulation of GLUT4-RBP4 system. The beneficial effects of bilberry extracts were also supported by a human trial demonstrating that insulin and postprandial glycaemia was significantly reduced in diabetic volunteers supplemented a bilberry extract (containing 36% (w/w) of ACNs which is equivalent to about 50 g of fresh bilberry) for 2 weeks, compared with the placebo group (a polysaccharide drink and equivalent to 75 g of glucose) (Hoggard et al. 2013). A longer intervention (4 weeks) with the extracts (providing 50 mg 3,4-caffeoylquinic (chlorogenic) acid, and 50 mg myricetin) from blueberry leaf has also shown that fasting plasma glucose was reduced significantly in diabetic volunteers (Abidov et al. 2006). However, other clinical studies have indicated that there were no significant differences in fasting blood glucose between the treatment and the control groups after dietary supplementation with ACNs for 12 (Qin et al. 2009) or 24 weeks (Zhu et al. 2013).

There are up to 27 different ACNs present in blueberry, however, only several specific ACNs exhibit strong hypoglycaemic capacity (Roopchand et al. 2013). Grace et al. (2009) observed that in diabetic C57BL/6J mice treated with 300 mg/kg of the pure ACN delphinidin-3-O-glucoside (D3G) or malvidin-3-O-glucoside (M3G), M3G decreased blood glucose to a greater extent compared to D3G. It is likely that the metabolism and bioavailability affects the magnitude of bioactivity in different types of ACNs. C3G is the predominant ACN in blueberries (Wang et al. 2016). Several studies have shown that isolated C3G improved insulin sensitivity and hyperglycaemia in animal models of diabetes (Sasaki et al. 2007; Guo, H. et al. 2012b; Liu et al. 2014). There are several pathways involved in these effects, such as the modulation of GLUT4-RBP4 system (Sasaki et al. 2007), the c-Jun N terminal kinase/forkhead box O1 (FoxO1) signalling pathway (Guo, H. et al. 2012a) and adiponectin activating cAMP-PKA-eNOS signalling pathways (Liu et al. 2014).

In animal studies, following supplementation with blueberry extracts or pure ACNs (C3G), ACNs were detected in the liver, blood, kidney and ocular tissues with an intact form suggesting that ACNs and/or their metabolites can be distributed to various tissues via blood and are therefore expected to regulate metabolic changes in the body (Tsuda et al. 1999; McGhie et al. 2003; Ichiyangi et al. 2006; Takikawa et al. 2010). An *in vitro* study has also reported that glucose uptake was increased in C₂C₁₂ cells treated with extracts from the root, leaf and stem of lowbush blueberry, and in 3T3-L1 cells only treated with extracts from root and stem of lowbush blueberry (Martineau et al. 2006). These results were consistent with an *in vivo* study that also demonstrated ACNs components in different fractions specifically contributed to improving hypoglycaemic activity in diabetic C57BL/6J mice (Grace et al. 2009). However, the fruit extract in lowbush blueberry did not show any effect on glucose-stimulated insulin secretion or glucose uptake in β TC-tet pancreatic β cells (Martineau et al. 2006). Since the ACNs composition extracted from the fruit are completely different,

compared to those extracted from the leaf, root and stem, the hypoglycaemic compounds from the blueberry *in vitro* studies perhaps do not have the same effect *in vivo* due to the different mechanisms of action.

1.5.6 Effect on lipid metabolism

1.5.6.1 Whole fruit and fruit juice

Diets enriched with blueberries have been reported to improve dyslipidaemia (Seymour et al. 2009; Seymour et al. 2011; Wu et al. 2013; Vendrame et al. 2014). Plasma TG and total cholesterol (TC) concentrations were significantly reduced in Obese Zucker rats supplemented with 8% wild blueberry for 8 weeks (Vendrame et al. 2014) or 2% blueberry powder for 13 weeks in both LFD and HFD groups compared with the control groups (Seymour et al. 2009). These observations were also supported by a reduction in serum TC and low density lipoprotein cholesterol (LDL-C), as well as the levels of liver TG and TC following consumption of blueberry juice, although the contents of liver lipids and cholesterol were not changed in C57BL/6 mice (Wu et al. 2013). The consumption of 1%, 2% and 4% blueberry-supplements for 8 weeks has significantly reduced the TC and LDL-C concentrations in pigs (Kalt et al. 2008).

The possible pathways involved in the anti-dyslipidaemic effect of blueberries include the regulation and expression of key enzymes such as lipoprotein lipase (LPL) (Wei et al. 2011), fatty acid synthase (Tsuda et al. 2005) and ATP-binding cassette transporter 1 (ABCA1) (Xia et al. 2005) which are involved in TG and cholesterol metabolism. Furthermore, the expression of transcription factors such as sterol regulatory element-binding transcription factor (SREBP) and peroxisome proliferator-activated receptor (PPAR) in bioactive tissues could also explain the observed effects of blueberry consumption on lipid profiles (Vendrame et al. 2014; Cutler et al. 2016). In a recent study, the expression of PPAR α and PPAR γ in

Obese Zucker rats were increased in the abdominal adipose tissue (AAT), while that of total SREBP-1 was decreased in both the liver and the AAT of the rats following consumption of a diet enriched with 8% wild blueberry for 8 weeks (Vendrame et al. 2014). The activation of PPAR α and PPAR γ following blueberry consumption could partly explain such an effect on lipid accumulation in blood and bioactive tissues. The activation of PPAR α is related to enhanced fatty acid uptake, conversion into acyl-CoA derivatives, and further catabolism (Pawlak et al. 2015); moreover, the activation of PPAR γ in adipose tissue is known to induce differentiation of preadipocytes and TG storage (Ferre 2004). The down-regulation of the expression of SREBP-1 also helps to explain the reduction in TG and TC in the Obese Zucker rats supplemented with blueberry diet, since SREBP-1 isoforms promote the synthesis and accumulation of TG and cholesterol via the induction of multiple enzymes (Horton et al. 2002). Similar results were also observed by Seymour et al. (2011) which showed blueberry intake increased PPAR α and PPAR γ activity in skeletal muscle in both HFD and LFD fed rats. In addition, the intake of blueberry significantly affected mRNA of several genes related to fat storage and glucose uptake, such as PPAR γ co-activator 1 α , Acyl-CoA oxidase, fatty acid synthase, fatty acid-CoA ligase, GLUT4 and insulin receptor substrate 1 (IRS-1) in both skeletal muscle and retroperitoneal abdominal fat in HFD induced rats (Seymour et al. 2011). With regards to improving lipid profile, clinical studies of blueberry supplementation have not supported those of animal studies with freeze-dried wild blueberries showing no effect on TG, TC, HDL-C and LDL-C levels in obese subjects (Basu et al. 2010), in subjects with developing CVD risk (Riso et al. 2013), and in healthy middle-aged male subjects (Wang et al. 2016).

1.5.6.2 Anthocyanins in blueberries

Mice that were fed a HFD and also had their drinking water supplemented with purified ACNs from blueberries, instead of whole blueberry, showed decreased serum TG and TC levels that were comparable with those of the lean control group (10% of kcal from fat) (Prior et al. 2009). This result indicated that sugars or other components in the whole fruits were possibly masking the benefits of ACNs and other components of blueberries. It should be noted that blueberry polyphenol was effective on serum TC level in C57BL/6 mice, which was 13.2% lower than in the control group (Roopchand et al. 2013). A human trial which investigated the effect of ACNs (from bilberry) supplementation on lipid profiles in dyslipidemic patients found that 160 mg of ACNs supplementation for 12 weeks increased cellular cholesterol efflux and HDL-C concentrations, as well as reduced the mass and activity of plasma cholesteryl ester transfer protein (CETP) and LDL-C concentrations, without affecting TC levels (Qin et al. 2009). Zhu et al. (2013) also found similar results, reporting that volunteers with hypercholesterolemia had greater reductions in LDL-C levels and greater increases in HDL-C after consuming 320 mg/day of purified ACNs for 24 weeks compared with controls. In an *in vitro* study, C3G reduced CETP activity in human HepG2 cells in a dose-dependent manner, suggesting that supplementation of ACNs may improve lipoproteins by increasing HDL-C concentrations and decreasing serum LDL-C partially due to the inhibition of CETP target (Zhu et al. 2013). Other possible mechanisms by which blueberry ameliorate lipid profile are possibly related to the intact assimilation of blueberry bioactivity such as ACNs, which exhibited the antioxidant properties in serum and other tissues (Mazza et al. 2002; McGhie et al. 2003). Studies have revealed that the high concentration of ACNs in wild blueberry is a major contributor to the antioxidant properties *in vitro*, instead of other antioxidant minerals, vitamins, or fibres (Prior et al. 1998). Moreover, the antioxidant properties of ACNs have been confirmed via other systems of

oxidation such as that for the prevention of LDL oxidation *in vitro* (Laplaud et al. 1997). It has been validated that ACNs can be absorbed intact in glycosylated and possibly acylated forms in male volunteers after the consumption of blueberries (Wu et al. 2002). Moreover, the presence of ACNs in the serum may be involved with a diet-induced increase in *ex vivo* serum antioxidant status (Mazza et al. 2002).

Taking all these data together, it can be concluded that blueberries and blueberry extracts may potentially improve dyslipidaemia by regulating TG, cholesterol and fatty acid metabolism through several signalling pathways. However, further studies are necessary to better clarify the mechanisms involved in these actions of bioactive components in blueberries.

1.5.7 Effect on inflammation and adipocytokine profile

Obesity is associated with systemic chronic inflammation, and this low-grade inflammation may play an important role in obesity associated insulin resistance, T2DM, and other complications (Gabay 2006; Giugliano et al. 2006; Calder et al. 2011; Chen et al. 2015). A diet enriched in vegetables and fruits is inversely related to inflammatory stress, compared with meals that are energy dense which induce an acute inflammatory status in both overweight and healthy adults (Manning et al. 2008; Calder et al. 2011; Root et al. 2012; Vendrame et al. 2013). Blueberries contain various anthocyanins, phenolic acid and other bioactive components recognized for their ability to provide and activate cellular antioxidant protection, scavenge free radicals, inhibit inflammatory gene expression, and consequently protect against oxidant-induced and inflammatory cell damage and cytotoxicity (Johnson et al. 2013; Nile and Park 2014; Kang et al. 2015).

Dietary supplementation with 8% blueberries to Obese Zucker rats for 8 weeks has been reported to decrease plasma concentrations of IL-6, TNF- α and CRP compared with the

control group (Vendrame et al. 2013). Furthermore, in this study, expression of TNF- α , IL-6 and NF- κ B was down-regulated in both the AAT and the liver, whereas CRP expression was down-regulated only in the liver (Vendrame et al. 2013). Similarly, supplementation with 4% whole blueberry powder decreased IL-10 and TNF- α mRNA expression in adipose tissue inflammation of HFD fed C57BL/6J mice, but no significant changes in other inflammatory biomarkers, such as nitric oxide synthase (iNOS), IL-6 and MCP-1 (DeFuria et al. 2009).

Bilberry consumption has also been demonstrated to attenuate pro-inflammatory responses induced by HFD in C57BL/6J mice fed with a 5% or 10% (w/w) of whole bilberries for three months, via reduction in MCP-1, IL-2, IL-1 β , IL-6 and TNF- α (Mykkanen et al. 2014). In particular, the levels of IL-15 and interferon gamma (IFN- γ) were increased in non-supplemented HFD fed animals and reduced to non-detectable levels in animals that were supplemented with bilberries (Mykkanen et al. 2014). In contrast, to the bilberry studies, dietary supplementation with a blueberry pomace by-product failed to alter mRNA expression of CD68 (an anti-inflammatory marker) and CRP in adipose tissue of Syrian Golden hamsters compared to controls (Kim et al. 2010). One explanation for the inconsistency in these findings may be associated with different components among blueberries, its fractions and its peel.

During the last few years a number of clinical trials have been carried out to assess the potential anti-inflammatory function of blueberry supplementation in subjects who are obese and have other disorders of metabolic syndrome (Table 2.5). Karlsen et al. (2010) reported that intake of bilberry juice could regulate inflammatory mediators such as, IL-6, IL-15 and CRP in men and women as well as improve the levels of plasma polyphenols. Furthermore, it was found that the decrease of these inflammatory mediators were associated with NF- κ B activation (Karlsen et al. 2010). In a preclinical study, dietary supplementation with 400 g of

bilberry for 8 weeks decreased serum IL-6, IL-12, high sensitivity-CRP (hsCRP) and LPS concentrations in obese individuals with low-grade inflammation (Kolehmainen et al. 2012). However, in another study where 110 female volunteers consumed 100 g of fresh blueberry fruits for 33 – 35 days, there were no differences observed in TNF- α between the baseline and treatment group at the end of the intervention (Lehtonen et al. 2011). Similarly no alterations in plasma IL-6 and CRP concentrations were observed in obese participants following consumption of freeze-dried blueberries (50 g) for 8 weeks (Basu et al. 2010). Another study demonstrated that consumption of blueberries (22.5 g) for 6 weeks did not affect the inflammatory biomarker profile including TNF- α , hsCRP and MCP-1 in obese, nondiabetic, and insulin-resistant volunteers (Stull et al. 2010). Perhaps the contradictions in the observed impacts on inflammatory markers in these clinical studies may at least in part be explained by the use of different species of berries [bilberry (Karlsen et al. 2010; Kolehmainen et al. 2012) vs. blueberry (Basu et al. 2010; Stull et al. 2010; Lehtonen et al. 2011)], the amount of berries consumed; type of serum samples used for measuring inflammatory biomarkers [fasting serum (Karlsen et al. 2010; Kolehmainen et al. 2012) vs. non-fasting serum (Stull et al. 2010)] or the status of these individuals [overweight subjects with 25.6 ± 6.1 of BMI (Karlsen et al. 2010) vs. obese subjects with 36.8 ± 0.9 of BMI (Stull et al. 2010) and 38.1 ± 1.5 of BMI (Basu et al. 2010)].

It has been reported that a purified ACN mixture exhibited higher anti-inflammatory activity compared to single ACN or whole berries *in vitro* and *in vivo* (Zhu et al. 2013). In that study, purified anthocyanin mixture (containing 17 ACN compounds from blueberries) produced a stronger inhibitory effect on IL-6, IL-1 β -induced CRP production in HepG2 cells and LPS-induced vascular cell adhesion molecule-1 (VCAM-1) secretion in endothelial cells, respectively, compared with the effects of single anthocyanin, D3G and C3G, which support the observations in human subjects (Zhu et al. 2013). These studies suggest that the various

ACNs in blueberry may act synergistically to inhibit the inflammatory response. Hence, consuming foods rich in different ACNs is likely to be more beneficial than consuming a single ACN supplement.

Blueberry and its extracts have also demonstrated potential benefits on the regulation of adipocytokines in animal and human studies. The concentration of adiponectin was higher in C57BL/6J obese mice fed HFD and genetically diabetic db/db mice with C3G supplementation, compared with mice only fed a HFD diet (Guo, H. et al. 2012b; Liu et al. 2014). Similarly, wild blueberry consumption in Obese Zucker rats resulted in a significant increase in circulating adiponectin level compared to the control group (+ 21.8%) (Vendrame et al. 2013). Adiponectin concentration, however has been demonstrated not to differ from the control groups following supplementation of blueberry or ACNs in several animal studies (Vuong et al. 2009; Takikawa et al. 2010; Roopchand et al. 2013; Wu et al. 2013; Mykkanen et al. 2014) and human trials (Qin et al. 2009; Basu et al. 2010; Kolehmainen et al. 2012). Lehtonen et al. (2011) demonstrated, however a decrease in adiponectin level after bilberry supplementation in overweight and obese women for 33 – 35 days. Therefore the exact effect of consumption of blueberries on adiponectin level is unclear.

Leptin secretion has been demonstrated to be inhibited by diets enriched with blueberry, both in genetic models of obesity and dietary-induced obese animal models (Prior et al. 2009; Prior et al. 2010; Wu et al. 2013). However, no significant effect was observed on leptin levels in other animal studies (Vuong et al. 2009; Mykkanen et al. 2014), or indeed in a human trial (Kolehmainen et al. 2012).

Resistin is a hormone secreted from adipose tissue and it has been implicated in the modulation of insulin action, energy, glucose and lipid homeostasis and also has been linked to the onset of insulin resistance and obesity-associated diabetes (Abate et al. 2014).

Mykkane et al. (2014) investigated the effect of blueberry supplementation (10% wild blueberry) in mice fed a HFD and indicated that serum resistin level was significantly reduced in the mice that were supplemented with blueberry for 12 – 14 weeks.

There are several potential mechanisms involved in the anti-inflammatory properties of blueberry. Firstly, antioxidants in blueberry, such as polyphenols and ACNs which exhibit the anti-inflammatory effect may be dependent on a reduction of pro-inflammatory cytokines and increase of anti-inflammatory mediators such as adiponectin (Guo, H. et al. 2012b). Secondly, oxidative stress, which leads to inflammation is reduced due to the strong antioxidant activity of blueberries and its extracts, which is subsequently involved in an increase of glutathione peroxidase 3 (a sensitive index of oxidative stress) gene expression (Lee et al. 2008). Thirdly, blueberry or its ACNs may be able to alter mitogen-activated protein kinase signalling, which modulate cell fate and inflammatory gene expression in various tissues and macrophages (Suganami et al. 2007). Finally the attenuation of NF- κ B activation could be related to the antioxidant capacity of blueberries or its extracts, thereby providing a potential mechanism with the observed anti-inflammatory effect of blueberry intake (Vendrame et al. 2013).

1.5.8 Conclusion

This review focused on blueberries and their bioactive components that influence obesity and its related comorbidities, although it is necessary to indicate that there are still a large number of phytonutrients in blueberries under exploration at present, especially ACNs. A major question to be addressed is whether a single purified component or constituent in blueberries such as C3G or ACNs, or multiple constituents in this fruit produced synergic effects on human health. In addition, there is a need for determining the bioactive constituents of blueberry and their metabolites, which may accumulate in the target tissues and exert

biological effects. Future studies could also focus on the interactions of nutrients and genes so we have a better understanding of the beneficial effects of blueberry at the molecular level, thus be able to develop effective intervention strategies and achieve better outcomes. According to the literature, the evidence suggests that several species of blueberries in the genus *Vaccinium* and their isolated compounds are potential contributors to the regulation of glucose, lipid metabolism, and improvement of inflammation. A deep understanding of the potential roles of blueberries in controlling body weight, regulating blood glucose, and attenuating dyslipidaemia and related chronic inflammation will guide further rigorous investigations on the underlying mechanisms of their beneficial effects on health.

Taken together, it has been reported that blueberry consumption may reduce body weight, blood lipids, blood pressure, and glucose levels. However, these findings are inconsistent in various animal studies and human trials. Available studies on yoghurt indicated that yoghurt consumption is beneficial to human health, including improving blood pressure and risk factors for cardiovascular disease, lowering risk of T2DM, and enhancing immunity. Consistently, the bio-active peptides extracted from yoghurt have been found to attenuate hypertension in both animal and human clinical studies. However, the underlying mechanisms associated with the health benefits of blueberry and yoghurt peptides are yet to be fully understood. Blueberries containing anthocyanins with strong antioxidant and free radical scavenger properties may prevent obesity and T2DM due to their glucose and lipid metabolism. Yoghurt and peptides with ACE inhibitory activity may have beneficial effects on the regulation of blood pressure and cardiovascular diseases via RAS. However, it is not known whether there is a synergistic effect on key markers of obesity and its related comorbidities, including T2DM and hypertension when blueberry and yoghurt are combined. The hypotheses of this research are that the combined supplementations with blueberries and yoghurt, and C3G and peptides would attenuate body weight and body fat mass, improve

glucose tolerance and/or decrease both diastolic and systolic blood pressure in obese mice. In addition, the expression of key genes involved in glucose and lipid metabolism may alter following dietary supplementation. It is expected that the combined supplementations would have more significant impacts on obesity and its related comorbidities, compared with the supplementation of each alone. Furthermore, through investigations on gene expression we would be able to provide mechanistic evidence on the potential synergistic effects of blueberry and yoghurt supplementation at molecular level.

1.6 Aims and hypothesis

1.6.1 Aims

The overall aim of this thesis was to determine the synergistic effects of blueberries and fermented yoghurt, as well as their bioactive components, C3G and peptides, alone or in combination on obesity and its comorbidities. To achieve this, human primary skeletal muscle myotubes obtained from obese and obese T2DM individuals and a HFHC induced obese mouse (C57BL/6) model were used.

1.6.2 Specific aims and hypotheses

- To determine the optimal fermentation conditions of peptides with ACE inhibitory activity from yoghurt with the incorporation of *Lactobacillus helveticus* and Flavourzyme. The primary outcome for this study was to produce peptides with high ACE inhibitory activity. It was hypothesized that yoghurt fermented by *L. helveticus* and Flavourzyme will produce bioactive peptides with ACE inhibitory activity.

- To determine the total anthocyanidin and cyanidin contents from different brands of blueberries. The primary outcome for this study was to choose one blueberry with high concentration of total anthocyanidin and cyanidin.
- To determine the effects of C3G and peptides, alone or in combination, on glucose uptake and metabolism in human primary skeletal muscle myotubes obtained from obese and obese T2DM individuals. The primary outcome for this cell culture study was the regulation of glucose uptake. It was hypothesized that the combination of peptides (ACE inhibitor) and C3G (an antioxidant) would enhance glucose uptake more than individual supplementation and cause more changes in the expression of multiple genes related to glucose metabolism, in human primary skeletal muscle myotubes obtained from obese and obese T2DM individuals.
- To determine the effects of blueberries and yoghurt, as well as their bioactive components, C3G and peptides alone or in combinations, on food and energy intake, body weight and composition, glucose tolerance, blood pressure and locomotion in diet induced obese C57BL/6 mice. The primary outcome of this animal study was to determine the impacts of various dietary treatments on intraperitoneal glucose tolerance and blood pressure. It was hypothesized that the combined supplementations with blueberries and yoghurt, and C3G and peptides would attenuate body weight and body fat mass, improve glucose tolerance and/or decrease both diastolic and systolic blood pressure in obese mice. Therefore, the combined supplementations would have more significant impacts on obesity and its related comorbidities, compared with the supplementation of each alone.
- To determine the effects of blueberries and yoghurt, as well as their bioactive components, C3G and peptides alone or in combinations, on the regulation of multiple genes involved in insulin signalling pathway and glucose metabolism in skeletal muscles (extensor digitorum longus and soleus) obtained from diet induced obese C57BL/6 mice. The primary outcome

for this study was regulation of the expression of AGTR-1, GLUT4 and IRS-1 in two kinds of skeletal muscles. It was hypothesized supplementation with blueberries, yoghurt, C3G, peptides alone or in combinations, would change the expression of multiple genes involved in insulin signally pathway and glucose metabolism in the skeletal muscle obtained obese mice.

CHAPTER 2: General materials and methods

This chapter describes the general materials and methodology used in the thesis. More information on experimental design, detailed procedures and techniques specific to each study are included in Chapters Four – Eight of the thesis.

2.1 Yoghurt fermentation and determination of ACE-I peptides

This section (2.1 Yoghurt fermentation and determination of angiotensin-converting enzyme inhibitory (ACE-I) peptides) has been published, see details in Appendix 2: **Effects of fermentation conditions on the potential antihypertensive peptides released from yoghurt fermented by *Lactobacillus helveticus* and Flavourzyme®** by Min Shi, Fatah Ahtesh, Michael Mathai, Andrew J. McAinch & Xiao Q. Su (2017) in the peer review journal, International Journal of Food Science and Technology, 52, 137–145. The content of this section is the same as the published paper (Appendix 2), and only the format has been changed here to match with the thesis.

2.1.1 Culture medium and reagents

Glycerol de Man, Rogosa, Sharpe (MRS) broth was purchased from Oxoid, Ltd., West Heidelberg, Victoria, Australia. Reconstituted skim milk (RSM) powder (52% lactose, 37% protein, 8.6% ash, and 1.2% fat) was obtained from Woolworths Ltd, Australia. M17 broth was purchased from Oxoid, Ltd, Hampshire, England. Flavourzyme (EC 3.4.11.1, an amino peptidase with an activity of 500 Leucine Amino-peptidase per gram), hippuryl-L-histidyl-L-leucine (HHL), ACE enzyme (from rabbit lung, 0.1 UN) and hippuric acid (HA) were purchased from Sigma-Aldrich Pty. Ltd., Melbourne VIC, Australia.

2.1.2 Bacteria storage and propagation

Lactobacillus helveticus ASCC 881315 (*L. helveticus*), *Streptococcus thermophiles* ASCC 1275 (*S. thermophiles*) and *Lactobacillus delbrueckii* subsp. *bulgaricus* 1466 (*L. bulgaricus*) were obtained from Dairy Innovation Australia Ltd, Werribee, Victoria, Australia. The *L. helveticus* strain was stored in MRS broth, while *S. thermophiles* and *L. bulgaricus* strains were kept in RSM (Woolworths Ltd, Australia) at – 80 °C. For activation of these strains, 100 µL of *L. helveticus* and *L. bulgaricus* strains were transferred separately into 9.9 mL of 40% MRS broth (Oxoid, Ltd., West Heidelberg, Victoria, Australia), whereas *S. thermophiles* was activated via transfer of 100 µL to 9.9 ml of 3.7% M17 broth (Oxoid, Ltd, Hampshire, England), containing 0.5% lactose. All media were autoclaved at 121 °C for 15 minutes before use. Following activation, all strains were incubated at 37 °C for 24 h.

2.1.3 Release of bioactive peptides from yoghurt

Yoghurt was prepared by dissolving skim milk powder (12%, w/w) in distilled water. RSM was heated to 90 °C for 30 min, then inoculated with 1% activated *S. thermophiles* and *L. bulgaricus* cultures (1: 1 of ratio) and fermented at 42 °C for 6 hours to produce yoghurt. Then the fermentation process was stopped by cooling at 4 °C overnight.

L. helveticus 881315 strain was incorporated into yoghurt with or without Flavourzyme (0.14%, Sigma-Aldrich Pty. Ltd., Melbourne VIC, Australia). Specifically, the yoghurt was re-incubated at 37 °C with stirring (200 rpm) for the purpose of a better homogenization between yoghurt and Flavourzyme during 16 h. After incubation for 4, 8, 12 and 16 h, with different inoculum sizes of *L. helveticus* (1%, 2%, 3% and 4% v/w), samples were taken for analysis. The fermentation process was terminated by heating the yoghurt at 90 °C for 20 min to stop enzyme activity. Subsequently, 25 mL of yoghurt sample was centrifuged by a Centrifuge (Beckman J-Avanti, Beckman Coulter, Inc. Indianapolis, United States) at 4000 ×

g at 4 °C for 30 min to separate proteins. The supernatant containing soluble peptides was freeze-dried (Freeze-drier, John Morris Scientific Pty Ltd, Melbourne VIC, Australia) for 72 h. The freeze-dried peptides powder was stored at - 20 °C for analysis.

2.1.4 Determination of ACE-Inhibitory activity

20 mg of the freeze-dried peptide powder from normal yoghurt with starter culture or yoghurt incorporated with *L. helveticus* without Flavourzyme was dissolved in 1 mL of Tris buffer (50 mM, pH 8.3) containing 300 mM sodium chloride (Sigma-Aldrich Pty. Ltd., Melbourne VIC, Australia), respectively. However for the peptides from yoghurt incorporated with *L. helveticus* and Flavourzyme, 2.5 mg/mL of powder was dissolved in the same Tris buffer, as these peptides showed 100% ACE-I activity, when the concentration was 20 mg/mL; therefore, lower concentration (2.5 mg/mL) was used to assess the production of ACE-I peptides under different fermentation conditions.

To determine the peptide fractions from yoghurt in the presence of Flavourzyme, freeze-dried powder (2.5 mg) was dissolved in Tris buffer (50 mM, pH 8.3) containing 300 mM sodium chloride to prepare the sample solutions of 62.5, 125, 250, and 500 µg/mL concentrations to calculate IC₅₀ value of different fractions. The IC₅₀ value was defined as the concentration of inhibitor required to inhibit 50% of ACE activity under the assayed conditions.

Evaluation of ACE-I activity was assayed using a method as previously described by Donkor et al (2007a). It is based on the hydrolysis HHL (Sigma-Aldrich Pty. Ltd., Melbourne VIC, Australia) by ACE to hippuric acid (HA, Sigma-Aldrich Pty. Ltd., Melbourne VIC, Australia) and histidyl-leucine (HL) as products. The HA released from HHL is directly related to the ACE activity. ACE enzyme (Sigma-Aldrich Pty. Ltd., Melbourne VIC, Australia) and HHL was prepared in Tris buffer. Briefly, the assay consisted of 300 µL of 3.0 mM HHL, 300 µL of 3.0 mU ACE enzyme, and 300 µL of peptide solution. The mixture was placed in a glass

tube and then incubated at 37 °C in a water bath for 0.5 h, mixed for 1 min, then returned to the water bath for another 0.5 h. The reaction was stopped by heating the mixture in an 85 °C water bath for 10 min in order to inactivate enzymes. The reaction mixture was stored at - 20 °C before further analysis of released hippuric acid by reversed-phase HPLC system (RP-HPLC, Varian Analytical Instruments, Santa Clara, CA, USA).

HA standard curve was prepared in five different concentrations (5, 10, 15, 20 and 25 µg/mL). The isocratic mobile phase composition was optimized to 12.5% acetonitrile (Merck, Bayswater, VIC, Australia) in MilliQ water (vol/vol) containing 0.1% trifluoroacetic acid (TFA, Sigma-Aldrich Pty. Ltd., Melbourne VIC, Australia), which was filtered (0.45 µm) prior to running through the column. The temperature of the column was kept at room temperature (~22 °C). An aliquot (10 µL) of the mixture was injected onto a VyDAC® C18 300 Å (250 mm x 4.6 mm, 5 µm) column (Grace Vydac, Hesperia CA, USA) using a Varian HPLC equipped with an auto sampler. The flow rate was set at 0.6 mL/min. The quantitative estimation of HA present was determined using a calibration curve of standard HA using a UV detector set at 228 nm.

The percent of ACE inhibition was calculated as follows:

$$\text{ACE inhibition (\%)} = \frac{C(\text{control}) - C(\text{sample})}{C(\text{control})} \times 100$$

Where: C (control) was the concentration of HA without the tested samples (active peptides), which contained 300 µL of HHL, 300 µL of ACE enzyme and 300 µL of Tris buffer, and C (sample) was the concentration of HA with the tested samples, which contained 300 µL of HHL, 300 µL of ACE enzyme and 300 µL of peptide solution.

2.1.5 Peptide profile of water-soluble extract

The RP-HPLC assay was developed for the profile of water-soluble peptides extracted from yoghurt as control, and yoghurt containing *L. helveticus* strain with or without Flavourzyme, respectively (Taruscio et al. 2004). The freeze-dried peptide powder (20 mg) was dissolved in 1 mL of 0.1% TFA in distilled water. All the supernatants thus obtained were filtered through a 0.45 µm membrane filter and stored at – 20 °C until assayed. Water soluble peptides were profiled by a RP- HPLC (SHIMADZU Corporation, Nakagyo-ku, Kyoto, Japan) using C-18 monomeric column (5 µm, 300 Å, 250 mm x 4.6 mm; Grace Vydac, Hesperia CA, USA). The injection volume was 10 µL. Solvent A was a mixture of water (99.9%) with 0.1% TFA (vol/vol) and solvent B contained acetonitrile (99.9%) with 0.1% TFA (vol/vol). Active peptides were eluted with a linear gradient of solvents B in A at concentrations from 0 to 100% over 30 min at a flow rate of 0.75 mL/min. The elution profile was monitored at 215 nm by UV-Vis detector at room temperature (~22 °C).

2.1.6 Peptide fractions

Bioactive peptide fractions from yoghurt with *L. helveticus* and Flavourzyme were collected using a column (Prep Nova-Pack HR C18, 60 Å, 250 mm x 10 mm, 10 µm, Phenomenex, Pty Ltd, Lane Cove, NSW, Australia) by preparative RP-HPLC. Solvent A was a mixture of water and TFA (1000:1, vol/vol), and solvent B contained acetonitrile and TFA (1000:1, vol/vol). The injection volume was 1 mL with 20 mg/mL of yoghurt peptides. The peptides were eluted with a linear gradient of solvent B in A ranged from 0% to 40% over 90 min, at a flow rate 4 mL/min. Detection was carried out at 215 nm by UV-Vis detector. According to the retention time of peaks, 7 fractions (F1 - F7) were collected respectively. All fractions were frozen dried under vacuum. IC₅₀ of ACE activity was determined for each fraction.

2.2 Determination of cyanidin 3-O- β -glucoside (C3G) from blueberries

2.2.1 Sample preparation

All fresh fruits were frozen at $-20\text{ }^{\circ}\text{C}$, freeze-dried for 4 – 6 days and grounded to a powder on dry ice. Freeze dried blueberry powder (50 mg) was suspended in water, vortexed for 30 seconds, and centrifuged at $4000 \times g$ for 30 min at $4\text{ }^{\circ}\text{C}$. Fractions were separated from the supernatant and freeze-dried for 72 hours using a freeze-drier (John Morris Scientific Pty Ltd, Melbourne VIC, Australia). The supernatant powder was stored in dark at $-20\text{ }^{\circ}\text{C}$ for determination of C3G and total anthocyanin content.

2.2.2 Determination of anthocyanins using reverse-phase high performance liquid chromatography (HPLC)

The total anthocyanins was measured according to previously published methodology (Dzamko and Steinberg 2009) with some minor modification, which is summarized in the subsequent sentences. 600 mg of supernatant powder was dissolved into 5 mL of acidified methanol (Merck, Bayswater, VIC, Australia) with 0.3% HCl (Life Technologies Australia Pty Ltd, Mulgrave, VIC, Australia). It was then vortexed for one minute, and filtered using $0.2\text{ }\mu\text{m}$ of filter for the anthocyanin determination using RP-HPLC. HPLC grade C3G (Polyphenols AS, Sandnes, Norway), as a standard, was treated with the same procedure, and a standard curve was prepared using five different concentrations (15, 30, 75, 150 and $300\text{ }\mu\text{g/mL}$). Samples or standard solutions were measured by RP-HPLC using a Prominence-i LC-2030 (Varian Analytical Instruments, Santa Clara, CA, USA) and a Prep Nova-Pack C18 column ($60\text{ }\text{\AA}$, $250\text{ mm} \times 10\text{ mm}$, $10\text{ }\mu\text{m}$, Phenomenex, Pty Ltd, Lane Cove, NSW, Australia). The temperature of the column was set at $30\text{ }^{\circ}\text{C}$. The mobile phase consisted of a mixture of aqueous methanol (phase A: 5% methanol; 0.1% 5M HCl) and aqueous

acetonitrile (phase B: 50%; 0.1% 5M HCl) and was pumped through the column at a flow rate of 0.7 mL/min. The following elution profile was used: start condition 5% phase B; then, 50% phase B from 5 minutes to 40 minutes with linear gradient, finally, 5% phase B from 40 minutes to 50 minutes for testing the next sample. The eluent was monitored by photodiode array detection at 520 nm and the injection volume was 10 μ L. The linearity of each calibration line was acceptable when $R^2 > 0.99$.

2.2.3 Determination of anthocyanidins using reverse-phase HPLC

To determine the content of total anthocyanidins from blueberries, blueberry powder (300 mg) was hydrolysed using 4 mL of acidified aqueous methanol (75% methanol; 2M HCl). Extracts were vortexed for 30 seconds. They were then filtered (0.45 μ m) and the supernatant was retained. After two further extractions of the remaining pellets, samples (~12 mL) were hydrolysed for 50 min at 90 °C. Following hydrolysis, samples were cooled to room temperature before centrifuging at 4000 x g for 10 min at 10 °C. 500 μ L was then used for RP-HPLC analysis. C3G was used as a standard to assess the stability of cyanidin under the same processing conditions and a standard curve was made using five different concentrations (25, 50, 100, 250 and 500 μ g/mL).

Hydrolysed samples or cyanidin solution were measured by HPLC using a Prominence-i LC-2030 (Varian Analytical Instruments, Santa Clara, CA, USA) and a Prep Nova-Pack C18 column (60 Å, 250 mm x 10 mm, 10 μ m, Phenomenex, Pty Ltd, Lane Cove, NSW, Australia). The temperature of column was held at 30 °C. The mobile phase consisted of a mixture of aqueous methanol (phase A: 5% methanol; 0.1% 5M HCl) and aqueous acetonitrile (phase B: 50%; 0.1% 5M HCl) and was pumped through the column at a flow rate of 0.7 mL/min. The following elution profile was used: start condition 5% phase B, then, 50% phase B from 5 minutes to 33 minutes with linear gradient, 5% phase B from 33 minutes

to 40 minutes. The eluent was monitored by photodiode array detection at 520 nm and injection volume was 10 μ L. The linearity of each calibration line was acceptable when $R^2 > 0.99$.

2.3 Cell culture

2.3.1 Materials and reagents

HPLC grade C3G was provided by Polyphenols AS (Sandnes, Norway). Pierce™ bicinchoninic acid (BCA) Protein Assay Kit, Diethylpyrocarbonate (DEPC) water, α -MEM, amphotericin B, horse serum, bovine serum albumin (BSA), fetal bovine serum (FBS), and trypsin/ EDTA were obtained from Life Technologies Australia Pty Ltd (Mulgrave, VIC, Australia). iQ™ SYBR Green and iScript™ cDNA Synthesis Kit were purchased from Bio-Rad Laboratories Pty Ltd (Gladesville, NSW, Australia). Liquid scintillation cocktail and deoxy-D-glucose, 2-[1,2-3H (N)]- were purchased from Perkin-Elmer, Inc. (Glen Waverley, VIC, Australia). All other chemicals, unless otherwise specified, were obtained from Sigma-Aldrich Pty. Ltd. (Castle Hill, NSW, Australia).

2.3.2 Participants

Human primary skeletal myotubes were previously obtained and established from obese donors (eight females and two males) and obese type 2 diabetic donors (eight males and two females) (Table 2.1). This research was approved by the Human Ethics Research committee of Victoria University (HRE08-158), and all participants have given their written informed consent. The minimum number of cell lines required was determined by statistical power analysis (two tailed t-test at the 0.05 significance level) based on the power of 90% of expected changes in glucose uptake reported in a previous study (Ho et al. 2017), the major measured outcome of the cell culture study. The minimum number of cell lines was four

according to data from the publication, in which 10 μM of C3G resulted in $138 \pm 2\%$ of glucose uptake in human skeletal muscle cells obtained from four healthy participants, compared with $131 \pm 3\%$ resulted from 1 μM of C3G. Due to more complicated characteristics of participants including obese and obese T2DM participants in this study, an additional six cell lines have been included in this study to account for greater variance than previously observed by Ho et al (2017) (healthy participants vs. obese and T2DM obese participants). Therefore, final number of cell lines was ten for each group.

Table 2.1: Characteristics of Participants

	Participants	
	Obese	Obese T2DM
Number (F/M)	8/2	8/2
Age (years)	49 ± 4	53 ± 2
BMI (kg/m^2)	40.42 ± 1.64	42.80 ± 2.38
Fasting plasma glucose (mM/L)	5.02 ± 0.13	8.61 ± 0.76 *
Hba1c (%)	5.59 ± 0.09	7.82 ± 0.47 *
Plasma insulin ($\mu\text{U}/\text{L}$)	10.17 ± 1.64	21.62 ± 2.53 *
Cholesterol (mmol/L)	5.74 ± 0.52	5.01 ± 0.37
Fasting triglyceride (mmol/L)	1.75 ± 0.31	2.36 ± 0.46

Values are expressed as means \pm SEM. * indicated a significant difference between obese and obese T2DM groups ($p < 0.05$).

2.3.3 Proliferation of human primary skeletal myotubes

Human primary skeletal myotubes were previously established according to the method of Blau and Webster (1981) and modified by Gaster et al. (2001). The process of the isolation of

cells has been previously detailed (Chen et al. 2005). Briefly, approximately 50 – 100 mg muscle samples were washed in α -MEM (Life Technologies Australia Pty Ltd., Mulgrave, VIC, Australia), minced and enzymatically dissociated with 0.05% trypsin/ EDTA (Life Technologies Australia Pty Ltd., Mulgrave, VIC, Australia). Cells were then collected through centrifugation and resuspended in growth medium [α -MEM supplemented with 10% FBS (vol/vol, Life Technologies Australia Pty Ltd., Mulgrave, VIC, Australia), 0.5% penicillin (vol/vol, Sigma-Aldrich Pty. Ltd., NSW, Australia), and 0.5% amphotericin B (vol/vol, Sigma-Aldrich Pty. Ltd., NSW, Australia)] in a 37 °C incubator (Heracell™ 240i, Thermo Fisher Scientific Inc., Scoresby, VIC, Australia) with 5% CO₂ throughout the incubation. These incubation conditions were used throughout the study. Cells were frozen with 10% dimethyl sulfoxide (DMSO, Life Technologies Australia Pty Ltd., Mulgrave, VIC, Australia) in the liquid nitrogen storage system (in which they were stored and suspended in freezing medium), as cells got 80% confluence.

The cells at passage three were obtained from the liquid nitrogen storage system for utilisation in this candidature. Cells were immediately thawed and placed into the growth medium (9 mL) and centrifuged at room temperature at 1600 x g for five minutes. Supernatant containing the freezing medium (10% DMSO) was discarded and the pellet of primary myotubes was resuspended and maintained in Minimum Essential Medium α (α -MEM), which contains 10% foetal bovine serum (vol/vol), 0.5% penicillin streptomycin (vol/vol), 0.5% Amphotericin B (vol/vol) and maintained in a cell culture incubator at 37 °C, 5% CO₂. For removing fibroblasts, cells were cultured on an uncoated flask (Greiner Bio-One North America Inc., North Carolina, the United States of America) for 30 min before transferring the cell media to a flask coated with ECM gel from Engelbreth-Holm-Swarm murine sarcoma (Sigma-Aldrich Pty. Ltd., NSW, Australia). Growth medium was changed every second day. On passage four, cells were resuspended in the growth medium and

transferred to ECM gel. They were then seeded into 6-well plates (Greiner Bio-One North America Inc., North Carolina, the United States of America) for mRNA expression analysis or 12-well plates (Greiner Bio-One North America Inc., North Carolina, the United States of America) for glucose uptake assay.

2.3.4 Differentiation of human primary myotubes

For the differentiation process, once the cells had reached ~ 80% confluence they were detached from the flask using trypsin-EDTA, centrifuged and resuspended immediately in differentiation medium (α -MEM containing 2% horse serum (vol/vol, Life Technologies Australia Pty Ltd., Mulgrave, VIC, Australia), 0.5% penicillin streptomycin (vol/vol) and 0.5% Amphotericin B (vol/vol)). The human primary skeletal myotubes were differentiated for six days with the media changed every second day.

2.3.5 Treatments

Following the differentiation period, human primary skeletal myotubes were washed three times with PBS. Subsequently, the cells were treated with the control (α -MEM with 0.1% BSA, Life Technologies Australia Pty Ltd., Mulgrave, VIC, Australia) or C3G in two concentrations, 10 μ M and 100 μ M, respectively; or peptides in two concentrations, 150 μ g/mL and 1500 μ g/mL, respectively; or a combination of C3G and peptides (10 μ M of C3G + 150 μ g/mL of peptides; 10 μ M of C3G + 1500 μ g/mL of peptides; 100 μ M of C3G + 150 μ g/mL of peptides and 100 μ M of C3G + 1500 μ g/mL of peptides) in various concentrations for 24 hours. The concentrations of C3G selected for the dose response were based on a previous study which has shown that 50 μ M of C3G increased adipocyte glucose uptake, and membrane translocation and expression of GLUT 4 (Scazzocchio et al. 2011). Furthermore, a previous study indicated that egg white ovotransferrin-derived tripeptides, Ile-Arg-Trp (a

peptide with ACE inhibitory activity) treatment (50, 100, and 150 μM) increased glucose uptake in L6 myoblasts (Son et al. 2018). In the current study, peptides with ACE inhibitory activity were extracted from fermented yoghurt, which were a mixture of different peptides, and a peptide stock solution was prepared (mg/mL). The stock solution of C3G, peptides or the combination of C3G and peptides was suspended in sterilised PBS, the treatment doses were then added to α -MEM medium containing only 0.1% BSA. 10 μL of the treatments were added to the 12-well plates to get 10 μM and 100 μM of C3G and 150 $\mu\text{g/mL}$ and 1500 $\mu\text{g/mL}$ of peptides as the final concentrations. The treatments (20 μL) were added to the 6-well plates to get 10 μM and 100 μM of C3G and 150 $\mu\text{g/mL}$ and 1500 $\mu\text{g/mL}$ of peptides as the final concentrations. See Figure 2.1 for a visual representation of the treatment dosages prepared for human primary skeletal myotubes experiment.

Each treatment was conducted in triplicates for glucose uptake assay and duplicates for gene expression. Figures 2.1 & 2.2 showed the treatment regime for the human primary skeletal muscle cell culture experiments across the 3 x 12-well plates for glucose uptake assay (Figure 2.1) and the 3 x 6-well plates for gene expression experiment (Figure 2.2).

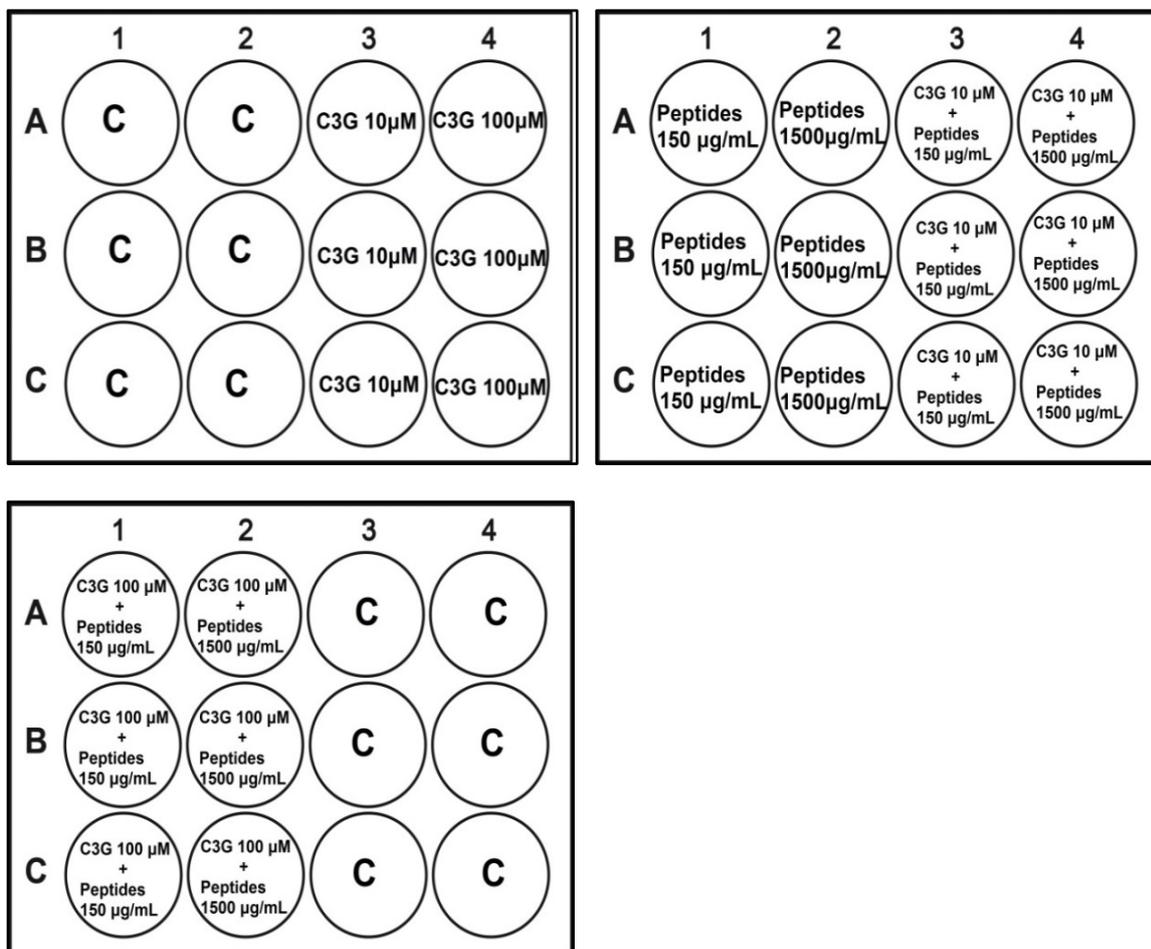


Figure 2.1: A visual representation of the treatment regime for glucose uptake assay in the human primary skeletal muscle cell culture experiment.

C, cells were incubated with α -MEM with 0.1% BSA. Cells in the treatment groups were incubated with C3G10 μ M, C3G 100 μ M, peptides 150 μ g/mL and peptides 1500 μ g/mL and the full combinations between G3G and peptides in α -MEM with 0.1% BSA for 24 hours.

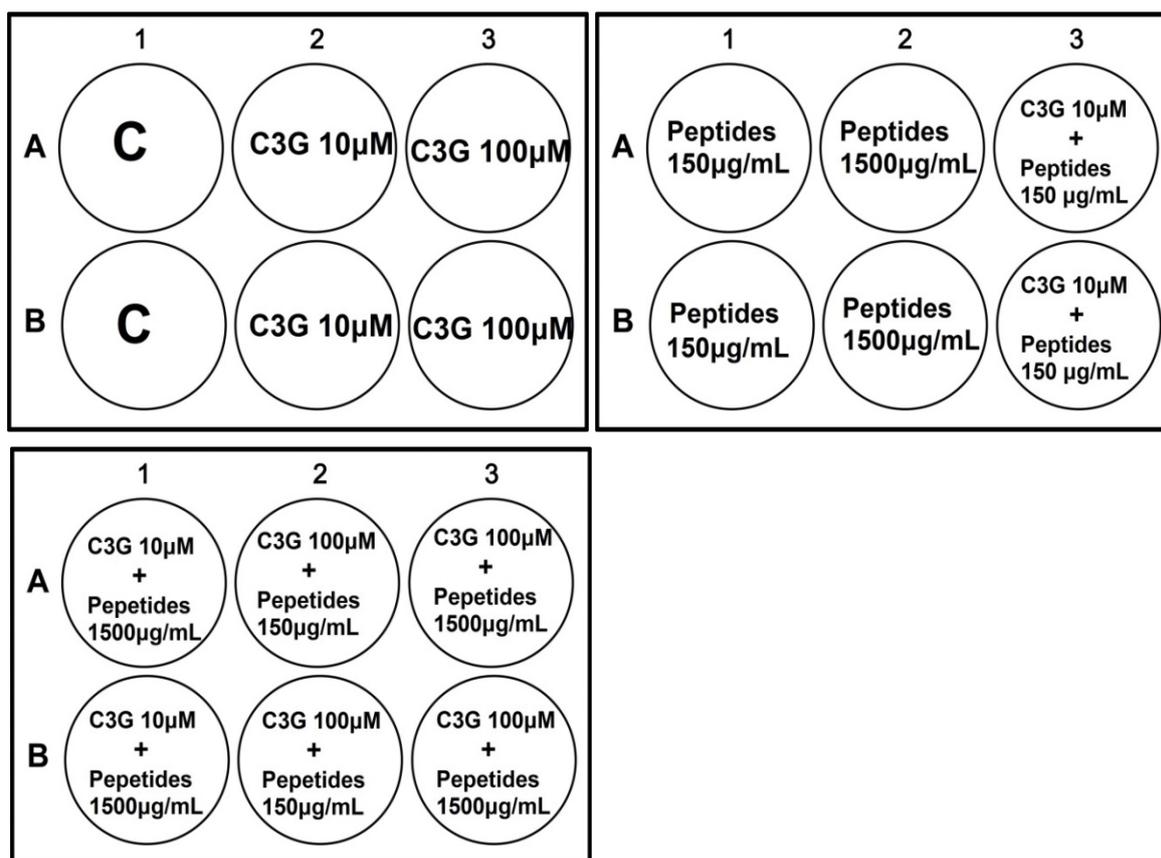


Figure 2.2: A visual representation of treatment regime for gene expression in the human primary skeletal muscle cell culture experiment.

C, cells were incubated with α -MEM with 0.1% BSA. Cells in the treatment groups were incubated with C3G10 μ M, C3G 100 μ M, peptides 150 μ g/mL, peptides 1500 μ g/mL and C3G cross with peptides in α -MEM with 0.1% BSA for 24 hours.

2.3.6 Glucose uptake assay

Differentiated human primary skeletal myotubes were treated with C3G cross with peptides with ACE inhibitory activity in various concentrations in isolation or in combination in the presence of serum-free medium for 24 hours. After the treatments, cells were rapidly washed three times at room temperature with warm glucose uptake buffer containing: 150 mM NaCl, 5 mM KCl, 1.2 mM MgSO₄, 2.5 mM NaH₂PO₄, 1.2 mM CaCl₂, 10 mM HEPES, and 0.1%

BSA (All of these chemicals were obtained from Sigma-Aldrich Pty. Ltd., Castle Hill, NSW, Australia). The cells were treated in the presence or absence of insulin 100 nM (10 μ L, Sigma-Aldrich Pty. Ltd., Castle Hill, NSW, Australia) at 37 °C for 30 min and incubated in the same buffer (1 mL) supplemented with 2-deoxyglucose(DOG)/2-[³H]DOG (10 μ M final concentration, 1 μ Ci/well, Perkin-Elmer, Inc., Glen Waverley, VIC, Australia) for 15 min at 37 °C. The reactions were stopped by aspirating the reaction mixture and the plate was rinsed four times with cold PBS (Sigma-Aldrich Pty. Ltd., Castle Hill, NSW, Australia). Cells were then solubilized by the addition of 0.5 mL of 0.3M NaOH (Sigma-Aldrich Pty. Ltd., Castle Hill, NSW, Australia) at room temperature for 30 min. An aliquot of 400 μ L was transferred to a scintillation vial and 4 mL of liquid scintillation cocktail was added. Glucose transport activity was measured by a scintillation counter (Tri-Carb 3180TR/SL; Perkin-Elmer, Inc.) and expressed as picomoles of DOG taken up per minute per milligram of total protein. The remaining fluid was removed to a 96-well plate (Greiner Bio-One North America Inc., North Carolina, the United States of America) for protein determination.

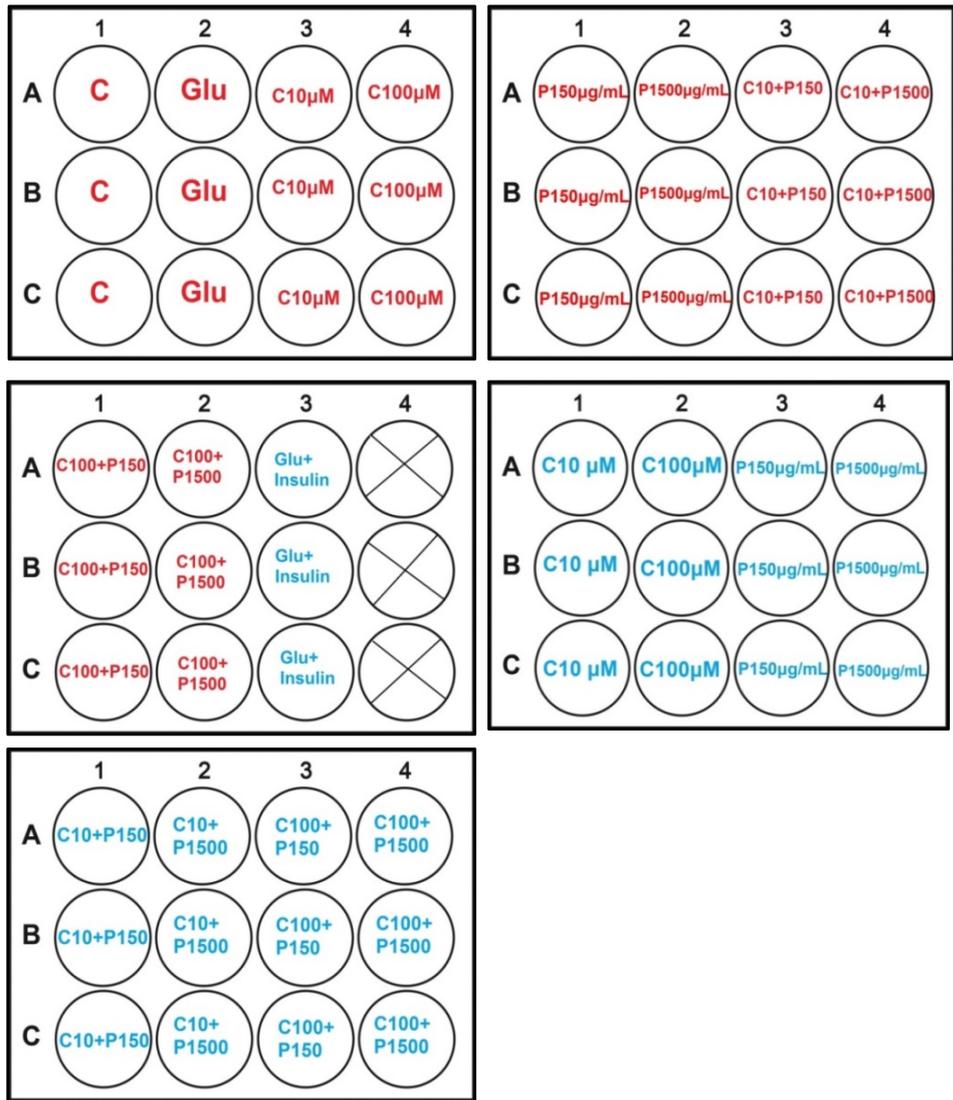


Figure 2.3: A visual representation of the treatment regime for glucose uptake assay in the human primary skeletal muscle cell culture experiment.

Red colour: cells were incubated in the absence of insulin; Blue colour: cells were incubated in the presence of insulin. C, cells were incubated with α -MEM with 0.1% BSA, as a blank; Glu, cells were incubated with 2-deoxyglucose(DOG)/2- 3 H]DOG as a control; Glu + Insulin, cells were treated in the present of insulin for 30 minutes, then incubated with 2-deoxyglucose(DOG)/2- 3 H]DOG as a positive control; Treatment Groups were α -MEM with 0.1% BSA and 10 μ M of C3G, 100 μ M of C3G, 150 μ g/mL of peptides and 1500 μ g/mL of peptides. All treatment groups were incubated in the presence or absence of 100 nM of insulin (10 μ L) for 30 min, then incubated in the same buffer supplemented with 2-deoxyglucose(DOG)/2- 3 H]DOG for 15 min.

2.3.7 Protein analysis

Total protein content was quantified using a Pierce™ BCA Protein Assay Kit (Life Technologies Australia Pty Ltd, Mulgrave, VIC, Australia) with albumin as a standard, according to the manufacturer's instructions. Cell culture lysate was diluted 1:10 with scintillation fluid and a series of nine standards (0, 12.5, 25, 50, 75, 100, 150, 200 and 250 µg/mL) were prepared via serial dilution of stock BCA using MilliQ water as diluent. To 200 µL of Pierce BCA protein assay reagents a 50:1 (vol/vol) ratio A to B, standards or 25 µL of fluid contained 400 µL of lysed cells and 4 mL of liquid scintillation were added into the 96-well plate and shook for 30 seconds. The plates were incubated at 37 °C for 30 minutes and cooled at room temperature. Absorbance was read at 562 nm with a Varioskan™ LUX Multimode Microplate Reader (Thermo Fisher Scientific Laboratory Equipment, Hudson, United States). A standard curve was prepared and protein content for each sample was calculated.

2.4 RNA extraction

2.4.1 Cell culture

After the treatments in six-well plates, human primary skeletal myotubes were rinsed with cold PBS twice. The cells were then lysed by 800 µL of TRIzol (Sigma-Aldrich Pty. Ltd., Castle Hill, NSW, Australia) at 4 °C and the homogenates were transferred to 1.5 mL Eppendorf tubes (Scientific Specialties, Inc., California, the United States of America) (McAinch et al. 2006). To each sample, 200 µL of chloroform (Sigma-Aldrich Pty. Ltd., Castle Hill, NSW, Australia) was added and briefly vortexed for approximately 10 seconds. Then the samples were incubated on ice for 5 minutes and followed by centrifuging at 13000 x g for 15 minutes at 4 °C. The clear upper layer was transferred to a fresh 1.5 mL Eppendorf

tube and an equal volume (approximately 600 μL) of 2-isopropanol (Sigma-Aldrich Pty. Ltd., Castle Hill, NSW, Australia) and 10 μL of 5 M NaCl made with DEPC water (Sigma-Aldrich Pty. Ltd., Castle Hill, NSW, Australia) were added prior to incubation on ice overnight at $-20\text{ }^{\circ}\text{C}$. Samples were centrifuged at $13000 \times g$ for 20 minutes at $4\text{ }^{\circ}\text{C}$ to separate the RNA. Supernatant was removed and the RNA pellet was carefully washed with 400 μL freshly prepared 75% (vol/vol) ethanol (Sigma-Aldrich Pty. Ltd., Castle Hill, NSW, Australia). Samples were centrifuged at $9000 \times g$ for 8 minutes at $4\text{ }^{\circ}\text{C}$. Ethanol was aspirated off and the RNA pellet was left to briefly air dry for approximately 5 minutes at room temperature. The pellet was then dissolved in 5 μL of DEPC water at $60\text{ }^{\circ}\text{C}$. Total RNA was quantified using a nanodrop spectrometry (NanoDrop 2000, Thermo Fisher Scientific, Wilmington, DE) at 260 nm. Samples were stored at $-80\text{ }^{\circ}\text{C}$ until analysis.

2.4.2 Tissues

800 μL of TRIzol was added to sterilised 2 mL lysis matrix tubes (MP Biomedicals, California, the United States of America) containing 1000 mg of sterilised ceramic beads and were then placed on ice (Vikman and Edvinsson 2006). Following this, the previously frozen skeletal muscle (the soleus and the EDL) was added to the 2 mL lysis matrix tubes containing the sterile ceramic beads and TRIzol.

The tubes were then transferred on ice to the Fast Prep (FP120, cell disruptor, Electron Corporation, Milford, the United States of America) and processed at the speed of 5.5 for 20 seconds, the samples were then placed on ice for 5 minutes and homogenised again. Following this the samples were centrifuged at $12,000 \times g$ for 15 minutes at $4\text{ }^{\circ}\text{C}$. The TRIzol and RNA containing supernatant was subsequently transferred to a sterile 1.5 mL Eppendorf tube containing 250 μL of chloroform, then vigorously mixed using a vortex and left on ice

for 5 minutes. From this step forward, the same process was followed for the cell culture RNA extraction procedure as detailed in section 2.4.1.

2.5 Reverse transcription of RNA samples

Reverse transcription is a process in which RNA is converted to complementary DNA (cDNA) using an enzyme called reverse transcriptase. The cDNA can then be used for 'Real Time' Polymerase Chain Reaction (PCR) quantification. During this process 0.5 µg of total RNA were diluted with DEPC treated water to a final volume of 7.5 µL. The RNA was then reverse transcribed into cDNA using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories Pty Ltd, Gladesville, NSW, Australia), including a master mix of the reaction mix and enzyme. 2 µL of iScript reaction mix and 0.5 µL of the enzyme reverse transcriptase were added to each 0.5 µg of RNA sample. Internal controls (using a mixture of some of the concentrated samples) were also required to determine the plate to plate variation during the 'Real Time' PCR quantification as well as a negative reverse transcription sample to ensure that the reverse transcription step was successful, in which 0.5 µL of DEPC treated water was added in place of the enzyme. The PCR amplification was performed using a MyCycler™ Thermo Cycler (Bio-rad Laboratories, Hurcules, California, the United States of America), set to the following program: 5 min at 25 °C, 20 min at 46 °C, 1 min at 95 °C for inactivating the reverse transcriptase, finally holding at 4 °C until storage at – 80 °C.

2.6 Dilution of cDNA

10 µL of cDNA (from human primary skeletal myotubes or mouse skeletal muscles) was diluted at the ratio of 1: 20 (190 µL of DEPC treated water was added to each sample) and stored at – 20 °C for subsequent 'Real Time' PCR mRNA quantification.

2.7 Real-Time polymerase chain reaction (PCR) analysis

2.7.1 Human and mouse oligonucleotide primer sequences

An oligonucleotide primer is a short nucleic acid sequence that is the initial point of DNA synthesis. Forward and reverse oligonucleotides were designed using Oligoperfect™ Suite (Invitrogen, Victoria, Australia, Victoria, Australia) and were purchased from Integrated DNA Technologies, Inc. (1710 Commercial Park, Coralville, Iowa 52241 USA). Selective gene homology for genes of interest was confirmed with BLAST (Basic Local Alignment Search Tool, National Centre for Biotechnology Information, Bethesda, MD) to determine homologous binding to the target mRNA sequence. The primer sequences used for both *Chapters Six* and *Eight* are detailed in Table 2.2: Human primer sequences used for ‘Real Time’ PCR analysis in human primary skeletal myotubes and Table 2.3: Mouse primer sequences used for ‘Real Time’ PCR analysis of skeletal muscles.

Table 2.2: Human primer sequences used for ‘Real Time’ PCR analysis of human primary skeletal myotubes

Gene	Accession Number	Sequence
AGTR-1	NM_009585.3	Forward (5' - 3') CTGATGCCATCCCAGAAAGT Reverse (5' - 3') CTTCCAGCTTTGGGACAATC
AMPK- α	XM_016919269	Forward (5' - 3') AACTGCAGAGAGCCATTCAC TTT Reverse (5' - 3') GGTGAAACTGAAGACAATGTGCTT
Cyclophilin	XM_024747056	Forward (5' - 3') CATCTGCACTGCCAAGACTGA Reverse (5' - 3') TTCATGCCTTCTTTCACTTTGC
FoxO1	XM_522749	Forward (5' - 3') TCATGGATGGAGATA CATTGGATT Reverse (5' - 3') TCCTGCTGTCAGACAATCTGAAG
GAPDH	XM_024733357	Forward (5' - 3') CAA CGA CCA CTT TGT CAA GC Reverse (5' - 3') TTA CTC CTT GGA GGC CAT GT
GLUT1	NM_006516.2	Forward (5' - 3') GGGCCAAGAGTGTGCTAAAG Reverse (5' - 3') TGCCGACTCTCTTCCTTCAT
GLUT4	NM_001042.2	Forward (5' - 3') CACCCTCACCACCCTCTG Reverse (5' - 3') CTTTTCCCAAGCCACTG
IRS-1	NM_000208	Forward (5' - 3') GTTTCCAGAAGCAGCCAGAG Reverse (5' - 3') TGAAATGGATGCATCGTACC
IRS-2	NM_003749.2	Forward (5' - 3') ACGCCAGCATTGACTTCTTGT Reverse (5' - 3') TGACATGTGACATCCTGGTGATAA
PI3K	NM_181504.3	Forward (5' - 3') GGAAGCAGCAACCGAAACAA Reverse (5' - 3') TTCGCCGTCCACCACTACA
PPAR- α	XM_024452253	Forward (5' - 3') GAAGCTGTCCTGGCTCAGAT Reverse (5' - 3') GGGGACCACAGGATAAGTCA

AGTR-1, Angiotensin II receptor type 1; AMPK- α , 5' AMP-activated protein kinase alpha; FoxO1, Forkhead box protein O1; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; GLUT1, Glucose transporter 1; GLUT4, Glucose transporter 4; IRS-1, Insulin receptor substrate 1; IRS-2, Insulin receptor substrate 2; PI3K, Phosphoinositide 3-kinase; PPAR- α , Peroxisome proliferator-activated receptor alpha.

Table 2.3: Mouse primer sequences used for ‘Real Time’ PCR analysis of skeletal muscles

Gene	Accession Number	Sequence
AGTR-1	NM_177322.3	Forward (5' - 3') TGGCTGGCATT TTTGTCTGGAT Reverse (5' - 3') TGCTTTTCTGGGTTGAGTTGGT
AMPK- α	NM_178143.2	Forward (5' - 3') GCCCAGATGAACGCTAAGAT Reverse (5' - 3') TGCATACAGCCTTCCTGAGA
FoxO1	NM_019739.3	Forward (5' - 3') ACCCTGTTCGCAGATCTACGA Reverse (5' - 3') AGGGACAGATTGTGGCGAAT
GLUT1	NM_011400.3	Forward (5' - 3') TTGCCCAGGTGTTTGGCTTA Reverse (5' - 3') GGCAGAAGGGCAACAGGATA
GLUT4	NM_009204.2	Forward (5' - 3') ACCAACTGGCCATCGTCATT Reverse (5' - 3') GGACAGAAGGGCAGCAGAAT
HPRT-1	NM_013556.2	Forward (5' - 3') GCAAAC TTTGCTTTCCCTGG Reverse (5' - 3') ACTTCGAGAGGTCCTTTTCACC
IRS-1	NM_010570.4	Forward (5' - 3') TCCAGAAGCAGCCAGAGGAT Reverse (5' - 3') CGTGAGGTCCTGGTTGTGAA
PI3K	NM_001024955.2	Forward (5' - 3') TGATGTGGCTGACGCAGAAA Reverse (5' - 3') CCACGTCTTCTCGTCATGGT

AGTR-1, Angiotensin II receptor type 1; AMPK- α , 5' AMP-activated protein kinase alpha; FoxO1, Forkhead box protein O1; GLUT1, Glucose transporter 1; GLUT4, Glucose transporter 4; HPRT-1, Hypoxanthine-guanine phosphoribosyltransferase; IRS-1, Insulin receptor substrate 1; PI3K, Phosphoinositide 3-kinase.

2.7.2 Real-Time PCR analysis

‘Real-time’ PCR was conducted using MyiQ™ multiplex ‘real-time’ PCR detection system (Bio-Rad Laboratories, Hercules, CA) with iQ™ SYBR Green Supermix (Bio-Rad Laboratories Pty Ltd, Gladesville, NSW, Australia). To compensate for variations in RNA

input amounts and reverse transcriptase efficiency, mRNA abundance of the housekeeping genes was also measured and quantified using the $2^{-\Delta\Delta CT}$ method (Godfrey et al. 2000). Data from treatment groups were expressed in relation to control treatment and all experiments were repeated at least three times. For the cell culture study, the 'real-time' PCR cycling parameters were as follows: initial denaturation and enzyme activation at 95 °C for 3 minutes, followed by 40 cycles of denaturation at 95 °C for 15 seconds, annealing and extension at 60 °C for 45 seconds. Relative changes in mRNA abundance was normalised to the average of two housekeeping genes [cyclophilin and glyceraldehydes-3-phosphate dehydrogenase (GAPDH)].

For skeletal muscle tissues, SsoAdvanced™ Universal SYBR Green Supermix (Bio-Rad Laboratories Pty Ltd, Gladesville, NSW, Australia) was used for 'Real-time' PCR detection. The 'real-time' PCR cycling parameters were as follows: 98 °C for 1 minute, followed by 40 cycles of denaturation at 95 °C for 15 seconds, annealing and extension at 60 °C for 45 seconds. Relative changes in mRNA abundance was normalised to the average of the housekeeping gene, hypoxanthine phosphoribosyltransferase-1 (HPRT-1).

2.7.3 Optimize primer concentrations

Primer optimisation was undertaken to determine the optimal concentration for primers utilised for the 'Real Time' PCR analysis in both cell culture and animal studies. This process involves different combinations of primer concentrations for forward and reverse primers run at 40 cycles. See Table 2.4 for details.

Table 2.4: Concentration combinations used for primer optimisations

Primer Concentration Combinations	
Forward	Reverse
3 μ M	3 μ M
3 μ M	9 μ M
9 μ M	3 μ M
9 μ M	9 μ M

2.8 Animals

From Chapter Six to Chapter Seven, six-week old male C57BL/6 mice were utilised, with a starting body weight between 19 – 21 grams. All the mice were purchased from the Animal Resources Centre (ARC), at Perth, WA, Australia.

C57BL/6 mice were selected due to their ability to gain weight on a high fat diet (Surwit et al. 1995; Nishikawa et al. 2007; Wang and Liao 2012). This mouse strain also showed the possibility of developing abnormalities including insulin resistance, glucose intolerance, hypertension and low grade (metabolic, meta-) inflammation in metabolic tissues following a period of high-fat diet consumption, which is a similar response to what was observed in humans following a long term high fat diet (Jayaprakasam et al. 2006; Winer and Winer 2012; Mykkanen et al. 2014; Elks et al. 2015; Kim and Park 2015).

Mice were housed at the Werribee Animal Facility, Victoria University, Australia. Ethical approval was obtained for this study from the Victoria University Animal Experimental Ethics Committee (AEC) # 16/005. The minimum number of animals required was determined by a statistical power analysis (two tailed t-test at the 0.05 significance level)

based on the power of 90% of expected changes in serum glucose level reported in a previous study (Guo, H. et al. 2012b), the major measured outcome of this study. The minimum number of mice was six according to data from the publication, in which serum glucose was 8 ± 1 mmol/L in HD groups supplemented with C3G (0.2%) in male C57BL/6J mice for 5 weeks, compared with 10 ± 1 mmol/L in HD control group. An additional four animals were included in this study to account for slightly greater variance than previously observed by Guo et al (2012b) and unforeseen incidences in the animals (i.e. illness). Therefore, final animal number was ten for each group.

All animals were housed in a stable environment maintained at $22 \pm 1^\circ\text{C}$ with a 12 hour light/dark cycle (7:00 – 19:00). Following four days acclimatisation, mice were randomly divided into groups, kept four per cage and fed with high fat diet (36% fat modification of AIN93G, 59% of total energy, mostly from lard; SF003-002; Speciality Feeds, Glen Forrest, WA, Australia) plus 30% fructose water, made by fructose powder (Consolidated Chemical Company, Dandenong South VIC, Australia), for eight weeks to induce obese with hypertension, hyperglycemia and inflammatory conditions in different metabolic tissues. Mice were then placed into divided cages (two in one cage) and provided with a separate diet for another eight weeks. Each mouse had its own *ad libitum* access to food and water during this period. Experimental procedures were approved by the Animal Ethics Committee of Victoria University (AEC number: 16/005).

2.8.1 Dietary intervention

The male C57BL/6 mice were randomly allocated into either high-fat-high-carbohydrate (HFHC) groups to induce obesity and other metabolic syndrome or a standard control (lean) group for eight weeks. The mice that were maintained on either a high fat (western) diet (36% fat modification of AIN93G, 59% of total energy from fat; SF003-002) and 30%

fructose water or the mouse standard chow pellet (5% fat) purchased from Speciality Feeds (Glen Forrest, WA, Australia). See Tables 2.5 – 2.6 for the ingredients and nutritional composition for both of the diets.

Table 2.5: Ingredients and nutrition composition of the standard chow pellets

Ingredients *	Wheat	
	Barley	
	Lupins	
	Soya meal	
	Fish meal	
	Mixed vegetable oils	
	Canola oil	
	Salt	
	Calcium carbonate	
	Dicalcium phosphate	
	Magnesium oxide	
Vitamin and trace mineral premix		
Calculated Nutritional Parameters	Protein	20.00%
	Total Fat	4.80%
	Crude Fibre	4.80%
	Acid Detergent Fibre	7.60%
	Neutral Detergent Fibre	16.40%
	Total Carbohydrate	59.40%
	Digestible Energy	14.0 MJ/kg
	% Total calculated digestible energy from lipids	12.00%
	% Total calculated digestible energy from protein	23.00%
Added Vitamins	Vitamin A (Retinol)	10 000 IU/kg
	Vitamin D (Cholecalciferol)	2 000 IU/kg
	Vitamin E (a Tocopherol acetate)	100 mg/kg
	Vitamin K (Menadione)	20 mg/kg
	Vitamin B1 (Thiamine)	80 mg/kg

	Vitamin B2 (Riboflavin)	30 mg/kg
	Niacin (Nicotinic acid)	100 mg/kg
	Vitamin B6 (Pryridoxine)	25 mg/kg
	Calcium Pantothenate	50 mg/kg
	Biotin	300 µg/kg
	Folic Acid	5.0 mg/kg
	Vitamin B12 (Cyancobalamin)	150 µg/kg
Calculated Amino Acids	Valine	0.87%
	Leucine	1.40%
	Isoleucine	0.80%
	Threonine	0.70%
	Methionine	0.30%
	Cystine	0.30%
	Lysine	0.90%
	Phenylanine	0.90%
	Tyrosine	0.50%
	Tryptophan	0.20%
	Histidine	0.53%
Added Trace Minerals	Magnesium	100 mg/kg
	Iron	70 mg/kg
	Copper	16 mg/kg
	Iodine	0.5 mg/kg
	Manganese	70 mg/kg
	Zinc	60 mg/kg
	Molybdenum	0.5 mg/kg
	Selenium	0.1 mg/kg

* Quantities of the diet ingredients are unknown.

Table 2.6: Ingredients and nutrition composition of the high fat diet (59% of SF03-002 total energy from fats)

Ingredients	Casein (Acid)	200 g/kg
	Sucrose	346 g/kg
	Canola Oil	60 g/kg
	Cocoa Butter	240 g/kg
	Hydrogenated Vegetable Oil (Copha)	60 g/kg
	Cellulose	50 g/kg
	DL Methionine	3.0 g/kg
	Calcium Carbonate	13.1 g/kg
	Sodium Chloride	2.6 g/kg
	AIN93 Trace Minerals	1.4 g/kg
	Potassium Citrate	2.5 g/kg
	Potassium Dihydrogen Phosphate	6.9 g/kg
	Potassium Sulphate	1.6 g/kg
	Choline Chloride (75%)	2.5 g/kg
	AIN93 Vitamins	10 g/kg
	Antioxidant (Oxicap E2)	0.04 g/kg
Calculated Nutritional Parameters	Protein	19.40%
	Total Fat	36.00%
	Crude Fibre	4.70%
	AD Fibre	4.70%
	Digestible Energy	22.8 MJ/kg
	% Total calculated digestible energy from lipids	59.00%
	% Total calculated digestible energy from protein	15.00%
Calculated Amino Acids	Valine	1.30%
	Leucine	1.80%
	Isoleucine	0.90%

	Threonine	0.80%
	Methionine	0.80%
	Cystine	0.06%
	Lysine	1.50%
	Phenylalanine	1.00%
	Tyrosine	1.00%
	Tryptophan	0.30%
	Histidine	0.60%
	Isoleucine	0.90%
	Threonine	0.80%
Calculated Total Minerals	Calcium	0.46%
	Phosphorous	0.32%
	Magnesium	0.09%
	Sodium	0.12%
	Chloride	0.16%
	Potassium	0.40%
	Sulphur	0.20%
	Iron	72 mg/kg
	Copper	7.0 mg/kg
	Iodine	0.2 mg/kg
	Manganese	18 mg/kg
	Cobalt	No data
	Zinc	51 mg/kg
	Molybdenum	0.15 mg/kg
	Selenium	0.3 mg/kg
	Cadmium	No data
	Chromium	1.0 mg/kg
Fluoride	1.0 mg/kg	

2.8.2 Jelly-dosing treatment and placebo protocol

Ten mice of the lean control group were fed standard chow pellets containing 5% fat, while the other 70 mice were randomised into seven groups (n = 10 per group) and fed a HFHC diet to induce obesity and associated metabolic abnormalities, such as diabetes and hypertension, for eight weeks. After the induced period, ten mice continued HFHC diet (HFHC negative control group) for another eight weeks, while the remaining 60 mice were randomised into six groups and each group was treated with one of the six dietary supplements for another eight weeks. See Figure 2.4 for the treatment regime used in the animal study.

All the supplements were formed into a powder. Blueberry and C3G were supplemented into HFHC groups at 6.4 and 0.02 g/kg body weight/day, respectively (DeFuria et al. 2009; Liu et al. 2014). Yoghurt and peptides extracted from yoghurt were supplemented at 3 and 0.2 g/kg body weight/day, respectively (Rodriguez-Figueroa et al. 2013). All the supplement powders were mixed with gelatine (Coles supermarket, Deer Park, VIC, Australia) to ensure the palatability. To keep consistency, mice in the lean and HFHC control groups were also provided jelly without dietary supplementation. Dietary powders and gelatine dissolved into sterile water to make jelly, and the same amount of fructose was added into jelly. The jelly was cut into small pieces and weighed before providing to animals. The amount of jelly was calculated according to the body weight of each animal. All animals were trialled for jelly feeding at the beginning of week nine prior to the commencement of designed treatments for eight weeks. Table 2.7 presents the calculations of treatment dosage and weight and volume of jelly.

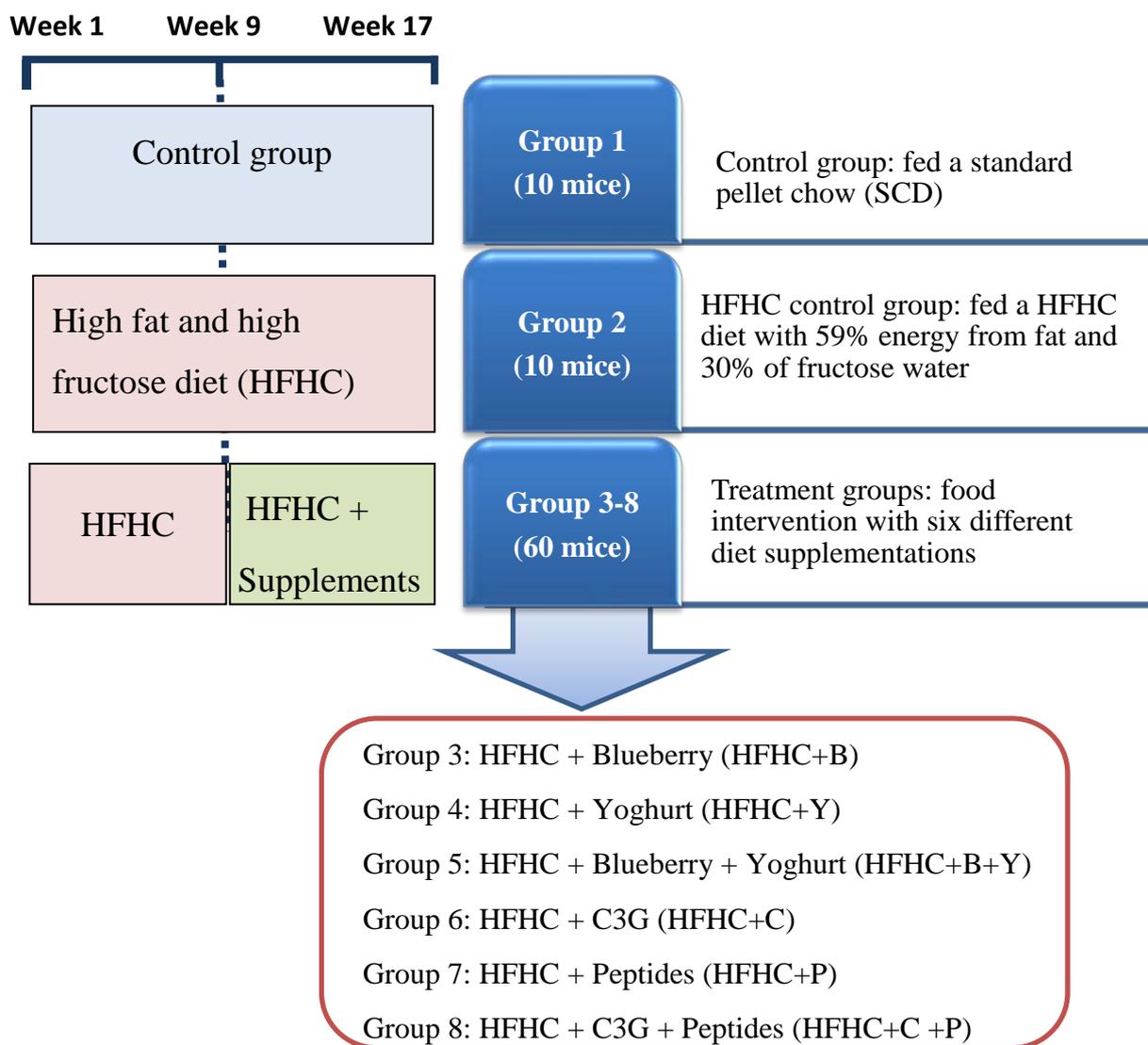


Figure 2.4: The treatment regime of animal study (Chapter 7) to determine the effect of dietary supplementations on attenuating obesity, diabetes and hypertension in a C57BL/6 mice model.

Table 2.7: The formulation of treatments with jelly

Group name	Supplements	Water (mL)	Gelatin e (g)	Fructose (g)	Dietary powder (g)	Amount of jelly/mouse/day (mg)
Control group (SCD)	None	18.5	1	0.5	0	BW* x 20
HFHC group (HFHC control group)	None	18.5	1	0.5	0	BW x 20
HFHC+B	Blueberry	18.5	1	0.5	6.4	BW x 26.4
HFHC+Y	Yoghurt	18.5	1	0.5	3	BW x 23
HFHC+B+Y	Blueberry + Yoghurt	18.5	1	0.5	6.4 g of Blueberry + 3 g of Yoghurt	BW x 29.4
HFHC+C	C3G	18.5	1	0.5	0.02	BW x 20.02
HFHC+P	Peptides	18.5	1	0.5	0.2	BW x 20.2
HFHC+C+P	C3G + Peptides	18.5	1	0.5	0.02 g of C3G + 0.2 g of Peptides	BW x 20.22

BW*: body weight

2.8.3 Food intake, water consumption and body weight

For the calculation of food intake, food was weighed before giving to mice, then the remaining pellets were weighed both the top (where food was presented daily) and inside of the cage for any remaining food that had not been consumed. The way of identifying water consumption was similar with food intake. Normal water or 30% fructose water including water bottle, was weighed before given to the animals, then the remaining liquid including water bottle was weighed to calculate water consumption. Bodyweight was measured by placing the animal in a bucket and weighed on the scale (Sartorius Practum3102-1S digital scale). Normal chow or high fat feed and water or fructose water changed weekly. Jelly and treatments were provided on daily basis. Food intake, water consumption and body weight were monitored twice per week and a weekly average for both measurements was calculated.

2.8.4 Echo magnetic resonance imaging (MRI)

In order to differentiate between lean tissue and fat mass in this study, Echo MRI™ system (Echo-MRI™ 900, Houston, TX, the United States of America) was utilised to determine the body composition of the mice. Echo MRI is a validated method to obtain body composition in rats and mice (Taicher et al. 2003). Echo MRI is a non-invasive method used to measure body composition of live mice and this procedure does not required anaesthesia. This involved calibrating the machine using canola oil. Animals were placed into a plastic tube (containing holes for breathing) and scanned three times in the Echo MRI to determine the body composition. Mice were acclimatised to this procedure at week one and week five, and then measurements were taken at week nine (baseline), week 13 (mid-treatment) and then at week 17 (end of the treatment). See Figure 2.5 for details on Echo MRI measurements over the treatment period.

2.8.5 Blood pressure

The systolic and diastolic blood pressure of animal were measured every four weeks using the CODA 8-Channel High Throughput Non-Invasive Blood Pressure system (Kent Scientific Corporation, Torrington, CT, the United States of America) at week one, five, nine (baseline), thirteen (mid-treatment) and seventeen (end of the treatment). The CODA tail-cuff system uses Volume Pressure Recording (VPR) to measure the blood pressure by determining the tail blood volume. A specially designed differential pressure transducer and an occlusion tail-cuff measured the total blood volume in the tail without the need to obtain the individual pulse signal. Special attention was afforded to the length of the occlusion cuff in order to derive the most accurate blood pressure readings. VPR can easily obtain readings on dark-skinned rodents, such as C57BL6 mice and is MRI compatible. The CODA system provides the measurements with six different blood pressure parameters, including systolic

and diastolic blood pressure, heart rate, mean blood pressure, tail blood flow, and tail blood volume. Measurements can be made on either awake or anesthetized mouse.

Before the actual test, mice were placed in the holder for 15 minutes for two consecutive days for acclimatization. A total of four mice were measured simultaneously. Specifically, each mouse was placed into a holder by picking up the animal by the tail and gently placing it into the rear of the holder which faces the open end of the nose cone. The rear hatch of the holder was carefully secured by turning the black screw cap on the rear hatch. During this process, pinching the tail or any other body parts while securing the rear hatch should be avoided. In order to limit the movement of the animal, the nose cone was slid toward the rear hatch, and the nose cone should be in a position to limit the animal from turning around while inside the holder. The incubator was used to warm the tail vein to increase circulation. The holder was placed into the incubator for 5 minutes at 32 °C for acclimation to the holder, once the animal was put into the holder. The mouse tail was threaded through the Occlusion Cuff and VPR Cuff, which were placed as close to the base of the tail as possible without force. The “Occlusion Cuff Tubing” was secured in the notch on the top rear of the holder, followed by attaching the cuffs to the CODA Controller. Twenty cycles were run by the software to ensure consistency and accuracy of the blood pressure measurements for 7 minutes. Data were analysed using CODA software. Mice were immediately removed from the cuffs and holders, and returned to the cage. See Figure 2.5 for details on the blood pressure measurement over the treatment period.

2.8.6 Measurement of metabolic variables

Metabolic variables including oxygen consumption (VO_2), carbon dioxide consumption (VCO_2), energy expenditure, respiratory quotient (VO_2/ VCO_2), movement and rest time were measured using the Promethion parallel continuous system (Sable Systems

International, North Las Vegas, NV, the United States of America), in which each cage is paired with its own flow generator and gas analyser to record changes in metabolic parameters. The Promethion system allows the measurement of precise food and water intake and access, wheel and ambulatory activity and the relative respirometry parameters, respiratory quotient, body weight, oxygen consumption, carbon dioxide release and water loss (Nishiyama et al. 2004). Prior to the use of the Promethion system, all sensors were calibrated, including the sensors of weight, oxygen, carbon dioxide and locomotion. Mice were placed into Promethion cages (one per cage), followed by connecting each cage with its sensor and setting up the parameters in the software. Data were recorded for 24 hours continuously. The metabolic variables for each mouse were measured every four weeks, in which the baseline was at week nine, mid-treatment was week thirteen and end of the treatment was week seventeen. Once data collection was completed, the mice were placed back to their home cages and monitored for 2 hours to ensure they were not stressed. See Figure 2.5 for details on the measurements of energy expenditure and locomotion obtained over the treatment period.

2.8.7 Intraperitoneal glucose tolerance test (ipGTT)

The glucose tolerance test is used to detect disturbances in glucose metabolism thus the diagnosis of diabetes (Bowe et al. 2014; Association 2015). In rodent research, this test is the most widely used test to determine whether a dietary-induced obese mouse has glucose intolerance/insulin resistance and diabetes (Furuyama et al. 2003; Andrikopoulos et al. 2008; Kousteni 2012). An ipGTT is usually performed to evaluate glucose tolerance and determine the effect of a glucose load on blood glucose concentrations over the treatment period. In this study, ipGTT was conducted at week nine (baseline) and week seventeen (towards the end of the treatment). Fructose water was replaced by normal water in HFHC control group and all

the treatment groups and then mice were fasted overnight for approximately 12 – 13 hours, with free access to water. A tail snip was performed and a drop of blood was gently obtained and dripped to a blood glucose strip (FreeStyle Optium Neo H, Abbott, USA) for the measurement of the baseline blood glucose concentration using a glucometer (FreeStyle Optium Neo H, Abbott, USA). Following this the mouse was injected with a dose of 1.5 g/kg body weight of glucose in 0.9% of sterile saline solution (Mcfarlane Medical, Surry Hills, Victoria, Australia). The solution was made under sterile conditions. For calculations, a 15% working solution was used, and the dosage (μL) administered. For example, if the body weight of a mouse was 30 g, then the amount of injected glucose was 0.045 g, thus the dosage of 15% glucose solution was 300 μL . A 1 mL insulin syringe with 27 gauge needle (McFarelene Medical, Surry Hills, Victoria, Australia) was utilised, due to the larger volume and viscosity of the liquid administered. Before snips, Eutectic mixture of local anaesthetic (EMLA) cream (2.5% lidocaine/ 2.5% prilocaine) was painted on the whole tail of the mouse. Following the delivery of the desired glucose load, subsequent blood glucose readings were taken by carefully brushing the scab of the previously performed tail snip to draw a droplet of blood.

The blood glucose concentrations were measured at baseline (0 minute), 15, 30, 45, 60, 90 and 120 min. Once this procedure was completed the mice were then given access to food and water again. See Figure 2.5 for details on ipGTT measurements obtained over the treatment period.

2.8.8 Blood collection

Following the intervention period, mice were deeply anaesthetised. Blood was immediately collected via cardiac puncture using an twenty three gauge needle (to help reduce red blood cell lysis, Mcfarlane Medical, Surry Hills, Victoria, Australia) and a 1 mL syringe (Mcfarlane

Medical, Surry Hills, Victoria, Australia), then transferred into 1.5 mL Eppendorf tube with ethylenediaminetetraacetic acid (EDTA) powder (2 µg, Sigma-Aldrich Pty. Ltd., Castle Hill, NSW, Australia), and subsequently placed on ice. Following this the blood tube was centrifuged at 4 °C and 4000 x g for 10 minutes to separate the plasma from the blood cells. The plasma layer was carefully aliquoted into 600 µL Eppendorf tubes (each tube with 50 µL of plasma) and stored at – 80 °C for further analysis. An aliquot of the lower layer containing red blood cells was also stored at – 80 °C for future analysis of inflammatory cytokines which was not within the scope of this thesis.

2.8.9 Anaesthesia and tissue collection

To determine the effects that the pharmacological compounds had on a number of organs involved in glucose metabolism, blood and a number of other tissues were collected for future analysis. When mice were deeply anaesthetised using isoflurane, skeletal muscle including soleus, tibialis anterior (TA) and extensor digitorum longus (EDL) were surgically collected, immediately following this while mice remained deeply anaesthetised, they were sacrificed via a cardiac puncture. Sequentially, heart, epididymal fat, mesenteric fat, liver and kidney were weighed and collected in cryotubes (Thermo Fisher Scientific, Wilmington, DE). Others adipose tissues, such as mesenteric white adipose tissue, omental depot and interscapular brown adipose tissues were also collected into cryotubes and immediately frozen in liquid nitrogen for RNA analysis.

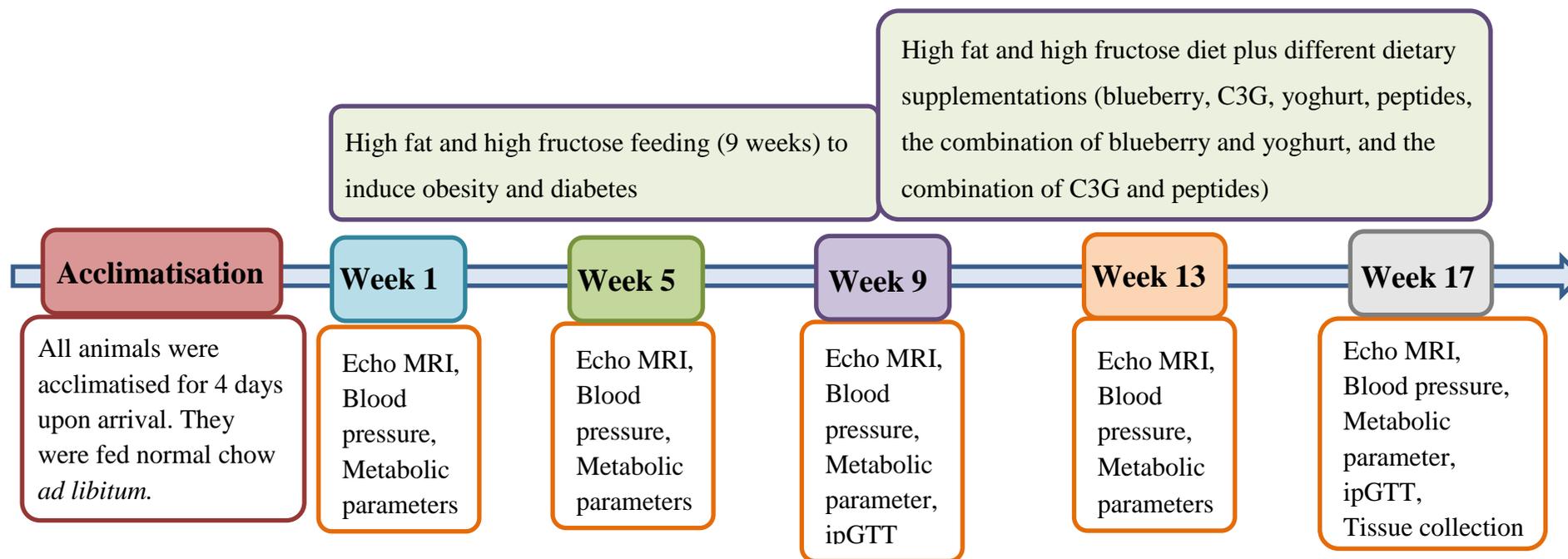


Figure 2.5: Time points of the measurements taken during the 17-week treatment period for animal study focusing on the effect of dietary supplementation on whole body energy homeostasis in a C57BL/6 mouse model (Chapter 6).

"This is the peer reviewed version of the following article: Shi, M., Ahtesh, F., Mathai, M., McAinch, A.J. and Su, X.Q. (2017), Effects of fermentation conditions on the potential anti-hypertensive peptides released from yogurt fermented by Lactobacillus helveticus and Flavourzyme®. Int J Food Sci Technol, 52: 137-145, which has been published in final form at <https://doi.org/10.1111/ijfs.13253>. This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Use of Self-Archived Versions."

CHAPTER 3: Antihypertensive activities of peptide from reconstituted yoghurt with *L. helveticus*

This chapter has been published, see Appendix 2: **Effects of fermentation conditions on the potential antihypertensive peptides released from yoghurt fermented by *Lactobacillus helveticus* and Flavourzyme®** by Min Shi, Fatah Ahtesh, Michael Mathai, Andrew J. McAinch & Xiao Q. Su (2017) in the peer review journal, International Journal of Food Science and Technology, 52, 137–145. The content of this chapter is the same as the published paper (Appendix 3), and only the format has been changed here to match with the thesis.

3.1 Summary

This study investigated the effects of fermentation conditions on the production of angiotensin-converting enzyme inhibitory (ACE-I) peptides in yoghurt by *Lactobacillus helveticus* 881315 (*L. helveticus*) in the presence or absence of Flavourzyme®, which is derived from a mold, *Aspergillus oryzae* and used for protein hydrolysis in various industrial applications. Optimal conditions for peptides with the highest ACE-I activity were 4% (v/w) inoculum size for 8 h without Flavourzyme® supplementation, and 1% inoculum size for 12 h when combined with Flavourzyme®. The yoghurt fermented by *L. helveticus* resulted in IC₅₀ values (concentration of inhibitor required to inhibit 50% of ACE activity under the assayed conditions) of 1.47 ± 0.04 and 16.91 ± 0.25 mg/mL with and without Flavourzyme®, respectively. Seven fractions of ACE-I peptides from the yoghurt incorporated with *L. helveticus* and Flavourzyme® were separated using the preparative high-performance liquid chromatography. Fraction (F3) showed the highest ACE-I activity with an IC₅₀ of $35.75 \pm$

5.48 µg/mL. This study indicates that yoghurt may be a valuable source of ACE-I peptides, which may explain the outcomes observed in the experimental and clinical studies and foresee the application of fermented milk proteins into functional foods or dietary supplements.

Keywords

Yoghurt, Peptides, Angiotensin-converting enzyme, Flavourzyme[®], *Lactobacillus helveticus*

3.2 Introduction

Yoghurt is generally made from milk by mixed cultures, including two types of homo-fermentative bacteria, *Streptococcus thermophilus* (*S. thermophilus*) and *Lactobacillus delbrueckii* subsp. *bulgaricus* (*L. bulgaricus*) (Radke-Mitchell and Sandine 1986; De Brabandere, A. G and De Baerdemaeker, J. G 1999). *Lactobacillus helveticus* (*L. helveticus*), is a lactic acid bacterium (LAB) and is used for the production of fermented milk beverages and some types of hard cheeses (Griffiths and Tellez 2013). In previous studies, fermented milk with *L. helveticus* exhibited anti-hypertensive effect in both animal and clinical studies due to its angiotensin-converting enzyme inhibitory (ACE-I) activity (Aihara et al. 2005; Jauhiainen et al. 2005; Griffiths and Tellez 2013; Chen, Y. et al. 2014). Angiotensin-converting enzyme (ACE), as a part of the renin-angiotensin system, has an important role in the regulation of blood pressure by converting angiotensin-I into a potent vasoconstrictor, angiotensin-II. Angiotensin-II induces the release of aldosterone and therefore increases the sodium retention and blood pressure (Muro Urista et al. 2011). Thus, inhibition of ACE can lower the blood pressure, and has potential health benefits to hypertensive patients (Tuomilehto et al. 2004; Aihara et al. 2005). The renin-angiotensin system has therefore become a key target for anti-hypertensive drugs (Miura et al. 2011), however, conventional

drugs targeting this system cause various side effects, such as headache, dizziness and cough (Coulter D 1987; Soleimani et al. 2015).

Anti-hypertensive peptides isolated from fermented dairy products such as fermented milk drink and cheese could represent a healthier and natural alternative for the ACE-I drugs as a non-pharmacological therapy to reduce the risk of hypertension (Minervini et al. 2003; Hannu and Anne 2006; Donkor et al. 2007b; Pan and Guo 2010). Previous studies showed that two tripeptides [Val-Pro-Pro (VPP), Ile-Pro-Pro (IPP)] released from fermented milk by several types of *L. helveticus* strains exhibited anti-hypertensive peptide effects due to high ACE-I activity in both human (Tuomilehto et al. 2004; Ishida et al. 2011b; Cicero et al. 2013) and rats (Jauhiainen et al. 2010a; Chen, Y. et al. 2014). However, little information is available on whether yoghurt is a better source of ACE-I peptides when it is further fermented by *L. helveticus* strains.

During yoghurt fermentation, bacteria growth and product synthesis depend on medium compositions and culture conditions such as temperature, pH, inoculum size and fermentation duration (Agyei et al. 2012). Inoculum size was an important factor for the growth rate of the bacterial culture. When the inoculum size is too low, it would take longer for bacteria to reach the logarithmic growth phase (Min et al. 2013). In contrast, a greater inoculum size could result in a quick consumption of nutrients required by bacterial strains, thus fermentation could be interrupted (Min et al. 2013). The optimisation of various fermentation parameters for maximising active peptide production by LAB is therefore a major research endeavour. Supplementation of some enzymes with strong proteolytic activity is an alternative method to improve functional peptide production during yoghurt fermentation (Donkor et al. 2007a; Shabboo and Ahmad 2011). One such enzyme, Flavourzyme® is an

important contributor to hydrolyse milk protein for the production of ACE-I peptides (Tsai et al. 2008).

Flavourzyme[®] contains enzymes that are generally recognized as safe for use in the food industry (Boschin et al. 2014). It is derived from a mold *Aspergillus oryzae* and used for protein hydrolysis in various industrial and research applications due to its high endoprotease and exopeptidase activities (Merz et al. 2015). Ahtesh *et al.* (2016 a, b) reported that Flavourzyme[®] can be used to increase the hydrolysis of the milk protein into further small molecular weight ACE-I peptides in 12% of reconstituted skim milk (RSM). However, the role of Flavourzyme[®] in the production of bioactive peptides from yoghurt is not well reported. Therefore, the aim of this study was to determine the optimal fermentation conditions for the production of ACE-I peptides from yoghurt fermented by *Lactobacillus* strains. Furthermore, we investigated whether the inclusion of Flavourzyme[®] is able to increase the release of ACE-I peptides in yoghurt media.

3.3 Material and methods

3.3.1 Culture medium and reagents

Glycerol de Man, Rogosa, Sharpe (MRS) broth was purchased from Oxoid, Ltd., West Heidelberg, Victoria, Australia. Reconstituted skim milk (RSM) powder (52% lactose, 37% protein, 8.6% ash, and 1.2% fat) was obtained from Woolworths Ltd, Australia. M17 broth was purchased from Oxoid, Ltd, Hampshire, England. Flavourzyme[®] (EC 3.4.11.1, an amino peptidase with an activity of 500 Leucine Amino-peptidase per gram), hippuryl-L-histidyl-L-leucine (HHL), ACE enzyme (from rabbit lung, 0.1 UN) and hippuric acid (HA) were purchased from Sigma-Aldrich Pty. Ltd., Melbourne VIC, Australia.

3.3.2 Bacteria storage and propagation

Lactobacillus helveticus ASCC 881315 (*L. helveticus*), *Streptococcus thermophiles* ASCC 1275 (*S. thermophiles*) and *Lactobacillus delbrueckii* subsp. *bulgaricus* 1466 (*L. bulgaricus*) were obtained from Dairy Innovation Australia Ltd, Werribee, Victoria, Australia. The *L. helveticus* strain was stored in MRS broth, while *S. thermophiles* and *L. bulgaricus* strains were kept in RSM at $-80\text{ }^{\circ}\text{C}$. For activation of these strains, $100\text{ }\mu\text{L}$ of *L. helveticus* and *L. bulgaricus* strains were transferred separately into 9.9 mL of 40% MRS broth, whereas *S. thermophiles* was activated via transfer of $100\text{ }\mu\text{L}$ to 9.9 mL of 3.7% M17 broth, containing 0.5% lactose. All media were autoclaved at $121\text{ }^{\circ}\text{C}$ for 15 minutes before use. Following activation, all strains were incubated at $37\text{ }^{\circ}\text{C}$ for 24 h.

3.3.3 Yoghurt preparation

Yoghurt was prepared by dissolving skim milk powder (12%, wt/ wt) in distilled water. RSM was heated to $90\text{ }^{\circ}\text{C}$ for 30 min, then inoculated with 1% activated *S. thermophiles* and *L. bulgaricus* cultures (1: 1 of ratio) and fermented at $42\text{ }^{\circ}\text{C}$ for 6 hours to produce yoghurt. Then the fermentation process was stopped by cooling at $4\text{ }^{\circ}\text{C}$ overnight.

3.3.4 Release of bioactive peptides from yoghurt

L. helveticus 881315 strain was incorporated into yoghurt with or without Flavourzyme[®] (0.14%). Specifically, the yoghurt was re-incubated at $37\text{ }^{\circ}\text{C}$ with stirring (200 rpm) for the purpose of a better homogenization between yoghurt and Flavourzyme[®] during 16 h. After incubation for 4, 8, 12 and 16 h, with different inoculum sizes of *L. helveticus* (1%, 2%, 3% and 4% vol/wt), samples were taken for analysis. The fermentation process was terminated by heating the yoghurt at $90\text{ }^{\circ}\text{C}$ for 20 min to stop enzyme activity. Subsequently, 25 mL of yoghurt sample was centrifuged at $4000 \times g$ at $4\text{ }^{\circ}\text{C}$ for 30 min to separate proteins. The

supernatant containing soluble peptides was freeze-dried (Freeze-drier, John Morris Scientific Pty Ltd, Melbourne VIC, Australia) for 72 h. The freeze-dried peptides powder was stored at -20 °C for analysis.

3.3.5 Determination of ACE-Inhibitory activity

20 mg of the freeze-dried peptide powder from normal yoghurt with starter culture or yoghurt incorporated with *L. helveticus* without Flavourzyme[®] was dissolved in 1 mL of Tris buffer (50 mM, pH 8.3) containing 300 mM sodium chloride, respectively. However for the peptides from yoghurt incorporated with *L. helveticus* and Flavourzyme[®], 2.5 mg/mL of powder was dissolved in the same Tris buffer, as these peptides showed 100% ACE-I activity, when the concentration was 20 mg/mL; therefore, lower concentration (2.5 mg/mL) was used to assess the production of ACE-I peptides under different fermentation conditions.

To determine the peptide fractions from yoghurt in the presence of Flavourzyme[®], freeze-dried powder (2.5 mg) was dissolved in Tris buffer (50 mM, pH 8.3) containing 300 mM sodium chloride to prepare the sample solutions of 62.5, 125, 250, and 500 µg/mL concentrations to calculate IC₅₀ value of different fractions. The IC₅₀ value was defined as the concentration of inhibitor required to inhibit 50% of ACE activity under the assayed conditions.

Evaluation of ACE-I activity was assayed using a reversed-phase HPLC system (RP-HPLC, from Varian Analytical Instruments, Santa Clara, CA, USA) previously described by Donkor et al (2007a). It is based on the hydrolysis HHL by ACE to hippuric acid (HA) and histidyl-leucine (HL) as products. The HA released from HHL is directly related to the ACE activity. ACE enzyme and HHL was prepared in Tris buffer. Briefly, the assay consisted of 300 µL of 3.0 mM HHL, 300 µL of 3.0 mU ACE enzyme, and 300 µL of peptide solution. The mixture was placed in a glass tube and then incubated at 37 °C in a water bath for 0.5 h, mixed for 1

min, then returned to the water bath for another 0.5 h. The reaction was stopped by heating the mixture in an 85 °C water bath for 10 min in order to inactivate enzymes. The reaction mixture was stored at -20 °C before further analysis of released hippuric acid by RP- HPLC.

HA standard curve was prepared in five different concentrations (5, 10, 15, 20 and 25 µg/mL). The isocratic mobile phase composition was optimized to 12.5% acetonitrile (Merck, Bayswater, VIC, Australia) in MilliQ water (vol/vol) containing 0.1% trifluoroacetic acid (TFA), which was filtered (0.45 µm) prior to running through the column. The temperature of the column was kept at room temperature (~22 °C). An aliquot (10 µL) of the mixture was injected onto a VyDAC® C18 300 Å (250 mm x 4.6 mm, 5 µm) column (Grace Vydac, Hesperia CA, USA) using a Varian HPLC equipped with an auto sampler. The flow rate was set at 0.6 mL/min. The quantitative estimation of HA present was determined using a calibration curve of standard HA using a UV detector set at 228 nm.

The percent of ACE inhibition was calculated as follows:

$$\text{ACE inhibition (\%)} = \frac{C(\text{control}) - C(\text{sample})}{C(\text{control})} \times 100$$

Where: C (control) was the concentration of HA without the tested samples (active peptides), which contained 300 µL of HHL, 300 µL of ACE enzyme and 300 µL of Tris buffer, and C (sample) was the concentration of HA with the tested samples, which contained 300 µL of HHL, 300 µL of ACE enzyme and 300 µL of peptide solution.

3.3.6 Peptide profile of water-soluble extract

The RP-HPLC assay was developed for the profile of water-soluble peptides extracted from yoghurt as control, and yoghurt containing *L. helveticus* strain with or without Flavourzyme®, respectively (Taruscio et al. 2004). The freeze-dried peptide powder (20 mg) was dissolved in

1 mL of 0.1% TFA in distilled water. All the supernatants thus obtained were filtered through a 0.45 µm membrane filter and stored at -20 °C until assayed. Water soluble peptides were profiled by a RP- HPLC (SHIMADZU Corporation, Nakagyo-ku, Kyoto, Japan) using C-18 monomeric column (5 µm, 300 Å, 250 mm x 4.6 mm; Grace Vydac, Hesperia CA, USA). The injection volume was 10 µL. Solvent A was a mixture of water with 0.1% TFA (vol/vol) and solvent B contained acetonitrile with 0.1% TFA (vol/vol). Active peptides were eluted with a linear gradient of solvents B in A at concentrations from 0 to 100% over 30 min at a flow rate of 0.75 mL/min. The elution profile was monitored at 215 nm by UV-Vis detector at room temperature (~22 °C).

3.3.7 Peptide fractions

Bioactive peptide fractions from yoghurt with *L. helveticus* and Flavourzyme® were collected using a column (Prep Nova-Pack HR C18, 60 Å, 250 mm x 10 mm, 10 µm, Phenomenex, Pty Ltd, Lane Cove, NSW, Australia) by preparative RP-HPLC. Solvent A was a mixture of water and TFA (1000:1, v/v), and solvent B contained acetonitrile and TFA (1000:1, v/v). The injection volume was 1 mL with 20 mg/mL of yoghurt peptides. The peptides were eluted with a linear gradient of solvent B in A ranged from 0% to 40% over 90 min, at a flow rate 4 mL/min. Detection was carried out at 215 nm by UV-Vis detector. According to the retention time of peaks, 7 fractions (F1-F7) were collected, respectively. All fractions were frozen dried under vacuum. IC₅₀ of ACE activity was determined for each fraction.

3.3.8 Statistical analysis

All results are expressed as mean ± standard deviation for each measurement (n = 3), including yoghurt fermentation, the pH, determination of ACE-I activity and calculation of the IC₅₀ value. The yoghurt fermentation was carried out on two separate occasions in accordance with the study design. The first phase of the study design was focused on the four

fermentation times (4, 8, 12 and 16 h) with 1% inoculum sizes of *L. helveticus*. The second phase was performed with different inoculum sizes of *L. helveticus* (1%, 2%, 3% and 4% v/w), after the optimal fermentation time was determined. Two-way ANOVA was performed using software SPSS version 22 (IBM Chicago, IL, USA) to analyse the significant differences in the treatments, which were fermentation time, inoculum sizes and presence or absence of Flavourzyme®. Fisher's (least significant difference; LSD) test was used to differentiate significant differences among the treatments. $P < 0.05$ was considered as significant.

3.4 Results and discussion

3.4.1 Effects of *L. helveticus* strains and Flavourzyme® on the pH of yoghurt

The pH of yoghurt containing *L. helveticus* strain with or without Flavourzyme® with different fermentation time and inoculation volume is shown in Figure 3.1. The pH of yoghurt fermented by *S. thermophiles* and *L. bulgaricus* was 4.71 ± 0.04 . The pH was decreased to 4.45 ± 0.04 when *L. helveticus* strains were incorporated alone into the yoghurt at 37 °C for 16 h ($P < 0.05$; Figure 3.1A). The pH was further decreased to 4.06 ± 0.03 when Flavourzyme® and *L. helveticus* strains were added in the yoghurt for 16 h of fermentation ($P < 0.01$; Figure 3.1 A). The decrease of the pH could be due to the production of lactic acid by lactic acid bacteria strains (Donkor et al. 2007a). The pH of yoghurt in the presence of Flavourzyme® was significantly lower than that without Flavourzyme® for the same fermentation time ($P < 0.01$; Figure 3.1A). The presence of Flavourzyme® could result in higher proteolysis and the release of more peptides, which was further supported by the experimental data on the fermentation time and peptides of ACE-I due to further hydrolysis of milk protein by Flavourzyme® to small size molecular weight of peptides (Fatah et al. 2016a; Fatah et al. 2016b). Previous research has demonstrated the same trends using these *L.*

helveticus strains in the 12 h fermentation of skim milk and the pH of the fermented product was 3.4 when Flavourzyme[®] was added compared with 5.0 without addition of Flavourzyme[®] (Fatah et al. 2016b). In the same study, it was also reported that the growth of all strains of *L. helveticus*, when mixed with Flavourzyme[®], was increased significantly at 8 h and declined after 8 h of fermentation at pH 3.4. This was possibly due to the low pH and heat treatment reducing available nutrients for growth (Dissanayake et al. 2013; Fatah et al. 2016b). The reason for the higher pH (4.15) in the present study may be attributed to the type of media. In this study, yoghurt was used as a media while RSM was used in the study by Ahtesh *et al* (2016b).

In the fermentation of yoghurt, the pH decreases steadily in correspondence with the milk acidification (two homofermentative bacteria transform fermented (milk) sugar into lactic acid) that underlies the fermentation process (De Brabandere, A.G and De Baerdemaeker, J.G. 1999). Many factors including starter culture, heat treatment, fermentation time, inoculum size and incubation temperature, affect the pH in yoghurt fermentation. In this study, the decrease in pH was observed in yoghurt combined with different inoculum sizes of *L. helveticus* ($P < 0.01$; Figure 3.1B). After fermentation, the pH of yoghurt decreased to 3.91 ± 0.04 without Flavourzyme[®], and 4.00 ± 0.03 with Flavourzyme[®] ($P < 0.05$; Figure 3.1B). The pH was lower with 1% inoculum of *L. helveticus* with Flavourzyme[®] than those without Flavourzyme[®] in the same inoculum size of *L. helveticus* ($P < 0.01$; Figure 3.1B). Then there were no significant changes with the increase of inoculum size of *L. helveticus* in the presence of Flavourzyme[®]. However, the pH declined with the increase of inoculum size of *L. helveticus* in absence of Flavourzyme[®].

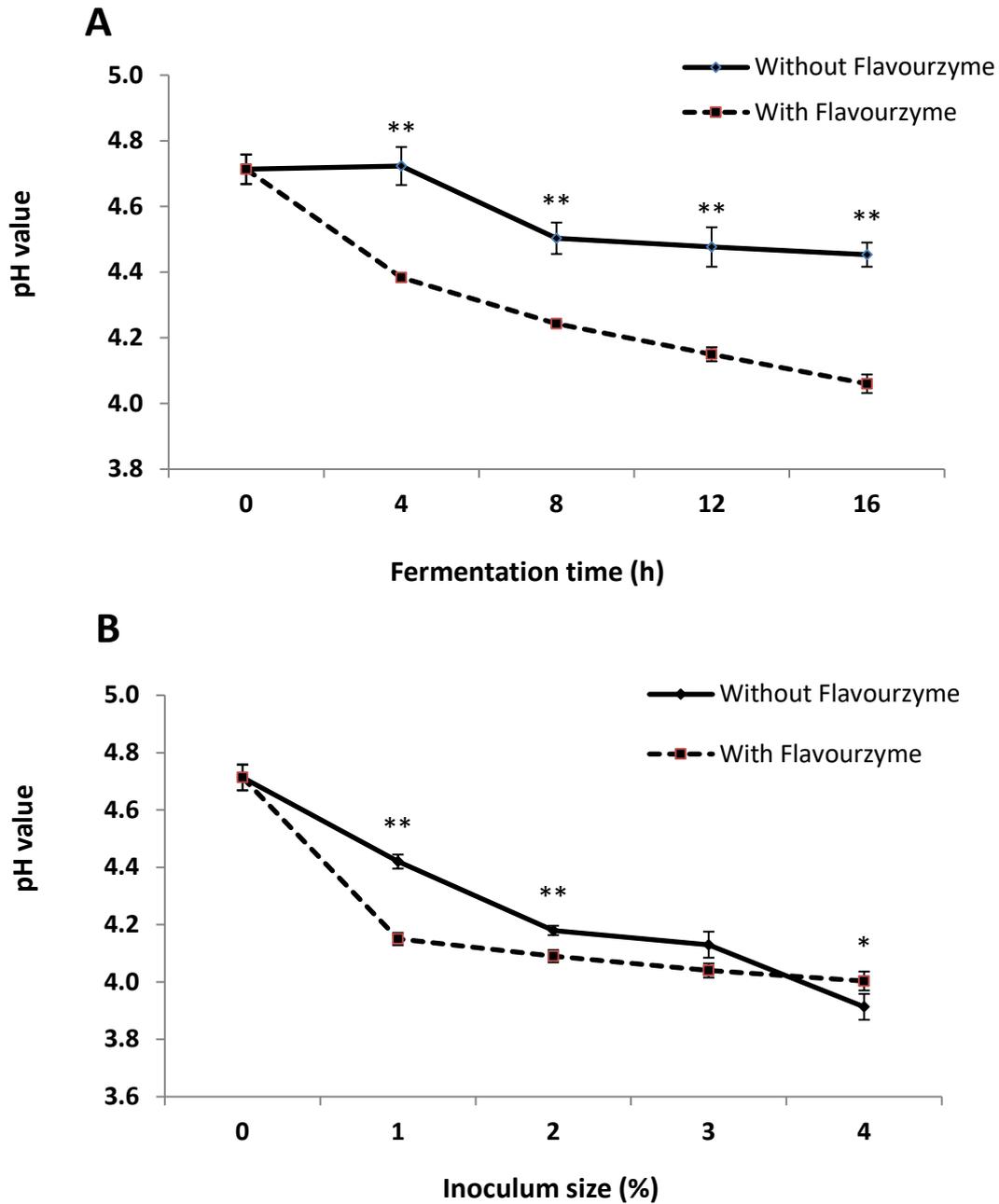


Figure 3.1: pH of yoghurt fermentation with *L. helveticus* during different fermentation times (A) and inoculum sizes (B) in the presence or absence of Flavourzyme®.

Fermentation times and inoculum sizes were varied by each other during the fermentation period. Data are shown in mean \pm standard deviation ($n = 3$). Significant difference between with and without Flavourzyme groups for the same fermentation time and inoculum size are indicated as * for $P < 0.05$ and ** for $P < 0.01$.

3.4.2 Effect of fermentation time on ACE-I activity with or without Flavourzyme®

A time course of peptide production showed that ACE-I activity of peptides was significantly different between the groups with and without Flavourzyme® at the same fermentation time ($P < 0.01$; Figure 3.2A). The results demonstrate that ACE-I activity showed an increasing trend with fermentation time, but this trend was not significant. Specifically, ACE-I activity in the absence of Flavourzyme® group increased significantly from $13.0 \pm 1.8 \%$ to $50.8 \pm 3.3\%$ (20 mg/mL) during 8 h fermentation, then mildly decreased at 12 h fermentation ($46.0 \pm 0.9\%$, $P = 0.12$; Figure 3.2A), but increased again with by 16 h fermentation back to similar levels seen at 8 h fermentation ($51.4 \pm 0.2\%$, $P = 0.81$; Figure 3.2A). These may be associated with the hydrolysis of ACE-I peptides, and the hydrolysed peptides can then be transported across the cell membrane of bacteria by several transporters (Law and Haandrikman 1997). Similar results were reported by Pan & Gao (2010) who showed that the optimal fermentation time to produce ACE-I activity from fermented sour milk was 8 h (Pan and Guo 2010).

A similar trend in ACE-I activity dependence on fermentation time was noted in the presence of Flavourzyme®, compared with the absence of Flavourzyme® (Figure 3.2A). The results showed that in the presence of Flavourzyme®, ACE-I activity increased gradually and reached the highest level in 12 h fermentation ($86.5 \pm 0.5\%$ at concentration of 2.5 mg/mL) ($P < 0.05$; Figure 4.2A). It then decreased after an additional 4 h fermentation ($P < 0.05$; Figure 3.2A). These results suggest that 12 h incubation was the optimal fermentation time to produce anti-hypertensive peptides from yoghurt, in combination with Flavourzyme®. These studies indicate that Flavourzyme® allows reaching high concentrations in ACE-I peptides in a short fermentation time, contrarily to formulation without Flavourzyme®. This was also confirmed by the change of pH levels in the presence or absence of Flavourzyme® ($4.06 \pm$

0.03 and 4.42 ± 0.02 , respectively, Figure 3.1). In agreement with this result, a previous study showed that ACE-I reached the highest level (approximate 70%) at 12 h fermentation of RSM by *L. helveticus* strain 881315 without Flavourzyme[®] addition; however, more than 70% ACE-I activity was obtained after only 4 h fermentation by the same strain with the supplementation of Flavourzyme[®] (Fatah et al. 2016b).

3.4.3 Effect of inoculum size on ACE-I activity with or without Flavourzyme[®]

Figure 3.2B shows the effects of inoculum size of *L. helveticus* strains on ACE-I activity with or without Flavourzyme[®] in yoghurt. Generally, the results showed a significant difference between the groups with and without Flavourzyme[®] in the same level of inoculum size ($P < 0.01$; Figure 3.2B). ACE-I activity increased gradually with increased inoculum size without Flavourzyme[®]. *L. helveticus* strains (4%) in the yoghurt exhibited the highest ACE-I activity, with $59.2 \pm 0.9\%$ at 20 mg/mL of the hydrolysate, for optimal fermentation time of 12 h ($P < 0.05$; Figure 3.2B). It has been shown that 4% of inoculum size produced ACE-I activity in sour milk fermented by *L. helveticus* LB10, possibly due to the high cell-envelope proteinase activity (Pan and Guo 2010), which may have also occurred in the present study.

It was found that, with the same inoculum size, ACE-I can be improved significantly when combined with Flavourzyme[®] ($P < 0.01$; Figure 3.2B). The highest ACE-I (86.5% at concentration of 2.5 mg/mL) was obtained with 1% of *L. helveticus* plus Flavourzyme[®] following a 12 h fermentation ($P < 0.01$; Figure 3.2B). Then, ACE-I activity showed slight decrease with increasing inoculum size. ACE-I activity of peptides reduced to 82% with 4% of *L. helveticus* combined with Flavourzyme[®], however this was still higher than those in the absence of Flavourzyme[®] ($59.17 \pm 0.88\%$).

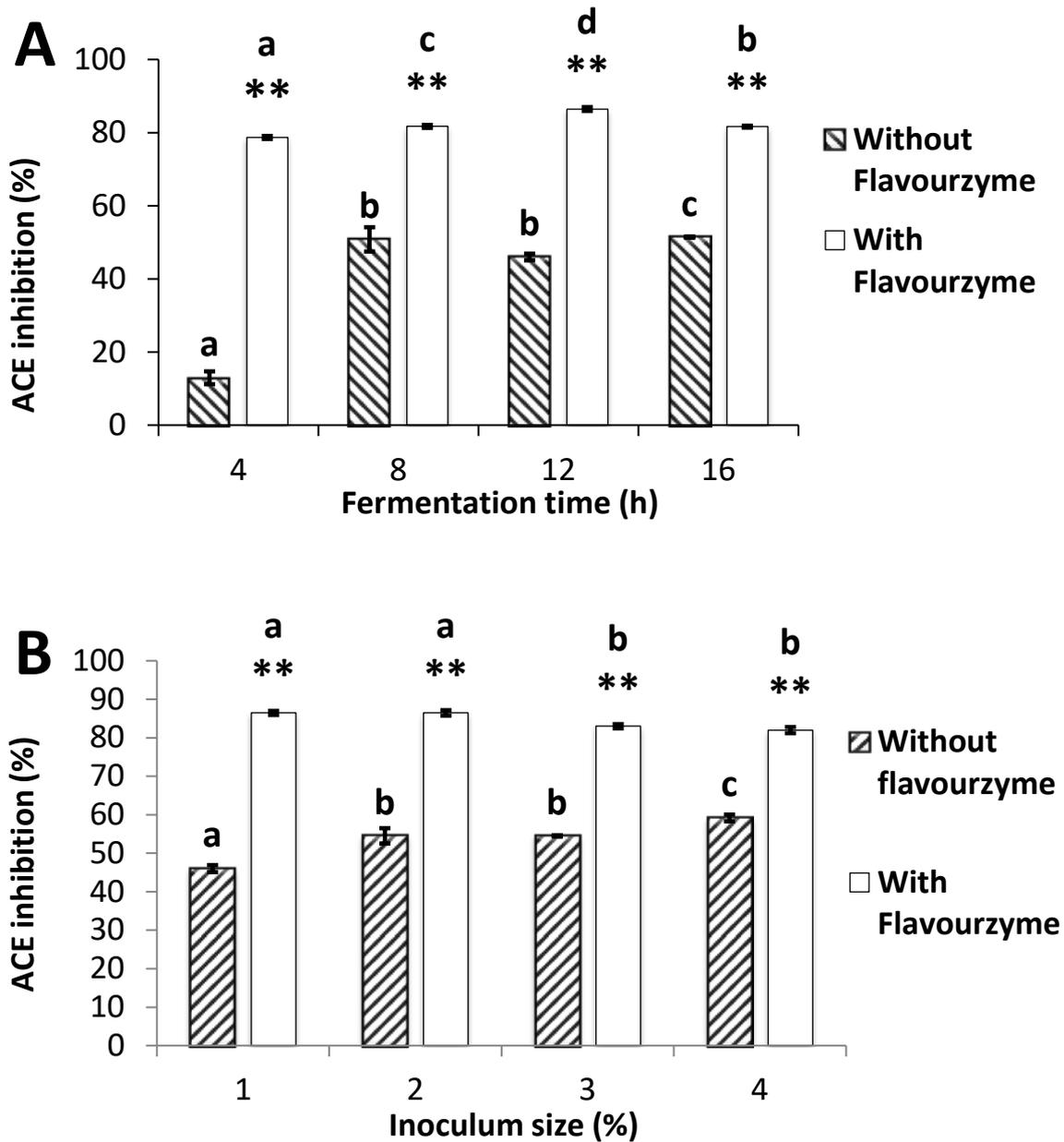


Figure 3.2: ACE inhibition of yoghurt added with *L. helveticus* in different fermentation time (A) and inoculum size (B) in the presence or absence of Flavourzyme®.

The concentrations of peptides from yoghurt with Flavourzyme® was 2.5 mg/mL, and the concentrations of peptides from yoghurt without Flavourzyme® was 20 mg/mL. Data are shown in terms of mean \pm standard deviation ($n = 3$). Different letters (a-d) above the bars indicate significant differences at the same concentration ($P < 0.05$). Significant difference between with and without Flavourzyme® groups for the same fermentation time and inoculum size are indicated as * $P < 0.05$ and ** $P < 0.01$.

3.4.4 ACE-inhibition activity

There were significant differences in ACE-I activity between yoghurt with starter culture and yoghurt with addition of *L. helveticus* and/or Flavourzyme®. Peptides from yoghurt showed the lowest activity of ACE-I, which was only $33.1 \pm 2.7\%$ at the concentration of 20 mg/mL ($P < 0.01$; Figure 3.3). In contrast, ACE-I activity was increased to $59.2 \pm 0.9\%$, when *L. helveticus* strain was added to the yoghurt at 37 °C for 12 h ($P < 0.01$; Figure 3.3). Furthermore, a 100% inhibition was observed when both *L. helveticus* strain and Flavourzyme® were added to the yoghurt (Figure 3.3).

The IC₅₀ value is an important indicator to assess the ACE-I potential. It can be used to classify individual bioactive peptides from different fermentation products based on their ACE-I capacity *in vitro*. In this study, the IC₅₀ value of yoghurt only fermented by starter cultures (30.45 ± 2.32 mg/mL) showed the lowest effect on ACE-I ($P < 0.01$; Figure 3.3). The hydrolysate extracted from yoghurt incorporated with *L. helveticus* strains demonstrated lower IC₅₀ (16.91 ± 0.25 mg/mL), compared with yoghurt without these strains ($P < 0.01$; Figure 3.3), which supported that *L. helveticus* has a potential to hydrolyse protein from yoghurt and produce anti-hypertensive peptides. In yoghurt supplemented with Flavourzyme®, significantly more ACE-I peptides were released with an IC₅₀ of 1.47 ± 0.04 mg/mL, which was 20-fold higher than yoghurt without *L. helveticus* strains, and 12-fold higher than yoghurt without Flavourzyme® ($P < 0.01$; Figure 3.3). In general, the effect of peptides on ACE-I depends on several factors, such as the type of bacteria strains, media used, fermentation conditions and enzymes used. Tsai *et al.* (2008) reported that, in milk fermented by lactic bacteria with Flavourzyme®, more active peptides were produced to exhibit higher ACE-I activity, with an IC₅₀ value of 0.226 mg/mL in Flavourzyme-facilitated fermentation in contrast to an IC₅₀ value of 0.515 mg/mL in the fermentation without

Flavourzyme®. The different media and fermentation condition could possibly contribute to the higher IC₅₀ values found in the study by Tsai et al. (2008), which used the mixture of 4.5% (wt/ vol) skimmed milk powder, 5.5% (wt/ vol) whole milk powder and 7% (wt/ vol) sucrose as a medium that was fermented by 0.1% (wt/ vol) of lactic acid bacteria powder. By contrast, 12% skimmed milk powder was used as a medium and it was fermented with 1% of activated lactic acid bacteria in the current study.

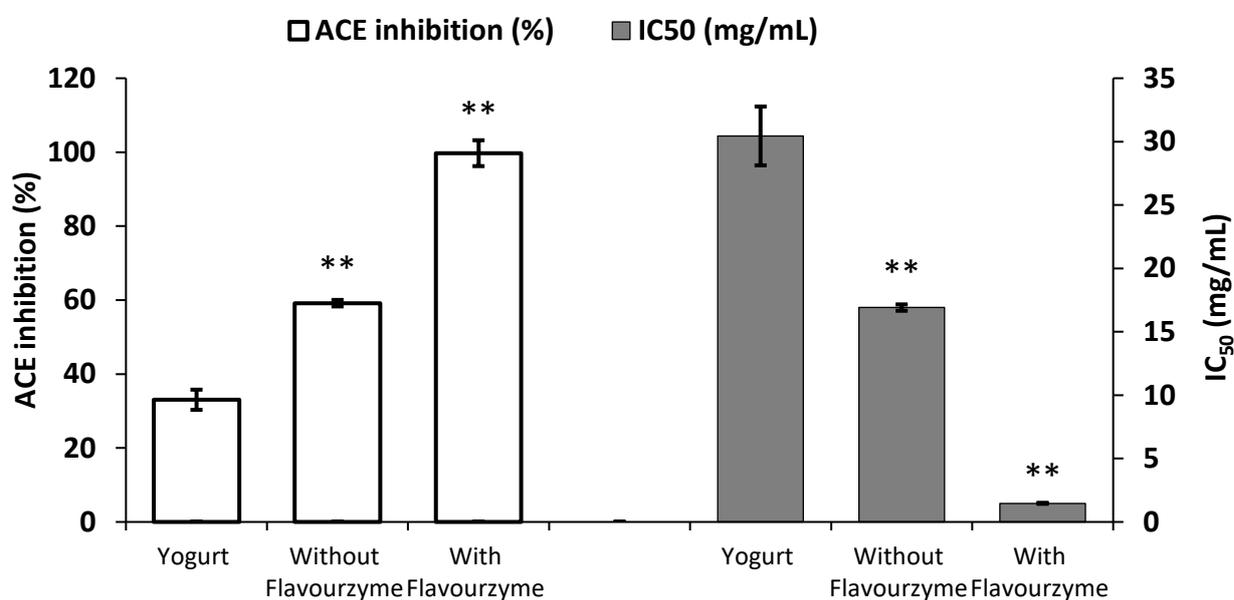


Figure 3.3: ACE inhibition of peptides from normal yoghurt (1% inoculum size for 6 h) and yoghurt added with *L. helveticus* strain with Flavourzyme® (1% inoculum size for 12 h) or without Flavourzyme® (4% inoculum sizes for 8 h) at the concentration of 20 mg/mL and their IC₅₀ value.

Data are shown in mean \pm standard deviation (n = 3). Significant difference between the peptides extracted from different yoghurt formulation was indicated as ** for $P < 0.01$.

3.4.5 Peptide profile of water-soluble extracts

The degree of hydrolysis at the higher ACE-I activity was determined by analysis of peptides from different yoghurts using RP-HPLC. The peptide profiles (20 mg/mL of the concentration) showed similar characteristics between yoghurt with starter culture and yoghurt incorporated with *L. helveticus* with or without Flavourzyme[®] (Figure 3.4). In particular, four major peaks appeared in the first eight minutes when the substantial hydrolysis took place. It was then followed by several minor peaks. Although the number of the proteolytic peptides in yoghurt fermented by *S. thermophiles* and *L. bulgaricus* was almost the same as those in yoghurt added with *L. helveticus* based on the peptide profiles (Figure 3.4A, B), ACE-I activity in yoghurt without *L. helveticus* was lower than that in yoghurt with *L. helveticus* (Figure 3.3). Many anti-hypertensive milk peptides have been isolated and identified (Hannu and Anne 2006). However, only specific peptides, especially those containing proline in the amino acid sequence at the C-terminal, such as VPP, IPP and LPP, have high ACE-I activity from fermented dairy products (Butikofer et al. 2008). In fermented milk, high amounts of VPP and IPP were obtained in most cases after fermentation with *L. helveticus* due to their higher proteolytic activity and their special capability to degrade the milk protein into these tripeptides, and also accumulate these tripeptides in the dairy products (Hu et al. 2011).

Yoghurt supplemented with *L. helveticus* and Flavourzyme[®] showed different peptide peaks (Figure 3.4C). More peaks were detected at retention times between 3 and 18 min due to the increase in milk protein hydrolyses by Flavourzyme[®], which led to the release of peptides with small molecular weight. These results were consistent with the recent study by Ahtesh *et al.* (2016b) using the same strains in the fermentation of 12% of RSM (Ahtesh *et al.*, 2016b. Tsai *et al.* (2008) indicated that Flavourzyme[®] has a potency to increase the content of

soluble protein (10-fold) and peptides (5-fold) in milk whey fermented by lactic acid bacteria (Tsai et al. 2008).

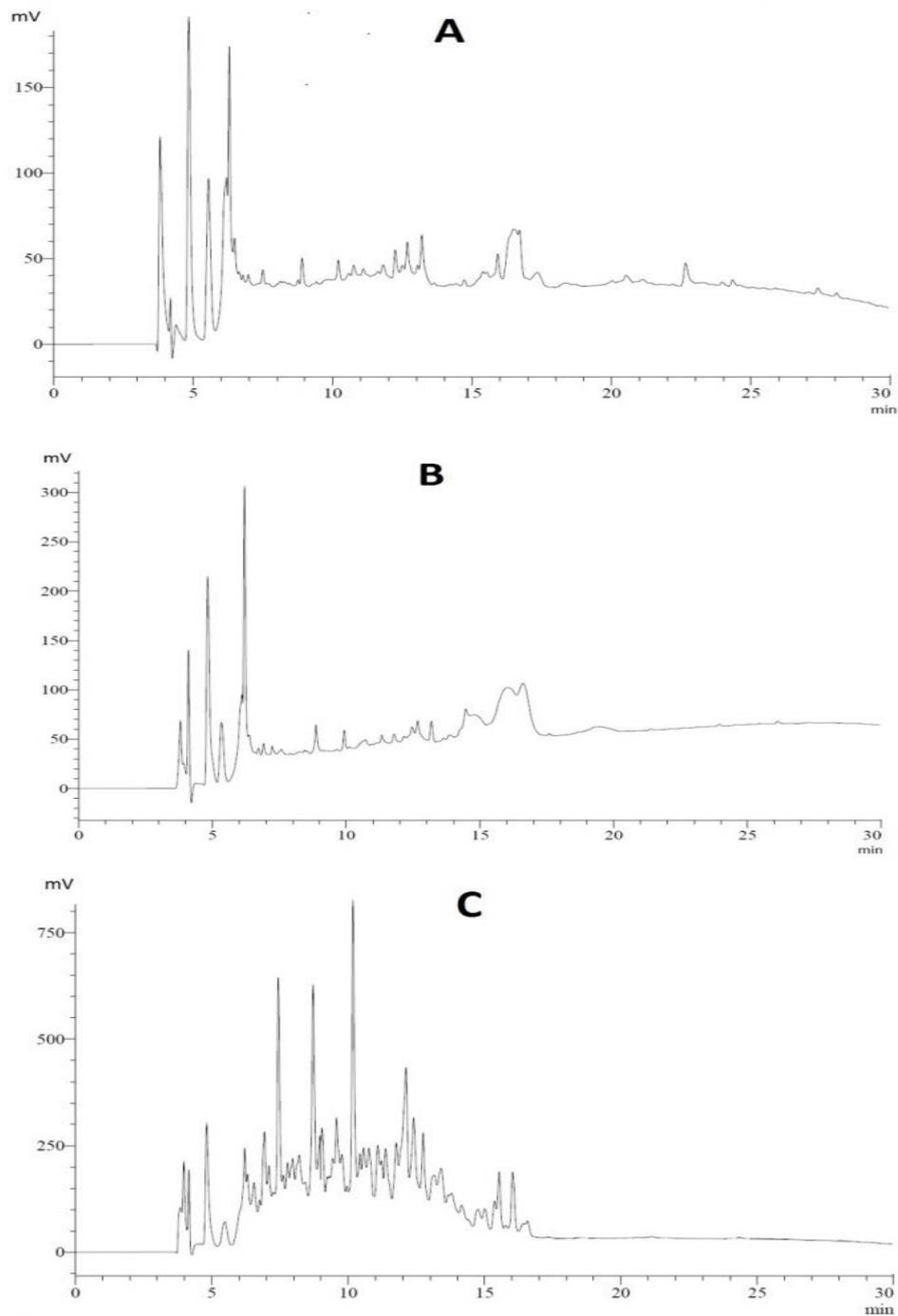


Figure 3.4: The HPLC chromatogram of peptide profile (20 mg/mL) obtained from normal yoghurt (A) and yoghurt added with *L. helveticus* without Flavourzyme® (B) and with Flavourzyme® (C) after 12 h fermentation at 37 °C.

3.4.6 ACE-I activity of fractions

In order to pinpoint the peptides bearing a potential ACE-I activity, peptides from yoghurt supplemented with Flavourzyme[®] were separated using preparative RP-HPLC, and seven fractions (F1- F7) were collected according to the elution time. Their ACE-I activity was then determined (Figure 3.5). The results implied that fractions F3- F6 were primarily responsible for ACE-I activities of peptides. Among them, fraction F3 exhibited the highest activity, corresponding to an IC₅₀ value of $35.75 \pm 5.48 \mu\text{g/mL}$, which was almost 42-fold lower than that found in the total hydrolysate ($1470 \pm 40 \mu\text{g/mL}$) ($P < 0.01$; Figure 3.5). Fraction F7 showed the lowest inhibitory effect on ACE, with an IC₅₀ value of $2211.22 \pm 213.41 \mu\text{g/mL}$. These findings implied that some small peptides separated and collected from yoghurt showed functional accumulation or synergism on ACE-I activities.

Six fractions were separated and collected based on the molecular weight of peptides extracted from fermented skim milk with *L. bulgaricus* LB340 in the study by Qian *et al* (2011), which revealed that fraction F2 showed the highest ACE-I activity (66.4%) at concentration of 100 mg/mL, with an IC₅₀ value of $67.71 \pm 7.62 \text{ mg/mL}$, which was higher compared with those in this study (Qian *et al.* 2011). The differences observed in ACE-I effect of peptides may be attributed to the variation in proteolytic activity of bacteria stains (*L. bulgaricus* LB340 versus *L. helveticus* 881315) and cultures (skim milk versus yoghurt), and therefore the variety of peptides present in the hydrolysates. The synthetic bioactive peptides, based on purification, characterization and identification of their amino acid sequence could be potentially used to develop functional foods that are beneficial to human health. Further work is required to identify the amino acid sequence of the ACE-I peptides extracted from yoghurt.

The HPLC chromatogram (Figure 3.5 A) clearly showed that each of the peptide fractions likely contains more than one single peptide as many peaks appeared in each fraction. Thus in future studies, a liquid chromatography mass spectrometry should be used to further identify these peptides, as the detector attached to the HPLC is not able to identify single peptide from the peptide fractions.

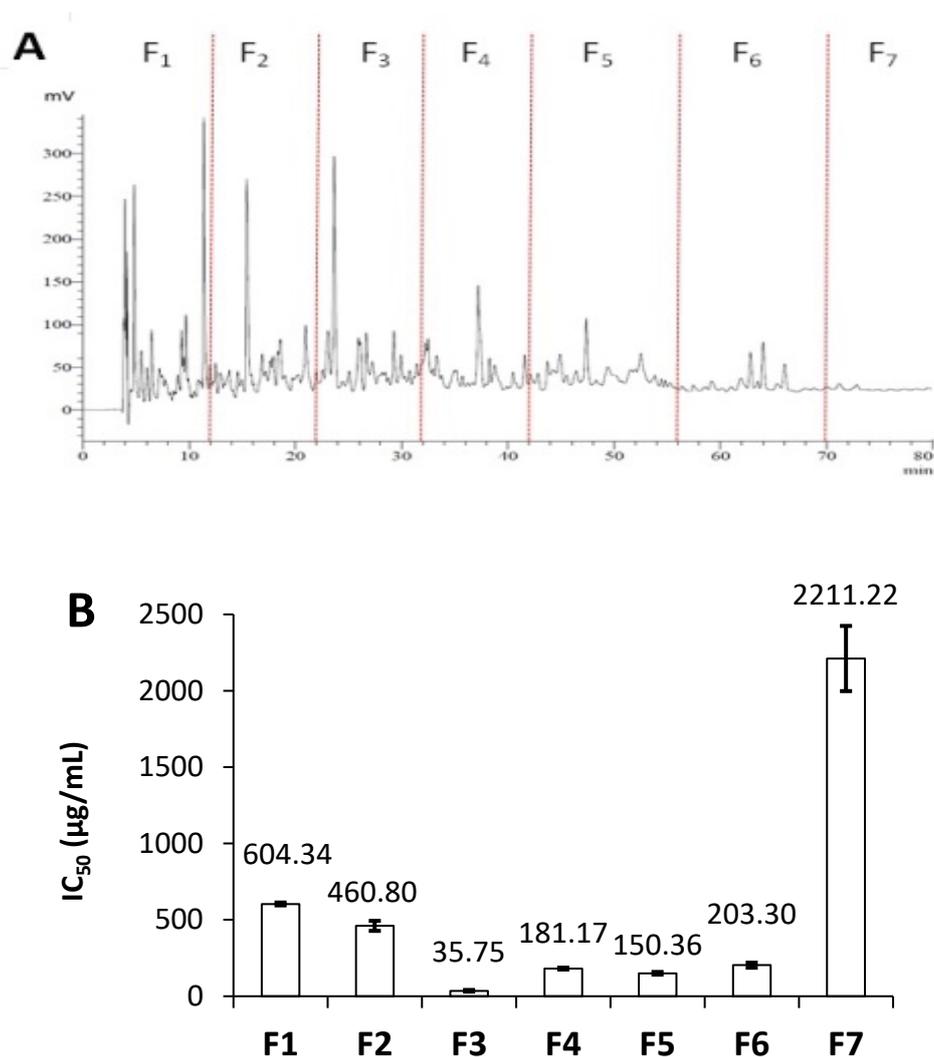


Figure 3.5: Typical chromatogram pertained to peptides fraction by preparative RP-HPLC obtained from yoghurt added with *L. helveticus* and Flavourzyme® (A), and corresponding ACE inhibitory effect of the resulting fractions (F1–F7) measured as IC₅₀ (B).

3.5 Conclusions

The bioactive peptides released from the aqueous extracts of yoghurt have ACE-I activity. The optimal fermentation conditions for producing ACE-I peptides were 1% inoculum size of *L. helveticus* strain 881315 for 12 h combined with Flavourzyme[®]. The IC₅₀ value was 1.47 ± 0.04 mg/mL, which was higher than yoghurt or yoghurt supplemented with *L. helveticus* strain without Flavourzyme[®]. The observation of peptide profiles also confirmed different quantities of peptides in different yoghurts, and yoghurt with or without Flavourzyme[®]. Among seven fractions of peptides, fraction F3 displayed the highest ACE-I effect. Therefore, yoghurts provide a good source for the generation of bioactive peptides with ACE-I activity in fermented dairy products. Although further *in vivo* study is required to confirm the ACE-I activity of yoghurt peptides and their potential anti-hypertensive properties, the outcomes of this study hold a promising potential as a complementary therapy for hypertension. It is necessary to identify the specific peptides or single peptide using liquid chromatograph – mass spectrometer in the yoghurt for future development of potential functional foods.

CHAPTER 4: Determination of C3G from blueberries

4.1 Summary

Blueberries are a rich source of anthocyanins. For separation and quantitation of anthocyanins, the most commonly used methods involve the high performance liquid chromatography (HPLC) with UV-Vis detector. However, due to the availability of reference compounds, the accurate identification of individual anthocyanin using conventional HPLC may not always be warranted. Therefore, an acid hydrolysis HPLC method, which can convert complex anthocyanins to anthocyanidins, for accurate measure of anthocyanidins in blueberry extracts has been developed. In this study, thirteen different brands of blueberry (fresh or frozen) were obtained from supermarkets in Melbourne (Coles, Woolworths and ALDI). The anthocyanins profile of these berries was analysed using a reverse-phase HPLC (RP-HPLC) and an acid hydrolysis HPLC respectively and results are compared. It was determined that RP-HPLC was not able to separate individual anthocyanin effectively with the presence of many peaks and some of them were separated incompletely and overlapped. This makes it difficult to calculate accurately the content of both the total anthocyanins and cyanidin 3-O- β -glucoside (C3G). In contrast, the acid hydrolysis HPLC method greatly simplified the analysis of the anthocyanin profile in blueberry samples and was able to convert anthocyanins to five major anthocyanidin aglycones including: delphinidin, cyanidin, petunidin, peonidin, and malvidin. Each of these aglycones could be separated completely. The concentration of the total anthocyanidin and cyanidin in blueberry samples was calculated against a commercially available external standard of anthocyanin, C3G, and expressed as its equivalents. Among thirteen brands of blueberry samples, the total content of anthocyanidins in blueberry fruit and dried blueberry powder ranged from 0.04% to 0.25%

and from 0.29% to 1.49%, respectively. The content of cyanidin in dried blueberry powder varied between 0.04% (Sweet Haven, labelled as A2) and 0.42% (Driscoll's, the sample labelled as C3). In the fruit samples, the greatest content of total cyanidin was from Driscoll's blueberries (0.05%, the sample labelled C3), followed by Bhatti & Manj and Woolworths Select blueberries (0.03%, the sample labelled C4 and W1), as well as the least content of total cyanidin was obtained from Senn Sational and Matilda's blueberries (less than 0.01%, the sample labelled C5 and C6). Based on these results, Bhatti & Manj blueberries (the sample labelled C4) was selected for future investigation in chapters Seven and Eight due to its higher content of total cyanidin and total anthocyanins for the further animal study to determine whether blueberries has the effect on obesity and its related comorbidities.

4.2 Introduction

Anthocyanins are a large subgroup of flavonoids, responsible for many of the red and blue colours present in nature (Burdulis et al. 2009). An anthocyanin molecule constitutes an aglycone (anthocyanidin), which in its natural state is glycosylated by one or more different sugars. Differences between the aglycone bodies are due to the number of hydroxyl groups and the degree of methylation of these groups (Ochmian et al. 2009). Anthocyanins are potent antioxidants (Kalt et al. 1999; Kalt et al. 2001; Borges et al. 2010; de Souza et al. 2014). The use of anthocyanin-containing foods as part of a healthy diet may be beneficial for health (Mazza et al. 2002; Hoggard et al. 2013; Castro-Acosta et al. 2016). Therefore, practical, accurate, and reliable methods to determine the anthocyanin content of foodstuffs are needed.

Blueberries are a rich source of anthocyanins and other polyphenolic compounds. Fruit quality of blueberries is associated with the levels of phenolics, flavonoids and overall antioxidant capacity. It has been shown that blueberries have among the highest overall antioxidant capacity of all the fruits and vegetables due to its anthocyanins and other

polyphenolic compounds (Gündüz et al. 2015). Multiple conditions, such as cultivars, location, ripeness season and storage method, could affect the fruit quality and phytochemical content (Michalska and Lysiak 2015). Therefore it is of significant importance to accurately measure not only the total concentration, but also the individual anthocyanins in various products available to the general public.

Several analytical methods for anthocyanins determination in powdered blueberry extracts (Cretu and Morlock 2014), blueberry fruit (Borges et al. 2010; de Souza et al. 2014), and blueberry wine (Liu, S. X. et al. 2015) have been published. High performance liquid chromatography (HPLC) is the most commonly used method for the analysis of anthocyanins profile. It is useful in verifying the identity of blueberries, monitoring the consistency of blueberry source, and quantifying the total anthocyanins (Nyman and Kumpulainen 2001; Comeskey et al. 2009; Borges et al. 2010). A major challenge in quantification of anthocyanins by HPLC methods is their unstable properties and unavailability of most standards on a commercial basis (Basiouny and Chen 1988). There are more than 250 naturally occurring anthocyanins which consist of one of six aglycones glycosylated with various sugar substitutes (Strack et al. 1994). Only a few anthocyanin reference compounds are commercially available. Fortunately, the complex anthocyanidin glycoside pattern can be reduced to six major anthocyanidins by acid hydrolysis. It has been reported that there are five major anthocyanidins in blueberry, which are delphinidin, cyanidin, petunidin, peonidin and malvidin (Nyman and Kumpulainen 2001; Cretu and Morlock 2014).

Cyanidin-3-O- β -glucoside (C3G), composition of cyanidin and its combination of sugar (glucoside), is a typical anthocyanin in blueberry. Several studies have shown that isolated C3G improved insulin sensitivity and hyperglycaemia in animal models of diabetes (Sasaki et al. 2007; Guo, H. et al. 2012b; Liu et al. 2014). Furthermore, *in vitro* studies reported that

C3G protected 3T3-L1 adipocytes against tumor necrosis factor- α induced insulin resistance (Guo et al. 2008), and eliminated the impacts of high-glucose on the induction of adipocyte lipolysis in 3T3-L1 adipocytes (Guo, H. et al. 2012a). C3G exerted these effects *in vitro* at various concentrations (Guo et al. 2008; Guo, H. et al. 2012b). The aim of this study was to determine the content of anthocyanidins and cyanidin in different blueberry samples using RP-HPLC and select a suitable brand of blueberry for further investigation to determine the efficacy of combined supplementation of blueberries and peptides extracted from yoghurt in obesity and diabetes.

4.3 Material and methods

4.3.1 Sources of blueberries

Thirteen brands of blueberries (fresh or frozen) were obtained from the supermarket, nine of them from Coles, two of them from Woolworths and another two from ALDI (Figure 4.1). Table 4.1 showed the detailed information of blueberries. The fresh blueberries were collected during the maturity of fruits (the middle of December, 2016 – the end of January, 2017). The frozen blueberries were collected from supermarkets, thus their collection period was unknown.



Figure 4.1: Commonly available blueberries in Melbourne analysed for its anthocyanin content.

Table 4.1: The details of thirteen brands of blueberries

Labelled name	Fresh/ Frozen	Brands	Sources (Supermarkets)	Place of manufacturing
C1	Fresh	Fresh Gourmet	Coles	New Zealand
C2	Fresh	Sodhi's	Coles	NSW, Australia
C3	Fresh	Driscoll's	Coles	VIC, Australia
C4	Fresh	Bhatti & Manj	Coles	NSW, Australia
C5	Fresh	Senn Sational	Coles	Australia
C6	Frozen	Matilda's	Coles	Australia
C7	Frozen	Coles	Coles	Chile
C8	Frozen	Organic	Coles	New Zealand
C9	Frozen	Greative Gourmet	Coles	Chile
W1	Frozen	Woolworth Select	Woolworths	Chile
W2	Frozen	Macro Organic	Woolworths	Chile
A1	Fresh	Driscoll's	ALDI	VIC, Australia
A2	Frozen	Sweet Haven	ALDI	Chile

4.3.2 Sample preparation

All fresh fruits were frozen at - 20 °C, freeze-dried for 4 – 6 days and ground to a powder on dry ice. Freeze dried blueberry powder (50 mg) was suspended in water, vortexed for 30 seconds, and centrifuged at 4000 x g for 30 min at 4 °C. Fraction separation from the supernatant was obtained and freeze-dried for 72 hours using a freeze-drier (John Morris Scientific Pty Ltd, Melbourne VIC, Australia). The freeze-dried supernatant powder was stored at -20 °C in the dark until analysis.

4.3.3 Anthocyanins profiling using RP-HPLC

The total anthocyanins was measured according to the previous report by (Corona et al. 2011), with a minor modification. 600 mg of supernatant powder was dissolved into 5 mL of acidified methanol with 0.3% HCl. It was vortexed for 1 minute, and then filtered using 0.2 μm filter for the total anthocyanin determination using RP-HPLC. C3G, as a standard, was treated with the same procedure, and a standard curve was prepared using five different concentrations (15, 30, 75, 150 and 300 $\mu\text{g}/\text{mL}$). Samples or standard solution were measured by RP-HPLC using a Prominence-i LC-2030 (Varian Analytical Instruments, Santa Clara, CA, USA) and a Prep Nova-Pack C18 column (60 \AA , 250 mm x 10 mm, 10 μm , Phenomenex, Pty Ltd, Lane Cove, NSW, Australia). The temperature of column was held at 30 $^{\circ}\text{C}$. The mobile phase consisted of a mixture of aqueous methanol (5% methanol; 0.1% HCl 5M) [phase A] and aqueous acetonitrile (50%; 0.1% HCl 5M) [phase B] and was pumped through the column at a flow rate of 0.7 mL/min. The following elution profile was used: start condition 5% phase B, then, 50% phase B from 5 minutes to 40 minutes with linear gradient, finally, 5% phase B from 40 minutes to 50 minutes for testing the next sample. The eluent was monitored by photodiode array detection at 520 nm and the injection volume was 10 μL . The linearity of each calibration line was acceptable ($R^2 > 0.99$).

4.3.4 Determination of anthocyanidins using a reverse-phase HPLC

To determine anthocyanidins from blueberries, blueberry powder (300 mg) was acid hydrolysed using 4 mL of acidified aqueous methanol (75% methanol; 2M HCl). The extracts were vortexed for 30 seconds, filtered (0.45 μm) and the supernatant retained. After two further extractions of the remaining pellets, samples (~ 12 mL) were hydrolysed for 50 min at 90 $^{\circ}\text{C}$. Following hydrolysis, samples were cooled to room temperature, centrifuged at 4000 X g for 10 min at 10 $^{\circ}\text{C}$ and 500 μL was subjected to RP-HPLC analysis. C3G was used as a

standard to assess the stability of cyanidin under these laboratory conditions and a standard curve was produced using five concentrations (25, 50, 100, 250 and 500 µg/mL).

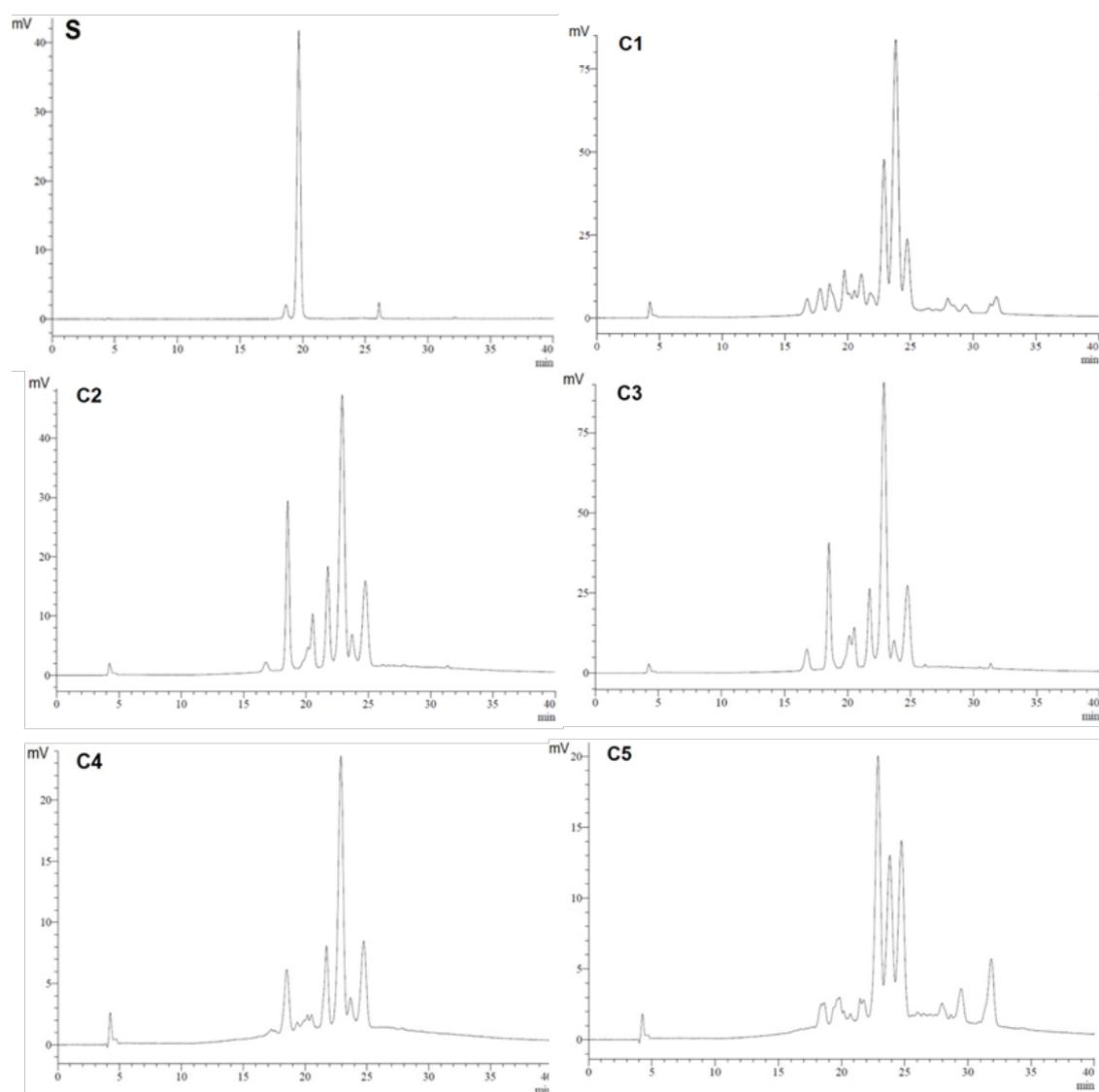
Hydrolysed blueberry samples or cyanidin solution were measured by the HPLC using a Prominence-i LC-2030 (Varian Analytical Instruments, Santa Clara, CA, USA) and a Prep Nova-Pack C18 column (60 Å, 250 mm x 10 mm, 10 µm, Phenomenex, Pty Ltd, Lane Cove, NSW, Australia). The temperature of column was held at 30 °C. The mobile phase consisted of a mixture of aqueous methanol (5% methanol; 0.1% HCl 5M) [phase A] and aqueous acetonitrile (50%; 0.1% HCl 5M) [phase B] and was pumped through the column at a flow rate of 0.7 mL/min. The following elution profile was used: start condition 5% phase B, then, 50% phase B from 5 minutes to 33 minutes with linear gradient, 5% phase B from 33 minutes to 40 minutes. The eluent was monitored by photodiode array detection at 520 nm and injection volume was 10 µL. The linearity of each calibration line was acceptable ($R^2 > 0.99$).

4.4 Results

4.4.1 Anthocyanin profile

Only one large peak was observed in the C3G standard, and the retention time was around 20 minutes (Figure 4.2S) and its area percentage was 93.00%. Another two small peaks, with 7.00% of total area percentage, were possibly other components mixed with C3G. The identification of anthocyanin was made by comparison of retention times of unknown substances from different blueberries and the standard. The anthocyanin profiles of different brands of blueberries are shown in Figure 4.2. All peaks from blueberry samples appeared before 25 minute of the retention time (Figure 4.2C1-A2). More than seven peaks were detected by the UV-vis detector in some brands of blueberries, such as Fresh Gourmet blueberries (the sample labelled C1), Senn Sational blueberries (the sample labelled C5) and

Driscoll's blueberries (the sample labelled A1), while others like Greative Gourmet blueberries (the sample labelled C9) and Sweet Haven blueberries (the sample labelled A2), showed less peaks. Due to the large number of anthocyanins peaks, overlapping and incomplete separation of some peaks on the chromatogram, plus the unavailability of required standards it was not possible to identify and quantify individual anthocyanins. In order to obtain accurate data, acid hydrolysis was used to convert anthocyanins to anthocyanidin aglycones.



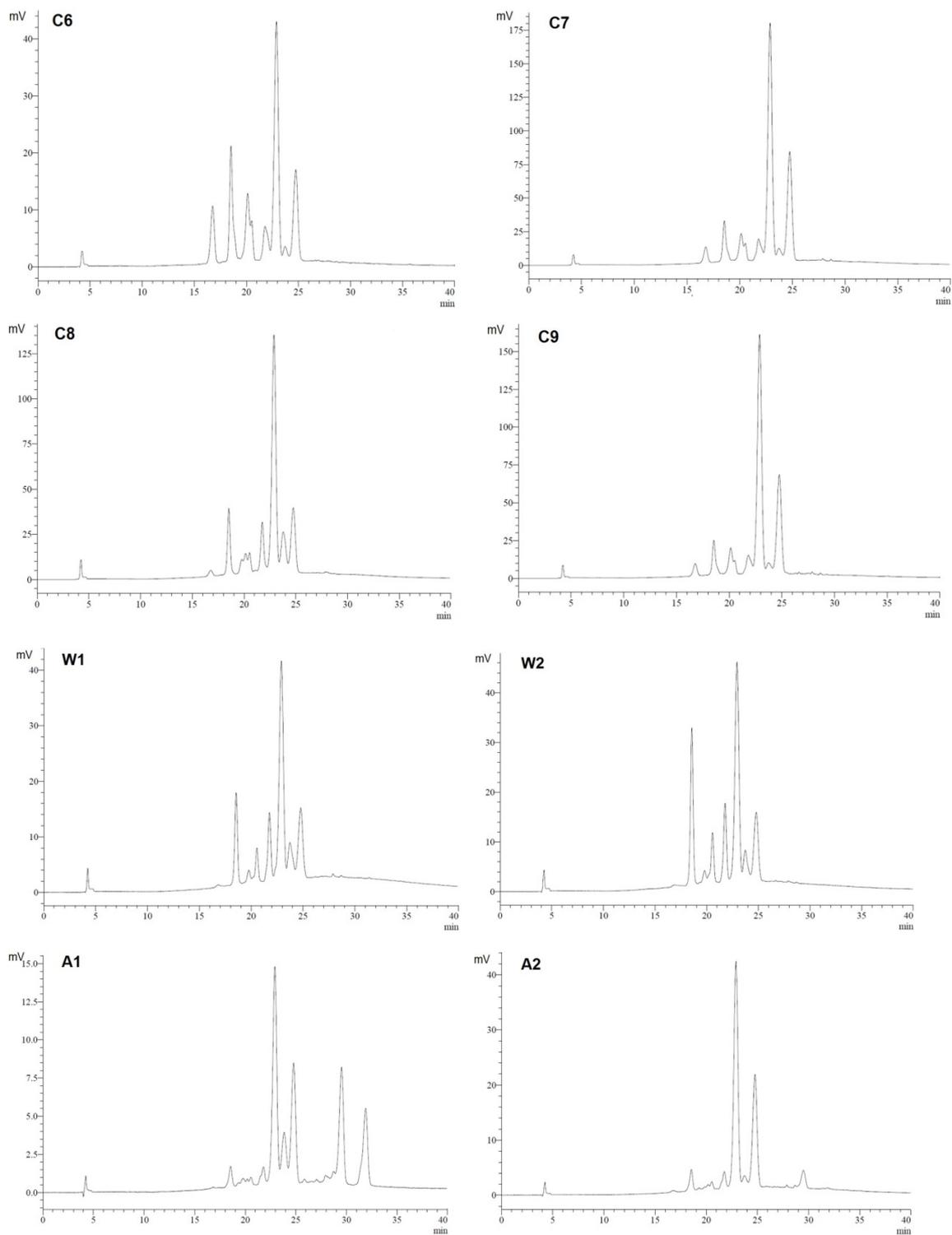


Figure 4.2: RP-HPLC chromatograms and spectra in anthocyanins of blueberries without hydrolysis.

S: commercial external standard, C3G; C: blueberries obtained from Coles; W: blueberries obtained from Woolworths; A: blueberries obtained from ALDI.

4.4.2 Anthocyanidin profile

Acid hydrolysis of the blueberry extract, allowed resolution of individual anthocyanidins, indicating that all the glycosidic forms present in blueberries had been effectively cleaved to yield their respective anthocyanidins. This technique allowed the characterisation of the major anthocyanidins and quantification of their total anthocyanidin content in blueberry samples (Cretu and Morlock 2014).

Figure 4.3 shows the chromatogram of the cyanidin standard at different concentrations. Only one large peak was detected with the consistent retention time at 23.8 minute. After acid hydrolysis, fewer peaks were shown with five major anthocyanidins peaks in total (vs seven peaks using conventional HPLC methodology) (Figure 4.4). Those peaks (from left to right) represent delphinidin, cyanidin, petunidin, peonidin and malvidin (Nyman and Kumpulainen 2001; Ochmian et al. 2009; Cretu and Morlock 2014). Blueberry species, methodological differences in sample collection, preparation, chromatographic separation, detection and data analysis can result in significant inter-laboratory variations (Wu et al. 2004). We presumed the elution order of the individual anthocyanidins was the same in the various blueberry species. The second peak at 24 minute of the retention time in thirteen blueberry samples was identified as cyanidin. This is consistent with the previous reports (Burdulis et al. 2009; Cretu and Morlock 2014). The anthocyanidin profile demonstrated that the area percentage of cyanidin ranged from 7.80% to 29.00%. Except cyanidin, delphinidin (the first peak) and malvidin (the last peak) were also common anthocyanidins in all brands of blueberry tested, whilst peonidin (the fourth peak) was the least common anthocyanidin (Figure 4.4).

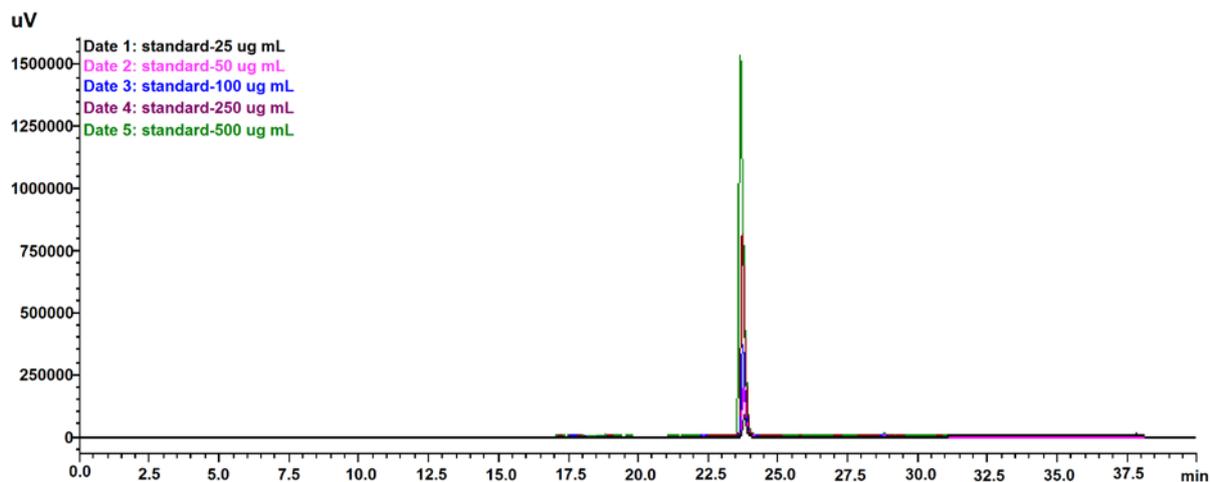
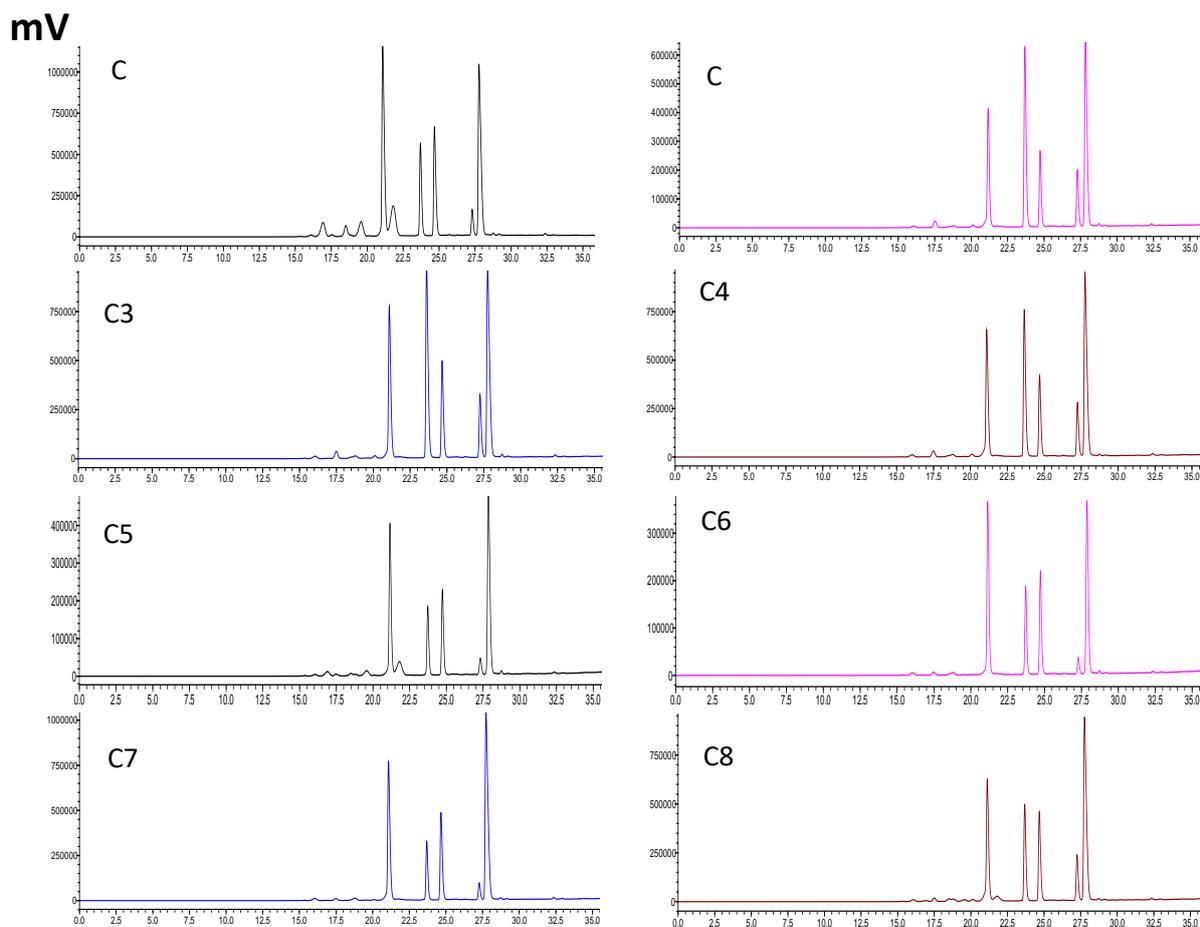


Figure 4.3: Chromatogram of cyanidin (hydrolysed C3G) at different concentrations (25, 50, 100, 250 and 500 $\mu\text{g/mL}$).



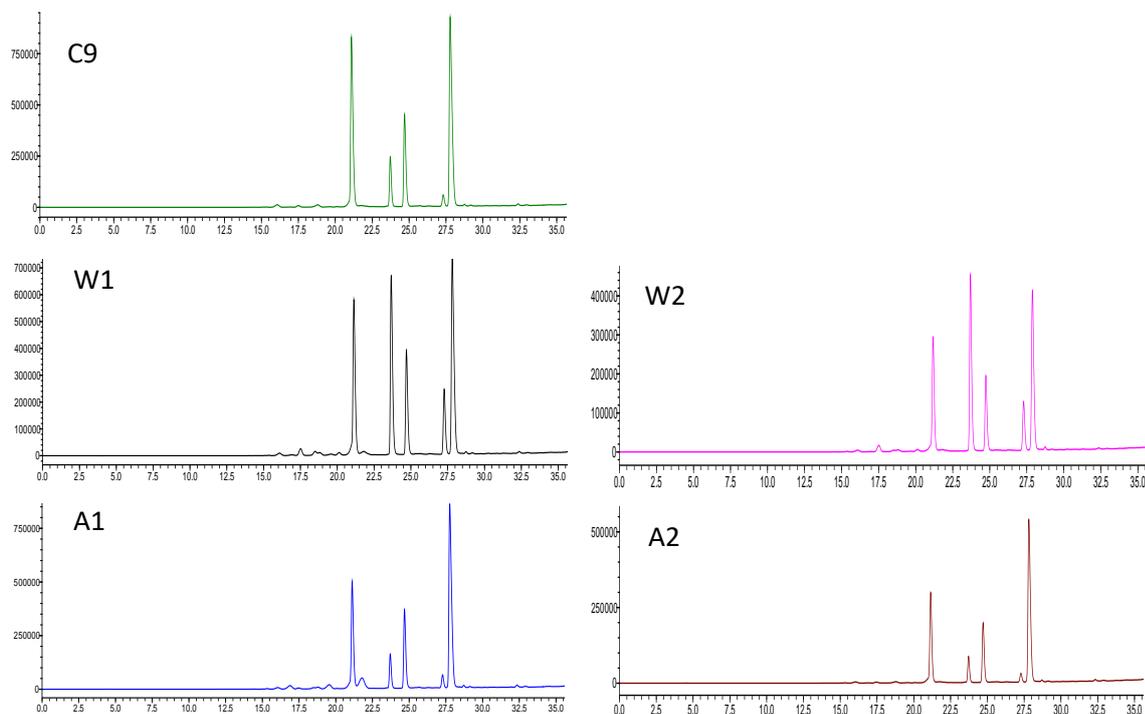


Figure 4.4: Chromatogram of anthocyanidin from thirteen brands of blueberries.

C: blueberries obtained from Coles; W: blueberries obtained from Woolworths; A: blueberries obtained from ALDI.

4.4.3 Determination of anthocyanidins and cyanidin in blueberry samples

In order to determine the accuracy of anthocyanidins and cyanidin contents in blueberry samples, the total anthocyanidins and cyanidin contents were calculated based on the area of chromatogram (Figure 4.3 and 4.4). C3G is usually used as a reference compound to calculate total anthocyanins and acidified C3G, as a standard, is used to calculate the total anthocyanidins as well (Basiouny and Chen 1988). The calibration formula was obtained based on the peak area and concentration gradient, and the corresponding correlation coefficient was 0.999 (Figure 4.5).

According to the calibration formula, the calculated total anthocyanidins and cyanidin contents in all blueberry samples on the basis of HPLC peak area with monitoring at 520 nm,

were presented in Table 4.2. Woolworths Select blueberries (the sample labelled W1) showed the highest extraction yield, and the dry matter was more than 20%, followed by Driscoll's blueberries (the sample labelled C3) and Bhatti & Manj blueberries (the sample labelled C4). Driscoll's blueberries (the sample labelled A1) contained the least dry matter, 5.64%. The total extracted anthocyanidins in blueberry fruit was from 0.04% to 0.25%. Furthermore, the total content of anthocyanidins in different dried blueberry powder was ranged from 0.29% to 1.49%. Regarding cyanidin, its content in the total anthocyanidins varied between samples, ranged from 6.27% to 29.00%. Correspondingly, a variation of the cyanidin content in dried blueberry powder was observed. The highest concentration of cyanidin in dried blueberry powder was detected in Driscoll's blueberries (the sample labelled C3), with 0.42%, which was approximately 10-times higher than in Sweet Haven blueberries (the sample labelled A2) dried blueberry powder. However, when the dry matter was combined, the variation of the cyanidin content was changed. For the total cyanidin content in blueberry fruits, the greatest content was still from Driscoll's blueberries (the sample labelled C3), with 0.05%, followed by Bhatti & Manj blueberries (the sample labelled C4) and Woolworths Select blueberries (the sample labelled W1). However, the least cyanidin content was from Senn Sational and Matilda's blueberries (the sample labelled C5 and C6), instead of Sweet Haven blueberries (the sample labelled A2).

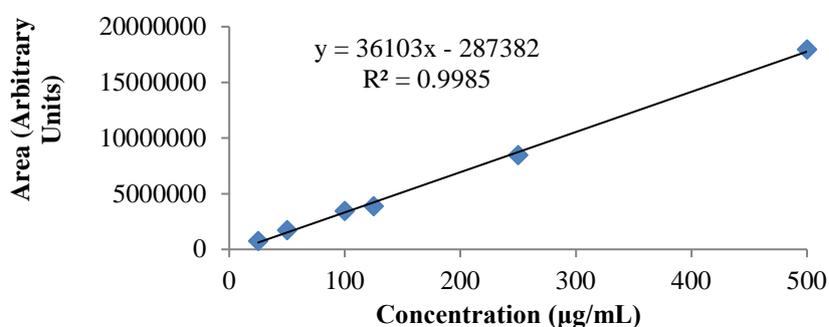


Figure 4.5: The calibration curve of cyanidin for the calculation of the total anthocyanidins and cyanidin in blueberry samples.

4.5 Discussion

In plant foods, anthocyanidins are present as glycosidic forms (anthocyanins) bound to glucose, galactose, arabinose, rutinose, sambubiose, or sophorose; sometimes with the addition of an acetyl, caffeoyl, dioxalyl, malonyl, coumaroyl, or succinyl moiety (Ochmian et al. 2009; Schroeter et al. 2010). Direct extraction and analysis of foods for their anthocyanin content often yields complex chromatograms with many peaks, thereby accurate quantification is difficult. In this study, HPLC analysis of un-hydrolysed blueberry samples resulted in chromatograms in which it was difficult to quantify individual anthocyanins and anthocyanidins due to the incomplete separation and overlapped peaks of anthocyanin compounds (Figure 4.2). In addition, as anthocyanins are relatively unstable, the availability of authentic anthocyanin standards is limited thus it is virtually impossible to achieve a complete and accurate quantification of the total anthocyanin content via the assessment of anthocyanins alone (Cretu and Morlock 2014). Therefore, it is necessary to convert various anthocyanins to several anthocyanidins. In this study we achieved an accurate quantification of the total anthocyanidin content of thirteen commercial brands of blueberries available in Melbourne, Australia during December 2016 to January 2017 via quantification of their major anthocyanidin content post hydrolysis of the extracted fruit.

The HPLC analysis provides information of the anthocyanin profile and is useful for the control of product quality, identification of sample origins, and examination of consistency in raw materials. The greatest disadvantage of anthocyanin quantitation via HPLC is the unavailability of reference compounds. In addition, the incomplete separation of all the anthocyanins in a single HPLC run makes the identification and quantification very difficult (Nyman and Kumpulainen 2001). HPLC combined with the mass spectrum is usually used to identify specific anthocyanins, as mass spectra data and information from the respective

literature can be used for tentative identification of the conjugated forms based on the value of m/z, which represents mass divided by charge number (Burdulis et al. 2009; Comeskey et al. 2009; Corona et al. 2011).

Table 4.2: The content of anthocyanidin and cyanidin in tested blueberry samples

Label	Dry matter (%)	ACNs* in blueberry fruit (%)	ACNs in dried blueberry powder (%)	Cyanidin in ACNs (%)	Cyanidin in blueberry fruit (%)	Cyanidin in dried blueberry powder (%)
C1	13.30	0.198	1.49	10.34	0.01	0.23
C2	15.36	0.10	0.67	25.27	0.02	0.25
C3	20.95	0.25	1.18	24.66	0.05	0.42
C4	19.93	0.20	1.00	21.37	0.03	0.31
C5	17.95	0.07	0.39	11.64	< 0.01	0.07
C6	12.13	0.04	0.29	14.81	< 0.01	0.07
C7	16.11	0.14	0.88	9.58	0.01	0.13
C8	18.27	0.17	0.92	14.59	0.02	0.20
C9	15.69	0.13	0.84	7.77	0.01	0.10
W1	21.30	0.16	0.74	23.76	0.03	0.26
W2	16.03	0.06	0.39	29.00	0.02	0.17
A1	5.64	0.04	0.63	6.27	0.01	0.07
A2	18.16	0.06	0.31	6.39	0.01	0.04

*ACNs: Anthocyanidin

In this study, the total content of anthocyanidins and cyanidin varied significantly in different brands of blueberry (Table 5.2). The greatest total cyanidin content in blueberry fruits was from C3, followed by C4 and W1. Based on the content of bioactive compounds and availability, C4 has been selected as a suitable source of anthocyanins and will be used in the further animal study. As previously reported, storage conditions play an important role in

retaining the anthocyanin content of fruits. Increases in anthocyanin content of two cultivars of rabbiteye blueberries *Vaccinium ashei* cv. 'Tifblue' and 'Bluegem' after storage for 45 days at 5 °C have been previously reported (Basiouny and Chen 1988). Kalt et al., (1996) also showed that the concentration of anthocyanin of lowbush blueberry increased by 18% after refrigerated storage for two weeks at 1 °C (Kalt and McDonald 1996). A slight increase of anthocyanins in blueberry was also found immediately after freezing (-18 °C), suggesting that the anthocyanins in frozen fruit become more easily extractable due to degradation of cell structures in the blueberry (Poiana et al. 2010). However, the same study has shown that storage of frozen fruit for 6 months slowed down anthocyanin degradation (loss in these compounds after 6 months was below 5% in blueberry) (Poiana et al. 2010). After 10 months there was an accelerated degradation of anthocyanins in blueberry up to 13% (Poiana et al. 2010). In the present study, the total anthocyanins in the fresh blueberries, such as Fresh Gourmet, Driscoll's and Bhatti & Manj blueberries (the sample labelled C1, C3 and C4), were higher than that of frozen blueberries, since the fresh blueberries were stored at -20 °C for less than two months, while the frozen blueberries may have been stored for longer periods. This indicates that the storage duration may be one of the important factors contributing to the total anthocyanins content of various blueberries.

Other factors, such as genes, taxonomy, geographical locations, agricultural conditions and ripeness of fruits may also affect the composition and content of anthocyanin in blueberry. Anthocyanins, as secondary metabolites, accumulate in ripe berry fruit and its accumulation continues in overripe berries. A previous study has shown that the content of anthocyanins of four highbush blueberries increased during successive harvest (Castrejón et al. 2008). Also Ochmian et al., (2009) indicated that substrates have an impact on anthocyanin synthesis and the total content of anthocyanin (Ochmian et al. 2009). In the same study, the blueberries that originated from peat- and cocoa husk grown plants showed higher total anthocyanin contents

(0.15% and 0.15%, respectively) than that in the fruit collected from sawdust-bedded bushes (0.12%) (Ochmian et al. 2009).

Furthermore, methodological differences in sample collection, preparation, chromatographic separation and detection, purity of reference standards and methods of data evaluation could result in significant inter-laboratory variations and represent a limitation with regards to anthocyanin composition and content (Cretu and Morlock 2014). For example, bilberry and blueberry were characterised for their anthocyanin content using a reliable high-performance thin-layer chromatography (HPTLC) method (Craciun et al. 2015). This has many advantages including simplified sample preparation, low operating costs, short analysis time and simultaneous analysis of several samples (Cretu and Morlock 2014). In the same study it was shown that the anthocyanin content in bilberry and blueberry powder were 59.50% and 2.50%, respectively, which were higher than that in the present study with the range of anthocyanin content varied between 0.29% - 1.49%. The method of measurements (HPTLC vs RP-HPLC) using different extraction solutions (ethyl acetate, toluene and 2-butanone vs methanol and acetonitrile) may contribute to these variations. Notably, due to the limited availability of facilities, the total anthocyanins content from different brands of blueberries can be measured only by RP-HPLC in the present study. An additional limitation is that each brand was bought at the one time point at the one shop and a variety of the same branded blueberries was not measured. In the future, a combination of RP-HPLC and mass spectra would allow the identification of the structure of conjugated anthocyanin compounds in blueberry samples. In addition, the same branded blueberries would be obtained at the different time point and the variety of the bioactive compounds would be involved for repeatability of the sample and confirmation of the results.

4.6 Conclusions

In summary, the HPLC analysis before hydrolysis of anthocyanins can provide data of anthocyanin profile. This is useful for the control of product quality and the identification and consistency of raw materials. However, the acid hydrolysis HPLC analysis is more suitable for quantitation of individual anthocyanidins. Thus both methods could be applicable to blueberry extracts. Furthermore, these two methods for the determination of the total content of anthocyanins and anthocyanidins using HPLC and the acid hydrolysis HPLC, respectively were investigated in more detail specifically in terms of the increasing focus on the impact on health promoting antioxidative compounds in fruits and vegetables. Moreover, data on anthocyanin composition of different brands of blueberries could provide useful information to general public and food industry.

CHAPTER 5: The effect of cyanidin-3-O- β -glucoside and peptides extracted from yoghurt on glucose uptake and gene expression in human primary skeletal muscle myotubes from obese and obese diabetic participants

This chapter has been published in the Journal of Functional Foods, see Appendix 3: Min Shi, Lannie O'Keefe, Anna C. Simcocks, Xiao Q Su, Andrew J McAinch. 2018. **The Effect of Cyanidin-3-O- β -Glucoside and Peptides Extracted from Yoghurt on Glucose Uptake and Gene Expression in Human Primary Skeletal Muscle Myotubes from Obese and Obese Diabetic Participants**. The content of this chapter is the same as the published paper (Appendix 3), and only the format has been changed here to match with the thesis.

5.1 Summary

Incidence of type II diabetes mellitus (T2DM) is rapidly increasing worldwide. This study aimed to investigate whether cyanidin-3-O- β -glucoside (C3G), or peptides with angiotensin converting enzyme (ACE) inhibitory activity, alone or in combination, alter glucose regulation in human primary myotubes derived from obese and obese T2DM participants. In the obese group, both low and high concentration of peptides and the combination of these peptides with high C3G concentration significantly enhanced glucose uptake in the presence or absence of insulin, and high concentration of peptide alone and its combination with low levels of C3G down-regulated the mRNA expression of angiotensin II receptor, type 1 (AGTR-1), and up-regulated the mRNA expression of insulin receptor substrate 1 (IRS-1), and glucose transporter 4 (GLUT4). However, only high peptide concentration increased

glucose uptake in the absence of insulin in the obese T2DM group, and the expression of AGTR-1 was decreased with high peptide and its combinations of C3G.

Keywords: cyanidin-3-O- β -glucoside, peptides, human primary skeletal muscle myotubes, glucose uptake, glucose metabolism.

5.2 Introduction

Skeletal muscle is a key site for insulin mediated glucose disposal in mammals and is therefore an important regulator of whole body glucose homeostasis. In this tissue, insulin binds to the insulin receptor (IR) and activates IR substrates (IRS-1 and IRS-2) (Krishnapuram et al. 2013). Activated IR substrates trigger downstream activation of the lipid kinase phosphatidylinositol-3 kinase (PI3K), and finally stimulate the translocation of glucose transporter 4 (GLUT4) to facilitate the transport of glucose into cells (Johnston et al. 2003; Guo et al. 2008). Additionally GLUT4 translocation can also be activated through the cellular energy sensor 5'adenosine monophosphate-activated protein kinase (AMPK) which regulates cell metabolism and integrates nutritional and hormonal signals (Alkhateeb and Qnais 2017; Naimi et al. 2017). Activation of AMPK has been viewed as a targeted approach to increase glucose uptake by the skeletal muscle and therefore improve blood glucose homeostasis. Impaired glucose uptake into skeletal muscle is attributable to insulin resistance, which is associated with several defects within the insulin signalling cascade, namely reduced IR substrates and PI3K response to insulin stimulation (Dos Santos et al. 2015). Insulin resistance can be associated with obesity and plays a key role in the development of type 2 diabetes mellitus (T2DM) (Bouzakri et al. 2005; Deshmukh 2016; Barazzoni et al. 2018).

Cyanidin-3-O- β -glucoside (C3G) is a predominant bioactive anthocyanin compound found in many edible plants and has been reported to be protective against T2DM by attenuating

multiple disorders *in vivo* and *in vitro* (Scazzocchio et al. 2011; Guo, H. et al. 2012a; Guo, H. et al. 2012b; Wang et al. 2016). It has been found that C3G increased glucose uptake in human skeletal muscle cells due to its strong antioxidant activity (Ho et al. 2017). C3G derived from black soybeans ameliorated T2DM in db/db mice through the induction of differentiation of 3T3-L1 preadipocytes into smaller and insulin-sensitive adipocytes, which subsequently resulted in activation of insulin signalling and increased glucose uptake (Matsukawa et al. 2015). Another *in vitro* study using L6 myotubes showed that C3G significantly induced AMPK activation and enhanced glucose uptake into the cells (Kurimoto et al. 2013). However, no information is available on the effect of C3G on glucose metabolism in human skeletal muscle cells, and the molecular mechanism/s underlying this action also remains unclear.

Angiotensin-converting enzyme (ACE) is a primary enzyme in the regulation of blood pressure through converting angiotensin-I into a potent vasoconstrictor, angiotensin-II (Ang II). Subsequently, angiotensin-II induces the release of aldosterone and therefore increases the sodium retention and blood pressure (Muro Urista et al. 2011). The blood pressure regulator ACE has also been shown to influence glucose uptake. Angiotensin- II (Ang II) has been shown to antagonize insulin-independent contraction-induced glucose uptake in skeletal muscle tissue (Rao 1994) via the Ang II type 1-receptor (AGTR-1) (Jones and Woods 2003). The chronic effects of Ang II on myocytes are associated with a reduced protein expression of GLUT4, suggesting limited availability of the glucose transporter to be partially responsible for the restricted glucose uptake (Shiuchi et al. 2004). In addition, Ang II inhibits insulin-mediated actions at multiple levels of the insulin signalling pathway including IR, IR substrates and PI3K (Shiuchi et al. 2004; Igarashi et al. 2007). Thus ACE inhibitors or AGTR-1 blockers could enhance whole-body and skeletal muscle insulin sensitivity, which is associated with increased expression of GLUT4 in skeletal muscle (Henriksen et al. 2001a).

It has previously been determined that the ACE inhibitor temocapril improves insulin resistance and glucose intolerance by increasing glucose uptake in skeletal muscle at least in part through GLUT4 translocation (Shiuchi et al. 2002). AGTR-1 blockade also increases insulin sensitivity and glucose uptake in skeletal muscle of KK-Ay mice, via stimulation of the insulin signalling cascade and increasing GLUT4 translocation to the plasma membrane (Shiuchi et al. 2004). However, no studies have elucidated the underlying mechanism of yoghurt peptides with high inhibition of ACE activity extracted from yoghurt in improving glucose uptake in human skeletal muscle cells.

We therefore aimed to investigate whether, in isolation and/ or in combination, C3G and peptides with ACE inhibitory activity enhanced glucose uptake, and to understand the related molecular mechanisms of the action. We hypothesized that the combinations of C3G and peptides with ACE inhibitory activity would improve insulin sensitivity by regulating glucose disposal in human primary skeletal muscle myotubes derived from obese or obese T2DM individuals.

5.3 Material and methods

5.3.1 Material and reagents

HPLC grade C3G was provided by Polyphenols AS (Sandnes, Norway). Pierce™ bicinchoninic acid (BCA) Protein Assay Kit, Diethylpyrocarbonate (DEPC) water, α -MEM, amphotericin B, horse serum, fetal bovine serum (FBS), and trypsin/EDTA were obtained from Life Technologies Australia Pty Ltd (Mulgrave, VIC, Australia). iQ™ SYBR Green and iScript™ cDNA Synthesis Kit were purchased from Bio-Rad Laboratories Pty Ltd (Gladesville, NSW, Australia). Liquid scintillation cocktail and deoxy-D-glucose, 2-[1,2-³H (N)]- were purchased from Perkin-Elmer, Inc. (Glen Waverley, VIC, Australia). All other

chemicals, unless otherwise specified, were obtained from Sigma-Aldrich Pty. Ltd. (Sunshine, VIC, Australia).

Peptides were extracted from yoghurt fermented by *Lactobacillus helveticus* 881315 in the presence of Flavourzyme for 12 hours. ACE inhibitory activity of peptides was determined using a reversed-phase-HPLC system (RP-HPLC, from Varian Analytical Instruments, Santa Clara, CA, USA) and the IC₅₀ value of peptides was 1.47 ± 0.04 mg/mL. The details on the process of fermentation, peptide extraction and determination of ACE inhibitory activity have previously been reported (Shi et al. 2017a). Subsequently, peptides derived from the same batch (with an IC₅₀ value of 1.47 ± 0.04 mg/mL) as we have previously reported were used in the present cell culture study (Shi et al. 2017a).

5.3.2 Participants

10 obese (eight female and two male), and 10 obese T2DM (eight female and two male) participants undergoing abdominal surgery were recruited for additional tissue collection (Table 5.1). After fasting for 12–18 hours, general anesthesia was induced with a short-acting propofol and maintained by a fentanyl and rocuronium volatile anesthetic mixture, and a biopsy of rectus abdominus muscle was removed as previously described (Chen et al. 2005). This research was approved by the Human Ethics Research committee of Victoria University (HRE08-158), and all participants gave written informed consent.

Table 5.1: Participant Characteristics

	Participants	
	Obese	Obese T2DM
n (F/M)	8/2	8/2
Age (years)	49 ± 4	53 ± 2
BMI (kg/m ²)	40.42 ± 1.64	42.80 ± 2.38
Fasting plasma glucose (mmol/L)	5.02 ± 0.13	8.61 ± 0.76 *
HbA1c (%)	5.59 ± 0.09	7.82 ± 0.47 *
Plasma insulin (μU/L)	10.17 ± 1.64	21.62 ± 2.53 *
Cholesterol (mmol/L)	5.74 ± 0.52	5.01 ± 0.37
Fasting Triglyceride (mmol/L)	1.75 ± 0.31	2.36 ± 0.46

Values are expressed as means ± SEM. * indicated a significant difference between obese and obese T2DM groups (p < 0.05).

5.3.3 Human primary skeletal muscle myotubes

Human primary skeletal muscle cell culture was established as previously described (Blau and Webster 1981; Gaster et al. 2001). Cells were thawed at passage four and grown to 70% confluence in growth media [α -MEM supplemented with 10% FBS (vol/vol), 0.5% penicillin (vol/vol) and 0.5% amphotericin B (vol/vol)]. Cells were then detached and resuspended in the growth medium and seeded on six-well and twelve-well plates (Greiner, Longwood, FL) coated with essential coating matrix (ECM) gel. Once at 80% confluence, the growth medium was replaced by the differentiation media [α -MEM, 2% horse serum, 0.5% penicillin (vol/vol) and 0.5% amphotericin B (vol/vol)] for 6 days for subsequent experimentation.

5.3.4 Glucose uptake bioassay

Glucose uptake assay in human primary skeletal muscle myotubes was established according to previously described methods (Boue et al. 2016) with minor modification. Following

differentiation cells were treated with C3G (10 and 100 μM) and peptides with ACE inhibitory activity (150 $\mu\text{g}/\text{mL}$ and 1500 $\mu\text{g}/\text{mL}$) in isolation and combination in the presence of serum-free medium for 24 hours. At the end of the treatment period the cells were treated in the presence or absence of insulin 100 nM at 37 °C for 30 min and supplemented with 2-deoxyglucose (DOG)/2- $[\text{}^3\text{H}]\text{DOG}$ (10 μM final concentration, 1 $\mu\text{Ci}/\text{well}$) for 15 min at 37 °C. The reactions were stopped by washing cells with cold PBS. Cells were then solubilized by the addition of 0.5 mL of 0.3 M NaOH. An aliquot of 400 μL was utilized for the determination of glucose transport activity by scintillation counter (Tri-Carb 3180TR/SL; Perkin-Elmer, Inc.) and expressed as picomoles of DOG taken up per minute per milligram of total protein. The remaining solution was utilized for determination of protein concentration via BCA assay using a Pierce™ BCA Protein Assay Kit based on the previous method (Bainor et al. 2011). Absorbance was read at 562 nm with a Varioskan™ LUX Multimode Microplate Reader (Thermo Fisher Scientific Laboratory Equipment, Hudson, United States). A standard curve was prepared and protein content for each sample was calculated.

5.3.5 RNA extraction

Following treatments, human primary myotubes were lysed using TRIzol and then stored at -80 °C prior to total cellular RNA extraction according to previously established methods (McAinch et al. 2006). Total RNA was quantified using NanoDrop spectrometry (NanoDrop 2000, Thermo Fisher Scientific, Wilmington, DE) at 260 nm. 0.5 μg of total RNA was reverse transcribed into cDNA using iScript™ cDNA Synthesis Kit according to the manufactures instructions using a MyCycler™ Thermo Cycler (Bio-rad Laboratories, Hercules, CA).

5.3.6 Real-Time PCR analysis

'Real-time' PCR was conducted using MyiQ™ multiplex 'real-time' PCR detection system (Bio-Rad Laboratories, Hercules, CA) with iQ™ SYBR Green Supermix. Forward and reverse oligonucleotide primers (Table 5.2) for the gene of interest were designed using OligoPerfect™ Suite (Invitrogen, Carlsbad, CA). Selective gene homology for genes of interest was confirmed with BLAST (Basic Local Alignment Search Tool, National Centre for Biotechnology Information, Bethesda, MD). To compensate for variations in RNA input amounts and reverse transcriptase efficiency, mRNA abundance of the housekeeping genes, cyclophilin and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) were also measured. The 'real-time' PCR cycling parameters were as follows: initial denaturation and enzyme activation at 95 °C for 3 minutes, followed by 40 cycles of denaturation at 95 °C for 15 seconds, annealing and extension at 60 °C for 45 seconds. Relative changes in mRNA abundance was normalised to the average of two housekeeping genes (cyclophilin and GAPDH) and then quantified using the $2^{-\Delta\Delta CT}$ method. Treatment groups were expressed relative to control treatment and all experiments were repeated at least three times.

Table 5.2: Human primer sequences used for ‘Real-time’ PCR analysis.

Gene	Forward Primer (5' - 3')	Reverse Primer (5' - 3')
AGTR-1	CTGATGCCATCCCAGAAAGT	CTTCCAGCTTTGGGACAATC
AMPK- α	AACTGCAGAGAGCCATTCAC TTT	GGTGAAACTGAAGACAATGTGCTT
Cyclophilin	CATCTGCACTGCCAAGACTGA	TTCATGCCTTCTTTCACTTTGC
FoxO1	TCATGGATGGAGATACATTGGATT	TCCTGCTGTCAGACAATCTGAAG
GAPDH	CAA CGA CCA CTT TGT CAA GC	TTA CTC CTT GGA GGC CAT GT
GLUT1	GGGCCAAGAGTGTGCTAAAG	TGCCGACTCTCTTCCTTCAT
GLUT4	CACCCTCACCACCCTCTG	CTTTTCCCAAGCCACTG
IRS-1	GTTTCCAGAAGCAGCCAGAG	TGAAATGGATGCATCGTACC
IRS-2	ACGCCAGCATTGACTTCTTGT	TGACATGTGACATCCTGGTGATAA
PI3K	GGAAGCAGCAACCGAAACAA	TTCGCCGTCCACCACTACA
PPAR- α	GAAGCTGTCCTGGCTCAGAT	GGGGACCACAGGATAAGTCA

5.3.7 Statistical analysis

All results were expressed as mean \pm standard error of the mean (SEM) for each measurement (n = 8-10). Two-way ANOVA was performed to determine the differences of glucose uptake rate between the presence and absence of insulin in all treatments using GraphPad Prism 7 (GraphPad Software, Inc, La Jolla, CA, USA). Student t-test was used to analyse the significant difference in characteristics of participants between obese and obese T2DM groups. One-way ANOVA was performed to analyse the significant differences in the mRNA expression of genes between control and treatments. Fisher’s (least significant difference; LSD) test was used to differentiate significant differences amongst the treatment groups. $P < 0.05$ was considered significant.

5.4 Results

5.4.1 Effect of C3G and peptides with ACE inhibitory activity on glucose uptake in human primary skeletal muscle myotubes of obese and obese T2DM participants.

As shown in Figure 5.1A, both low and high C3G concentrations (10 and 100 μM), and the combinations of low C3G with peptides (both low and high concentrations) did not alter glucose uptake in the absence of insulin in human skeletal muscle myotubes from obese participants. However, glucose uptake in human myotubes from obese participants treated with high C3G (100 μM) with insulin stimulation was significantly increased 1.41 fold ($P = 0.044$), compared to cells treated with insulin alone. Furthermore, high C3G concentration (100 μM) combined with peptides at the concentrations of 150 $\mu\text{g/mL}$ and 1500 $\mu\text{g/mL}$ in the presence or absence of insulin significantly enhanced glucose uptake ($P < 0.001$), compared with their respective controls. Furthermore, compared with high C3G concentration alone, the combinations of C3G and peptides significantly increased glucose uptake ($P < 0.001$, Figure 5.1A) in the absence of insulin. The high peptide concentration (1500 $\mu\text{g/mL}$) alone resulted in the highest glucose uptake with 1.87 ± 0.59 fold being recorded in the absence of insulin, compared with the control (Figure 5.1 A). Similarly, neither low C3G concentration alone nor its combination with low peptide altered glucose uptake in the presence of insulin in the obese group, compared with the control (Figure 5.1A). In contrast, glucose uptake was improved when treated with low C3G concentration combined with high peptide concentration ($P = 0.011$, Figure 5.1A) in the presence of insulin, compared to control. In addition, the other treatments including high C3G concentration, low and high peptide concentrations alone and their combinations resulted in a significant increase in glucose uptake in the presence of insulin, compared with the control in the obese group.

Figure 5.1B illustrates that, only high peptide (1500 $\mu\text{g}/\text{mL}$) significantly enhanced glucose uptake (by 1.39 ± 0.23 fold) in the absence of insulin in the obese T2DM group, compared with the control group ($P = 0.002$). Glucose uptake was not altered by any of the treatments in the presence of insulin in the obese T2DM group.

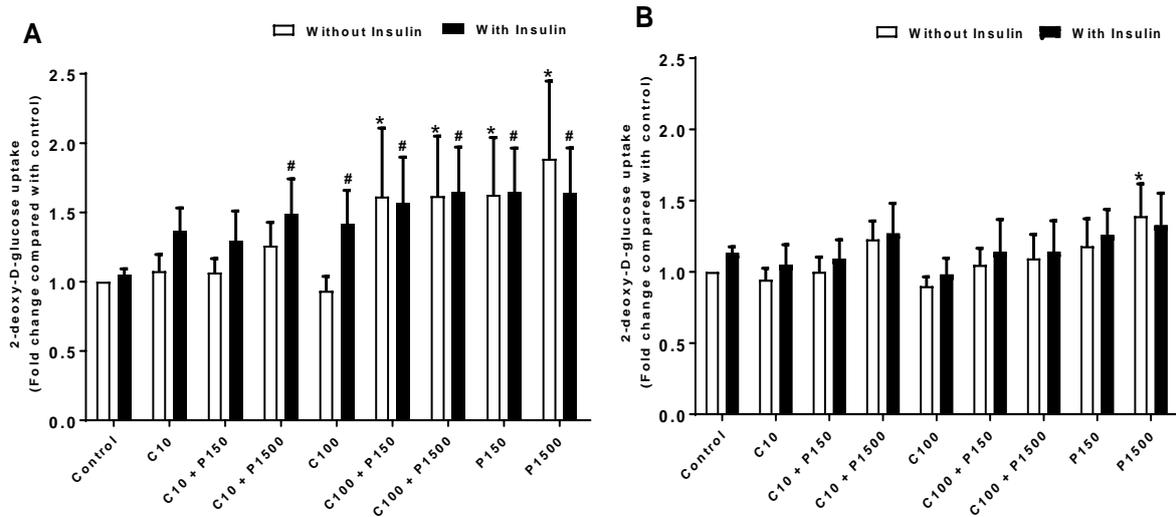


Figure 5.1: The effect of C3G and peptides on glucose uptake in human primary skeletal muscle myotubes derived from obese participants (A) and obese T2DM participants (B).

Cells were treated with BSA (as a vehicle control), 10 μM or 100 μM of C3G, 150 $\mu\text{g}/\text{mL}$ or 1500 $\mu\text{g}/\text{mL}$ of peptides and combinations of C3G/ peptides in the presence or absence of insulin for 24 hours. Data was normalised to control with or without insulin, respectively. Data was expressed as mean \pm SEM ($n = 10$). * $P < 0.05$ compared with control without insulin stimulation; # $P < 0.05$ compared control with insulin stimulation.

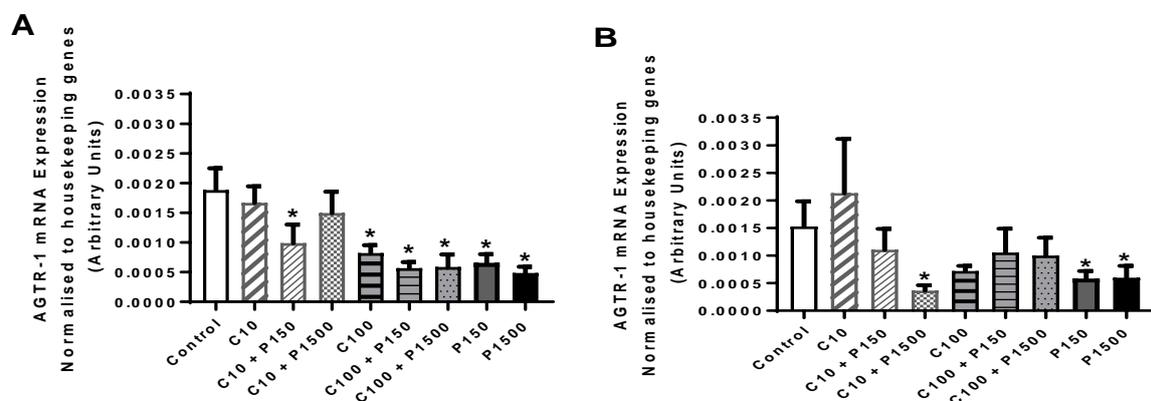
5.4.2 Expression of AGTR-1, GLUT4, IRS-1 and forkhead box protein O1 (FoxO1) in primary human skeletal muscle myotubes of obese and obese T2DM participants.

In this study, we investigated the effects of C3G and peptides in isolation and in combination on the expression of multiple genes associated with obesity and T2DM including AGTR-1, GLUT4, IRS-1 and FoxO1 by 'Real-Time' PCR analysis. As shown in Figure 5.2A, human primary skeletal muscle myotubes from obese participants treated with high C3G (100 μ M), low and high peptide concentrations as well as their combinations exhibited significantly lower AGTR-1 expression compared to control treatment ($P < 0.05$, $n = 8$). The similar results were also observed in the obese T2DM group. AGTR-1 expression was significantly reduced ($P < 0.05$), in myotubes from obese T2DM participants following the treatment with both low and high peptide concentrations and the combination of high peptide (1500 μ g/mL) with low C3G concentration (10 μ M) for 24 hours ($n = 9$, Figure 5.2B). However, high C3G concentration alone and its combination with peptides did not alter AGTR-1 expression in human primary myotubes obtained from obese T2DM participants, compared with control treatment (Figure 5.2B).

1500 μ g/mL of peptide combined with 10 μ M of C3G up-regulated the mRNA expression of GLUT4 in the obese group (Figure 5.2C). For the obese T2DM group, high C3G, high and low peptide concentrations alone, as well as their combinations significantly increased the mRNA expression of GLUT4. However, low C3G concentration (10 μ M) did not change the expression of this gene (Figure 5.2D). GLUT4 mRNA was significantly up-regulated when 10 μ M of C3G was combined with 150 μ g/mL ($P = 0.011$, $n = 10$) and 1500 μ g/mL of peptide ($P = 0.008$, $n = 10$), compared with low C3G concentration (10 μ M) alone in the obese T2DM group (Figure 5.2D).

The mRNA expression of IRS-1 was significantly increased in myotubes derived from obese participants following treatments with high peptide concentration alone as well as combined with low C3G concentration ($P < 0.05$, $n = 10$, Figure 5.2E). However, in the obese T2DM group, only high peptide treatment increased the expression of IRS-1 compared with control treatment ($P = 0.016$, $n = 10$, Figure 5.2F).

Low C3G and peptide concentrations alone as well as in combination did not alter FoxO1 mRNA expression significantly in human skeletal muscle myotubes. A similar result was also observed following the treatment of high C3G concentration (Figure 5.2G, H). In contrast, high peptide concentration alone or in combination with both low and high C3G concentrations ($P < 0.05$, $n = 10$) resulted in a significant reduction in the mRNA expression of FoxO1 in both obese and obese T2DM groups, compared with the control treatment.



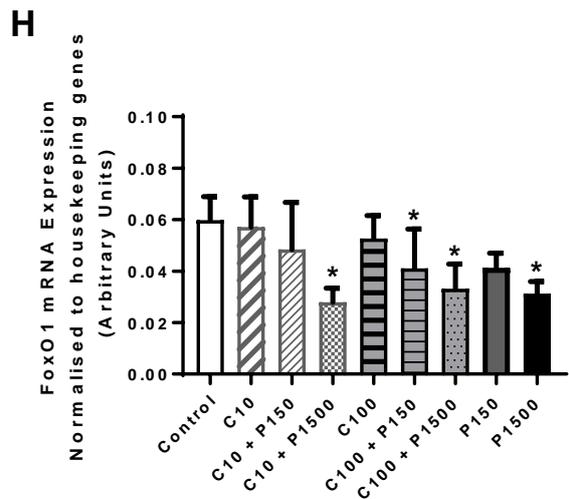
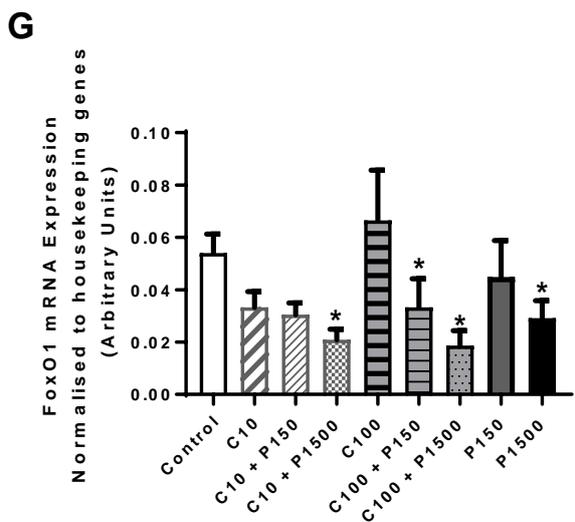
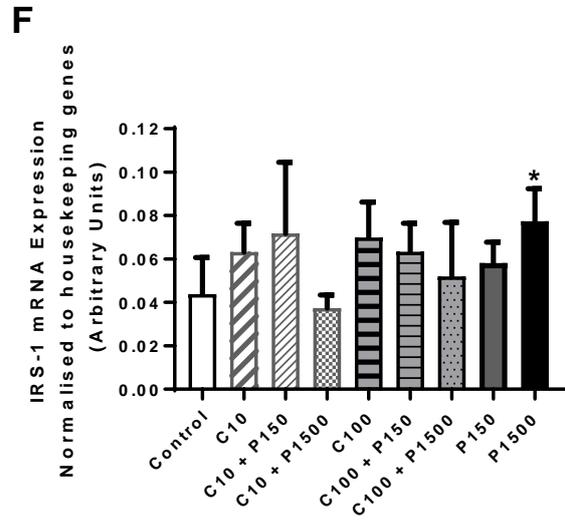
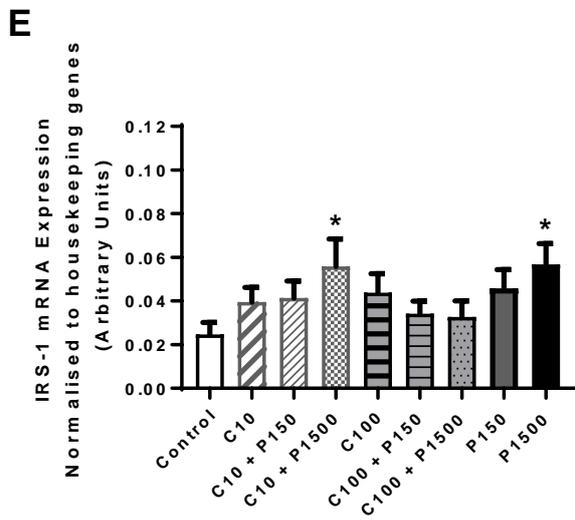
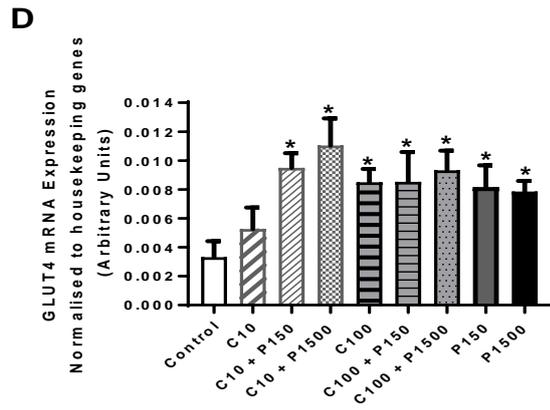
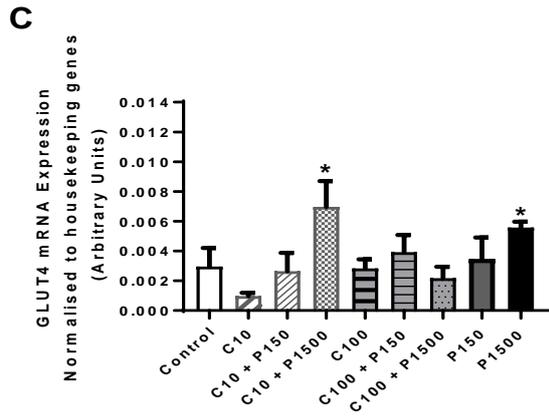


Figure 5.2: The expression of AGTR-1, GLUT4, IRS-1 and FoxO1 in human primary skeletal muscle myotubes derived from obese and obese T2DM participants.

Cells were treated with 10 μ M and 100 μ M of C3G, 150 μ g/mL and 1500 μ g/mL of peptides, and combinations of C3G / peptides for 24 hours. All genes were normalised to the average of two housekeeping genes, GAPDH and cyclophilin. Data was expressed as mean \pm SEM (n = 8-10). Significance is indicated by * $P < 0.05$. The expression of (A) AGTR-1, (C) GLUT4, (E) IRS-1 and (G) FoxO1 in human primary skeletal muscle myotubes derived from obese participants. The expression of (B) AGTR-1, (D) GLUT4, (F) IRS-1 and (H) FoxO1 in human primary skeletal muscle myotubes derived from obese T2DM participants.

5.4.3 ‘Real-time’ PCR analysis of the expression of others genes related to glucose metabolism in human primary skeletal muscle myotubes obtained from obese and obese T2DM participants

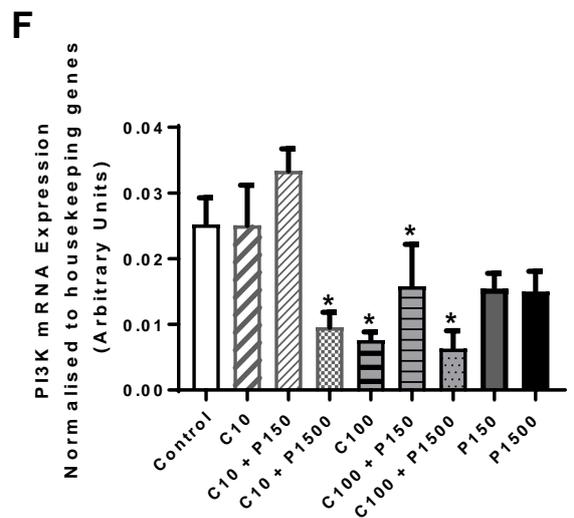
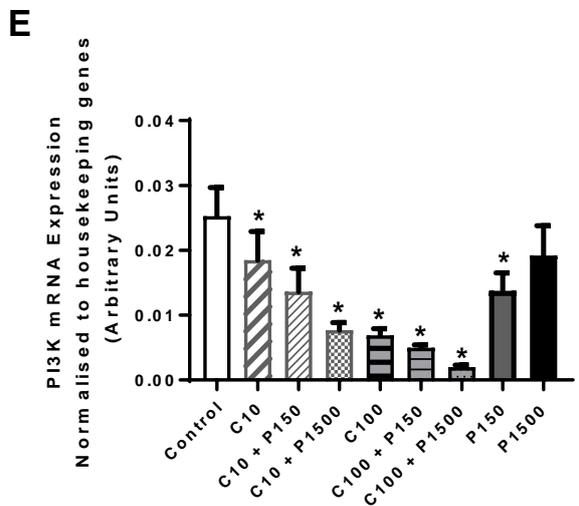
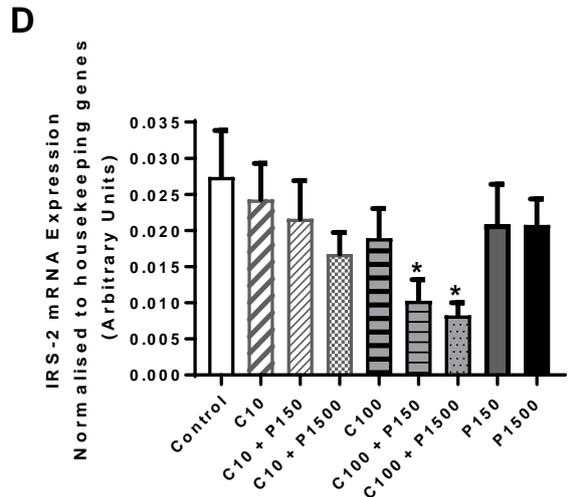
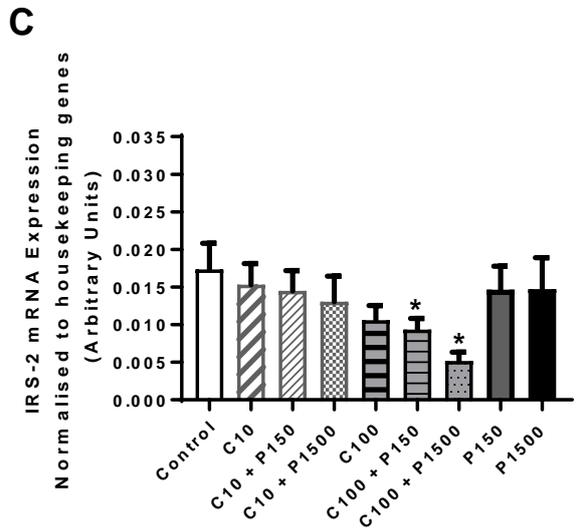
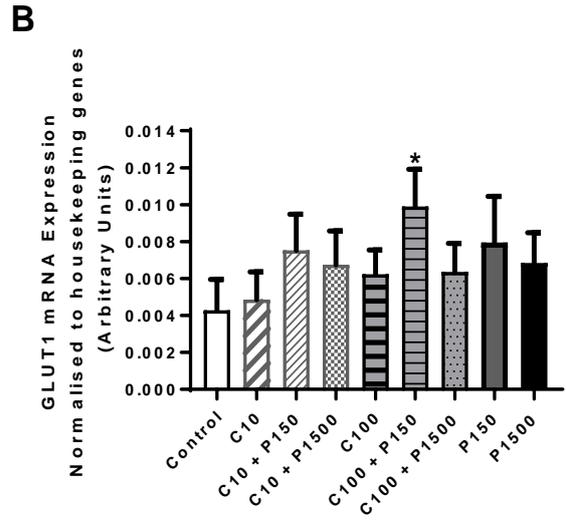
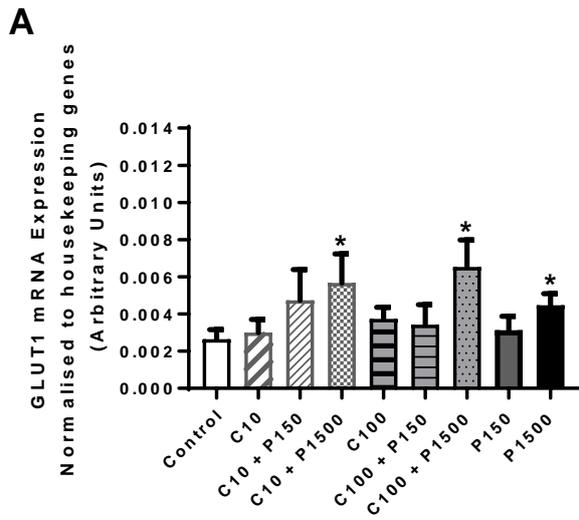
Figure 5.3A showed that GLUT1 mRNA expression was up-regulated by the high peptide concentration (1500 μ g/mL) in the obese group, compared with the control treatment. Treatments with C3G or peptides alone did not alter GLUT1 mRNA expression significantly in human skeletal muscle myotubes obtained from obese T2DM participants (Figure 5.3B). In contrast, the combination of 100 μ M of C3G and 150 μ g/mL of peptide ($P = 0.012$, n = 8, Figure 5.3B) significantly increased the expression of GLUT1 mRNA in myotubes from T2DM participants, compared with the control treatment.

C3G or peptides alone did not alter IRS-2 mRNA expression in the obese group (Figure 5.3C) or obese T2DM group (Figure 5.3D), however it was down-regulated in both groups by the combinations of high C3G concentration with both low and high doses of peptides (150 μ g/mL and 15000 μ g/mL; $P < 0.05$, Figure 5.3C, D).

All treatments including low and high C3G concentrations and their combinations with low and high peptide concentrations ($P < 0.05$, Figure 5.3E), but not high peptide concentration alone, significantly reduced the mRNA expression of PI3K in the obese group. High C3G concentration and its combinations with peptides (both of low and high concentrations) resulted in a significant reduction in mRNA expression of PI3K in the obese T2DM group, and the combination of low C3G (10 μM) and high peptide concentration (1500 $\mu\text{g/mL}$) ($P < 0.05$, Figure 5.3F). The other treatments did not alter mRNA expression of PI3K in the obese T2DM group.

There was no change in the mRNA expression of AMPK- α following the treatments of C3G and peptides alone, as well as the combinations of low C3G concentration with peptides in the human primary skeletal muscle myotubes from obese participants. But peptides combined with high C3G concentration resulted in a down-regulation in the mRNA expression of AMPK- α in the same group (Figure 5.3G). On the other hand, no treatment caused significant changes in the mRNA expression of AMPK- α , compared with control in the obese T2DM group (Figure 5.3H).

C3G and peptide treatments alone and their combinations did not alter the mRNA expression of PPAR- α in the obese group (Figure 5.3I). However, high C3G concentration (100 μM) alone and its combination with peptides (both low and high concentrations) ($P < 0.05$, Figure 5.3I) down-regulated the mRNA expression of PPAR- α , compared with the control treatment. In the obese T2DM group, the mRNA of PPAR- α was not significantly different following the treatments of low C3G alone or peptides alone (both concentrations, Figure 5.3J). In contrast, the expression of PPAR- α was decreased with the treatments of high peptide concentration combined with both concentrations of C3G, as well as high C3G alone ($P < 0.05$, Figure 5.3J).



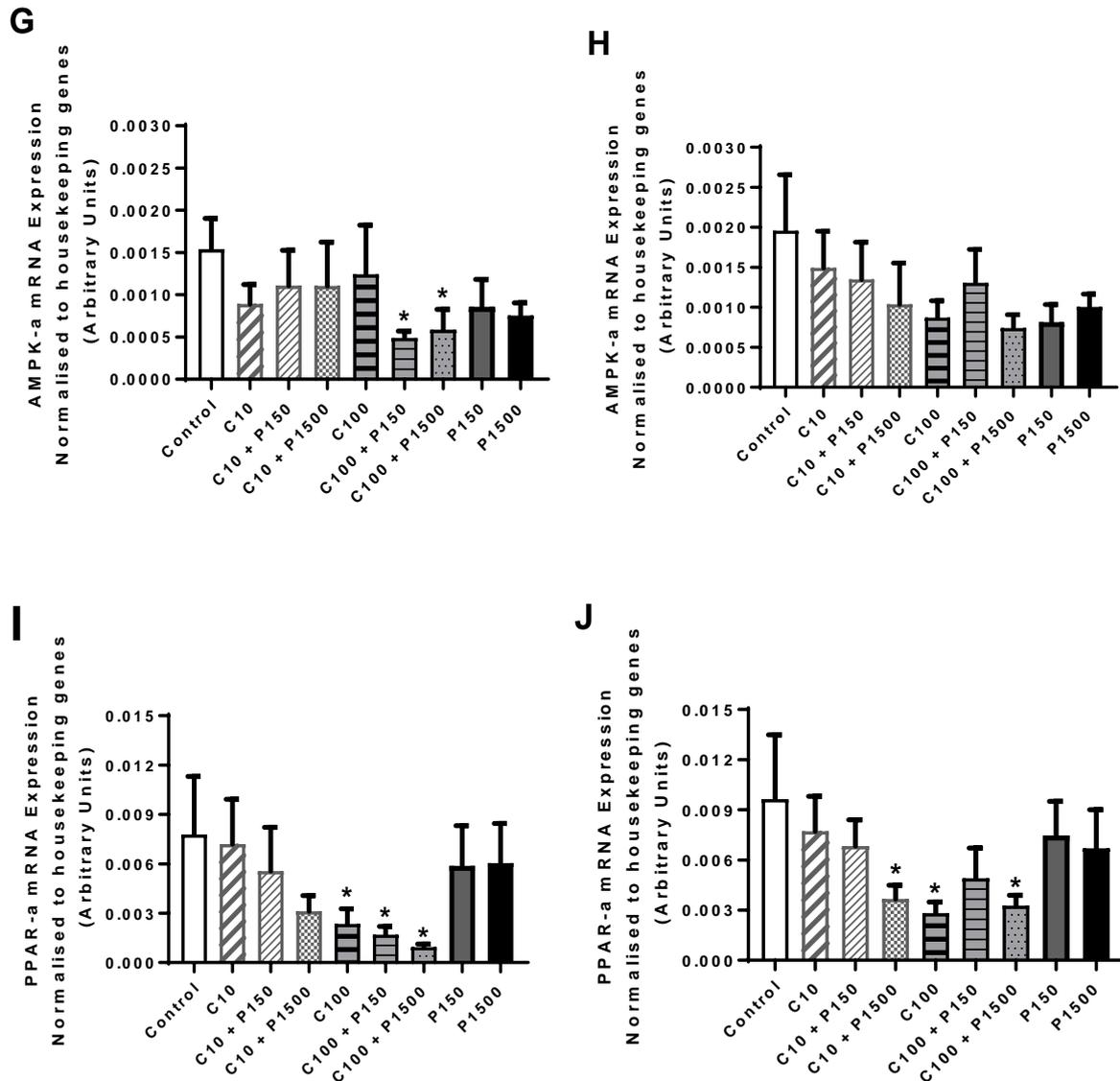


Figure 5.3: The expression of GLUT1, IRS-2, PI3K, AMPK- α and PPAR- α in human primary skeletal muscle myotubes derived from obese and obese T2DM participants.

Cells were treated with 10 μ M and 100 μ M of C3G, 150 μ g/mL and 1500 μ g/mL of peptides, and combinations of C3G/ peptides for 24 hours. All genes were normalised to the average of two housekeeping genes, GAPDH and cyclophilin. Data are expressed as mean \pm SEM. Significance is indicated by * $P < 0.05$ compared to control treatment (n = 8-10). The expression of (A) GLUT1, (C) IRS-2, (E) PI3K, (G) AMPK- α and (I) PPAR- α in human primary skeletal muscle myotubes derived from obese participants. The expression of (B) GLUT1, (D) IRS-2, (F) PI3K, (H) AMPK- α and (J) PPAR- α in human primary skeletal muscle myotubes derived from obese T2DM participants.

5.4 Discussion

Anthocyanins extracted from plants have previously been shown to increase glucose uptake in both L6 and C₂C₁₂ myotubes as well as in 3T3-L1 adipocytes (Martineau et al. 2006; Harbilas et al. 2009; Rojo, L. E. et al. 2012). ACE inhibitors have also been found to enhance the whole-body glucose disposal and glucose transport activity in the skeletal muscle, in a variety of insulin-resistant animal models (Henriksen et al. 2001a; Sugiishi et al. 2013) and insulin-resistant humans with essential hypertension (Kinoshita et al. 2002). The present study is the first to look at the synergistic effects of C3G and peptides with ACE inhibitory activity on glucose uptake in human primary myotubes derived from obese and obese T2DM participants.

In this study, high concentration of C3G in the presence of insulin significantly increased glucose uptake, compared with insulin treatment alone in the obese group, but low C3G concentration in the presence or absence of insulin did not change glucose uptake in either the obese or obese T2DM groups. In agreement with our result, it has previously been demonstrated that an increase in glucose uptake of L6 myotubes was obtained with higher concentrations of anthocyanin combined with insulin (Rojo, L. E. et al. 2012). It is well known that human primary myotubes express obese or diabetic phenotypes when they are established from participants with obesity or diabetes (Berggren et al. 2007; Thingholm et al. 2011). A previous study showed that a reduction of glucose transport stimulated by insulin was observed in myotubes only from obese T2DM participants, not from nondiabetic obese participants, indicating that differentiated myotubes raised from patients with T2DM remain insulin resistant in vitro (Henry et al. 1995). In this current study, only donors who had diabetes demonstrated the characteristics of diabetes with elevated plasma insulin levels and high fasting blood glucose, while donors who were obese but not diabetic did not display any

evidence of hyperglycaemia (Table 6.1). Therefore, differential effects of the treatments on glucose uptake between obese and obese T2DM groups were possibly associated with human primary myotubes preserving their phenotype. In addition, the variation of fibre types, mitochondrial function and mitochondrial capacity in myotubes from different groups may have also exacerbated the differences following the treatments (Minet and Gaster 2010; Thingholm et al. 2011). The differences observed in the current study between obese and T2DM cell lines are however consistent with previous studies, such as those reported with globular adiponectin with variations in a dose effect on AMPK activity in human myotubes obtained from both obese and obese T2DM participants (Chen et al. 2005). This finding also suggests that high concentration of peptides with ACE inhibitory activity have a potential to improve insulin sensitivity and attenuate insulin resistance in both obese and obese T2DM patients, however this may be dose dependent in the obese T2DM state. It has been reported that ACE inhibitors on skeletal muscle glucose uptake were associated with insulin signalling up-regulation, including enhanced IRS-1 tyrosine phosphorylation and PI3K activity (Dal Ponte et al. 1998; Henriksen and Jacob 2003b), which was consistent with our findings that high peptide concentration improved the expression of IRS-1 in human primary myotubes in both obese and obese T2DM groups. *In vivo* studies have reported that the ACE inhibitors captopril (Dal Ponte et al. 1998) trandolapril (Steen et al. 1999) or imidapril (Nawano et al. 1999) could improve oral glucose tolerance and whole-body insulin sensitivity in obese Zucker rats. In this study, peptides with ACE inhibitory activity were extracted from yoghurt fermented by *L. helveticus*. It has been reported that *L. helveticus*, one of the most efficient lactic acid bacteria, possess an efficient proteolytic system capable of producing short peptides from the casein matrix (Sadat-Mekmene et al. 2011b). Furthermore, the small size peptides that were produced, contained proline and hydroxyproline residues, and can usually resist breakdown by digestive enzymes thus they can arrive to intestine (Seppo et al. 2003).

Therefore, these small peptides that exerted the regulatory function observed on glucose metabolism in the current study may be able to exert their function in vivo due to their bioavailability.

To further explore the mechanisms underlying the efficacy of C3G and peptides with ACE inhibitory activity in promoting glucose uptake in human primary myotubes, multiple genes involved in glucose metabolism were assessed. To the best of our knowledge, this is the first study to explore the mRNA expression of AGTR-1 in human primary myotubes following the treatments of C3G and/or peptides that have ACE inhibitory activity. Based on our findings that C3G and/or peptide treatments significantly down-regulated mRNA expression of AGTR-1, and significantly increased the mRNA expression of IRS-1, in both obese and obese T2DM groups, may suggest a possible crosstalk between Ang II and insulin-signalling pathways. Ang II induces insulin resistance via tyrosine phosphorylation of IRS-1 associated with the AGTR-1, thereby attenuating insulin-induced activation of PI3K associated with IRS-1 (Folli et al. 1997; Dal Ponte et al. 1998; Ogihara et al. 2002; Fujimoto et al. 2004). ACE inhibitors also up-regulate the mRNA expression of GLUT4 in skeletal muscle (Carnagarin et al. 2015). Consistent with these observations our results also revealed that the expression of GLUT4 was significantly increased by all treatments except low C3G concentration alone in the obese T2DM group, although the increase in mRNA expression, was not translated into an increase in glucose uptake with the exception of the high peptide concentration in isolation. The mechanisms by which C3G and/ or peptides improved glucose uptake in human primary myotubes remains unclear and requires further analysis. However a down-regulation of AGTR-1 mRNA and an up-regulation of GLUT4 mRNA expression may indicate that these bioactive components have a role in glucose metabolism.

FoxO1, which belongs to the Forkhead family of transcription factors, has a role in skeletal muscle insulin and glucose homeostasis. The over expression of FoxO1 has been shown to impair glycaemic control as a result of reduced skeletal muscle mass (Sugiishi et al. 2013). The activated FoxO1, as a negative regulator of insulin sensitivity, plays a critical role in muscle insulin resistance (Wang et al. 2017). Previous studies have reported that insulin dramatically reduced the FoxO1 nuclear level in insulin-stimulated vehicle-treated myotubes (Southgate et al. 2005), significantly decreasing the stability of FoxO1 protein (Matsuzaki et al. 2003), as well as suppressing FoxO1 transcriptional activity. A recent study has shown that the reduction in FoxO1 could increase GLUT4 expression in transfected C₂C₁₂ cells with siRNA-FoxO1 (Wang et al. 2017). In the present study, mRNA expression of FoxO1 was suppressed by high peptide concentration and its combination with C3G in both obese and obese T2DM groups, suggesting that this inhibition may be attributed to, at least partially, to peptides and C3G having insulin-like properties. Further analysis however, is required to elucidate the mechanisms that caused this observation in human primary myotubes.

It has been established that activation of AMPK increases glucose uptake in skeletal muscle and helps regulate blood glucose homeostasis (Naimi et al. 2017). In the current study, the expression of AMPK- α was decreased with the treatments of high C3G combined with both low and high peptide concentrations in the obese group, while no change was observed in the obese T2DM group. These results are in conflict with previous published findings in which fermented blueberry juice (that had *Serratia vaccinii* bacterium added to it) increased glucose uptake by 48% in C₂C₁₂ myotubes as a result of AMPK activation (Vuong et al. 2007). It has also been demonstrated that dietary bilberry extract significantly activated AMPK in skeletal muscle, and enhanced glucose uptake into skeletal muscle tissue (Takikawa et al. 2010). The different experimental design, such as species (human primary vs mouse immortalised cell-line), phenotype of myotubes (human primary myotubes obtained from obese and T2DM

participants *vs* C₂C₁₂ myotubes), the treatments (single compound *vs* mixture including various anthocyanins and experimental system (*in vivo vs in vitro*) could account for the differences observed between previously reported data and the current study.

In this study, only mRNA of AMPK- α and GLUT4 were measured, but future determination of phosphorylation of AMPK- α and the translocation of GLUT4 would be of value to ensure the changes in mRNA were translated into functional changes within the cell. Numerous studies however, have shown that mRNA of AMPK- α and GLUT4 are correlated with phosphorylation of AMPK- α and the translocation of GLUT4 (Huang et al. 2010; Chen, M. H. et al. 2014; Han et al. 2015; Dhanya et al. 2017). Thus mRNA expression of AMPK and GLUT4 still can be recognized as suitable markers to assess glucose metabolism in human primary myotubes.

5.5 Conclusions

In summary, the results presented here demonstrate that high C3G concentration in combination with peptides with high ACE inhibitory activity extracted from yoghurt (both low and high concentrations) can enhance glucose uptake with or without insulin stimulation in human primary myotubes from obese participants. Only high peptide concentration however, increased glucose uptake in myotubes derived from obese T2DM participants in the absence of insulin. It is possible that the peptides extracted from yoghurt with high ACE inhibitory activity increased glucose uptake through the inhibition of AGTR-1 mRNA expression, and subsequently the increase of IRS-1 and GLUT4 mRNA expression. C3G also demonstrated its potentially anti-diabetic activity, at least in part, in an insulin-dependent signalling pathway, associated with the increase of IRS-1 mRNA expression and GLUT4 mRNA expression. However, the combinations of C3G and peptides appear to have not had an additive effect on either glucose uptake or the regulation of multiple genes. The results

suggest that C3G and peptides with ACE inhibitory activity provide a novel insight into the potential implications of natural anti-diabetic compounds for the regulation of glucose metabolism in obese and obese T2DM participants. Further research is required to elucidate the mechanisms of these active compounds involved in the signalling pathways via regulation of the related protein expression and translocation of GLUT4 to the cell membrane. Moreover, the efficacy of C3G and peptides on glucose metabolism will need to be validated through human clinical trials.

CHAPTER 6: The effects of supplementation with blueberry, cyanidin-3-O- β -glucoside, yoghurt and its peptides on obesity related comorbidities in a diet-induced obese mouse model

This chapter has been published in Journal of Functional Foods, see Appendix 4: Min Shi, Michael L Mathai, Guoqin Xu Andrew J McAinch, Xiao Q Su. 2018. **The Effects of Supplementation with Blueberry, Cyanidin-3-O- β -Glucoside, Yoghurt and Its Peptides on Obesity and Related Comorbidities in a Diet-Induced Obese Mouse Model**. The content of this chapter is the same as the published paper, and only the format has been changed here to match with the thesis.

6.1 Summary

It is widely acknowledged that type 2 diabetes mellitus (T2DM) is associated with obesity, insulin resistance and hypertension. Cyanidin-3-O- β -glucoside (C3G), an anthocyanin in blueberry, and peptides with angiotensin converting enzyme (ACE) inhibitory activity derived from yoghurt are potentially beneficial for numerous health conditions including improving insulin resistance and glucose intolerance. In this study, the synergistic/additive effects of combined supplementations with blueberry and yoghurt, and C3G and peptides were determined. Blueberry and yoghurt alone, and the combination of C3G and peptides significantly reduced both systolic and diastolic blood pressure in diet-induced obese mice. Yoghurt supplementation significantly reduced body weight, percentage body fat and improved intraperitoneal glucose tolerance. Furthermore, peptides and the combination of peptides and C3G resulted in a significant reduction of percentage body fat and improved intraperitoneal glucose tolerance. As widely available, safe and nutritious foods, blueberry

and yoghurt showed therapeutic potential in the treatment of obesity, diabetes and hypertension.

Keywords: blueberry, cyanidin-3-O- β -glucoside, yoghurt, peptides, diabetes

6.2 Introduction

Obesity is a serious problem in industrialized and developing countries worldwide. As the prevalence of obesity has increased, so has the prevalence of obesity-related comorbidities, including type 2 diabetes mellitus (T2DM), cardiovascular diseases, hypertension, stroke, certain forms of cancer and chronic inflammation (Landsberg 2000; Chan et al. 2002; Crujeiras et al. 2012). In recent years much attention has been focused on food components that may have beneficial effects against dysfunction of obesity and its related metabolic diseases (Basu et al. 2010; Stull et al. 2010).

Consumption of blueberries has been shown to prevent obesity and its related comorbidities in several animal models, such as Obese Zucker rats, KKAY mice, C57BL/6J mouse and Sprague-Dawley rats (Vuong et al. 2009; Seymour et al. 2011; Elks et al. 2015). Specifically, long-term feeding of blueberry-enriched diet lowered blood pressure (BP) and improved redox status in kidneys of hypertensive rats and concomitantly demonstrated the potential to delay or attenuate development of hypertension-induced renal injury (Elks et al. 2011). Another study on the obesity-prone Zucker rats, fed a high-fat (45% fat) diet found that supplementation with whole blueberry powder (2% w:w) appeared to reduce fat accumulation and improve insulin sensitivity (Seymour et al. 2009). On the contrary, other studies indicated that administering purified anthocyanins from blueberry via drinking water prevented the development of dyslipidemia and obesity in mice, but feeding diets containing whole berries did not alter the development of obesity (Prior et al. 2008; Prior et al. 2009).

However, it is not clear from these studies whether the anti-obesity and anti-diabetic effects of blueberries were due to the anthocyanins or the whole fruits of blueberry.

Cyanidin-3-O- β -glucoside (C3G), one of the most widely distributed anthocyanins in fruits and vegetables, is an important component in blueberries (Olivas-Aguirre et al. 2016; Wang et al. 2016). It has been reported that C3G ameliorates insulin resistance, hyperglycaemia and obesity-associated inflammation with potent anti-obesity and anti-diabetes properties (Sasaki et al. 2007; Guo, H. et al. 2012b; Liu et al. 2014). Furthermore, *in vitro* studies have also shown that C3G protected 3T3-L1 adipocytes against tumor necrosis factor- α induced insulin resistance (Guo et al. 2008), and eliminated the impacts of high-glucose on the induction of lipolysis in 3T3-L1 adipocytes (Guo, H. et al. 2012a), although the potential mechanisms are still unclear.

Yoghurt, a fermented milk product by lactic acid bacteria (LAB), not only provides energy and nutrients but has also been demonstrated to be beneficial in obesity and diabetes (Honda et al. 2012; Mekkes et al. 2013; Tsai, Yueh-Ting et al. 2014; Evivie et al. 2017). The diet supplemented with yoghurt fermented by probiotic *Lactobacillus acidophilus* and *Lactobacillus casei* significantly delayed the onset of glucose intolerance, hyperglycemia, hyperinsulinemia, dyslipidemia, and oxidative stress in high fructose-induced diabetic rats, indicating a lower risk of diabetes and its complications (Yadav et al. 2007). Furthermore, yoghurt is an excellent source of bioactive peptides, including the antihypertensive peptides or angiotensin-converting enzyme (ACE) inhibitors (Kajimoto et al. 2002; Papadimitriou et al. 2007). Many studies confirmed the importance of ACE inhibition in relation to the prevention of obesity and obesity-related diseases (Manikkam et al. 2016). Carter et al. (2004) suggested that ACE inhibition in aged rats improved their body composition and physical performance compared to control rats (Carter et al. 2004). Another study has shown

that peptides with ACE inhibitory activity (derived from bovine α -lactalbumin) significantly reduced body weight, blood glucose and insulin levels, and downregulated inflammation-related gene expression in adipose tissues of high-fat-diet (HFD)-fed C57BL/6J mice (Gao et al. 2018). Furthermore, our previous study has shown that peptides with ACE inhibitory activity can be extracted from yoghurt fermented by *Lactobacillus helveticus* 881315 in the presence of Flavourzyme (Shi et al. 2017a). However, there is no data available on the effect of combining C3G and antihypertensive peptides for the prevention of obesity and its related disorders.

In this study, we determined the effects of supplementation with blueberry, yoghurt, C3G and yoghurt peptides in isolation and combination on obesity related comorbidities. Following the eight-week supplementation period, changes in the body weight, body fat mass, energy expenditure, glucose tolerance and blood pressure in the diet-induced obese C57BL/6 mice were assessed. We hypothesised that combinations of blueberries and yoghurt, as well as peptides and C3G would have synergistic/additive effects on obesity and its related comorbidities.

6.3 Materials and methods

6.3.1 Chemicals and Reagents

C3G (purity > 97%) was provided by Polyphenols AS (Sandnes, Norway). Fresh blueberries (Rabbiteye blueberry) were obtained from Bhatti and Manj Australian Blueberries (Woolgoolga, NSW, Australia). Fructose was purchased from Consolidated Chemical Company (Dandenong, South VIC, Australia). 36% Fat Modified Rodent Diet (59% of total energy from fat) was obtained from Specialty Feeds (Glen Forrest, WA, Australia). All other

chemicals, unless otherwise specified, were obtained from Sigma-Aldrich Pty. Ltd. (Sunshine, VIC, Australia).

Skim milk was fermented by *Streptococcus thermophiles* ASCC 1275 and *Lactobacillus delbrueckii* subsp. *bulgaricus* 1466 to produce the yoghurt. The yoghurt was further fermented by *Lactobacillus helveticus* 881315 in the presence of Flavourzyme and the end product was used in the animal study. Peptides with ACE inhibitory activity were extracted from the yoghurt and the IC₅₀ value was 1.47 ± 0.04 mg/mL. The details on the process of fermentation, peptide extraction and determination of ACE inhibitory activity have previously been reported (Shi et al. 2017a). Subsequently, peptides derived from the same batch (with an IC₅₀ value for ACE inhibition of 1.47 ± 0.04 mg/mL) as we have previously reported (Shi et al. 2017a) were used in the present animal study.

6.3.2 Animal feeding

Six-week-old male C57BL/6 mice were obtained from Animal Resources Centre (Perth, WA, Australia). As male mice are more likely to develop diabetes than females (Wang and Liao 2012), and also considering the fluctuation of female hormones associated with heat, male mice were used in this study. All animals were housed in a stable environment maintained at 22 ± 1 °C with a 12 hour light/dark cycle (7:00 – 19:00). The mice had *ad libitum* access to food (standard chow) and water for four days prior to commencement of the experimental procedures. Mice were separated into eight groups (n = 10 for each group) and fed different diets: standard chow with 5% fat (SCD), high-fat high-carbohydrate (59% of total energy from fat plus 30% fructose water, diet alone (HFHC) and HFHC with six dietary supplements including blueberry (HFHC+B), C3G (HFHC+C), yoghurt (HFHC+Y), peptides extracted from yoghurt (HFHC+P), combined blueberry and yoghurt (HFHC+B+Y) and combined C3G and peptides (HFHC+C+P). For the first 8 weeks, ten mice (control group) were fed a

SCD diet, while 70 mice were fed a HFHC diet. At week 9, mice of control group continued with SCD diet, while the remaining 70 mice were randomised into seven groups (n=10 per group), in which 60 mice were fed with six dietary supplements, and one group (10 mice) continued with HFHC diet without supplementation for another 8 weeks. The supplements were added to the mixture of gelatine and water at a temperature just below 40 °C. The set supplemented jelly was then fed to mice separately to their HFHC diet. The dose of supplementations were 6.4 g/kg body weight (BW)/ day of blueberries, 0.02 g/kg BW/ day of C3G, 3 g/kg BW/ day of yoghurt and 0.2 g/kg BW/ day of peptides. The amount of blueberries and peptides were chosen based on the previous studies (Nakashima et al. 2002; Mykkanen et al. 2014). The supplemented dosage of C3G was matched with its content in blueberries (0.31% of cyanidin in blueberry powder). Furthermore, the dosage of yoghurt was matched with the amount of peptides it contained (7% in yoghurt powder). Mice were housed individually in divided cages and provided a separate diet (blueberries, C3G, yoghurt or peptides, or the combination of blueberries and yoghurt, or C3G and peptides) with gelatine during the 8-week treatment period. To keep consistency, mice in SCD and HFHC groups were also fed gelatine but without supplementation. Diet supplements were prepared fresh and stored at 4 °C for a maximum of 3 to 6 days following preparation. The standard chow and high fat diets were purchased from Speciality Feeds (Glen Forrest, WA, Australia). Food intake, water consumption, body weight and total energy intake were monitored twice per week and a weekly average for both measurements was calculated. All animal experiments were carried out in accordance with Australian Animal Welfare Act 1992. Experimental procedures were approved by the Animal Ethics Committee of Victoria University (AEC NO: 16/005).

6.3.3 Echo MRI

Echo MRI™ system (Echo-MRI™ 900, Houston, TX, United States of America) was utilised to determine the body composition. Calibration of the machine was undertaken using the canola oil before measurement according to the manufacturer instructions. Animals were scanned three times in the Echo MRI to determine body composition. Mice were acclimatised to this procedure at week one and week five, and then measurements were taken at the 9th week (baseline), 13th week (mid-treatment) and then at the 17th week (end of the treatment).

6.3.4 Blood pressure measurement

Systolic and diastolic blood pressure (BP) were measured every four weeks using a volumetric tail-cuff blood pressure analyser (CODA 8-Channel, Kent Scientific Corporation, CT, USA). Specifically, each mouse was placed into a holder; the holder was then placed into a warming chamber for 5 minutes at 32 °C for acclimation. The mouse tail was threaded through the sensor. Both systolic and diastolic BP were measured for twenty times and data were analysed using the CODA software. Upon completion of the procedure, mice were immediately removed from the cuffs and holder, and returned to their home cage.

6.3.5 Measurement of metabolic variables

Metabolic variables including oxygen consumption (VO_2), carbon dioxide production (VCO_2), energy expenditure, respiratory quotient (VO_2/VCO_2), movement and rest time were monitored using the Promethion system (Sable Systems International, North Las Vegas, NV, United States of America) once every four weeks. Data were recorded for 24 hours continuously.

6.3.6 ipGTT

ipGTT was conducted at week nine (pre-supplementation baseline) and week seventeen (end of treatment). Fructose water was replaced by normal water in the HFHC control and all the treatment groups and mice were fasted overnight for approximately 12-13 hours, with free access to water. A tail snip was performed and a drop of blood (~ 50 μ L) was gently obtained and placed onto a blood glucose strip (FreeStyle Optium Neo H, Abbott, USA) for the measurement of the baseline blood glucose concentration using a glucometer (FreeStyle Optium Neo H, Abbott, USA). Following this, the mouse was injected i.p. with a dose of 1.5 g/kg body weight of glucose/0.9% sterile saline solution with 150 mg/mL of glucose (Mcfarlane Medical, Surry Hills, Victoria, Australia). The blood glucose concentration was measured at 15, 30, 45, 60, 90, 120 mins following glucose injection. Once this procedure was completed the mice were given access to food and water again.

6.3.7 Anaesthesia and Tissue Collection

After the 8-week supplementation period, mice were deeply anaesthetised using isoflurane. They were then sacrificed via a cardiac puncture. Sequentially, heart, epididymal fat, mesenteric fat, liver and kidney were collected and weighed.

6.3.8 Statistical Analysis

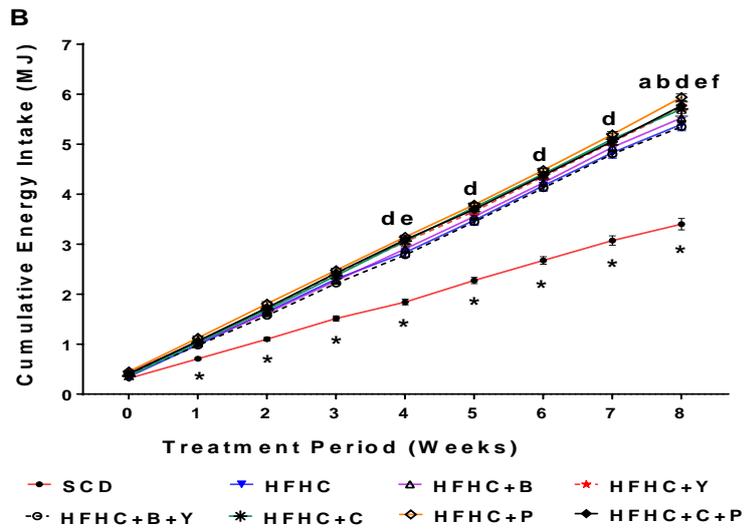
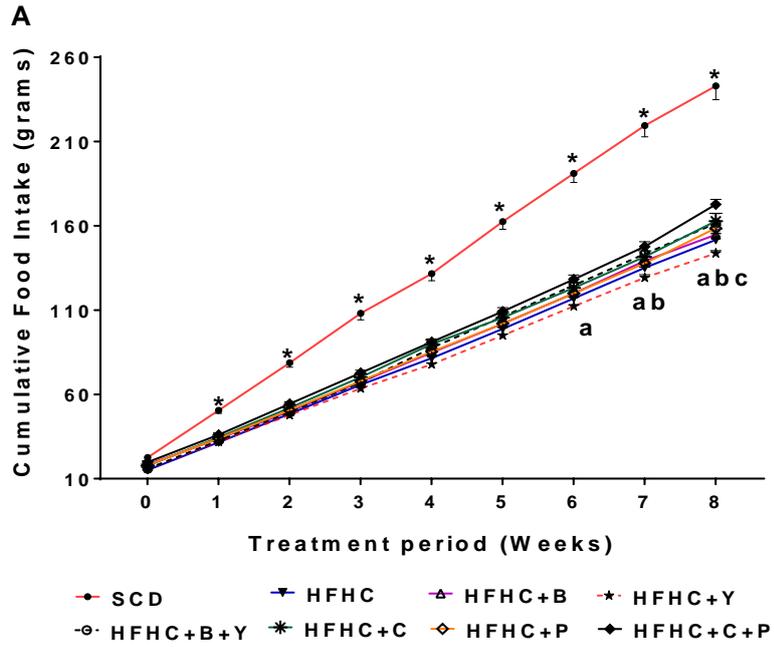
All results were expressed as mean \pm standard error of the mean (SEM, n = 9-10). Two-way ANOVA was performed to determine the differences in cumulative food intake, blood pressure, body composition, and blood glucose level between the treatment groups at various time points using GraphPad Prism 7 (GraphPad Software, Inc, La Jolla, CA, United States of America). Post-Hoc analysis was conducted using Tukey HSD test for multiple comparisons. $P < 0.05$ was considered significant.

6.4 Results

6.4.1 Cumulative food and energy intakes

Cumulative weekly food intake (in grams) was significantly higher in the control group (SCD diet) than in other groups at the beginning of dietary supplementation (Figure 6.1A). The group supplemented with the combination of C3G and peptides (HFHC+C+P) showed a significantly lower cumulative food intake in the last two weeks of treatment compared to the obese control (HFHC) group, while other treatment groups did not show significant differences in food intake from to the HFHC control group. Furthermore, at the end of treatment, the HFHC+Y group showed lower cumulative food intake compared to the HFHC+P group and the HFHC+B+Y group, respectively.

The mice fed the SCD had the least cumulative energy intake ($P < 0.05$) compared with other groups (including the HFHC group) from week one onwards (Figure 6.1B). The animals supplemented with the peptides (HFHC+P) showed a significantly higher cumulative energy intake from week four onwards compared to the obese control group (HFHC, Figure 6.1C). At the end of treatment, the effects of blueberries or blueberries plus yoghurt on cumulative energy intake (HFHC+B and HFHC+B+Y) were not significantly different to the obese control group (HFHC), while the other supplemented groups showed an increased cumulative energy intake compared to the obese control group (HFHC, Figure 6.1C).



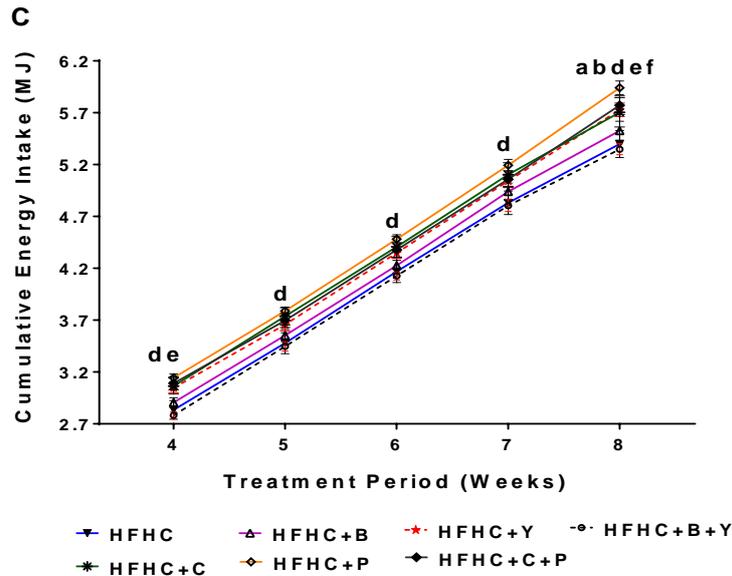


Figure 6.1: Cumulative food intake and energy intake during the treatment period.

Food intake and energy intake were measured every week during the eight-week treatment period, (A) Cumulative food intake (g), (B) cumulative energy intake (MJ) during the whole treatment period and (C) cumulative energy intake (MJ) excluding SCD group from week 4 to week 8 of the treatment. Data were reported as mean \pm SEM. Significant differences ($P < 0.05$) between groups are indicated as follows: * HFHC vs SCD control; ^a represents HFHC+Y vs HFHC+B+Y; ^b represents HFHC vs HFHC+C+P, and ^c represents HFHC+Y vs HFHC+P; ^d represents HFHC vs HFHC+P, HFHC+B vs HFHC+P, HFHC+B+Y vs HFHC+C, HFHC+B+Y vs HFHC+P; ^e represents HFHC+B+Y vs HFHC+C+P; ^f represents HFHC vs HFHC+Y, HFHC vs HFHC+C.

6.4.2 Changes in the body weight

As shown in Figure 6.2A, the HFHC group had a higher body weight than the SCD group from week five. Following the treatment of yoghurt for six weeks, body weight was significantly reduced compared to the HFHC group with no significant difference from the SCD group. However, supplementation with blueberries (HFHC+B) did not seem to protect against the diet-induced weight gain. At the end of the 8-week treatment period, obese mice

supplemented with blueberries (HFHC+B) showed a significant increase in the body weight compared with HFHC control mice (Figure 6.2B). In addition, mice supplemented with blueberries (HFHC+B) were heavier compared to mice supplemented with C3G (HFHC+C, $P < 0.05$, Figure 6.2B).

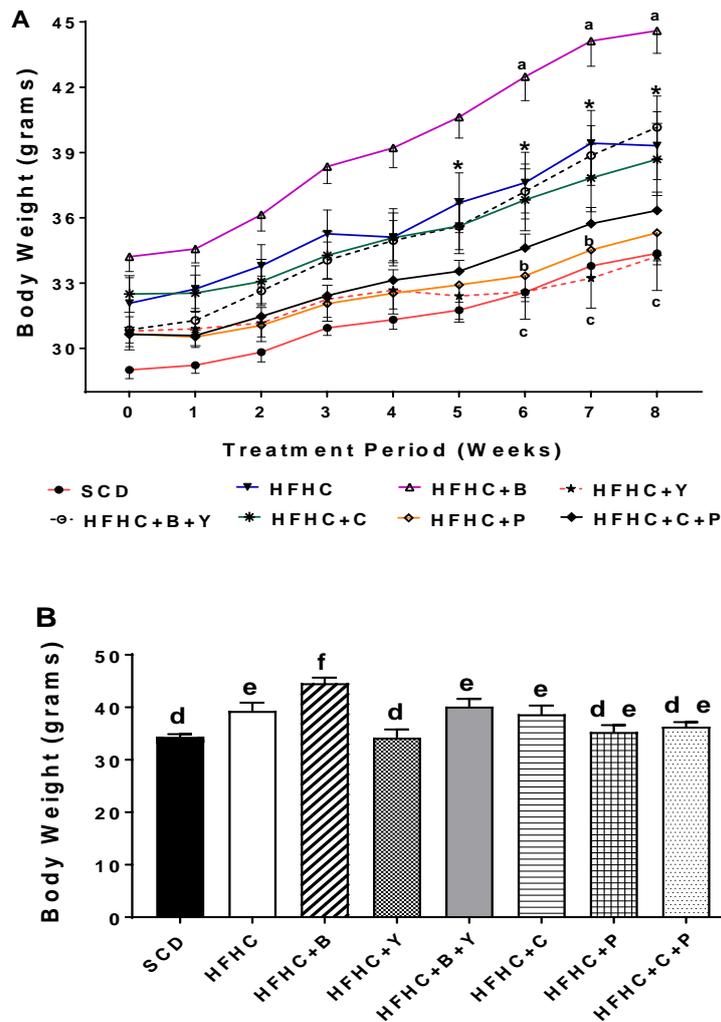


Figure 6.2: The changes of body weight during the treatment period.

Body weight of each group was measured every week during the eight-week treatment period, (A) body weight (g) during the treatment period and (B) body weight (g) at the end of treatment. Data were reported as mean \pm standard error of mean (SEM). Significant differences ($p < 0.05$) between groups are indicated as follows: * HFHC vs SCD control; ^a represents HFHC vs HFHC+B; ^b represents HFHC vs HFHC+P and ^c represents HFHC vs HFHC+Y in Figure 2A. Figure 2B different letters indicate significant differences between groups ($P < 0.05$).

6.4.2 Body composition

At the end of the 8-week treatment, both the weight of body fat and the percentage body fat in the HFHC group were higher than those in the SCD group (Table 6.1). Peptide treatment has reduced both the weight and percentage of body fat significantly compared to the HFHC group, and resulted in a comparable outcome to the SCD group. However, other treatment groups showed no significant differences from the HFHC group.

The weight of body fat in all groups was significantly increased throughout the supplementation period, with the exception of HFHC + Y group in which no significant change was found (Table 6.1). When the whole body weight was considered, the percentage body fat in the HFHC+Y group did not significantly alter in the first four weeks, while a significant increase was recorded in the following four weeks compared to baseline and week four. Based on the data of percentage changes in body composition from baseline, it was noticed that blueberry supplementation resulted in a significant increase in body fat compared with the HFHC group in weeks 4 and 8 (data not shown). In addition, the combination of blueberry and yoghurt supplementation (HFHC+B+Y) for 8 weeks also significantly increased body fat compared to the HFHC group while no other groups showed significant differences (data not shown).

6.4.3 Tissue weights

At the end of the 8-week treatment period, the percentage of heart weight was significantly higher in the SCD, HFHC+Y and HFHC+P groups compared to all other groups with the exception of the HFHC+C+P group (Table 6.1).

As expected, the epididymal fat was higher in the obese HFHC mice compared to the SCD group (Table 6.1). The supplementation of yoghurt (HFHC+Y) significantly decreased

epididymal fat compared with the HFHC group, and no alterations compared to the SCD group.

The percentage of mesenteric fat in the HFHC+B group was greater than in all other groups except the HFHC and HFHC+B+Y groups (Table 6.1). Mesenteric fat in mice with the other supplements did not differ from both the SCD and the HFHC groups.

The percentage of liver weight was unchanged in all groups (Table 6.1). No significant differences in the percentage of kidney weight were observed in all treatment groups compared to both the SCD and the HFHC groups (Table 6.1). However it was significantly decreased in the HFHC+B group compared with the HFHC+Y, HFHC+P and HFHC+C+P groups (Table 6.1).

Table 6.1: Effects of dietary supplementation on body composition, tissue index and metabolic variables of C57BL/6 mice

	SCD	HFHC	HFHC+B	HFHC+Y	HFHC+B+Y	HFHC+C	HFHC+P	HFHC+C+P
Body composition								
Baseline								
Body fat (g)	4.07 ± 0.39 ^a	8.32 ± 0.57 ^b	8.30 ± 0.63 ^b	5.17 ± 0.93 ^{ab}	6.01 ± 0.70 ^{ab}	7.23 ± 0.76 ^{ab}	4.26 ± 0.48 ^a	5.67 ± 0.49 ^{ab}
Body fat (%)	11.48 ± 0.14 ^a	24.76 ± 1.05 ^b	24.63 ± 1.50 ^b	17.45 ± 1.99 ^{ab}	18.98 ± 2.00 ^{ab}	22.20 ± 2.01 ^b	13.95 ± 1.35 ^a	18.42 ± 1.54 ^{ab}
Week Four								
Body fat (g)	4.42 ± 0.52 ^a	9.65 ± 0.55 ^{bc}	13.11 ± 0.74 ^{b*}	5.75 ± 0.98 ^a	8.60 ± 0.93 ^c	8.66 ± 1.11 ^{cd}	4.84 ± 0.52 ^a	6.25 ± 0.68 ^{ad}
Body fat (%)	12.49 ± 0.59 ^a	26.56 ± 0.80 ^{bc}	33.35 ± 1.35 ^{b*}	17.97 ± 2.17 ^{ad}	24.50 ± 2.24 ^{cd*}	24.25 ± 2.55 ^{cd}	14.77 ± 1.26 ^a	18.79 ± 1.85 ^{ac}
Week Eight								
Body fat (g)	7.26 ± 0.58 ^{a*}	13.71 ± 1.07 ^{bc*#}	17.57 ± 0.82 ^{b*#}	7.09 ± 1.43 ^{ac}	13.14 ± 1.26 ^{cd*#}	11.84 ± 1.44 ^{c*#}	7.77 ± 1.05 ^{ad*#}	9.36 ± 1.06 ^{ac*#}
Body fat (%)	19.92 ± 1.09 ^{a*#}	33.80 ± 1.44 ^{bc*#}	38.99 ± 1.12 ^{b*#}	21.07 ± 2.82 ^{a*#}	32.52 ± 2.30 ^{bd*#}	29.90 ± 2.59 ^{cd*#}	21.51 ± 2.07 ^{a*#}	25.44 ± 2.46 ^{ad*#}
Tissue index (% BW)								
Heart	0.43 ± 0.01 ^a	0.35 ± 0.01 ^{bc}	0.31 ± 0.01 ^b	0.43 ± 0.02 ^a	0.35 ± 0.01 ^b	0.36 ± 0.02 ^{bc}	0.43 ± 0.01 ^a	0.41 ± 0.02 ^{ac}
Epididymal fat	2.43 ± 0.16 ^a	3.88 ± 0.24 ^b	3.18 ± 0.20 ^{ab}	2.30 ± 0.37 ^a	3.35 ± 0.18 ^{ab}	3.22 ± 0.27 ^{ab}	2.64 ± 0.31 ^{ab}	3.14 ± 0.38 ^{ab}
Mesenteric fat	0.78 ± 0.07 ^a	1.23 ± 0.11 ^{ac}	1.61 ± 0.15 ^{bc}	0.79 ± 0.12 ^a	1.13 ± 0.11 ^{ab}	1.04 ± 0.09 ^a	0.92 ± 0.12 ^a	1.03 ± 0.13 ^a
Liver	4.55 ± 0.11 ^a	4.61 ± 0.12 ^a	4.92 ± 0.33 ^a	4.44 ± 0.19 ^a	4.44 ± 0.08 ^a	4.91 ± 0.16 ^a	4.40 ± 0.09 ^a	4.79 ± 0.23 ^a
Kidney	1.21 ± 0.03 ^{abc}	1.17 ± 0.03 ^{abc}	1.02 ± 0.03 ^a	1.35 ± 0.05 ^{bc}	1.19 ± 0.04 ^{abc}	1.17 ± 0.04 ^{ab}	1.37 ± 0.06 ^c	1.34 ± 0.07 ^{bc}
Metabolic variables (Week 8)								
VO ₂ (mL/min)	1.39 ± 0.03 ^a	1.77 ± 0.04 ^b	1.94 ± 0.04 ^b	1.77 ± 0.03 ^b	1.90 ± 0.05 ^b	1.84 ± 0.05 ^b	1.84 ± 0.04 ^b	1.80 ± 0.03 ^b
VCO ₂ (mL/min)	1.15 ± 0.03 ^a	1.51 ± 0.04 ^b	1.65 ± 0.04 ^b	1.51 ± 0.02 ^b	1.59 ± 0.04 ^b	1.59 ± 0.04 ^b	1.59 ± 0.03 ^b	1.57 ± 0.03 ^b
Energy expenditure (kcal/day)	9.73 ± 0.20 ^a	12.46 ± 0.29 ^b	13.65 ± 0.29 ^b	12.45 ± 0.20 ^b	13.33 ± 0.36 ^b	12.99 ± 0.32 ^b	13.00 ± 0.27 ^b	12.71 ± 0.23 ^b
Respiratory quotient (VO ₂ /VCO ₂)	0.82 ± 0.01 ^a	0.85 ± 0.01 ^{abc}	0.85 ± 0.01 ^{abc}	0.85 ± 0.01 ^{abc}	0.84 ± 0.01 ^{ab}	0.86 ± 0.01 ^{bc}	0.86 ± 0.01 ^{bc}	0.87 ± 0.01 ^c
Movement (Meters)	181.62 ± 11.97 ^a	205.47 ± 7.07 ^{ab}	252.94 ± 17.94 ^{bc}	230.72 ± 11.24 ^{ab}	247.24 ± 14.78 ^{bc}	229.80 ± 9.70 ^{ac}	246.72 ± 18.77 ^{bc}	218.94 ± 14.93 ^{ac}
Rest time (hrs)	17.82 ± 0.35 ^a	17.39 ± 0.21 ^{ab}	16.32 ± 0.33 ^{ab}	17.76 ± 0.48 ^{ab}	15.89 ± 0.27 ^{ab}	15.80 ± 0.22 ^b	17.55 ± 0.52 ^{ab}	16.46 ± 0.20 ^{ab}

Data are reported as mean \pm SEM. * indicate a significant difference to baseline for the respective group ($P < 0.05$). # indicates a significant difference to week four data for the respective group ($P < 0.05$). Different letters indicate significant differences between groups for the corresponding time point ($P < 0.05$).

BW, body weight; VO_2 , oxygen consumption; VCO_2 , carbon dioxide production; SCD, standard chow with 5% fat; HFHC, high-fat diet (59% of total energy from fat) plus 30% fructose water; HFHC+B, HFHC diet supplemented with blueberries; HFHC+Y, HFHC diet supplemented with yoghurt; HFHC+B+Y, HFHC diet supplemented with blueberries and yoghurt; HFHC+C, HFHC diet supplemented with cyanidin-3-O- β -glucoside; HFHC+P, HFHC diet supplemented with peptides; HFHC+C+P, HFHC diet supplemented with cyanidin-3-O- β -glucoside and peptides.

6.4.4 ipGTT

As expected, HFHC diet impaired glucose tolerance in obese mice compared to SCD after eight weeks feeding (Figure 6.3A, B). There was no difference in glucose clearance in all groups prior to introduction of the intervention diets except the SCD group (Figure 6.3B). Following the 8-week supplementation, mice supplemented with yoghurt (HFHC+Y), peptides (HFHC+P) and the combination of C3G and peptides (HFHC+C+P) showed a significant improvement in the intraperitoneal glucose tolerance compared to the HFHC group, and the outcomes were comparable with that in the SCD group (Figure 6.3C, D). In contrast, supplementation with blueberries (HFHC+B) resulted in the same level of glucose tolerance as in the HFHC group, suggesting that blueberries did not attenuate glucose tolerance, as shown in Figure 6.3D.

6.4.5 Blood pressure

As shown in Figure 6.4A, none of the supplements altered systolic BP in the first four weeks compared with the HFHC or SCD groups. A significantly lower systolic BP was however observed in all supplement groups except the C3G group (HFHC+C) by the end of the feeding period compared with the HFHC group.

Mice fed HFHC diet for eight weeks showed significantly higher diastolic BP, compared with the SCD group (Figure 6.4B). Obese mice supplemented with blueberries (HFHC+B), yoghurt (HFHC+Y) and the combination of C3G and peptides (HFHC+C+P) had significantly decreased diastolic BP, compared to the HFHC group at the end of the supplement period.

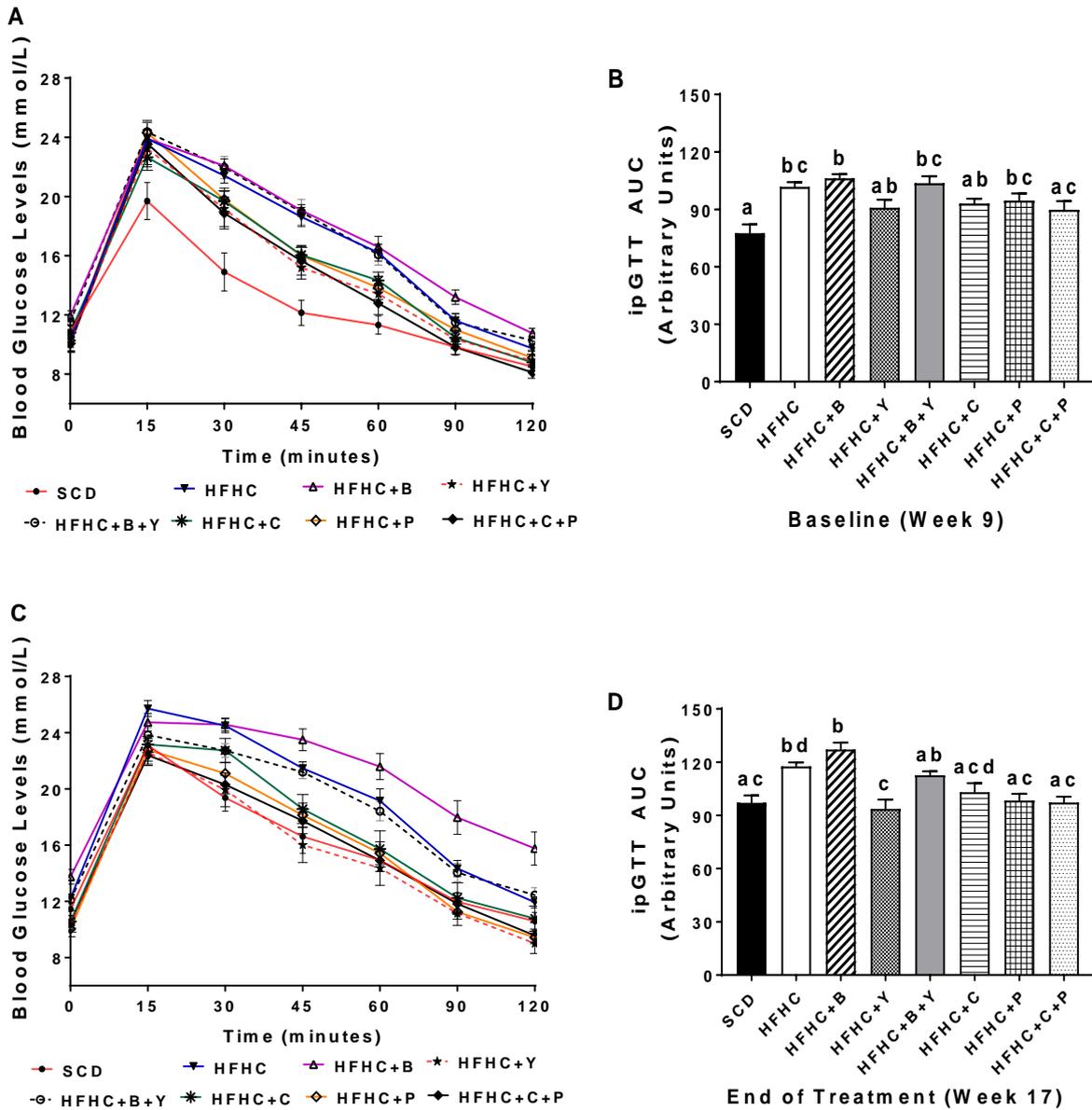


Figure 6.3: Changes in response to blood glucose level as shown in the ipGTT.

Blood glucose concentrations of mice were measured at pre- and post-treatment with different diets. (A) Pre-treatment ipGTT (intraperitoneal glucose tolerance test) blood glucose concentration (mmol/ L) (week 9); (B) Pre-treatment ipGTT AUC (area under the curve, arbitrary units) (week 9); (C) ipGTT blood glucose concentrations (mmol/ L) at the end of treatment (week 17); (D) Post-treatment ipGTT AUC (arbitrary units) at the end of treatment (week 17). Data were reported as mean \pm SEM. Different letters indicate a significant difference between groups ($P < 0.05$).

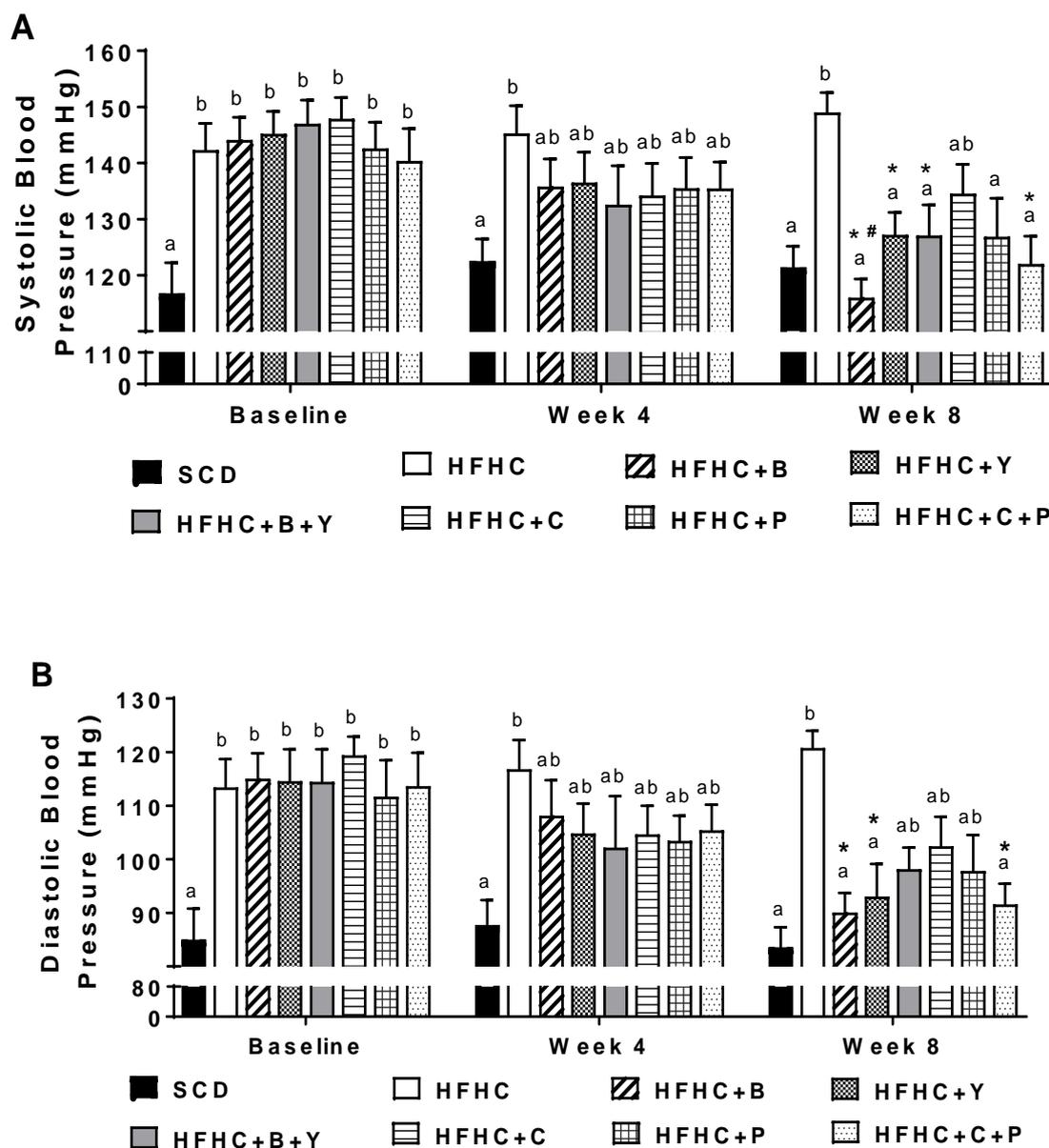


Figure 6.4: Changes in blood pressure following dietary supplementation.

(A) Systolic blood pressure and (B) Diastolic blood pressure of C57BL/6 mice were measured every four weeks during the treatment period. Data were reported as mean \pm SEM. SCD (n = 10), HFHC (n = 9), HFHC+B (n = 10), HFHC+Y (n = 10), HFHC+B+Y (n = 10), HFHC+C (n = 10), HFHC+P (n = 10), HFHC+C+P (n = 10). * indicates a significant difference to baseline for the respective group ($P < 0.05$). # indicated a significant difference to week four data for the respective group ($P < 0.05$). Different letters indicate significant differences between groups ($P < 0.05$).

6.4.6 Metabolic parameters

VO₂, VCO₂, and energy expenditure were significantly increased in the HFHC group, compared to these in the SCD group. Furthermore, VO₂, VCO₂, and energy expenditure were higher in all supplemented groups than the SCD group, while there was no significant difference between all the supplemented groups and the HFHC group throughout the treatment period (Table 6.1).

No significant differences were observed in respiratory quotient between the SCD and the HFHC groups. There were also no differences between all treatment groups and the HFHC group (Table 6.1). Respiratory quotient in the groups supplemented with C3G (HFHC+C), peptides (HFHC+P) and the combination of C3G and peptides (HFHC+C+P) was higher than in the SCD group.

None of the treatments altered movement compared with the HFHC control group, while mice supplemented with blueberries (HFHC+B), peptides (HFHC+P) and the combination of blueberries and yoghurt (HFHC+B+Y) had increased movement compared with the SCD diet group (Table 6.1).

At the end of treatments, no significant difference in the resting time was observed between the SCD and the HFHC groups. The resting time in the HFHC+C group was significantly decreased compared to the SCD group. There were no significant differences observed between any supplementation group and the HFHC or the SCD groups (Table 6.1).

6.5 Discussion

Blueberry is known to be particularly rich in anthocyanins, which have been found to have beneficial effects for people with obesity and metabolic syndrome (Lehtonen et al. 2011; Wu et al. 2013; Shi et al. 2017b). In addition, our previous study showed that bioactive peptides

extracted from yoghurt fermented by *L. helveticus* had high ACE inhibition activity, which indicates its potential anti-hypertensive properties. The current study demonstrated that both systolic and diastolic BP were significantly reduced following supplementations of blueberry, yoghurt and the combination of peptides and C3G groups; a significant inhibition of body weight gain from the HFHC diet following yoghurt supplementation; and a significant reduction in body fat following peptide supplementation. In addition, an improvement in glucose tolerance was observed following supplementations of yoghurt, peptides and the combination of C3G and peptides.

In agreement with our results that eight weeks of blueberry supplementation reduced both systolic and diastolic BP in HFHC induced obese mice, diets enriched with blueberries have been reported to improve vascular tone and decreased BP in spontaneously hypertensive rats, compared with control animals (Shaughnessy et al. 2009; Kristo et al. 2010). Feeding a 3% blueberry diet for six weeks reduced plasma ACE activity in the spontaneously hypertensive stroke-prone rats (Wiseman et al. 2011). A human trial also showed that consumption of 50 mL of 1.5 mM total polyphenols per day for two-weeks resulted in a 36% reduction in serum ACE activity and a 5% reduction in systolic BP of hypertensive patients (Aviram and Dornfeld 2001). Therefore, the anti-hypertensive effect of blueberry was at least partially due to inhibition of ACE activity in the blood. Interestingly a pure anthocyanin, C3G, at the same dose as what was found in blueberries did not alter the BP in HFHC induced obese mice, suggesting that positive outcomes of the whole blueberry may be associated with other compounds, instead of C3G. Further studies are required to determine all the active components in blueberries and the underlying mechanisms associated with their anti-hypertensive properties.

Yoghurt fermented milk by LAB has received much attention as a functional food and nutrient. Consumption of yoghurt has been demonstrated to reduce fasting and postprandial 2 h blood glucose levels in db/db mice (Yun et al. 2009), while consumption of a probiotic yoghurt also improved fasting blood glucose in type 2 diabetic patients (Ejtahed et al. 2012). In agreement with previous research, the current study showed that feeding yoghurt and its peptides with ACE inhibition for eight weeks improved glucose tolerance in obese mice compared with the HFHC control group. It has been suggested that yoghurt regulates blood glucose due to LAB, which possess anti-diabetic propensity on their hosts and thus can play a crucial role in human health care (Evivie et al. 2017). The anti-diabetic activity of LAB differed depending on the bacterial strain and whether the bacteria are able to hydrolyse protein to produce short peptides with possible resistance to physiological digestion after ingestion (Matsui et al. 2002; Honda et al. 2012). It has been reported that *L. helveticus*, one of the most effective LAB, possess an efficient proteolytic system capable of producing short peptides from the casein matrix (Sadat-Mekmene et al. 2011b). Furthermore, the small size peptides produced by *L. helveticus*, containing proline and hydroxyproline residues, can usually resist breakdown by digestive enzymes, thus they can reach the intestine and can be detected in faecal samples after ingestion (Takano 1998; Seppo et al. 2003; Saito et al. 2004). It has been reported that peptides with ACE inhibitory activity containing proline and hydroxyproline residues are absorbed intact and present in the bloodstream (Segura-Campos et al. 2011). Taken together, in this study, the dietary supplementations with yoghurt fermented by *L. helveticus* displayed their anti-diabetic effect, which was most likely related to its LAB, *L. helveticus*, and the bioavailability of these bioactive peptides.

Milk contains high quality proteins including whey protein and casein which can contribute to appetite control by increasing satiety and suppressing food intake (Akhavan et al. 2010; Akhavan et al. 2014; Nabavi et al. 2015). The results from the present study are in agreement

with the impact of dairy proteins on appetite as the cumulative food intake was reduced significantly following yoghurt supplementation, compared with peptides supplementation. Moreover, the body weight, the percentage of body fat and epididymal fat of obese mice were reduced significantly in the yoghurt supplemented group, compared with the HFHC control group, although there was no significant difference in cumulative food intake between these two groups. Other possible mechanisms underlying the role of yoghurt in attenuating obesity, as suggested by Tsai et al. (2014) and Evivie et al. (2017), include modulation enzymes involved in processing of dietary polysaccharides and the regulation of energy metabolism (Tsai, Yueh-Ting et al. 2014; Evivie et al. 2017). It appears that peptides have no effect on obesity, and this may be partially due to increased energy intake compared with the HFHC group.

The proteolytic system of *L. helveticus* has been widely studied due to its ability to release peptides containing proline during milk fermentation with ACE inhibitory activity (Seppo et al. 2003; Jauhiainen et al. 2005; Beganovic et al. 2013; Chen, Y. et al. 2014). However, the health benefits of fermented yoghurt by adding *L. helveticus* have not previously been investigated. Our study found that feeding fermented yoghurt for eight weeks significantly reduced both systolic and diastolic BP in HFHC induced obese mouse, which may be due to the ACE-inhibitory peptides extracted from yoghurt. In order to confirm the mechanisms of BP regulation following yoghurt supplementation, peptides with ACE inhibitory activity, extracted from yoghurt were also included in the animal study with the same dosage as in the yoghurt group. It is interesting to note that purified peptides significantly reduced only the systolic BP compared to the HFHC control group. The effect on hypertension in the peptide group was not fully matched with that of the yoghurt group, possibly due to other active components in yoghurt. In addition, peptides failed to reduce diastolic BP, and this may be due to the low dosage. Several reports have shown that the anti-hypertensive effect of milk

peptides is dose-dependent (De Leeuw et al. 2009; Hirota et al. 2011). The present dose of peptides (0.2 g/kg BW/ day) maybe insufficient to change the BP in HFHC-induced obese C57BL/6 mice by a single oral administration daily. These findings suggested that yoghurt might be more effective to regulate BP, which related to the synergistic action with other components, such as protein, amino acids and the minerals, in addition to the peptides with ACE inhibitory activity. Cell wall fragments from LAB in yoghurt may be another possibility for the better effect of yoghurt on hypertension as suggested by Sawada et al (Sawada et al. 1990). It is unclear why alone both blueberry supplementation and yoghurt supplementation decreased diastolic BP, but when combined there was not an equal reduction or synergistic/additive effect observed. As both of these supplements contain a number of potential active components any number of these could have had accumulative or antagonistic effects on diastolic BP. Further research is warranted to uncover these complex interactions.

In this study, lower body weight and body fat mass were observed in the C3G group, compared to the blueberry group. This is consistent with the previous findings by Prior et al (2019), which showed that consumption of purified anthocyanins from blueberries reduced obesity, but not the whole blueberry, which did not prevent and may have actually increased obesity in the C57BL/6J mouse model (Prior et al. 2008). DeFuria et al. (2009) also found that blueberry supplementation (4% wt:wt whole blueberry powder) did not affect HFD-associated alterations in energy intake, metabolic rate, body weight, or adiposity in C57Bl/6J mice (DeFuria et al. 2009). In this study, C3G content supplemented to obese mice was matched between the blueberry group and C3G group. The discrepancy in the effects of blueberries and C3G on body weight and fat mass, thus implicates that blueberries are not as effective as the C3G in protecting against diet induced obesity. This is possibly due to the amount of simple sugars presented in the whole fruit as it is well known that simple sugars,

e.g., fructose and sucrose are closely associated with body weight gain and obesity (Surwit et al. 1995; Messier et al. 2007; Ronn et al. 2013).

Several studies have demonstrated that blueberries have anti-diabetic properties and supplementation or consumption of blueberry cause changes in glucose metabolism and improve insulin sensitivity (Seymour et al. 2009; Vuong et al. 2009). Supplementation of 2% (w:w) freeze-dried blueberry powder improved glucose clearance, as measured by the glucose tolerance test in the obesity-prone Zucker fatty rat for 13 weeks (Seymour et al. 2009). Conversely, Prior et al. (2008) reported that long term supplementation with freeze-dried whole blueberry powder (10% diet) did not affect the results of a glucose tolerance test in C57BL/6J obese mice. A similar observation was found in our study. Blueberry supplementation did not affect glucose metabolism and showed no significant difference in glucose tolerance from the HFHC control animals. To eliminate the interference of sugar and other components in the whole blueberry, an extract of its active component, anthocyanin C3G (with the same dosage in blueberries) was also used in the current study. A previous study showed that blueberry polyphenols improved oral glucose tolerance in hyperglycemic C57BL/6 mice fed HFD for seven weeks (Roopchand et al. 2013). Furthermore, dietary C3G (0.160–0.3 g/kg BW/ day) reduced fasting glucose level and markedly improved the insulin sensitivity in HFD fed C57BL/6J mice, compared with the untreated control (Guo, H. et al. 2012b). However the present study found that glucose tolerance was significantly improved by pure anthocyanin, C3G, compared with the whole blueberry fruit, although there was no difference between C3G and HFHC groups. This may be due to the small amount of C3G (0.02 g/kg BW/ day) which was administrated. Moreover mouse strain maybe another factor that may have contributed to the discrepancies in response to glucose following the treatments. For example, for a genetic diabetic mouse (db/db mouse), treatment with low

dose of C3G (0.01 g/kg BW/ day) for 12 weeks improved the abnormalities of glucose metabolism as evidenced by a decrease in both glucose and insulin levels (Qin et al. 2018).

Based on the findings from this study, the equivalent human doses of treatments would be 0.52 g/kg BW/ day for blueberry supplementation and 0.243 g (3/12.3)/kg BW/ day for yoghurt supplementation (Nair and Jacob 2016). This indicates that, for a young male adult with approximately 70 kg body weight, 36.4 g of dried blueberry powder and 17.1 g of dried yoghurt powder would be required daily, which equal to 182 g of fresh blueberries (20% of dry matter in the fresh blueberry) and 142.5 g of normal yoghurt (12% of dry matter in normal yoghurt). These doses are feasible for the general public (ie less than one small punnet of blueberries and one small tube of yoghurt), thus the study highlights the potential benefits of regular blueberry and yoghurt consumption, and the potential for future manufacturing/cultivation processes to enhance the effectiveness of these food products.

It is acknowledged that the study had several limitations which include that despite attempting to standardise several baseline measurements within the animals following the diet induced obese period, animals in different treatment groups began the treatment period with variations with baseline body composition. In addition, only one dose of C3G (0.02 g/kg BW/ day) was included in the study. This low dose of C3G, despite being chronically administered may not have been high enough to alter a number of measurements undertaken in the current study. Further studies with higher doses of C3G are warranted to verify the health effects of C3G.

6.6 Conclusions

This study demonstrated that both systolic and diastolic BP were significantly reduced in obese mice supplemented with blueberries. However, the active component of blueberry,

C3G did not reduce the blood pressure compared to the HFHC fed animals, although its content was fully matched with that in the blueberries. This implicates that the anti-hypertensive effect of blueberries may be associated with other active components or it may be related to the synergistic effect of multiple components in the whole fruit. In addition, yoghurt fermented by *L. helveticus* possesses protective effects against HFHC induced metabolic disturbances associated with developing obesity, diabetes and hypertension in C57BL/6 mice. Peptides extracted from yoghurt also showed anti-obesity effects with significant reduction in the percentage of body fat and improvement of glucose tolerance being observed in obese mice. Our findings attest that yoghurt is promising candidates for the prevention of obesity related diabetes and hypertension, and blueberries showed anti-hypertensive activity, although the responsible mechanisms behind the beneficial effects require further investigation.

CHAPTER 7: The effect of dietary supplementation with blueberry, cyanidin-3-O- β -glucoside, yoghurt and its peptides on gene expression associated with glucose metabolism in skeletal muscle tissue obtained from a high-fat-high-carbohydrate diet induced obesity model

7.1 Summary

Obesity is a leading global health problem contributing to various chronic diseases, including type II diabetes mellitus (T2DM) and hypertension. Current conventional medical interventions for the treatment of these diseases are limited with varied efficacy. Our previous animal study found that yoghurt, peptides and the combination of cyanidin-3-O- β -glucoside (C3G) and peptides are capable of the improvement in the intraperitoneal glucose tolerance and the outcomes are comparable with that in the lean mice. Due to the significant role that skeletal muscle plays in glucose homeostasis, it is important to understand the potential mechanisms of blueberries, yoghurt, C3G and peptides supplementation on the regulation of glucose metabolism in skeletal muscle obtained from obese mice. The aim of this study was therefore to investigate whether blueberries, yoghurt, and their respective bioactive components, C3G and peptides alone or in combinations, alter the expression of genes related to glucose metabolism in skeletal muscles from diet-induced obese mice.

In extensor digitorum longus (EDL), yoghurt alone up-regulated the expression of AMPK, IRS-1, PI3K and GLUT4, and down-regulated the expression of angiotensin II (ANG II) receptor, type 1 (AGTR-1). The combination of blueberries and yoghurt down-regulated the

mRNA expression of AGTR-1 and FoxO1 in the EDL. Furthermore, the other combined treatments, C3G and peptides also down-regulated the mRNA expression of AGTR-1 and up-regulated the mRNA expression of GLUT4 in the EDL. In the soleus, it was found that supplementation with blueberries and yoghurt alone, and their combination down-regulated the mRNA expression of AGTR-1 and up-regulated the mRNA expression of GLUT4. Moreover, peptides alone lowered the mRNA expression of AGTR-1, and increased the mRNA expression of IRS-1 in the soleus. The mRNA expression of AMPK was up-regulated by C3G and its combination with peptides in the soleus. Therefore, this study revealed that blueberries and yoghurt with their bioactivity components as alternatives to pharmaceutical interventions regulated multiple genes associated with glucose metabolism in mouse skeletal muscles.

7.2 Introduction

Skeletal muscle is the major site for uptake and storage of plasma glucose after a meal, and the process is mediated by the glucose transporter proteins GLUT1 and GLUT4 (Gamboa et al. 2010). The GLUT1 isoform is believed to support basal glucose transport, whereas the GLUT4 isoform increases glucose transport in response to insulin (Zhang et al. 1999; Manolescu et al. 2007). Insulin induces the translocation of GLUT4 from intracellular storage vesicles to the plasma membrane either by binding to its receptor, leading to increased receptor tyrosine kinase activity, phosphorylation of IRS-1, downstream activation of the lipid kinase, PI3K and the serine/threonine kinase Akt (Thurmond and Pessin 2001; Huang and Czech 2007; Gannon et al. 2015; Mokashi et al. 2017; Naimi et al. 2017). Apart from the insulin signalling pathway, an effective means to stimulate GLUT4 translocation to the plasma membrane is through the activation of AMPK (Alkhateeb and Qnais 2017). Activation of AMPK has been viewed as a targeted approach to increase glucose uptake by

skeletal muscle and control blood glucose homeostasis (Naimi et al. 2017). The EDL and the soleus muscles have often been used as representative of fast-twitch and slow-twitch muscles, respectively (James et al. 1995). Different fiber-types in these two muscles have shown different effects on insulin signal transduction pathway (Bassel-Duby and Olson 2006). Slow-twitch oxidative skeletal muscle (the soleus) has greater insulin binding capacity as well as increased insulin receptor kinase activity and autophosphorylation compared with fast-twitch glycolytic skeletal muscle (Song et al. 1999). Furthermore, muscles with a greater percentage of oxidative myofibers have a higher content of GLUT4, which is associated with the improvement of glucose uptake (Henriksen et al. 1990).

Defects in the expression of critical elements of insulin signalling are known to cause insulin resistance in mammalian skeletal muscle, and these impairments in insulin action are associated with the development of prediabetes and T2DM. Moreover, insulin resistance is associated with the renin-angiotensin system (RAS), in which the initial action of renin cleaves angiotensinogen to angiotensin I (ANG I), then ANG I converts to ANG II by the angiotensin converting enzyme (ACE) (Dietze and Henriksen 2008). In skeletal muscle, ANG II, by acting on its receptor (AGTR-1), can induce insulin resistance by increasing cellular oxidative stress, leading to impaired insulin signalling and insulin-stimulated glucose transport activity (Archuleta et al. 2009; Henriksen and Prasannarong 2013). Therefore, interventions that target RAS overactivity, including ACE inhibitors and ANG II receptor blockers, are effective in both reducing hypertension and in improving whole-body and skeletal muscle insulin action. Previous studies have shown an association between the consumption of fermented milk or yoghurt containing an abundance of LAB, and reduced obesity and T2DM (Tsai, Y.T. et al. 2014; Salas-Salvadó et al. 2017). Our previous study has demonstrated that peptides extracted from yoghurt fermented by *Lactobacillus helveticus* 881315 in the presence of Flavourzyme displayed high ACE inhibitory activity. However, the

mechanism triggered by yoghurt and its peptides involved in the regulation of glucose metabolism and improvement of insulin resistance is not fully elucidated.

Both *in vitro* and *in vivo* studies have suggested that consumption of blueberry products and their bioactive components, such as C3G, have potential health benefits in regulating glucose metabolism and subsequently ameliorating the development of prediabetes and T2DM (Martineau et al. 2006; Sasaki et al. 2007; Vuong et al. 2009; Seymour et al. 2011). However, blueberries and C3G did not attenuate glucose tolerance in our previous study (Chapter 6). Thus the efficacy of blueberries and C3G on glucose metabolism is controversial and underlying mechanisms are still unclear. We have previously showed that yoghurt and peptides with ACE inhibitory activity improved glucose clearance, as measured by a glucose tolerance test in the obese mice (Chapter 6). Therefore due to skeletal muscles role in glucose regulation we aimed to determine the role of blueberries, yoghurt, C3G and yoghurt peptides, in isolation or combination on mRNA expression of markers involved in glucose metabolism in two kinds of skeletal muscles, the EDL and the soleus in an obese mouse model induced by HFHC diet. Understanding these changes in gene expression may lead to the development of effective therapeutic strategies for obesity and associated comorbidities such as T2DM.

7.3 Material & Methods

7.3.1 Animal and feeding regime

Six-week old male C57BL/6 mice were utilised, with a starting body weight between 19 – 21 grams. All the mice were purchased from the Animal Resources Centre (ARC), at Perth, WA, Australia, and acclimatised for four days. Mice were randomly divided into groups of four per cage and fed with high fat diet (59% of total energy from fat) plus 30% fructose water for eight weeks to induce obesity and diabetes. Mice were then placed into divided cages (two in

one cage) for dietary supplementations for another eight weeks. Details of feeding regime and supplementation were provided in Chapter Six. Briefly, mice were separated into eight groups (n = 10 for each group) and fed different diets: standard chow with 5% fat (SCD), high-fat high-carbohydrate [59% of total energy from fat plus 30% fructose water, diet alone (HFHC)] and HFHC with six dietary supplements including blueberries (HFHC+B), C3G (HFHC+C), yoghurt (HFHC+Y), peptides (HFHC+P) extracted from yoghurt, combined blueberries and yoghurt (HFHC+B+Y) and combined C3G and peptides (HFHC+C+P). Each mouse had its own *ad libitum* access to food and water during the experimental period. Experimental procedures were approved by the Animal Ethics Committee of Victoria University (AEC NO: 16/005). For further details refer to section 2.8.

7.3.2 Skeletal muscle tissue collection

The mice underwent a number of tests to determine metabolic parameters throughout the duration of the study which have been detailed in Chapters Two and Six. Following the eight week treatment period and upon the completion of the study the mice were deeply anaesthetised using isoflurane. During this time the soleus and the EDL were surgically collected into cryotubes and immediately frozen in liquid nitrogen for RNA analysis. Following this mice were sacrificed via a cardiac puncture while they remained deeply anaesthetised. For further details refer to section 2.8.9.

7.3.3 Real-time PCR analysis of skeletal muscle

RNA was extracted from the soleus and the EDL of the left leg of mice utilising a TRIzol based method according to the manufacturer's instruction and previously described (Vikman and Edvinsson 2006). 0.5 µg of total RNA was reverse transcribed into cDNA using the iScriptTM cDNA Synthesis Kit (Bio-Rad Laboratories Pty Ltd, Gladesville, NSW, Australia),

according to manufactures instructions. Subsequently, cDNA was diluted in 1:20 for further analysis of all interested genes related to glucose metabolism and stored at – 20 °C.

Oligonucleotide primers were designed using the Oligoperfect™ Suite (Invitrogen, Victoria, Australia, Victoria, Australia) and were purchased from Integrated DNA Technologies, Inc. (1710 Commercial Park, Coralville, Iowa 52241 USA). Selective gene homology for genes of interest was confirmed with BLAST (Basic Local Alignment Search Tool, National Centre for Biotechnology Information, Bethesda, MD) to determine homologous binding to the target mRNA sequence. The primer sequences used for the genes of interest are detailed in Table 2.3.

To quantify mRNA expression in the soleus and the EDL, ‘Real Time’ PCR was utilised using SYBR Green Supermix (Bio-Rad Laboratories Pty Ltd, Gladesville, NSW, Australia) and MyiQ™ multiplex ‘real-time’ PCR detection system (Bio-Rad Laboratories, Hercules, CA). The ‘real-time’ PCR cycling parameters were as follows: 98 °C for 1 minute, followed by 40 cycles of denaturation at 95 °C for 15 seconds, annealing and extension at 60 °C for 45 seconds. Relative changes in mRNA abundance was normalised to the average of the housekeeping gene, hypoxanthine phosphoribosyltransferase 1 (HPRT-1), then quantified using the $2^{-\Delta\Delta CT}$ method (Godfrey et al. 2000). For further details refer to sections 2.4 to 2.7.

7.3.4 Statistical analysis

GraphPad Prism Software 7.0 (GraphPad Software, Inc, La Jolla, CA, United States of America) was utilised for statistical analysis. All results were expressed as mean \pm standard error of the mean (SEM) for each measurement (n = 8-10). One-way ANOVA was performed to analyse the significant differences in the mRNA expression of genes amongst treatment groups. Post-hoc analysis was conducted using Fisher’s (least significant difference; LSD) test for multiple comparisons amongst all groups. $P < 0.05$ was considered significant.

7.4 Results

7.4.1 'Real-time' PCR analysis of the expression of genes related to glucose metabolism in the EDL

Obese mice supplemented with blueberries and yoghurt, alone or in combination; as well as the combination of C3G and peptides exhibited significantly lower AGTR-1 expression in the EDL compared to the obese control group (Figure 7.1A; $P < 0.05$), and it was comparable with that in the lean control group (SCD). While AGTR-1 expression was not changed by either C3G alone or peptides alone in the EDL compared to the obese control group (Figure 7.1A; $P > 0.05$). Mice supplemented with blueberries showed a lower AGTR-1 expression than the C3G group (HFHC+C). Furthermore, the combination of C3G and peptides (HFHC+C+P) significantly reduced AGTR-1 expression than C3G alone.

AMPK mRNA expression in EDL was significantly up-regulated in HFHC+Y and HFHC+C groups, compared with the HFHC group (Figure 7.1B; $P < 0.05$). The other supplementation groups showed no significant differences compared with either the SCD or the HFHC groups.

The supplementation with blueberries and peptides alone, as well as the combination of blueberries and yoghurt significantly decreased FoxO1 mRNA expression in the EDL compared to the obese control group (HFHC), and to the comparable level as in the lean group (SCD) (Figure 7.1C; $P < 0.05$).

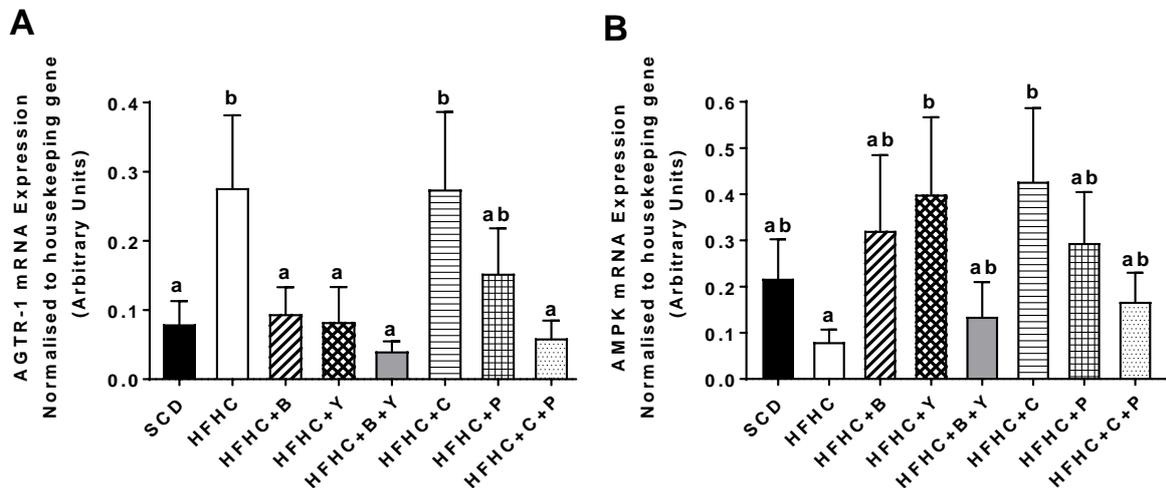
There was no change in the mRNA expression of GLUT1 in all groups (Figure 7.1D; $P < 0.05$).

Yoghurt alone (HFHC+Y) and the combination of C3G and peptides (HFHC+C+P) significantly increased GLUT4 mRNA expression in the EDL compared to the obese control

group (Figure 7.1E; $P < 0.05$). The other groups did not show a significant difference in GLUT4 mRNA expression compared to either the SCD or the HFHC groups. Furthermore, the combined supplementation groups (HFHC+B+Y or HFHC+C+P) did not show any difference in GLUT4 mRNA expression compared to the single supplementation of blueberries (HFHC+B), yoghurt (HFHC+Y), C3G (HFHC+C) or peptides (HFHC+P).

The expression of IRS-1 in the EDL was increased only following the yoghurt supplementation compared with the obese control group; and the other supplementations did not result in any difference in the expression of IRS-1, compared to either the SCD or the HFHC groups (Figure 7.1F; $P < 0.05$).

Obese mice supplemented with yoghurt and C3G alone significantly increased the expression of PI3K in the EDL compared to obese control mice (Figure 7.1G; $P < 0.05$).



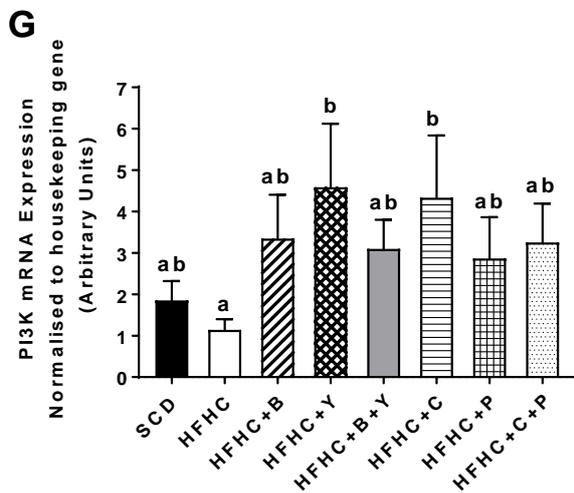
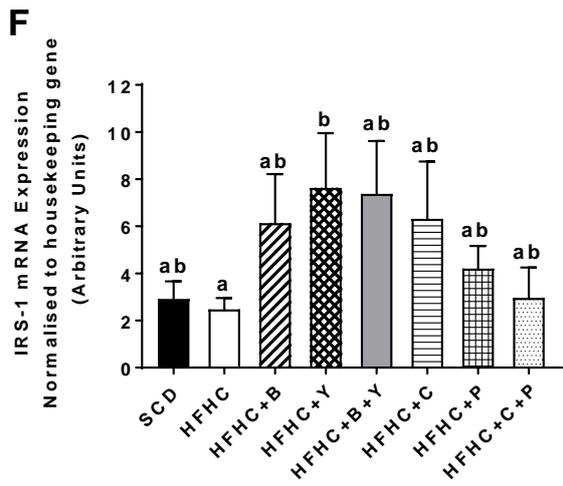
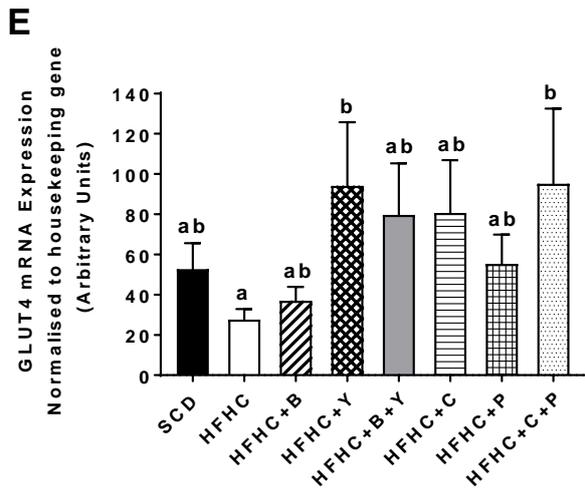
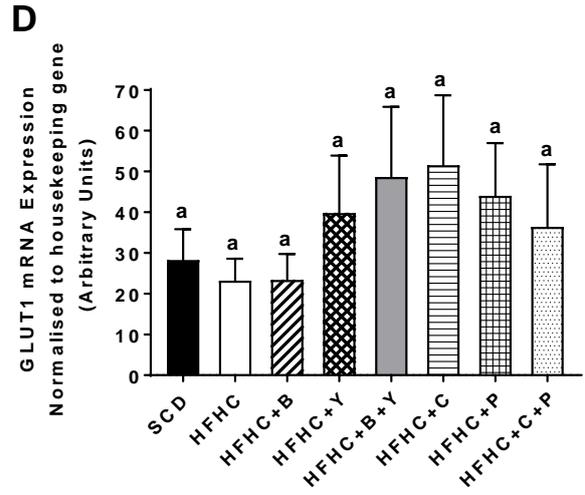
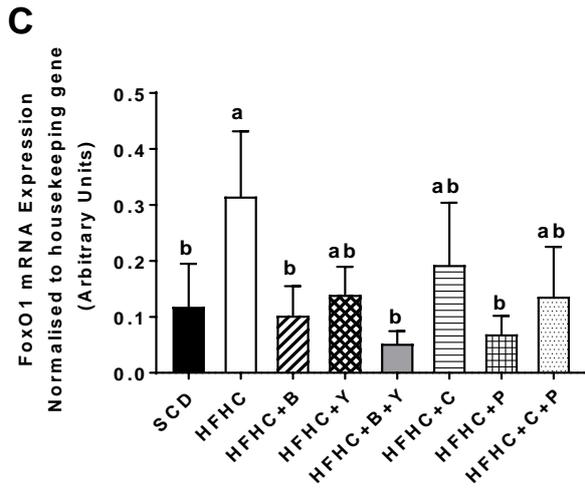


Figure 7.1: The expression of (A) AGTR-1; (B) AMPK; (C) FoxO1; (D) GLUT1; (E) GLUT4; (F) IRS-1 and (G) PI3K in the extensor digitorum longus (EDL) obtained from animals following various dietary treatments.

Obese mice were treated with blueberries, C3G, yoghurt, peptides alone, and combinations of blueberries / yoghurt and C3G / peptides for eight weeks. All genes were normalised to the housekeeping gene, HPRT-1. ■ SCD (n = 9), □ HFHC (n = 9), ▨ HFHC+B (n = 10), ▩ HFHC+Y (n = 10), ▪ HFHC+B+Y (n = 10), ▭ HFHC+C (n = 9), ▮ HFHC+P (n = 10), ▯ HFHC+C+P (n = 10). Data were expressed as mean ± SEM. Different letters indicate a significant difference between groups ($P < 0.05$).

7.4.2 ‘Real-time’ PCR analysis of the expression of multiple genes related to glucose metabolism in the soleus

Four supplementation groups (HFHC+B, HFHC+Y, HFHC+B+Y and HFHC+P) significantly increased the mRNA expression of AGTR-1 compared to the HFHC control group, while the other supplementation groups (HFHC+C and HFHC+C+P) did not show alterations in the mRNA expression of AGTR-1 in the soleus compared to the HFHC control group (Figure 7.2A; $P < 0.05$). Moreover, blueberry supplementation resulted in a lower mRNA expression of AGTR-1 in the soleus when compared to C3G.

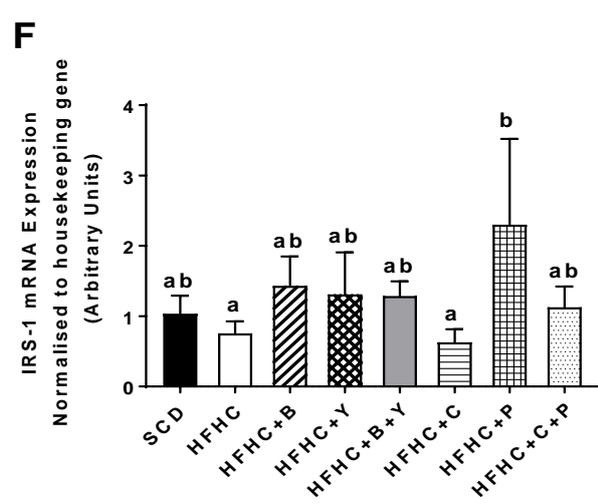
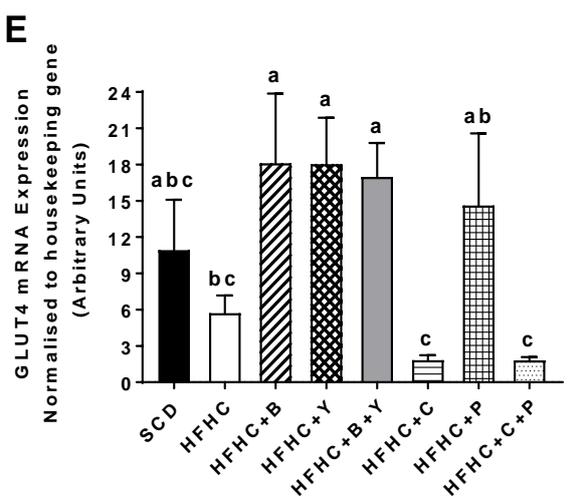
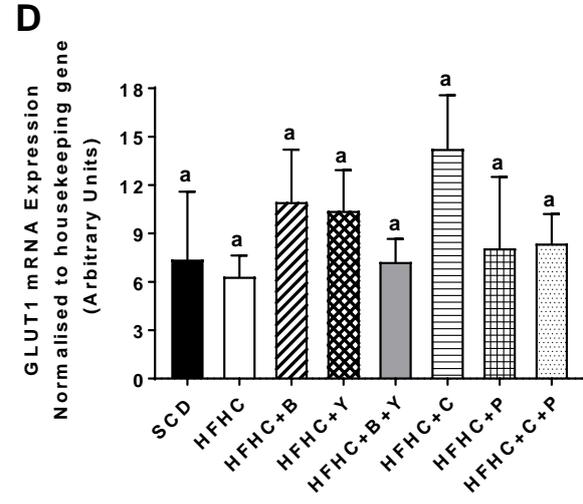
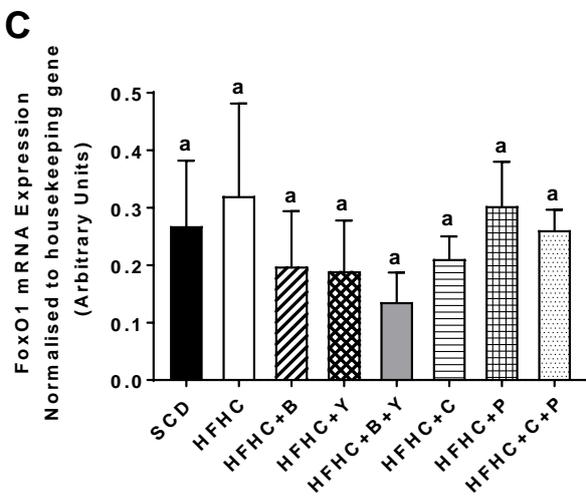
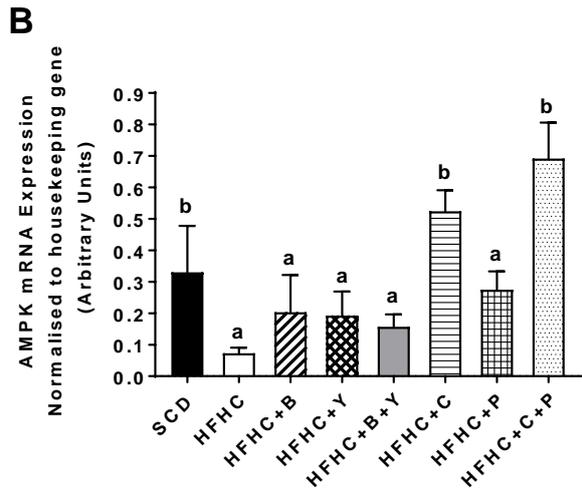
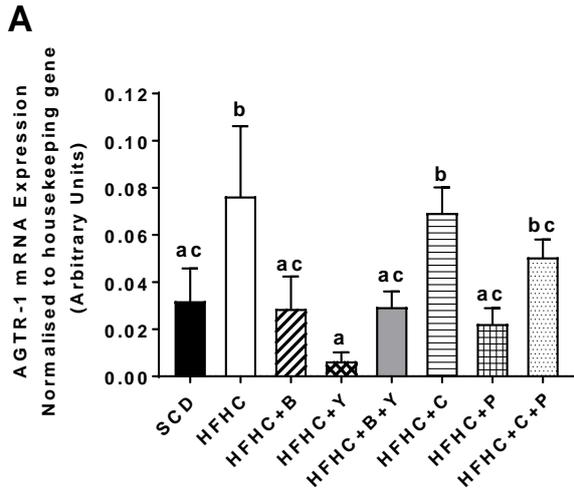
The expression of AMPK in the soleus was significantly increased in mice supplemented with C3G and its combination with peptides compared to the obese control group (HFHC) and other supplementation groups (Figure 7.2B; $P < 0.05$). The other supplementations did not alter the expression of AMPK compared to the HFHC group.

No supplement caused significant changes in the mRNA expression of FoxO1 and GLUT1 in the soleus, compared with either the SCD or the HFHC group (Figure 7.2C, and Figure 7.2D; $P < 0.05$).

A higher mRNA expression of GLUT4 in the soleus was observed following supplementation of blueberries (HFHC+B), yoghurt (HFHC+Y) and their combination (HFHC+B+Y) compared with the HFHC group (Figure 7.2E; $P < 0.05$). All supplementations resulted in a higher level of mRNA expression of GLUT4 comparable with the SCD group. Moreover, the HFHC+B group showed a significantly higher expression of GLUT4 than in the HFHC+C group.

The expression of IRS-1 in the soleus was increased only in HFHC+P group compared to the HFHC group. Other supplementation did not cause alternations in the expression of IRS-1 compared to either SCD or HFHC groups (Figure 7.1F; $P < 0.05$).

Yoghurt (HFHC+Y) supplementation exhibited a significant increase in the mRNA expression of PI3K in the soleus compared to the HFHC group (Figure 7.2G). However, no difference was observed between other supplementation groups and the HFHC group.



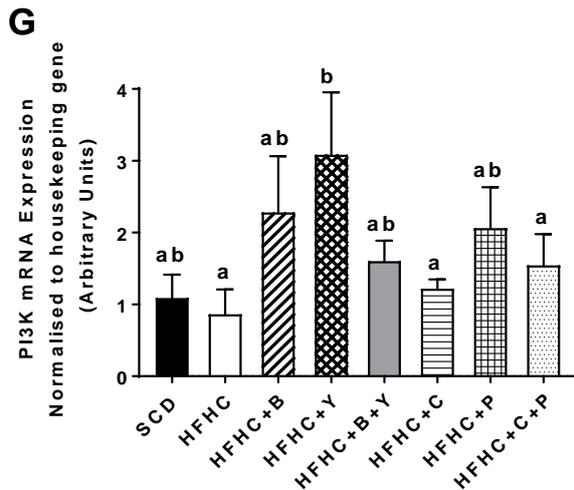


Figure 7.2: The expression of (A) AGTR-1; (B) AMPK; (C) FoxO1; (D) GLUT1; (E) GLUT4; (F) IRS-1 and (G) PI3K in the soleus obtained from animals following various dietary treatments.

Obese mice were treated with blueberries, C3G, yoghurt, peptides alone, and combinations of blueberries / yoghurt and C3G / peptides for eight weeks. All genes were normalised to the housekeeping gene, HPRT-1. ■ SCD (n = 10), □ HFHC (n = 9), ▨ HFHC+B (n = 10), ▩ HFHC+Y (n = 10), ▭ HFHC+B+Y (n = 10), ▮ HFHC+C (n = 10), ▯ HFHC+P (n = 10), ▰ HFHC+C+P (n = 10). Data were expressed as mean ± SEM. Different letters indicate a significant difference between groups ($P < 0.05$).

7.5 Discussion

As described in chapter six, 8-week supplementations with yoghurt and peptides, alone and the combination of C3G and peptides have resulted in an improvement of glucose tolerance in obese mice induced by HFHC diet. Due to the role of skeletal muscle on glucose regulation, this chapter focuses on the impact of supplementation with blueberries and yoghurt, as well as their respective bioactive components on skeletal muscle mRNA expression of various genes related to glucose metabolism. This is the first study looking at

the synergistic anti-diabetic effects of blueberries and yoghurt, as well as their bioactive components C3G and peptides on glucose metabolism in the EDL and the soleus skeletal muscle.

The major finding of this study is that eight weeks of supplementation with yoghurt increased the expression of multiple genes related to insulin-dependent (IRS-1/PI3K/GLUT4) and insulin-independent (AMPK) signalling pathways in skeletal muscle. These findings supported the previous observation (Chapter six) of yoghurt showing anti-diabetic activity with the improvement of glucose tolerance in obese mice. Moreover, these results were consistent with a recent study, which showed that a LAB, *Lactobacillus plantarum* Ln4 administration induced the up-regulation of hepatic mRNA levels, including IRS-2, Akt2, and AMPK, and subsequently improved systemic insulin resistance in mice (Lee, E. et al. 2018). Similar results were also found in the study of Alauddin et al. (2016) in which they observed that fermentation of rice bran with fungi and LAB (*Lactobacillus brevis*, *Lactobacillus rhamnosus*, and *Enterococcus faecium*) improved glucose metabolism via regulation of AMPK activity in stroke-prone spontaneously hypertensive rat (Alauddin et al. 2016). Moreover, an AGTR-1 blocker, telmisartan treatment improved insulin sensitivity in obese db/db mice fed a high-fat diet, as well as *in vitro* treatment with telmisartan led to increased level of AMPK phosphorylation, and an increase in the mRNA levels of GLUT4 in C₂C₁₂ myocytes (Shiota et al. 2012). Chronic administration of ACE inhibitors to insulin-resistant rodents has also been reported to increase protein expression of GLUT4 in skeletal muscle (Henriksen et al. 2001b; Henriksen and Jacob 2003a). Therefore yoghurt increased the mRNA levels of AMPK and GLUT4 possibly due to its peptides with ACE inhibitory activity and acted as an ACE inhibitor.

It has been proposed that blockade of RAS may improve insulin sensitivity and prevent T2DM in metabolic tissues like liver, muscle and pancreas (Sharma et al. 2002; Aksnes et al. 2006). A previous study showed that ANG II increased the phosphorylation of insulin subunit at serine in vascular smooth muscle cells, and inhibits the phosphorylation of IRS-1 at tyrosine residues, suggesting that ANG II might negatively modulate insulin-mediated actions by regulating multiple levels of the insulin signalling cascade such as the IR, IRS, and PI3K (Folli et al. 1997). However, another study demonstrated that ANG II signalling contributes to glucose metabolism and inhibition of the insulin signalling pathway through AGTR-1 in both non-diabetic and diabetic vascular smooth muscle cells (Igarashi et al. 2007). Furthermore, a recent study has shown that tripeptides with ACE inhibitory activity improved insulin resistance in rat-derived L6 skeletal muscle cells, at least partially via reduced AGTR-1 expression and its antioxidative activity with attenuating reactive oxygen species (ROS) in L6 cells (Son et al. 2018). Yoghurt fermented by *L. helveticus* included bioactive peptides with a high ACE inhibitory activity (Chapter 3). In the present study, yoghurt decreased AGTR-1 mRNA expression in both the EDL and the soleus obtained from HFHC induced obese mice possibly due to its peptides with ACE inhibitory activity, thereby attenuated glucose tolerance in obese mice observed in the previous chapter (Chapter 6).

L. helveticus is a LAB with a strong proteolytic system and *L. helveticus* strains are considered to be one of the most efficient in production of anti-hypertensive peptides and aromatic compounds from caseins in fermented milk (Beganovic et al. 2013; Chen, Y. et al. 2014; Giraffa 2014). In the present study, peptides with ACE inhibitory activity extracted from yoghurt fermented by *L. helveticus* up-regulated IRS-1 and down-regulated AGTR-1 expression in the soleus. The same results were also observed in human primary skeletal muscle myotubes (Chapter 5). Consistently, the findings in the previous chapters have shown that peptides could increase glucose uptake in human primary skeletal muscle myotubes

(Chapter 5) and improve glucose tolerance in obese mice (Chapter 6). Fructose-fed rats treated with Angiotensin-(1-7), an ACE inhibitor, resulted in an increased glucose uptake via a mechanism involved in the modulation of insulin signalling, through the IR/IRS-1/PI3K/Akt pathway in skeletal muscle, liver, and adipose tissue, as well as increased levels of IRS-1 phospho-Ser³⁰⁷ in skeletal muscle and adipose tissue (Giani et al. 2009). An *in vitro* study by Liu et al also showed that Angiotensin-(1-7) improved glucose uptake and decreased ROS production in 3T3-L1 adipocytes (Liu et al. 2012). It has been reported that ANG II significantly decreased 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside (AICAR)-activated glucose uptake of the soleus muscles, and AGTR-1 blocker cancelled the effect of ANG II, suggesting acute inhibition of the AGTR-1 improves glucose metabolism not via an insulin pathway but via an AMPK pathway (Shinshi et al. 2007). However, in the present study, peptides increased IRS-1 expression and reduced AGTR-1 expression but did not change AMPK expression in the soleus. Animal models, treatment period, different supplementations and diet induced disease symptom may contribute to the different results. It is still unclear how ANG II is involved in glucose metabolism, and further studies are needed to clarify the correlation among RAS, insulin pathway and AMPK pathway.

During milk fermentation, LAB hydrolyse lactose producing tagatosem, which has low caloric value and is poorly degraded by the human body making it an interesting anti-hyperglycaemic agent (Rhimi et al. 2011). Furthermore, an enzyme with ferulic acid esterase activity isolated from *Lactobacillus johnsonii* showed potential effect on diabetes due to ferulic acid stimulating insulin production and alleviating symptoms caused by diabetes (Lai et al. 2009). Food intake with weak organic acids, including lactic acid, formic acid, pyruvic acid and acetic acid, could increase insulin sensitivity and alleviate the insulin resistance in diabetes mellitus due to weak organic acids with the lowered interstitial fluid pH values (Marunaka 2018). *L. helveticus* has a strong proteolytic system, which is capable of

producing not only short peptides but also liberating amino acids from the casein matrix (Genay et al. 2009). This system is composed of (i) cell envelope proteinases that hydrolyse caseins into oligopeptides, (ii) transport systems that allow uptake of oligopeptides, and (iii) various intracellular peptidases with differing and partly overlapping specificities, leading to a pool of free amino acids (Kunji et al. 1996; Savijoki et al. 2006). Therefore, the anti-diabetic activity of yoghurt fermented by *L. helveticus* could be not only related to anti-hypertensive peptides, but also organic acid enzymes and free amino acid produced during the fermentation by *L. helveticus*. The results from this chapter indicate that yoghurt and its peptides played an important role in the regulation of glucose metabolism in muscle and maybe a potential therapeutic candidate in the prevention of T2DM through IR/IRS-1/PI3K/GLUT4, AMPK and AGTR-1 pathways.

In the present study, we also showed that blueberries down-regulated AGTR-1 expression in both the EDL and the soleus, FoxO1 expression in the EDL, and up-regulated GLUT4 expression in the soleus obtained from HFHC induced obese mice, although blueberries did not alter glucose tolerance in obese mice (Chapter 6). Two percent freeze-dried whole highbush blueberry powder has been reported to increase the expression of IRS-1 and GLUT4 in the adipose and skeletal muscle tissues in both Zucker fatty rats and Zucker lean rats (Seymour et al. 2011). Furthermore, fermented blueberry juice increased the phosphorylation of AMPK in C₂C₁₂ cells and 3T3-L1 cells, but treatment with non-fermented juice did not affect total AMPK content in either cell line (Vuong et al. 2007). Consistently, the present study also showed that blueberry supplementation did not alter mRNA expression of AMPK in either the EDL or the soleus. A recent study showed that blueberry supplementation improved markers of insulin sensitivity, including the normalized hepatic IRS1 Ser³⁰⁷ phosphorylation and reduced hepatic malondialdehyde, a marker of oxidative stress in high-fat-diet-fed rats supplemented with blueberries (Lee, S. et al. 2018). However,

neither blueberry supplementation nor C3G supplementation altered mRNA expression of IRS-1 in both skeletal muscle tissues. These inconsistent results may be due to different tissues or animal species.

In order to determine which components in blueberries showed the major bioactivity on the regulation of genes related to glucose metabolism, cyanidin-3-O- β -glucoside (C3G), a typical anthocyanin in blueberries was also investigated in this study. Several studies have shown that C3G regulates glucose metabolism via stimulating AMPK activation in skeletal muscle, visceral adipose, HepG2 cells and high glucose-incubated adipocytes, as well as attenuating high-glucose-promoted O-glycosylation of transcription factor FoxO1 in 3T3-L1 adipocytes (Wei et al. 2011; Guo, H. et al. 2012a; Guo, Honghui et al. 2012). These findings were in agreement with the present study, in which C3G was found to increase the mRNA expression of AMPK in both the EDL and the soleus, and PI3K in the EDL. However, C3G did not attenuate glucose tolerance in obese mice (Chapter 6), suggesting that the upregulation of AMPK and PI3K mRNA in skeletal muscle do not appear to be sufficient to regulate glucose metabolism. In addition, the similar results were also observed in the previous chapter (Chapter 5) which showed that either C3G in isolation or in combination with peptides resulted in an up-regulation in the mRNA expression of AMPK and PI3K in human primary skeletal myotubes. Previous reports showed that C3G has significant potency in an anti-diabetic effect related to GLUT4, including the increase of GLUT4 membrane translocation and the increase of GLUT4 expressions in murine adipocyte 3T3-L1, as well as through the regulation of GLUT4-retinol binding protein (RBP4) system (Sasaki et al. 2007; Scazzocchio et al. 2011). Therefore, blueberries and C3G regulated key molecules related to glucose metabolism may be through AMPK pathway and PI3K/AKT/GLUT4 pathway as well as the inhibition of AGTR-1 and FoxO1 expression, although neither blueberries nor C3G supplementation showed improvement in glucose tolerance in obese mice (Chapter 6).

Interestingly, the two combination treatments did not appear to show an additive effect on the regulation of multiple genes related to glucose metabolism in the skeletal muscle of obese mice. Further investigation is required to determine the possible interactions of various bioactive components in blueberries and yoghurt.

7.6 Conclusions

In summary, supplementation with blueberries resulted in a reduction of the mRNA expression of AGTR-1 in the EDL and the soleus. However, a key bioactive component of blueberries, C3G displayed a role in the regulation of different genes, with a consequential increase in the mRNA expression of AMPK in both the EDL and the soleus of obese mice suggesting that the anti-diabetic mechanism of blueberries was different from C3G and the anti-diabetic properties of blueberries may be related to the bioactive components other than C3G, or the synergistic effects of C3G and those components. Furthermore, yoghurt showed its potentially anti-diabetic activity involved an insulin-dependent signalling pathway associated with an increase in the mRNA expression of PI3K, IRS-1 and GLUT4; and an insulin-independent signalling pathway associated with an increase in the mRNA expression of AMPK and a decrease in the mRNA expression of AGTR-1. Peptides extracted from yoghurt showed similar effects on the regulation of multiple genes in skeletal muscle of obese mice. This study demonstrated that yoghurt has the potential to be further developed as a functional food for consumers with diabetes and hypertension based on the results from Chapter 3 (yoghurt fermented by *L. helveticus* showed anti-hypertensive effect due to its peptides with ACE inhibitory activity) and Chapter 6 (yoghurt improved glucose tolerance in HFHC induced obese mice), as well as the present chapter (yoghurt showed anti-diabetic activity related to the regulation of multiple gene expressions in skeletal muscles). Further research is required to elucidate the mechanisms of its active compounds (peptides, protein,

enzyme, amino acids and so on) involved in the signalling pathways via regulation of protein expression and translocation of GLUT4 to the cell membrane. Moreover, the efficacy of yoghurt and its peptides on glucose metabolism will need to be validated through human clinical trials.

CHAPTER 8: General discussion and future research direction

8.1 General summary

The studies accomplished within this thesis provide an insight into the use of C3G and peptides for the improvement of glucose uptake with or without insulin in human primary skeletal myotubes obtained from obese and obese T2DM individuals. This thesis also investigated the molecular mechanisms associated with the effects of C3G and peptides on glucose uptake through analysis of mRNA expression of multiple genes involved in glucose metabolism. Furthermore, this thesis determined the role of blueberries and yoghurt, and their respective bioactive components, C3G and peptides in isolation and combination on obesity related comorbidities in HFHC induced obese mice. Additionally the potential mechanisms of these functional foods involved in the regulation of genes related to glucose metabolism in skeletal muscles have been investigated. The results from this thesis suggest that the combined supplementations (the combined blueberries with yoghurt, and the combination of C3G and peptides) did not show synergistic impact compared with individual supplementations. Furthermore, the consumption of yoghurt provides more health benefits compared with consumption of peptides extracted from these foods.

8.2 General discussion

Current conventional medical interventions for the treatment of obesity and its related comorbidities are limited with varied efficacy and associated side effects, therefore complementary dietary therapies have become increasingly popular. It is generally accepted that an intake of fruits is beneficial to human health (Fan et al. 2013; Mekkes et al. 2013). However, the mechanism of action of various fruits and their bioactive components in the

human body still remains unclear. Bioactive peptides derived from fermented yoghurt have been considered as functional and alternative therapeutic agents with numerous potential health benefits including improving insulin resistance and glucose intolerance, and suppressing the activity of ACE (Carter et al. 2004; Gao et al. 2018; Manikkam et al. 2016). However, the potential mechanism of yoghurt and its peptides have yet to be fully explored. Furthermore, due to the complexity of *in vitro* experiment, the synergistic effects of C3G and peptides on glucose uptake in human primary skeletal muscle myotubes compared with the single treatment have not been covered. Also the synergistic effects of combined supplementations (blueberries + yoghurt, and C3G + peptides) on obesity and related comorbidities have not been studied. This chapter aims to integrate and make concluding remarks about the studies contained within the thesis, and attempt to provide recommendations for future research in this area. Cell culture and animal studies in this thesis showed that C3G and peptides may enhance glucose uptake associated with insulin-dependent and -independent signalling pathways, and blueberries and yoghurt, including their bioactive compounds, C3G and peptides, may attenuate hyperglycemia and hypertension. These results are likely to have biological effect with the potential benefits of blueberry and yoghurt consumption for regulation of glucose metabolism and blood pressure in humans. This could lead to a development of future manufacturing/cultivation processes to enhance the effectiveness of these food products in obesity and T2D prevention and management.

Based on the results from animal study in this thesis, the equivalent human dose of treatments would be 0.52 g/kg BW/day for blueberry supplementation and 0.243 g (3/12.3)/kg BW/ day for yoghurt supplementation. For instant, a young male adult with approximately 70 kg body weight, 36.4 g of dried blueberry powder and 17.1 g of dried yoghurt powder would be required daily, which was equivalent to 182 g of fresh blueberries (20% of dry matter in the

fresh blueberry) and 142.5 g of normal yoghurt (12% of dry matter in normal yoghurt). Thus the dosage of both blueberries and yoghurt is feasible for the general public demonstrating, in conjunction with the observed physiological and genetic alterations, that this level of consumption would produce a meaningful biological effect.

8.2.1 The protective role of blueberries in diabetes and hypertension

Blueberries showed the a potential properties to regulate genes related to glucose metabolism based on the results obtained from Chapter Seven of this thesis, in which blueberries down-regulated AGTR-1 and FoxO1 expression, and up-regulated GLUT4 expression in skeletal muscles obtained from HFHC induced obese mice, although blueberries did not alter the glucose tolerance in obese mice (Chapter Six). Other potential mechanisms of blueberries protecting against diabetes were also observed including the changes in PPAR gene transcripts in adipose and muscle tissue, and reversion of adiponectin levels in plasma (Vuong et al. 2009; Seymour et al. 2011).

Previous studies reported that body weight and total fat weight were significantly reduced in Obese Zucker rats fed a LFD combined with 2% (w/w) whole highbush blueberry powder (Seymour et al. 2009; Seymour et al. 2011). However, other studies demonstrated that both low dose (2%) and high dose (4%) blueberry supplementation did not affect the body weight and adiposity in a rat model or a mouse model (Ahmet et al. 2009; DeFuria et al. 2009). The current thesis (Chapter Six) also showed that blueberries did not inhibit body weight gain. One explanation for the inconsistency in these findings may be due to the species of blueberries used, location, soil conditions and harvest season for planting blueberries (Usui et al. 1994; By Barbie Cervoni 2019).

A blueberry-enriched diet exhibited lower systolic and diastolic BP in male spontaneously hypertensive rats and in participants with metabolic syndrome (BMI: 37.8 kg/m²) (Basu et al.

2010; Elks et al. 2011). The results presented in the Chapter Six also showed that blueberry supplementation decreased systolic and diastolic BP in HFHC induced obese mice. However, these findings are not consistent with some previous studies demonstrating that blueberry supplementation (2% w/w) had no effect on blood pressure in a rat model of myocardial infarction following 12-month supplementation. (Ahmet et al. 2009; Basu et al. 2010; Elks et al. 2011). This discrepancy may be due to the type of blueberry used, seasonal variation in blueberries, different doses of blueberry supplement and the duration of supplementation, as well as types of animal model. Therefore, the efficacy of blueberries on obesity and associated comorbidities is still a challenge and needs further study.

8.2.2 The protective role of C3G in obesity and associated comorbidities

Previous studies indicated that consumption of anthocyanins could possibly ameliorate the function of adipocytes, and thus may prevent overweight and obesity (Tsuda et al. 2003; He and Giusti 2010). It was reported that supplementation with black soybean anthocyanins effectively reversed or suppressed the body weight gain of HFD fed rats to the same level as that in the control group (Kwon et al. 2007). In addition, mice supplemented with C3G-rich purple corn colour for 12 weeks significantly suppressed the HFD-induced increase in body weight gain, and white and brown adipose tissue weights (Tsuda et al. 2003). The results provided in Chapter Six of this thesis did not support the hypothesis that supplementation with a typical anthocyanin, C3G would reduce obesity. C3G supplement did not significantly elicit beneficial effects on body weight and body fat gain, as well as epididymal fat, mesenteric fat and liver weight in the HFHC-induced obese mouse model. These findings are inconsistent with previous studies, partially due to the supplementation with single anthocyanin, C3G, instead of total anthocyanins suggesting that multiple anthocyanins may present synergistic effect and be more effective on obesity and diabetes than single C3G.

Another possibility may be related to the dosage of C3G (0.02 g/kg body weight/ day) supplemented in obese mice. This low dose of C3G, despite being chronically administered may not have been high enough to alter a number of measurements undertaken in the current study.

Anthocyanins extracted from plants have previously been shown to increase glucose uptake in several cell culture studies, including L6 myotubes, C₂C₁₂ myotubes and 3T3-L1 adipocytes (Martineau et al. 2006; Harbilas et al. 2009; Rojo, Leonel E et al. 2012). However, the effects of anthocyanins on glucose metabolism in human primary skeletal myotubes and the related mechanisms have not been reported yet. The present study in Chapter Five showed for the first time that C3G increases glucose uptake in human primary skeletal myotubes and demonstrated its potentially anti-diabetic activity, at least in part, in an insulin-dependent signalling pathway, associated with the increase of IRS-1 mRNA expression and GLUT4 mRNA expression. The reason that C3G supplementation did not lead to significant attenuation of glucose tolerance in HFHC induced obese mice in the present thesis (Chapter Six) is still unclear. It may be due to the low dose of C3G consumed by obese mice or administration duration based on a previous study showed that supplementation with high dose of C3G (0.2%) decreased the fasting glucose levels in both HFD and db/db mice and improved glucose homeostasis compared with their respective untreated controls (Guo, H. et al. 2012b).

In addition, results observed in Chapter Six showed that C3G at the same dose as what was found in blueberries did not alter the blood pressure in HFHC induced obese mice, suggesting that positive outcomes of the whole blueberry may be associated with other compounds. For instance, others important anthocyanins (myricetin and quercetin), phenolic acid and flavonoids have demonstrated their anti-obesity and anti-diabetic properties, although some

of them are not major compounds in blueberries (Manzano and Williamson 2010; Wu et al. 2016; Mokashi et al. 2017).

8.2.3 The protective role of yoghurt in obesity, diabetes and hypertension

The findings in Chapter Six showed that yoghurt significantly inhibited body weight gain, and the increase of body fat percentage was much low in the yogurt group compared with the other groups. Numerous human trials have demonstrated a significant inverse relationship between calcium, or dairy consumption, and both body weight and body fat (Mckinley 2005). Thus the effect of yoghurt on body weight may possibly be related to calcium in this dairy product. Other possible mechanisms underlying the role of yoghurt in the management of body weight may have related to the modulatory enzymes involved in processing dietary polysaccharides and the regulation of energy metabolism (Tsai, Y.T. et al. 2014; Evivie et al. 2017). In addition, peptides with ACE inhibitory activity extracted from yoghurt were associated with a lower percentage of body fat, but do not alter body weight gain and the percentage of metabolic tissues (Chapter Six), which may be partially due to increased energy intake in peptides group.

It has been reported that consumption of yoghurt improved fasting blood glucose level in both animals and humans (Yun et al. 2009; Ejtahed et al. 2012). In agreement with the previous results, the effects described in Chapter Six demonstrated that both yoghurt and peptide supplements significantly improved the intraperitoneal glucose tolerance in HFHC induced obese mice. This is further supported by the finding from Chapter Seven, in which yoghurt increased the expression of multiple genes related to insulin-dependent (IRS-1/PI3K/GLUT4) and insulin-independent (AMPK) signalling pathways in skeletal muscle. The anti-diabetic activity of yoghurt may be attributed to multiple factors, such as the LAB strain for the fermentation of yoghurt, the capability of hydrolysis to produce short peptides,

contained bioactive peptides and their bioavailability (Matsui et al. 2002; Seppo et al. 2003; Segura-Campos et al. 2011; Honda et al. 2012). In addition, peptides enhancing glucose uptake in human primary skeletal myotubes as shown in Chapter Five and peptides up-regulating IRS-1 expression and down-regulating AGTR-1 expression in the soleus as shown in Chapter Seven also supported the anti-diabetic properties of peptides with improvement of glucose tolerance in obese mice.

The results presented in Chapter Six showed that yoghurt supplement significantly decreased both systolic and diastolic BP, while peptides supplement only decreased systolic BP compared with the HFHC control group, suggesting that other active components, including protein, amino acids and the minerals in yoghurt may also play a role in the regulation of blood pressure. A previous report also indicated polysaccharide-glycopeptide complexes found in autologous *Lactobacillus casei* cell with anti-hypertensive activity, in which 1 mg/kg BW of polysaccharide-glycopeptide complexes decreased systolic BP by 10-20 mmHg after 6 to 12 hours administration to spontaneously hypertensive rats (SHR) and renal hypertensive rats (Sawada et al. 1990).

8.2.4 The impacts of combined supplementations with blueberries, yoghurt, C3G and peptides on glucose metabolism and obesity associated comorbidities

In order to investigate whether multiple supplements show synergistic effects on obesity and associated comorbidities, the combination of C3G and peptides on glucose uptake in human skeletal muscle myotubes was explored, as well as two combination groups (the combination of blueberries and yoghurt, and the combination of C3G and peptides) were investigated in HFHC diet induced obese mice. However, both combinations did not show any synergistic effects on glucose uptake in human primary skeletal muscle myotubes or body weight, blood pressure and glucose tolerance in obese animals, compared with the single supplement. It is

unclear why combined supplementation did not achieve an equal reduction or synergistic/additive effects. As both of blueberries and yoghurt contain a number of potential active components any number of these could have had accumulative or antagonistic effects on those parameters. Similarly C3G and peptides may interact and resulted in antagonistic effects. Further research is warranted to uncover these complex interactions.

8.3 Limitations

There are several limitations to the research conducted in this thesis. The first limitation is the dosage of peptides. The crude peptides (1.47 mg/mL of IC₅₀ value) was selected for both cell culture study and animal study, because only a lower dose (less than 100 µg) of peptides with the highest ACE inhibitory activity (35.75 µg/mL of IC₅₀ value) was available. This low dose may have impacted the outcomes of animal study, in which the peptide did not alter diastolic blood pressure in HFHC induced obese mice compared with the control group, possibly due to its low ACE activity.

Another limitation of the study is that some animals did not develop obesity following eight-week consumption of HFHC diet. In order to maintain the statistical power, the animals with obesity tolerance were not excluded in the subsequent experiment and data analysis, which may have disturbed the results. Furthermore, despite attempting to standardise several baseline measurements within the animals following the diet induced obese period, animals in different treatment groups began the treatment period with variations with baseline body composition. Therefore it is difficult to explain the effects of peptides on body composition and verify whether it was due to 8-weeks supplementation or low body composition at the baseline. There are many important factors to be considered to induce obesity in animal models. The most important is perhaps the strain of the mouse. C57BL/6 is the most widely used mouse strain for HFD-induced obesity because they exhibit abnormalities similar to

human metabolic syndrome when fed the HFD (Collins et al. 2004; Kanasaki and Koya 2011). It has been reported that C57BL/6 mice fed with a HFD displayed increased body weight, had more overall fat, particularly increased adipose tissue and developed hyperglycemia, hyperinsulinemia and hypercholesterolemia (Jayaprakasam et al. 2006; Dissard et al. 2013; Wooten et al. 2016; De Sousa Rodrigues et al. 2017). Other considerations are the variety of HFD, the duration of the HFD feeding, the age of mice, formulation of the HFD and so on. A number of studies have found that diets high in saturated fat resulted in increased body weight and obesity as well as impaired glucose tolerance (Alsaif and Duwaihyy 2004; Messier et al. 2007). For example, Petro et al.(2004) showed that mice fed on a high fat diet (58% kcal fat) for 11-weeks gained significantly more weight and had significantly higher blood glucose levels than those fed on a low-fat diet (Petro et al. 2004). It remains unclear as to the causes of obesity tolerance. A longer duration of the HFHC feeding regime may help to establish an obese phenotype.

With regards to the mechanism(s) of action, the studies within this thesis describe the ability of blueberries and yoghurt to regulate multiple genes related to glucose metabolism in skeletal muscle (the EDL and the soleus) and that this may play a critical role in preventing obesity associated diabetes. Furthermore, C3G and peptides improved glucose uptake in human skeletal muscle myotubes and regulated multiple genes related to insulin-independent or dependent pathways. However, these were based only on mRNA expression data and there is a lack of data for protein expression confirming that blueberries- and yoghurt-associated patterns of gene expression are manifested as functionally significant alterations in the protein expression related to obesity and diabetes, and then subsequently metabolic pathways.

Lastly, due to the limited availability of facilities, the total anthocyanins content from different brands of blueberries can be measured only by RR-HPLC in the present study.

Combination of HPLC and mass spectrometry is a better and more accurate method to determine the total anthocyanins content and single anthocyanin content (Grace et al. 2009). In addition to this, each brand of blueberries was purchased at only one time point from one supplier.

8.4 Recommendations for future research arising from this thesis

From this thesis, it is evident that blueberries and yoghurt have potential beneficial effects on obesity, T2DM and hypertension. To extend on the observations contained within this thesis, further investigation on the effects of blueberries and yoghurt and their bioactive components for their potential impacts on human health with animal models and human trials is required, and attempt to provide recommendations for future research in this area.

Blueberry and yoghurt supplementation have resulted in reduced systolic and diastolic blood pressure in HFHC induced obese mice, but the associated anti-hypertensive mechanisms remain unanswered. Yoghurt demonstrated the effects on blood pressure possibly due to its bioactive peptides with blood pressure-lowering activity attributed to the role of ACE in the conversion of angiotensin I to angiotensin II, which causes the contraction of blood vessels and increases blood pressure (Butikofer et al. 2008). In addition, ROS contribute to endothelium-dependent contraction and to increased vascular resistance. On the other hand antioxidants can possibly restore endothelial function and hence decrease blood pressure (Weseler and Bast 2010; Olmez and Ozyurt 2012). Increased serum ACE activity was associated with enhanced susceptibility to lipid peroxidation and hence, the inhibitory effect on serum ACE activity could further contribute to an antioxidant environment and attenuate atherosclerotic risk (Aviram and Dornfeld 2001). Moreover, a previous study showed that a blueberry-enriched diet attenuated nephropathy in a rat model of hypertension via reduction in oxidative stress (Elks et al. 2011). It is also important to understand the role of specific or

target organs in the regulation of blood pressure. For example, the renin–angiotensin system (RAS) plays a central role in the regulation of blood pressure, volume and electrolyte homeostasis and is associated with the development and progression of cardiovascular diseases and diabetes (Li et al. 2004; Murca et al. 2012). In addition, obesity associated with hypertension can cause marked changes in systemic hemodynamics as well as structural adaptations in blood vessels and heart (Zhang and Reisin 2000). Taken together, the proposed analyses include: (1) analysing serum ACE activity of obese mice using a spectrophotometric method (Rahimi et al. 2011); (2) determining lipid profile in serum, including total cholesterol and triglycerides concentrations using the peroxidase-antiperoxidase method, HDL-cholesterol and LDL-cholesterol concentrations using the clearance method (Qin et al. 2009); (3) determining the liver lipid content using gas chromatography (Airanthi et al. 2011); (4) determining the generation of ROS including superoxide anion radical ($O^{\bullet-}_2$) in blood vessels and the heart using electron paramagnetic resonance spectroscopy and H_2O_2 using dichlorodihydrofluorescein diacetate (Griendling et al. 2016); (5) histological study for the determination of lipids, inflammatory cells infiltration and fibrosis in liver and left ventricle in obese mice by using various stains (Kohli et al. 2010).

The previous studies in the literature showed that the efficacy of blueberry supplementation on obesity, hyperglycemia and hypertension was inconsistent (Prior et al. 2008; DeFuria et al. 2009; Vuong et al. 2009; Zhu et al. 2011; Rodriguez-Mateos et al. 2014; Zhu et al. 2017). Furthermore, consuming yoghurt may have beneficial effects for people with obesity and metabolic syndrome (Jauhiainen et al. 2005; Yun et al. 2009; Khaledi et al. 2014; Evivie et al. 2017). Therefore, the effects of combined supplementation of blueberries and yoghurt on body weight, fat mass, blood glucose, as well as BP were investigated in this thesis using an obese mouse model. The results showed that (1) the combination of blueberries and yoghurt supplementation reduced hypertension; (2) the combination of blueberries and yoghurt

exhibited significantly lower body fat in both the total weight and the percentage, compared to blueberries alone; (3) yoghurt alone had beneficial effects on obesity, hyperglycemia and hypertension; and (4) blueberry consumption alone had the efficacy on lowering blood pressure. However, it is not known whether the beneficial effects observed in this research will be translated in humans. More controlled human clinical trials over adequate time scales are required to validate these outcomes and elucidate the association between these supplementations and risk factors of relevant disorders. The potential factors that may impact the outcomes of supplementation should be considered include patients' characteristics, baseline indicators, species and geographical locations of blueberries and type of probiotics in the yoghurt, as well as dose of consumption.

Obesity is related to chronic inflammation due to an increased infiltration of inflammatory cells into tissues such as liver and adipose tissue (Jung and Choi 2014). It has been reported that regular bilberry consumption reduced low-grade inflammation indicating decreased cardio-metabolic risk in the long term due to decreased serum high-sensitivity C-reactive protein, IL-6, IL-12, and lipopolysaccharide (LPS) concentrations in participants (Kolehmainen et al. 2012). Furthermore, C3G has exhibited anti-inflammatory effects with lowered serum concentrations of inflammatory cytokines (TNF- α , IL-6, and MCP-1) (Guo, H. et al. 2012b). *In vitro* studies reported that C3G significantly inhibited the production of pro-inflammatory cytokines (TNF- α , IL-6, and IL-1 β) in supernatants of human umbilical vein endothelial cells (Ma et al. 2015), and significantly inhibited LPS-stimulated TNF- α and IL-6 mRNA expression and secretion of these proteins in human monocyte/macrophage THP-1 cells (Zhang et al. 2010). Therefore, additional work focusing on serum pro-inflammatory adipokines, cytokines and chemokines such as MCP-1, IL-6, NF- κ B and TNF- α could be beneficial to fully understand the health benefiting properties of blueberries and C3G.

There is a close link established between adipose tissue metabolism, differential fat distribution and disorders such as obesity and diabetes (Ibrahim 2010). Due to time constraint, the animal study presented in this thesis has not analysed all the fat depots that are associated with metabolic risk such as omental, mesenteric, brown adipose tissue and epididymal fat. Future animal research could focus on the assessment of metabolically active adipose tissue in order to better elucidate the anti-obesity effects and the reduction of blood glucose level of yoghurt and its peptides.

In addition, the total contents of anthocyanidins and C3G of thirteen types of blueberries were analysed in this thesis (Chapter Four). However, the analysis was undertaken only once. It is unknown how much the content of these bioactive components in blueberries varies in different season. Furthermore, we assumed that there was no degradation when blueberry fruits, blueberry powder and C3G were stored at -80 degree for one month, three months and two years, respectively. Determination of total contents of anthocyanidins and C3G from blueberries harvested in different season and stored for different duration would provide useful data for future studies.

As previously discussed in Chapter Seven, C3G did not show anti-obesity properties. This is possibly due to the low dose. Further studies are required to assess the effect of ACNs consumption at a series of doses, especially high dose in order to establish the specific concentration of ACNs required for ameliorating the development of obesity. In addition, it is important to determine the effects of other principal anthocyanins in blueberries such as myricetin and quercetin, in addition to C3G, as well as elucidate the potential mechanisms associated with the effects of blueberries on obesity and diabetes. Besides, further human studies would be beneficial to fully understand the mechanism underlying the anti-hypertensive properties of blueberries and yoghurt.

The obese mice supplemented with blueberries and yoghurt exhibited significantly lower body fat in both the total weight and the percentage, compared to the obese mice supplemented with blueberries alone. Furthermore, the combination of C3G and peptides significantly reduced AGTR-1 expression, compared to C3G alone in EDL, and the same combination significantly increased the mRNA expression of GLUT4, compared to peptides alone in soleus. Thus it would be beneficial to consume products containing yoghurt mixed with blueberries rather than blueberries alone. As the relationship between the dosage of bioactive components in blueberries and the effects on obesity and its related comorbidities has not been established yet, the exact amount of blueberries to be added in yoghurt in order to achieve maximum health benefits will need further investigation. Other limitations in manufacture/production of food products containing mixture of yoghurt and blueberries include: (1) the forms of blueberry product (juice, powder or the whole fruit) added into yoghurt; (2) how to consistently formulate yoghurt with blueberries without agitation or with low-speed agitation, as normally yoghurt is not stirred or agitated during the fermentation phase; and (3) whether and how blueberries influence the growth of LAC, such as change in pH value. Future research focusing on these areas would provide further evidence for developing novel food products that can boost the health impacts of yoghurt and blueberries.

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APPENDIX 1: Blueberry as a source of bioactive compounds for the treatment of obesity, type 2 diabetes and chronic inflammation



Blueberry as a source of bioactive compounds for the treatment of obesity, type 2 diabetes and chronic inflammation



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ABSTRACT

Recent experimental and clinical studies suggest that consumption of blueberry products has potential health benefits in ameliorating the development of obesity and its related comorbidities, including type 2 diabetes (T2D) and chronic inflammation. Blueberry fruits are enriched with numerous bioactive components such as vitamins, phenolic acid and anthocyanins which could contribute to these protective effects. Possible mechanisms by which blueberries exert their beneficial properties include counteracting oxidative stress, regulating glucose metabolism, improving lipid profile, and lowering inflammatory cytokine levels in animal models and preliminary human trials. This review focuses on the potential role of blueberries as a functional food in the prevention and treatment of obesity and its comorbidities. Although the current evidence is promising, further randomized controlled studies in the longer term are needed to evaluate the role of blueberries and blueberry extracts to support human health.

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1. Introduction

Obesity is a medical condition in which excess body fat has accumulated to the extent that it may have a negative effect on health, causing reduced life expectancy and/or increased health problems (Haslam & James, 2005). Obesity causes a dysfunction in the metabolic system via a number of mechanisms, including initiation of endothelial dysfunction, increasing free radical production, lipid peroxidation and production of inflammatory cytokines (Chen, Chen, Wang, & Liang, 2015; Neale, Batterham, & Tapsell, 2016). Obesity predisposes to various diseases, especially obstructive sleep apnoea, cardiovascular disease (CVD), type 2 diabetes (T2D) and certain cancers (Haslam & James, 2005). Obesity is caused by excessive energy intake coupled with a lack of physical activity, however the complex interplay between genetics and environmental factors means that obesity is difficult to treat.

Obesity increases the risk of developing T2D, a disease that is characterised by hyperglycaemia with an antecedent phase of insulin resistance (Musso, Gambino, & Cassader, 2010; Zierath et al., 1996). Uncontrolled or poorly managed T2D can cause changes in the structure and function of major organs and tissues, including blood vessels, heart, nerves, eyes and kidneys which can lead to further serious and life threatening complications such as cardiac dysfunction, atherosclerosis, and nephropathy (Musso et al., 2010; Zierath et al., 1996). In the early stages of T2D (or pre-diabetes), the pancreatic β -cells respond to hyperglycaemia by secreting increased amounts of insulin to facilitate the cellular uptake of the excess plasma glucose. Over time, insulin dependent cells become desensitised to insulin, resulting in β -cell dysfunction, insulin resistance and chronic hyperglycaemia if left untreated (Hajjaghaalipour, Khalilpourfarshbafi, & Arya, 2015). Furthermore, dyslipidemia and an increase in pro-inflammatory cytokines have been shown to be associated with insulin resistance (Guo, Xia et al., 2012). Oxidative stress is another factor that can cause β -cell dysfunction, impaired glucose tolerance, insulin resistance and eventually T2D (Evans, Goldfine, Maddux, & Grodsky, 2003). Many studies have demonstrated that dietary antioxidants are effective in neutralizing or trapping reactive oxygen species (ROS) and thus antioxidants may be useful anti-diabetic agents (Defuria et al., 2009; Laplaud, Lelubre, & Chapman, 1997; Martineau et al., 2006; Poudyal, Panchal, & Brown, 2010).

It is well known that obese and diabetic patients often present with dyslipidemia, characterized by elevated triglycerides (TG), low high density lipoprotein cholesterol (HDL-C) and predominance of small-dense low density lipoprotein (LDL) particles (Chan, Barrett, & Watts, 2014). Dyslipidaemia in visceral obesity is principally the result of insulin resistance, which perturbs the kinetics of both apolipoprotein B- (apoB) and apolipoprotein A- (apoA) containing lipoproteins (Chan et al., 2002; Martinez-Fernandez, Laiglesia, Huerta, Martinez, & Moreno-Aliaga, 2015). Effective management of dyslipidaemia in obesity and T2D therefore often requires lipid regulation.

Obesity is related to chronic inflammation due to an increased infiltration of inflammatory cells into tissues such as liver and adipose tissue (Jung & Choi, 2014). Excess body fat, especially central adiposity, is correlated with a concomitant and persistent increase in low grade inflammation, which results in increased pro-inflammatory adipokines, cytokines and chemokines such as monocyte chemoattractant protein-1 (MCP-1), interleukin (IL)-6, nuclear factor-kappa B (NF- κ B) and tumor necrosis factor alpha (TNF- α), and reduced production of anti-inflammatory adipokines, including adiponectin (Joseph, Edirisinghe, & Burton-Freeman, 2014; Matsuzawa, 2010).

Dietary and/or complementary strategies to alleviate the metabolic complications of obesity and its related metabolic conditions

have aroused considerable interest and are now under exploration as alternatives to pharmaceutical interventions. This paper will review the possible health benefits of one such dietary component, blueberries and blueberry extracts, emphasizing emerging evidence for its potential to ameliorate the impacts of obesity, T2D and chronic inflammation. Moreover, data collected from studies on bioactive compounds of blueberries, in particular phytochemical constituents are included. The mechanisms of action of blueberries, as well as mechanistic and signalling pathways involved in the effects of blueberries on obesity and its related chronic diseases are also discussed. Fig. 1 shows the proposed effects of blueberries on obesity and its related comorbidities, as well as associated metabolic and molecular pathways.

2. Bioactive constituents in blueberries

Blueberries are perennial flowering plants with indigo-coloured berries from the family Ericaceae within the genus *Vaccinium* (Luby, Ballington, Draper, Pliska, & Austin, 1999). Many species of blueberry come predominantly from North America, however they are now produced in almost all countries, including Australia, New Zealand and European countries. Depending on the growing season and harvesting time, several types of blueberries are commonly available, including highbush blueberry plants (*Vaccinium corymbosum* L.), the rabbiteye blueberry (*Vaccinium ashei* Reade), lowbush blueberry plants or wild blueberry (*Vaccinium angustifolium* Aiton), and bilberry (*Vaccinium myrtillus* L.) (Maatta-Riihinen, Kamal-Eldin, Mattila, Gonzalez-Paramas, & Torronen, 2004; Michalska & Lysiak, 2015). Bilberry is a European wild blueberry that contains a higher content of anthocyanins (ACNs) than cultivated blueberry species (Chu, Cheung, Lau, & Benzie, 2011). Blueberries are nutritious fruits as they are rich sources of carbohydrates, vitamins and minerals (Liu et al., 2015b). Blueberries are also a good source of dietary fibres that constitutes 3–3.5% of fruit weight (Michalska & Lysiak, 2015). In addition, blueberries have a high content of several phytochemicals, including ascorbic acid and phenolics. Many of the proposed beneficial health effects associated with blueberry consumption are linked to the bioactive properties of the phytochemical constituents. The predominant bioactive components contained in blueberries are ascorbic acid, flavonols (including kaempferol, quercetin and myricetin), hydroxycinnamic acids (including caffeic acids, ferulic acids and coumaric acids), hydroxybenzoic acids (including gallic acids and protocatechuic acids), pterostilbene, resveratrol, and ACNs. The potential benefits of blueberry for human health have received much attention in recent years due to these bioactive components (Chen, Li, & Xu, 2010; Koupy, Kotolova, & Kucerova, 2015).

2.1. Ascorbic acid

Blueberries are rich in ascorbic acid, which is a water-soluble compound that fulfils several roles in living systems, including enhancing immunity and reducing inflammation (Liu et al., 2015a; Nile & Park, 2014). Ascorbic acid is an antioxidant vitamin and is widely distributed in various blueberry species and varieties. On average 100 g of blueberries provide 10 mg of ascorbic acid, which is equal to one third of the daily recommended dietary intake (Capra, 2006; Prior et al., 1998), however varying amounts of ascorbic acid have been reported in different species. The content of ascorbic acid in highbush blueberries (total eight species) ranged from 5 to 15 mg/100 g of fresh fruit, compared with 16.4 mg/100 g in lowbush blueberry (Prior et al., 1998). Fresh bilberry only contains small quantities of ascorbic acid (3 mg/100 g) (Graff & Upton, 2001). Rabbiteye blueberries contain different amounts of ascorbic acid due to the variety of species. Six species

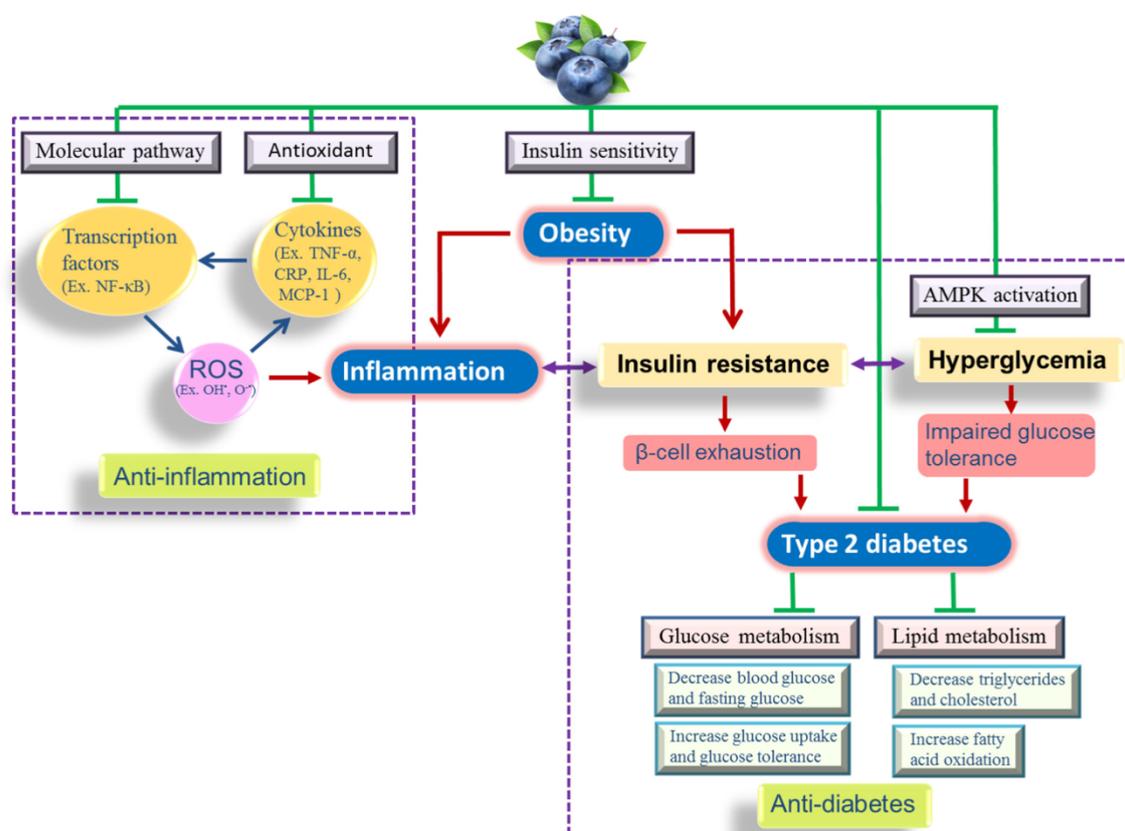


Fig. 1. Proposed metabolic pathways involved in the effects of blueberry on obesity and related comorbidities. Blueberry possibly inhibits obesity and type 2 diabetes via improving hyperglycaemia through the activation of 5' adenosine monophosphate-activated protein kinase (AMPK), and the increase of insulin sensitivity. These inhibitions possibly include the reduction of blood glucose, triglyceride and total cholesterol, and increase of glucose uptake, glucose tolerance and fatty acid oxidation. Blueberry consumption may also have anti-inflammatory potential through the inhibition of reactive oxygen species (ROS) production, for example, hydroxyl radical (OH[•]) and superoxide radical (O₂^{•-}); and inflammatory cytokines including tumor necrosis factor (TNF- α), C-reactive protein (CRP), interleukin-6 (IL-6), and monocyte chemoattractant protein-1 (MCP-1) due to the strong antioxidant activity of blueberry and its extracts. Nuclear factor-kappa B (NF- κ B) is a main signally pathway.

of rabbiteye blueberry were found to have a lower amount of ascorbic acid (6–10 mg/100 g) compared to the average content (Prior et al., 1998). However, it has been reported that the concentration of ascorbic acid was high and up to 41 mg/100 g in fresh Ochlockonee fruit, belonging to the rabbiteye species, and 25 mg/100 g in fresh highbush blueberry (Gündüz, Serçe, & Hancock, 2015). There are also other contributors to the potential variation in ascorbic acid in blueberries, such as cultivation, climate, weather conditions and storage time. The concentration of ascorbic acid decreases when conditions such as oxygen level and temperature are suboptimal during storage. Moreover, after storage for 8-days at 20 °C the content of ascorbic acid in fresh fruit decreases by 27% (Kalt, Forney, Martin, & Prior, 1999).

2.2. Phenolics

Phenolic compounds belong to a wide and heterogeneous group of chemical substances that possess one or more aromatic rings with a conjugated aromatic system and one or more hydroxyl groups. Phenolic compounds occur in free or conjugated forms with sugars, acids, and other biomolecules as water-soluble (phenolic acids, flavonoids and quinones) or water-insoluble com-

pounds (condensed tannins) (Skrovankova, Sumczynski, Mlcek, Jurikova, & Sochor, 2015). The total content of phenolic compounds in blueberries is highly variable, with variation upwards of 10-times higher or lower (e.g. ranges from 48 up to 304 mg/100 g of fresh fruit weight (up to 0.3%) (Ehlenfeldt & Prior, 2001; Moyer, Hummer, Finn, Frei, & Wrolstad, 2002) depending on the cultivar (Taruscio, Barney, & Exon, 2004), growing conditions and maturity (Castrejón, Eichholz, Rohn, Kroh, & Huyskens-Keil, 2008), and its estimation may vary depending on the method of analysis (De Souza et al., 2014; Maatta-Riihinen et al., 2004). Phenolic compounds presented in blueberries contain stilbenoids, tannins [hydrolyzable tannins (gallotannins and ellagitannins) and condensed tannins (proanthocyanidins)], and flavonoids, including flavan-3-ols, ACNs, and their polymeric condensation products, flavanones, flavonols (i.e., kaempferol, quercetin, myricetin) and flavones (Borges, Degeneve, Mullen, & Crozier, 2010; Seeram, 2008; Taruscio et al., 2004). High amounts of phenolics are found in blueberry and account for 50–80% of the total polyphenol content, which can reach a concentration of up to 3000 mg/kg fresh weight (Kuntz et al., 2015; Muller, Schantz, & Richling, 2012).

Tannins are a unique group of phenolic metabolites with molecular weights between 500 and 30,000 Da, which are widely

distributed in all berry species and specific berries may contain an abundance of a particular group of tannins (Ferreira, Gross, Kolodziej, & Yoshida, 2005; Serrano, Puupponen-Pimia, Dauer, Aura, & Saura-Calixto, 2009). It has been suggested that tannins may have therapeutic potential in the treatment of diabetes, mainly through two ways; (i) they may lower glucose levels by delaying intestinal glucose absorption and an insulin-like effect on insulin-sensitive tissues, and (ii) they may delay the onset of insulin-dependent T2D by regulating the antioxidant environment of pancreatic β -cells (Serrano et al., 2009). Previous studies showed that tannins were an effective inhibitor of intestinal α -glucosidase activity (Mcdougall et al., 2005; Toda, Kawabata, & Kasai, 2001), and they also inhibited glucose uptake in intestinal cells (Song et al., 2002). Proanthocyanidins, known as condensed tannins, are the most widely represented products of plant secondary metabolism throughout nature, after lignins (Gu et al., 2003). Blueberries contain predominantly proanthocyanidins, compared with other berries, such as blackberries, black raspberries, red raspberries, and strawberries, which contain predominantly ellagitannins (Seeram, 2008). Therefore, the unique biological properties of blueberries may be associated with the specific chemical structures of tannins. The distinct biological effects of blueberries on neuronal function in different regions of the brain and behaviour in aging animals may be due to the effects of individual classes of tannins (Shukitt-Hale, Carey, Jenkins, Rabin, & Joseph, 2007).

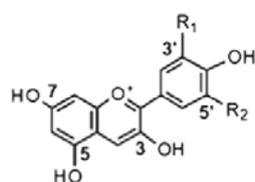
Flavonoids are a large heterogenic group of benzo- γ -pyron derivatives, which are abundantly present in food products and beverages derived from fruits and vegetables (Heo & Lee, 2004). Many physiological benefits of flavonoids have been attributed to their antioxidant and free radical scavenging properties to exert positive health effects on chronic disease, including cancer and neurodegenerative disorders (Lau, Bielinski, & Joseph, 2007; Neto, 2007; Nile & Park, 2014). Blueberries have also been demonstrated to contain high levels of flavanoid compounds, ranking them among the foods showing the highest antioxidant activity (Barberis et al., 2015; Borges et al., 2010; Moyer et al., 2002).

The predominant flavonoids in blueberries are quercetin glycosides (quercetin-3-galactoside, quercetin-3-glucoside, quercetin-3-rutinoside) and myricetin glycosides (myricetin-3-glucoside, myricetin-3-rhamnoside) (Skrovankova et al., 2015). Quercetin,

one of the most frequently researched flavonoids, has shown antioxidative and anti-carcinogenic activities to protect against oxidative stress (Heo & Lee, 2004). The content of quercetin in blueberry and bilberry were 24 and 30 mg/kg fresh fruit, respectively, which were accounted to 50% and 60% of total flavonoids (Hakkinen, Karenlampi, Heinonen, Mykkanen, & Torronen, 1999). Several *in vitro* studies indicated its efficacy in the prevention of different types of cancer induced by potent carcinogens, such as benzo(a)pyrene, azoxymethane, and N-nitrosodiethylamine (Kamaraj et al., 2007; Seufi, Ibrahim, Elmaghaby, & Hafez, 2009; Volate, Davenport, Muga, & Wargovich, 2005) and its anti-cancer capability has also been demonstrated in animal models (Caltagirone et al., 2000; Devipriya, Ganapathy, & Shyamaladevi, 2006). Myricetin is a bioflavonoid abundant in berries and it was reported that the anti-diabetic effectiveness of myricetin is due to its anti-inflammatory activity (Fu et al., 2013; Wang et al., 2010; Wu, Zheng, Gong, & Li, 2016). The content of total flavonoids in blueberries ranged from 2.5 to 387.48 mg/100 g fresh fruit (Hakkinen et al., 1999; Sellappan, Akoh, & Krewer, 2002), depending on the species and the method used (Borges et al., 2010; Buran et al., 2014; Taruscio et al., 2004). Taruscio et al. (2004) reported the contents of flavonols extracted from eight blueberry species, including three species of highbush blueberry, three species of half-highbush blueberry and two species of bilberry. The HPLC analytical results showed that myricetin and quercetin were the principal flavonols in blueberries (Taruscio et al., 2004). Bilberry contained the highest level of quercetin (163.6 $\mu\text{g/g}$ in frozen fruit) followed by half-highbush blueberry (102.5 $\mu\text{g/g}$ in frozen fruit) and highbush blueberry (86.4 $\mu\text{g/g}$ in frozen fruit) (Taruscio et al., 2004). Bilberry also contained the highest content of myricetin (200 $\mu\text{g/g}$ in frozen fruit) at the level of nearly 10 and 15-fold higher, compared to half-highbush blueberry (19.8 $\mu\text{g/g}$ in frozen fruit) and highbush blueberry (12.9 $\mu\text{g/g}$ in frozen fruit) (Taruscio et al., 2004).

Anthocyanins (ACNs), pigments that contribute to the intense colours in blueberry, have been shown to exhibit numerous bioactive properties, such as anti-inflammatory, antioxidant and anti-cancer activities (Faria et al., 2010; Vendrame, Daugherty, Kristo, Riso, & Klimis-Zacas, 2013; Zepeda et al., 2012). The most common anthocyanidin aglycones are peonidins, pelargonidins, malvidins, delphinidins, cyanidins and petunidins (Li, Wang, Guo, & Wang, 2011). These then combine with organic acids and sugars to generate various ACNs (Fig. 2) (Rodriguez-Mateos, Heiss, Borges, & Crozier, 2014). Muller et al. (2012) found that malvidin and delphinidin are the main components and constitute almost 72% of all identified anthocyanins (Muller et al., 2012). However, other studies reported less concentrations of malvidin (22–33%) and delphinidin (27–40%) in five genotypes of blueberries (Cho, Howard, Prior, & Clark, 2004). There are up to 27 different ACNs found in blueberries (Prior et al., 1998). The content and type of ACNs depend on the species, fruit size, ripening stage, as well as on climatic, pre-harvest environmental conditions and storage (Muller et al., 2012; Scibisz & Mitek, 2007). The concentration of ACNs is up to 800 mg/100 g fresh weight in highbush species and more than 1000 mg/100 g fresh fruit in lowbush species (Cho et al., 2004; Hosseinian & Beta, 2007). The high content of ACNs in different *Vaccinium* species is a main contributor to their antioxidant activity and is responsible for about 84% of total antioxidant capacity (Borges et al., 2010). Whereas ascorbic acid was only found to contribute to 10% of the antioxidant capacity despite being present in a significant amount (Barberis et al., 2015).

Although structural and categorical diversity can be noticed among bioactive constituents in blueberries, other factors influence this diversity including, but are not limited to, species and genetic makeup of blueberries, agricultural practices, growing



Anthocyanidin	R ₁	R ₂
Pelargonidin	H	H
Cyanidin	OH	H
Delphinidin	OH	OH
Peonidin	OCH ₃	H
Petunidin	OCH ₃	OH
Malvidin	OCH ₃	OCH ₃

Fig. 2. Structures of the major anthocyanidins in blueberry.

Table 1
Summary of the effects of blueberry and its extract on obesity and comorbidities in animal studies.

Type of blueberry	Amount (per day)	Animals	Days	Obesity Food intake (FI); Metabolic variables	Body weight (BW) or fat (BF)	Tissue weight (g) or fat (%)	Type 2 diabetes		Lipid profiling	Inflammatory cytokines (Serum marker)	Reference
							Glucose	Insulin			
Lowbush BB	8% (w/w)	Obese Zucker rat	56	Nil FI	Nil BW	↓weight of liver, AT fat	Nil fasting glucose, JBG	↓PI	↓serum TC, TG	↑adiponectin, ↓IL-6, TNF- α , CRP-1	Vendrame et al. (2013)
Lowbush BB powder	8% (w/w)	Zucker Fatty rats	56	Nil FI	Nil BW	↓weight of liver, AT fat	Nil fasting glucose, JBG	↓PI	Nil plasma HDL-C; ↓plasma TG, TC	Nil TNF- α , CRP-1	Vendrame, Daugherty, Kristo and Khamis-Zacas (2014)
Highbush BB powder, freeze-dried	2% (w/w)	Zucker Fatty rats	90	Nil FI	Nil BW	↓weight of liver, AT fat	Nil fasting glucose, BG	↓PI	Nil serum TG, TC, HDL-C	Nil IL-6, TNF- α	Seymour et al. (2011)
BB powder	4% (w/w)	C57BL/6j mice	56	Nil energy intake, heat produce	Nil BW	Nil AT fat	Nil fasting glucose, BG	Nil FI, energy intake, heat produce	Nil serum free fatty acids	Nil IL-6, TNF- α , MCP-1	Defuria et al. (2009)
Bilberry, freeze-dried	5% (w/w)	C57BL/6j mice	84	Nil FI, energy intake	Nil BW gain	Nil BG	Nil BG	Nil BW gain, BF	Nil serum free fatty acids	Nil TNF- α , MCP-1	Mykkanen et al. (2012)
Bilberry	5% or 10% (w/w)	C57BL/6j mice	90	Nil FI, energy intake, heat produce	Nil BW gain, BF	Nil BG	Nil fasting glucose	Nil BF, ↓BW, ↓BF	Nil serum TC	Nil TNF- α , IL-1 β , MCP-1	Mykkanen et al. (2014)
BB powder, freeze-dried	10% (w/w)	C57BL/6j mice	84	Nil energy intake, heat produce	↓BW gain, BF	↓TAT weight	Nil BG	↓BW gain, BF	Nil serum TC, liver TC, TG; ↓serum TG, liver TC; ↓serum TC	Nil leptin, ↓resistin, TNF- α , IL-1 β , MCP-1	Prior et al. (2009)
Purified ANCS from BB	10% (w/w)	C57BL/6j mice	92	Nil energy intake, heat produce	↓BW, BF	Nil weights of liver; ↓weights of kidney, heart; TAT fat	Nil BG	↓BW, BF	Nil liver TG; ↓serum TG, liver TC; ↓serum TC	Nil leptin, ↓resistin, TNF- α , IL-1 β , MCP-1	Prior et al. (2008)
BB juice	3.75 mg	C57BL/6j mice	70	Nil energy intake, heat produce	Nil BW; ↓BF	Nil weights of kidney, liver, heart	Nil BG	Nil BW; ↓BF	Nil liver lipids, cholesterol; ↓TC, liver TG	Nil leptin; Nil adiponectin	Wu et al. (2013)
BB juice	ad libitum	C57BL/6j mice	84	Nil energy intake, heat produce	Nil BW; ↓BF	Nil weights of heart, liver, kidney, AT	Nil BG	Nil BW; ↓BF	Nil serum cholesterol, TG	Nil leptin	Prior et al. (2010)
Purified ANCS (0.2 or 1.0 mg/mL)	ad libitum	C57BL/6j mice	72	Nil energy intake; ↓FI	Nil BF; ↓BW	Nil weights of heart, liver, kidney, AT; ↓total fat, AT fat	Nil fasting glucose	Nil BF, ↓BW	Nil serum cholesterol, TG	Nil leptin	Prior et al. (2010)
BB juice	(80 ml/kg)	Obese KKAY mice	28	Nil energy intake	↓BW gain	Nil weights of kidney; ↓weight of liver, AT fat	JBG; ↓glucose tolerance	↓BW gain	Nil serum cholesterol, TG	Nil leptin, adiponectin	Vuong et al. (2009)
BB polyphenol	ad libitum	Obese C57BL/6j mice	91	Nil FI	↓BW gain	Nil weights of liver, AT fat	↓fasting glucose	↓BW gain	Nil serum cholesterol, TG	Nil leptin, adiponectin, RBP4	Roopchand et al. (2013)
BB, ANCS, or phenolic-fractions ACNs (10 g/kg in diet)	500 mg/kg	Diabetic C57BL/6j mice	42	Nil FI, energy intake	Nil BW, ↓BW gain	Nil weights of AT; ↓weights of liver	JBG	Nil BW, ↓BW gain	Nil serum TG; ↓serum cholesterol	Nil leptin, adiponectin, RBP4	Grace et al. (2009)
BB peel extracts	15.8% (w/w)	Sprague-Dawley rats	35	Nil FI	↓BW, BW gain	↓weight of AT	↓serum TC, TG; ↓serum HDL-C	↓BW, BW gain	↓total lipid, TC, TG of liver, serum TG, serum TC	Nil adiponectin	Takikawa et al. (2010)
											Song et al. (2013)

Table 1 (continued).

Type of blueberry	Amount (per day)	Animals	Days	Obesity Food intake (FI); Metabolic variables	Body weight (BW) or fat (BF)	Tissue weight (g) or fat (%)	Type 2 diabetes		Lipid profiling	Inflammatory cytokines (Serum marker)	Reference
							Glucose	Insulin			
BB pomace by-products	8% or 10% (w/w)	Golden Syrian hamsters	21	↑FI	Nil BW, BW gain	Nil weights of liver, AT		Nil hepatic total lipid, free cholesterol, TG		Kim et al. (2010)	
C3G	0.2% (w/w)	Diabetic db/db mice C57BL/6J obese mice	35	Nil FI	Nil BW	Nil weights of kidney, heart, AT fat; ↓weights of liver; ↑AT fat	↓BG, fasting glucose	↓hepatic TC	Tadiponectin ↓IL-6, TNF-α, CRP-1	Goto, Xia et al. (2012)	
C3G	2% (w/w)	Diabetic KKAY mice	35	Nil FI	Nil BW, AT weight	Nil weights of kidney, heart, AT fat; ↓weights of liver; ↑AT fat	↓fasting glucose, BG	↓hepatic TG	Nil IL-6; ↑adiponectin; ↓MCP-1, TNF-α	Sasaki et al. (2007)	
C3G (2 g/kg in diet)	ad libitum	Diabetic db/db mice	56				↓BG	Nil serum: TG, TC	Tadiponectin	Liu et al. (2014)	

Nil: no statistically significant differences between the blueberry and control groups. ACNs: anochyanins; AT, adipose tissue; BB, blueberry; BF, body fat (%); BG, blood glucose (mmol/L); BW, body weight (g); C3G, cyanidin-3-glucoside; CRP, high levels of C-reactive protein (mg/L); FI, food intake (g); HDL-C, high-density lipoprotein cholesterol (mg/dL); IR, insulin resistant; IL-1β, interleukin-1 beta (ng/L); IL-6, interleukin-6 (ng/L); MCP-1, monocyte chemoattractant protein-1 (ng/L); PI, plasma insulin (pmol/L); RBP4, retinol binding protein 4; TC, total cholesterol (mg/dL); TG, triglycerides (mg/dL); TNF-α, tumor necrosis factor (ng/L).

condition, season of harvest, irrigation, and storage of the fruits (Castrejón et al., 2008; Scibisz & Mitek, 2007).

2.3. Phenolic acid

Phenolic acid, in general, describes phenols that possess one carboxylic acid functionality (Robbins, 2003). Phenolic acids account for approximately one-third of the dietary phenols present in plants (Zadernowski, Naczka, & Nesterowicz, 2005). Researchers have become increasingly interested in phenolic acids and their derivatives due to their high nutritional and antioxidant properties in foods (Chalas et al., 2001; Zadernowski et al., 2005). Clifford (1999) estimated that the average amount of phenolic acids consumed is between 25 mg and 1 g daily (Clifford, 1999). In blueberries, only a minor fraction of phenolic acid exists as free forms, with the majority of phenolic acid existing in conjugated forms, which are linked with esters, amides and glycosides (Robbins, 2003). Vanillic acid, hydroxycinnamic acids, ferulic acid, caffeic acid, chlorogenic acid, p-coumaric acid, gallic acid and salicylic acid are the principal phenolic acids in blueberry (Zadernowski et al., 2005). Among them, chlorogenic acid is the most abundant in blueberry species (Kang, Thakali, Jensen, & Wu, 2015), however its content was highly variable between species with highbush and lowbush blueberry varieties ranging from 34.3 to 113.8 mg/100 g fresh weight (Rodriguez-Mateos, Cifuentes-Gomez, Tabatabaee, Lecras, & Spencer, 2012). This high concentration of chlorogenic acid present in blueberries is likely to contribute to the anti-inflammatory effects of blueberries (Santos, Almeida, Lopes, & De Souza, 2006). A previous study showed that seven phenolic acid mixture including hydroxycinnamic acid, hippuric acid, 3-(3-hydroxyphenyl)propionic acid, 3-(4-hydroxyphenyl) propionic acid, hydroxyphenylacetic acid, hydroxybenzoic acid and ferulic acid from blueberry inhibited lipopolysaccharide (LPS)-induced production of pro-inflammatory cytokine, IL-6 and TNF-α by the reduction of mitogen-activated protein kinase, Jun amino-terminal kinases (JNK), p38 and Erk1/2 phosphorylation in murine macrophage cell line RAW 264.7 (Xie et al., 2011).

3. Effects on body weight and fat mass

The anti-obesity effects of blueberries and blueberry extracts have been investigated in both clinical studies and also several animal models, such as Obese Zucker rats, KKAY mice, C57BL/6J mouse and Sprague-Dawley rats (Prior et al., 2010; Seymour et al., 2009, 2011; Vuong et al., 2009). Tables 1 and 2 summarise the impacts of consumption of blueberries and blueberry extracts on obesity in animal models and human trials.

3.1. Whole fruit or juice

It has been reported that body weight, liver weight, and total fat weight were significantly reduced in Obese Zucker rats fed a low-fat diet (LFD) combined with 2% (w/w) whole highbush blueberry powder (Seymour et al., 2009, 2011). These results are consistent with the study of Prior et al. (2010) who reported that supplementation with blueberry juice (0.2 mg/mL) prevented weight gain in C57BL/6J mice that were fed a HFD (45% of kcal from fat). Furthermore, Vuong et al. (2009) showed that incorporating blueberry juice in drinking water significantly reduced weight gain in obese KKAY mice. These positive results possibly related to the improved glucose tolerance and enhanced insulin sensitivity seen in these animals (Vuong et al., 2009). Contrary to these results, blueberry supplementation did not affect the body weight of C57BL/6J mice fed a HFD (60% of energy) with 4% (w/w) whole blueberry powder for 8 weeks (Defuria et al., 2009) or of Sprague-Dawley rats

Table 2
Summary of the effects of blueberry on obesity and its comorbidities on human trials.

Type of blueberry	Amount (per day)	Population	Days	Obesity		Circumference of weight, hip (WC, HC); Waist-to-hip ratio (W/H)	Body weight, fat (BW, BF); Body mass index (BMI)	Type 2 diabetes		Lipid Profiling (in serum)	Serum marker	Inflammatory cytokines (in plasma)	Reference
				Daily intake (DI); Energy intake (EI)	Daily intake (DI); Energy intake (EI)			Glucose	Insulin				
Lowbush BB powder, (freeze-dried)	100 g	Healthy	7							Nil TG	↓TEAC; ↑TORAC		Mazza et al. (2002)
Bilberry fruit (frozen)	100 g	Metabolic syndrome	40		↓WC		↓BW	↑PI		Nil TG, TC, HDL-C, LDL-C, free fatty acids	↓adiponectin	↓VCAM-1, TNF- α , JhsCRP, IL-6, IL-12, LPS	Lehtonen et al. (2011) Kolehmainen et al. (2012)
Bilberry fruit (fresh)	400 g	Metabolic syndrome	126	Nil DI, EI		Nil WC		Nil fasting BG		Nil TG, TC	Nil ORAC, FRAP	Nil MCP-1, IL-1 β ; JCRP, IL-6, IL-15, MIG; ↑TNF- α	Karlsen et al. (2010)
Bilberry juice	330 mL	Cardiovascular disease	28				Nil BW, BMI			Nil TG, TC, HDL-C, LDL-C, Tox-LDL	Nil	Nil CRP, IL-6	Basu et al. (2010)
BB beverage	480 ml (twice)	Obese	56	Nil DI		Nil	Nil BW, BMI	Nil IR		Nil TG, TC, HDL-C, LDL-C	adiponectin	Nil TNF- α , CRP, MCP-1	Stull et al. (2010)
BB bioactives	22.5 g (twice)	Obese, nondiabetic, IR	42	Nil EI			Nil BW, BF, BMI	Nil PI; ↑IS		Nil TG, TC, HDL-C, LDL-C			Stull et al. (2010)
Bilberry extract	0.47 g (36% ACNs)	T2D	14					↓BG	↓PI		Nil TEAC, FRAP	Nil MCP-1	Hoggard et al. (2013)
Blueberin (250 g BB + 50 mg myricetin)	300 mg (three times)	T2D	28					↓fasting BG				↓CRP	Abidov et al. (2006)
ACNs	160 mg (twice)	dyslipidemic	84	Nil EI		Nil WC, HC, W/H	Nil BW, BMI	Nil BG		Nil TG, TC; ↑HDL-C; ↓LDL-C	Nil		Qin et al. (2009)
ACNs	160 mg (twice)	Hypercholesterolemia	168	Nil DI				Nil BG	Nil PI	Nil TG, TC; ↑HDL-C; ↓LDL-C	adiponectin	Nil TNF- α , JhsCRP, IL-1 β , VCAM-1	Zhu et al. (2013)

Nil: no statistically significant differences between the blueberry and control groups.

ACNs, anthocyanins; AT, adipose tissue; BB, blueberry; BF, body fat (%); BG, blood glucose (mmol/L); BMI, body mass index (kg/m²); BW, body weight (g); CRP, high levels of C-reactive protein (ng/L); CVD, cardiovascular disease; DI, daily intake (g); EI, energy intake (kcal/d); FRAP, ferric-reducing ability of plasma ($\mu\text{mol/L}$); HC, hip circumference (cm); HDL-C, high-density lipoprotein cholesterol (mg/dL); hsCRP, high sensitivity C-reactive protein (ng/L); IL-1 β , interleukin-1 beta (ng/L); IL-6, interleukin-6 (ng/L); IR, insulin resistant; IS, insulin sensitivity; LDL-C, low-density lipoprotein cholesterol (mg/dL); LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein-1 (ng/L); MIG, monokine induced by INF- γ ; ORAC, oxygen radical absorbance capacity (μmol of Trolox equiv/L); ox-LDL, oxidized low-density lipoprotein ($\mu\text{mol/L}$); PI, plasma insulin ($\mu\text{mol/L}$); T2D, type 2 diabetes; TC, total cholesterol (mg/dL); TEAC, trolox equivalent antioxidant capacity (μmol of Trolox equiv/L); TG, triglycerides (mg/dL); TNF- α , tumor necrosis factor (ng/L); VCAM-1, vascular cell adhesion molecule-1; WC, weight; circumference (cm); W/H, waist-to-hip ratio.

supplemented with 10% freeze-dried whole blueberry for 3 weeks (Seymour et al., 2009). Another study found no significant differences in weight gain after the 12 weeks of feeding C57BL/6 J mice with 5% bilberry compared with mice fed a HFD (45% kcal fat) (Mykkanen et al., 2012). Conversely, Prior et al. (2008) demonstrated that diets supplemented with 10% whole blueberry powder increased adiposity and body weight in C57BL/6J mice fed a HFD. However, blueberry-fed mice in this study consumed approximately 12% more energy/day than the control HFD group, which may have contributed to these outcomes (Prior et al., 2008).

Most of the clinical studies have shown that dietary supplementation with whole blueberry or blueberry juice failed to reduce body weight and waist circumference (Basu et al., 2010; Qin et al., 2009; Stull, Cash, Johnson, Champagne, & Cefalu, 2010). This has been demonstrated in a randomised controlled trial with 48 obese participants (4 males and 44 females) in which participants consumed a freeze-dried blueberry beverage (50 g freeze-dried blueberries equivalent to 350 g of fresh blueberries) or water for 8 weeks. There were no significant differences observed in waist circumference, body weight or dietary intakes between the treatment group and the control group (Basu et al., 2010). Similar results were also observed in non-diabetic obese participants who were supplemented with either 22.5 g blueberry powder or a placebo twice daily for 6 weeks in that there were no significant differences observed between the treatment and control groups in body weight, adiposity and energy or macronutrient consumption (Stull et al., 2010). Overall there is limited evidence to suggest that blueberry supplementation alone affects adiposity in obese or overweight individuals. Future studies are encouraged to focus on calorie restriction and longer intervention periods in conjunction with supplementation, however whether this will result in clinically significant improvements in weight loss compared to calorie restriction alone is uncertain.

3.2. Extracts of blueberries

Several studies have examined the effects of blueberry extracts, particularly ACNs from fresh blueberry fruit, juice and peel on control of body weight and have indicated that the anti-obesity capability of blueberry extract is quite different to whole fruit or juice (Prior et al., 2008, 2009, 2010). Although the reasons for these disparities are not clear, one possible explanation is that there are different types and amounts of bioactive constituents contained in blueberry and its products, which might change the response to extracts from blueberry, compared with purification or single components. For instance, blueberry juice contains not only ACNs but also other components such as procyanidins, chlorogenic acid, and other water-soluble compounds including sugars (Prior et al., 2010). Another possibility is that there are some specific components, such as uronic acids, neutral sugars, noncellulosic sugars including xylose and arabinose, or other factors as an obstruction in whole blueberry to counteract the potential benefit of blueberry consumption (Vicente et al., 2007). Wu et al. (2013) showed that ACNs from blueberry juice decreased body weight up to 7.3% in dietary-induced models of obesity. Dietary-induced weight gain, perirenal adipose tissue and epididymal weights were significantly lowered in male Sprague-Dawley rats fed a HFD supplemented with blueberry peel extracts for 5 weeks compared to an equivalent control group. It has been reported that blueberry peel extracts may potentially affect obesity by a reduction of adipogenesis and inhibition of fat accumulation through the PI3K/Akt/GSK3 β pathway in 3T3-L1 preadipocytes (Song et al., 2013).

Further studies are required to assess the effect of ACNs consumption at various doses to establish the specific concentration of ACNs required for ameliorating the development of obesity. According to a previous study conducted by Prior et al. (2010),

the low concentration of ACNs (0.2 mg/mL) decreased retroperitoneal and epididymal fat (% body weight) by 31% and 25%, respectively in mice fed a LFD, and 26% and 29%, respectively in mice fed a HFD for 72 days. However, retroperitoneal and epididymal fat levels were not decreased in HFD-fed mice treated with higher concentration of ACNs (1.0 mg/mL) but were similar to, or slightly higher than the HFD mice without ACNs (Prior et al., 2010). ACNs intake was measured as 0.6 and 3.4 mg/day for each mouse fed a LFD, and 0.5 and 1.8 mg/day for each mouse fed a HFD, according to liquid intake with the low concentration (0.2 mg/mL) and high concentration (1.0 mg/mL) of ACNs (Prior et al., 2010). This indicated that low concentrations of ACNs are potentially more beneficial compared to higher doses; however the exact reasons for this observation are unknown. Conversely, another independent study has demonstrated that supplementation of a higher concentration (2.8 mg/day/mouse) of purified ACNs for 92 days significantly prevented the development of obesity, but 3.75 mg/day/mouse failed to prevent body weight gain in HFD induced obese mouse model (Prior et al., 2008). Thus it appears to be no clear dose dependent effect and further investigation is needed to define the effective dose of ACNs or blueberries for body weight control in cases of obesity.

4. Effect on glucose metabolism and insulin signalling

Animal models (Table 1) and clinical studies (Table 2) have demonstrated that supplementation or consumption of blueberry or blueberry bioactive compounds cause changes in glucose metabolism and improve insulin sensitivity.

4.1. Whole fruit or juice

Supplementation of 2% freeze-dried blueberry powder for 13 weeks in Obese Zucker rats have demonstrated significant reductions in glucose, fasting insulin and insulin resistance, as indicated by the Homeostasis Model Index of Insulin Resistance (HOMA-IR) (Seymour et al., 2009, 2011). Likewise, Vuong et al. (2009) showed that fermented blueberry juice by the *Serratia* *vaccinii* bacterium significantly reduced blood glucose levels and maintained the glycaemia of pre-diabetic KKAY mice to a normal level. These results indicate that blueberry intake could reduce phenotypes of diabetes in obesity-prone rats by regulating glucose metabolism. Conversely, Prior et al. (2008) reported that long term supplementation with freeze-dried whole blueberry powder did not affect the results of a glucose tolerance test that were administered to C57BL/6J obese mice. These inconsistent results mainly depend on the variation of animal models, the duration of the treatment, and the dose of bioactivity components in blueberry. Furthermore, clinical studies have also reported that blueberry supplementation did not show the impact on fasting serum glucose (Basu et al., 2010; Kolehmainen et al., 2012; Stull et al., 2010). Specifically, Basu et al. (2010) documented that a freeze-dried blueberry beverage (50 g freeze-dried blueberries equivalent to 350 g of fresh blueberries) for 8 weeks to 48 obese participants (4 males and 44 females) was not able to significantly change their serum glucose concentration. Also, glucose and insulin responses did not differ between the bilberry group (400 g fresh fruit) and the control group, when obese individuals consumed a diet rich in bilberries for 8 weeks (Kolehmainen et al., 2012). Likewise, no changes was observed in serum glucose during the intervention with 22.5 g blueberry bioactive twice daily for 6 weeks, although insulin sensitivity was improved significantly more in the blueberry group compared to the placebo group in participants who were obese, nondiabetic, and insulin resistant (Stull et al., 2010). *In vitro* studies have however consistently shown that blueberry

improves glucose uptake. For instance, 6-h incubation of fermented blueberry juice with and without insulin enhanced glucose uptake into the adipocyte and muscle cells and increased the phosphorylation/activation of proteins in the insulin-independent pathway (i.e., AMP-activated protein kinase) but had no effect on phosphorylation of key proteins in the insulin-dependent pathway (i.e., AKT and ERK1/2) (Vuong, Martineau, Ramassamy, Matar, & Haddad, 2007). These findings showed that the bioactive components in fermented blueberry improved glucose uptake into the cells via an insulin-independent mechanism. These positive cellular mechanistic studies provide evidence on the improvement of insulin sensitivity *in vitro*, however why the variation in the *in vivo* studies remains to be determined.

4.2. Extracts from blueberries

While the effects of blueberry juice on glucose tolerance *in vivo* is varied, supplementation with ACNs appear to have a more positive effect as it has been previously indicated that fasting serum glucose concentrations were decreased and oral glucose tolerance was increased in mice fed a HFD supplementation with ACNs compared to blueberry juice (Prior et al., 2010). This result is possibly attributed to other constituents in blueberry juice such as procyanidins, chlorogenic acid, and other water-soluble compounds including sugars, which are not present in ACNs. It is possible that this beneficial effect of ACNs on glucose tolerance may be due to a direct effect on the liver as blueberry ACNs (0.05–10 mg/mL) have been demonstrated to significantly reduce glucose production by 24–74% in H4IIE hepatocytes (Roopchand, Kuhn, Rojo, Lila, & Raskin, 2013). In addition, diabetic C57BL/6J mice supplemented with 500 mg/kg body weight of a phenolic-rich fraction or an anthocyanin-rich fraction showed reductions in blood glucose levels by 33% and 51%, respectively. In these fractions, 287 mg/g ACNs was in a phenolic-rich fraction, while 595 mg/g ACNs (cyanidin-3-glucoside equivalents) was in an anthocyanin-rich fraction, which suggested that higher ACNs concentration in different fractions may contribute to more hypoglycaemic activity of the extracts (Grace et al., 2009).

Bilberry extract also reduces blood glucose level and enhances insulin sensitivity in diabetic KKAY mice (Sasaki et al., 2007). Furthermore, in the same study, the glucose transporter 4 (Glut4) was upregulated and retinol binding protein 4 (RBP4) was downregulated in the white adipose tissue in bilberry extract group (Sasaki et al., 2007). These results indicated that bilberry extract has a potent effect on glucose metabolism through the regulation of Glut4-RBP4 system. The beneficial effects of bilberry extracts are also supported in a human trial demonstrating that insulin and postprandial glycaemia was significantly reduced in diabetic volunteers supplemented a bilberry extract (containing 36% (w/w) of ACNs which is equivalent to about 50 g of fresh bilberry) for 2 weeks, compared with the placebo group (a polysaccharide drink and equivalent to 75 g of glucose) (Hoggard et al., 2013). A longer intervention (4 weeks) with the extracts (providing 50 mg 3,4-caffeoylquinic (chlorogenic) acid, and 50 mg myricetin) from blueberry leaf has also shown that fasting plasma glucose was reduced significantly in diabetic volunteers (Abidov, Ramazanov, Jimenez Del Rio, & Chkhikvishvili, 2006). However, other clinical studies have indicated that there were no significant differences in fasting blood glucose between the treatment and the control groups after dietary supplementation with ACNs for 12 (Qin et al., 2009) or 24 weeks (Zhu et al., 2013).

There are up to 27 different ACNs present in blueberry, however, only several specific ACNs exhibit strong hypoglycaemic capacity (Roopchand et al., 2013). Grace et al. (2009) observed that in diabetic C57BL/6J mice treated with 300 mg/kg of the pure ACN delphinidin-3-O-glucoside (D3G) or malvidin-3-O-glucoside

(M3G), M3G decreased blood glucose to a greater extent compared to D3G. It is likely that the metabolism and bioavailability affects the magnitude of bioactivity in different types of ACNs. Cyanidin-3-glucoside (C3G) is the predominant ACN in blueberries (Wang, Zhao, Wang, Huo, & Ji, 2016). Several studies have shown that isolated C3G improved insulin sensitivity and hyperglycaemia in animal models of diabetes (Guo, Xia et al., 2012; Liu, Li, Zhang, Sun, & Xia, 2014; Sasaki et al., 2007). There are several pathways involved in these effects, such as the modulation of Glut4-RBP4 system (Sasaki et al., 2007), the c-Jun N terminal kinase/forkhead box O1 signalling pathway (Guo, Guo, Jiang, Li, & Ling, 2012) and adiponectin activating cAMP-PKA-eNOS signalling pathways (Liu et al., 2014).

In animal studies, following supplementation with blueberry extracts or pure ACNs (C3G), ACNs were detected in the liver, blood, kidney and ocular tissues with an intact form suggesting that ACNs and/or their metabolites can be distributed to various tissues via blood and are therefore expected to regulate metabolic changes in the body (Ichiyanagi, Shida, Rahman, Hatano, & Konishi, 2006; Mcghee, Ainge, Barnett, Cooney, & Jensen, 2003; Takikawa, Inoue, Horio, & Tsuda, 2010; Tsuda, Horio, & Osawa, 1999). An *in vitro* study has also reported that glucose uptake was increased in C₂C₁₂ cells treated with extracts from the root, leaf and stem of lowbush blueberry, and in 3T3-L1 cells only treated with extracts from root and stem of lowbush blueberry (Martineau et al., 2006). These results were consistent with an *in vivo* study that also demonstrated ACNs components in different fractions specifically contributed to improving hypoglycaemic activity in diabetic C57BL/6J mice (Grace et al., 2009). However, the fruit extract in lowbush blueberry did not show any effect on glucose-stimulated insulin secretion or glucose uptake in β TC-tet pancreatic β cells (Martineau et al., 2006). Since the ACNs composition extracted from the fruit are completely different, compared to those extracted from the leaf, root and stem, the hypoglycaemic compounds from the blueberry *in vitro* studies perhaps do not have the same effect *in vivo* due to the different mechanisms of action.

5. Effect on lipid metabolism

5.1. Whole fruit and fruit juice

Diets enriched with blueberries have been reported to improve dyslipidaemia (Seymour et al., 2009, 2011; Vendrame, Daugherty, Kristo, & Klimis-Zacas, 2014; Wu et al., 2013). Plasma TG and total cholesterol (TC) concentrations were significantly reduced in Obese Zucker rats supplemented with 8% wild blueberry for 8 weeks (Vendrame et al., 2014) or 2% blueberry powder for 13 weeks in both LFD and HFD groups compared with the control groups (Seymour et al., 2009). These observations were also supported by a reduction in serum TC and low density lipoprotein cholesterol (LDL-C), as well as the levels of liver TG and TC following consumption of blueberry juice. Although the contents of liver lipids and cholesterol were not changed in C57BL/6 mice (Wu et al., 2013). The consumption of 1%, 2% and 4% blueberry-supplements for 8 weeks has significantly reduced the TC and LDL-C concentrations in pigs (Kalt et al., 2008).

The possible pathways involved in the anti-dyslipidaemic effect of blueberries include the regulation and expression of key enzymes such as lipoprotein lipase (LPL) (Wei et al., 2011), fatty acid synthase (Tsuda, Ueno, Kojo, Yoshikawa, & Osawa, 2005) and ATP-binding cassette transporter 1 (ABCA1) (Xia et al., 2005) which are involved in TG and cholesterol metabolism. Furthermore, the expression of transcription factors such as sterol regulatory element-binding transcription factor (SREBP) and peroxisome proliferator-activated receptor (PPAR) in bioactive tissues could

also explain the observed effects of blueberry consumption on lipid profiles (Cutler, Petersen, & Anandh Babu, 2016; Vendrame et al., 2014). In a recent study, the expression of PPAR α and PPAR γ in Obese Zucker rats were increased in the abdominal adipose tissue (AAT), while that of total SREBP-1 was decreased in both the liver and the AAT of the rats following consumption of a diet enriched with 8% wild blueberry for 8 weeks (Vendrame et al., 2014). The activation of PPAR α and PPAR γ following blueberry consumption could partly explain such an effect on lipid accumulation in blood and bioactive tissues. The activation of PPAR α is related to enhanced fatty acid uptake, conversion into acyl-CoA derivatives, and further catabolism (Pawlak, Lefebvre, & Staels, 2015); moreover, the activation of PPAR γ in adipose tissue is known to induce differentiation of preadipocytes and TG storage (Ferre, 2004). The down-regulation of the expression of SREBP-1 also helps to explain the reduction in TG and TC in the Obese Zucker rats supplemented with blueberry diet, since SREBP-1 isoforms promote the synthesis and accumulation of TG and cholesterol via the induction of multiple enzymes (Horton, Goldstein, & Brown, 2002). Similar results were also observed by Seymour et al. (2011) which showed blueberry intake increased PPAR α and PPAR γ activity in skeletal muscle in both HFD and LFD fed rats. In addition, the intake of blueberry significantly affected mRNA of several genes related to fat storage and glucose uptake, such as PPAR γ co-activator 1 α , Acyl-CoA oxidase, fatty acid synthase, fatty acid-CoA ligase, Glut4 and insulin receptor substrate 1 in both skeletal muscle and retroperitoneal abdominal fat in HFD induced rats (Seymour et al., 2011). With regards to improving lipid profile, clinical studies of blueberry supplementation have not supported those of animal studies with freeze-dried wild blueberries showing no effect on TG, TC, HDL-C and LDL-C levels in obese subjects (Basu et al., 2010), in subjects with developing CVD risk (Riso et al., 2013), and in healthy middle-aged male subjects (Wang et al., 2016).

5.2. Anthocyanins in blueberries

Mice that were fed a HFD and also had their drinking water supplemented with purified ACNs from blueberries, instead of whole blueberry, showed decreased serum TG and TC levels that were comparable with those of the lean control group (10% of kcal from fat) (Prior et al., 2009). This result indicated that sugars or other components in the whole fruits were possibly masking the benefits of ACNs and other components of blueberries. It should be noted that blueberry polyphenol was effective on serum TC level in C57BL/6 mice, which was 13.2% lower than in the control group (Roopchand et al., 2013). A human trial which investigated the effect of ACNs (from bilberry) supplementation on lipid profiles in dyslipidemic patients found that 160 mg of ACNs supplementation for 12 weeks increased cellular cholesterol efflux and HDL-C concentrations, as well as reduced the mass and activity of plasma cholesteryl ester transfer protein (CETP) and LDL-C concentrations, without affecting TC levels (Qin et al., 2009). Zhu et al. (2013) also found similar results, reporting that volunteers with hypercholesterolemia had greater reductions in LDL-C levels and greater increases in HDL-C after consuming 320 mg/day of purified ACNs for 24 weeks compared with controls. In an *in vitro* study, C3G reduced CETP activity in human HepG2 cells in a dose-dependent manner, suggesting that supplementation of ACNs may improve lipoproteins by increasing HDL-C concentrations and decreasing serum LDL-C partially due to the inhibition of CETP target (Zhu et al., 2013). Other possible mechanisms by which blueberry ameliorates lipid profile are possibly related to the intact assimilation of blueberry bioactivity such as ACNs, which exhibited the antioxidant properties in serum and other tissues (Mazza, Kay, Cottrell, & Holub, 2002; Mcghee et al., 2003). Studies have revealed that the high

concentration of ACNs in wild blueberry is a major contributor to the antioxidant properties *in vitro*, instead of other antioxidant minerals, vitamins, or fibres (Prior et al., 1998). Moreover, the antioxidant properties of ACNs have been confirmed via other systems of oxidation such as that for the prevention of LDL oxidation *in vitro* (Laplaud et al., 1997). It has been validated that ACNs can be absorbed intact in glycosylated and possibly acylated forms in male volunteers after the consumption of blueberries (Wu, Cao, & Prior, 2002). Moreover, the presence of ACNs in the serum may be involved with a diet-induced increase in *ex vivo* serum antioxidant status (Mazza et al., 2002).

Taking all these data together, it can be concluded that blueberries and blueberry extracts may potentially improve dyslipidaemia by regulating TG, cholesterol and fatty acid metabolism through several signalling pathways. However, further studies are necessary to better clarify the mechanisms involved in these actions of bioactive components in blueberries.

6. Effect on inflammation and adipocytokine profile

Obesity is associated with systemic chronic inflammation, and this low-grade inflammation may play an important role in obesity associated insulin resistance, T2D, and other complications (Calder et al., 2011; Chen et al., 2015; Gabay, 2006; Giugliano, Ceriello, & Esposito, 2006). A diet enriched in vegetables and fruits is inversely related to inflammatory stress, compared with meals that are energy dense which induce an acute inflammatory status in both overweight and healthy adults (Calder et al., 2011; Manning et al., 2008; Root et al., 2012; Vendrame et al., 2013). Blueberries contain various anthocyanins, phenolic acid and other bioactive components recognized for their ability to provide and activate cellular antioxidant protection, scavenge free radicals, inhibit inflammatory gene expression, and consequently protect against oxidant-induced and inflammatory cell damage and cytotoxicity (Johnson, De Mejia, Fan, Lila, & Yousef, 2013; Kang et al., 2015; Nile & Park, 2014).

Dietary supplementation with 8% blueberries to Obese Zucker rats for 8 weeks has been reported to decrease plasma concentrations of IL-6, TNF- α and CRP compared with the control group (Vendrame et al., 2013). Furthermore, in this study, expression of TNF- α , IL-6 and NF- κ B was down-regulated in both the AAT and the liver, whereas CRP expression was down-regulated only in the liver (Vendrame et al., 2013). Similarly, supplementation with 4% whole blueberry powder decreased IL-10 and TNF- α mRNA expression in adipose tissue inflammation of HFD fed C57BL/6J mice, but no significant changes in other inflammatory biomarkers, such as nitric oxide synthase (iNOS), IL-6 and MCP-1 (Defuria et al., 2009).

Bilberry consumption has also been demonstrated to attenuate pro-inflammatory responses induced by HFD in C57BL/6J mice fed with a 5% or 10% (w/w) of whole bilberries for three months, via reduction in MCP-1, IL-2, IL-1 β , IL-6 and TNF- α (Mykkanen et al., 2014). In particular, the levels of IL-15 and interferon gamma (IFN- γ) were increased in non-supplemented HFD fed animals and reduced to non-detectable levels in animals that were supplemented with bilberries (Mykkanen et al., 2014). In contrast, to the bilberry studies, dietary supplementation with a blueberry pomace by-product failed to alter mRNA expression of CD68 (an anti-inflammatory marker) and CRP in adipose tissue of Syrian Golden hamsters compared to controls (Kim, Bartley, Rimando, & Yokoyama, 2010). One explanation for the inconsistency in these findings may be associated with different components among blueberries, its fractions and its peel.

During the last few years a number of clinical trials have been carried out to assess the potential anti-inflammatory function of

blueberry supplementation in subjects who are obese and have other disorders of metabolic syndrome (Table 2). Karlsen et al. (2010) reported that intake of bilberry juice could regulate inflammatory mediators such as, IL-6, IL-15 and CRP in men and women as well as improve the levels of plasma polyphenols. Furthermore, it was found that the decrease of these inflammatory mediators were associated with NF- κ B activation (Karlsen et al., 2010). In a preclinical study, dietary supplementation with 400 g of bilberry for 8 weeks decreased serum IL-6, IL-12, high sensitivity-CRP (hsCRP) and LPS concentrations in obese individuals with low-grade inflammation (Kolehmainen et al., 2012). However, in another study where 110 female volunteers consumed 100 g of fresh blueberry fruits for 33–35 days, there were no differences observed in TNF- α between the baseline and treatment group at the end of the intervention (Lehtonen et al., 2011). Similarly no alterations in plasma IL-6 and CRP concentrations were observed in obese participants following consumption of freeze-dried blueberries (50 g) for 8 weeks (Basu et al., 2010). Another study demonstrated that consumption of blueberries (22.5 g) for 6 weeks did not affect the inflammatory biomarker profile including TNF- α , hsCRP and MCP-1 in obese, nondiabetic, and insulin-resistant volunteers (Stull et al., 2010). Perhaps the contradictions in the observed impacts on inflammatory markers in these clinical studies may at least in part be explained by the use of different species of berries [bilberry (Karlsen et al., 2010; Kolehmainen et al., 2012) vs. blueberry (Basu et al., 2010; Lehtonen et al., 2011; Stull et al., 2010)], the amount of berries consumed; type of serum samples used for measuring inflammatory biomarkers [fasting serum (Karlsen et al., 2010; Kolehmainen et al., 2012) vs. non-fasting serum (Stull et al., 2010)] or the status of these individuals [overweight subjects with 25.6 ± 6.1 of BMI (Karlsen et al., 2010) vs. obese subjects with 36.8 ± 0.9 of BMI (Stull et al., 2010) and 38.1 ± 1.5 of BMI (Basu et al., 2010)].

It has been reported that a purified ACN mixture exhibited higher anti-inflammatory activity compared to single ACN or whole berries *in vitro* and *in vivo* (Zhu et al., 2013). In that study, purified anthocyanin mixture (containing 17 ACN compounds from blueberries) produced a stronger inhibitory effect on IL-6, IL-1 β -induced CRP production in HepG2 cells and LPS-induced vascular cell adhesion molecule-1 (VCAM-1) secretion in endothelial cells, respectively, compared with the effects of single anthocyanin, D3G and C3G, which support the observations in human subjects (Zhu et al., 2013). These studies suggest that the various ACNs in blueberry may act synergistically to inhibit the inflammatory response. Hence, consuming foods rich in different ACNs is likely to be more beneficial than consuming a single ACN supplement.

Blueberry and its extracts have also demonstrated potential benefits on the regulation of adipocytokines in animal and human studies. The concentration of adiponectin was higher in C57BL/6J obese mice fed HFD and genetically diabetic db/db mice with C3G supplementation, compared with mice only fed a HFD diet (Guo, Xia et al., 2012; Liu et al., 2014). Similarly, wild blueberry consumption in Obese Zucker rats resulted in a significant increase in circulating adiponectin level compared to the control group (+21.8%) (Vendrame et al., 2013). Adiponectin concentration, however has been demonstrated not to differ from the control groups following supplementation of blueberry or ACNs in several animal studies (Mykkanen et al., 2014; Roopchand et al., 2013; Takikawa et al., 2010; Vuong et al., 2009; Wu et al., 2013) and human trials (Basu et al., 2010; Kolehmainen et al., 2012; Qin et al., 2009). Lehtonen et al. (2011) demonstrated, however a decrease in adiponectin level after bilberry supplementation in overweight and obese women for 33–35 days. Therefore the exact effect of consumption of blueberries on adiponectin level is unclear.

Leptin secretion has been demonstrated to be inhibited by diets enriched with blueberry, both in genetic models of obesity and dietary-induced obese animal models (Prior et al., 2009, 2010; Wu et al., 2013). However, no significant effect was observed on leptin levels in other animal studies (Mykkanen et al., 2014; Vuong et al., 2009), or indeed in a human trial (Kolehmainen et al., 2012).

Resistin is a hormone secreted from adipose tissue and it has been implicated in the modulation of insulin action, energy, glucose and lipid homeostasis and also has been linked to the onset of insulin resistance and obesity-associated diabetes (Abate et al., 2014). Mykkanen et al. (2014) investigated the effect of blueberry supplementation (10% wild blueberry) in mice fed a HFD and indicated that serum resistin level was significantly reduced in the mice that were supplemented with blueberry for 12–14 weeks.

There are several potential mechanisms involved in the anti-inflammatory properties of blueberry. Firstly, antioxidants in blueberry, such as polyphenols and ACNs which exhibit the anti-inflammatory effect may be dependent on a reduction of pro-inflammatory cytokines and increase of anti-inflammatory mediators such as adiponectin (Guo, Xia et al., 2012). Secondly, oxidative stress, which leads to inflammation is reduced due to the strong antioxidant activity of blueberries and its extracts, which is subsequently involved in an increase of glutathione peroxidase 3 (a sensitive index of oxidative stress) gene expression (Lee et al., 2008). Thirdly, blueberry or its ACNs may be able to alter mitogen-activated protein kinase signalling, which modulate cell fate and inflammatory gene expression in various tissues and macrophages (Suganami et al., 2007). Finally the attenuation of NF- κ B activation could be related to the antioxidant capacity of blueberries or its extracts, thereby providing a potential mechanism with the observed anti-inflammatory effect of blueberry intake (Vendrame et al., 2013).

7. Conclusion

This review focused on blueberries and their bioactive components that influence obesity and its related comorbidities, although it is necessary to indicate that there are still a large number of phytonutrients in blueberries under exploration at present, especially ACNs. A major question to be addressed is whether a single purified component or constituent in blueberries such as C3G or ACNs, or multiple constituents in this fruit produced synergic effects on human health. In addition, there is a need for determining the bioactive constituents of blueberry and their metabolites, which may accumulate in the target tissues and exert biological effects. Future studies could also focus on the interactions of nutrients and genes so we have a better understanding of the beneficial effects of blueberry at the molecular level, thus be able to develop effective intervention strategies and achieve better outcomes. According to the literature, the evidence suggests that several species of blueberries in the genus *Vaccinium* and their isolated compounds are potential contributors to the regulation of glucose, lipid metabolism and improvement of inflammation. A deep understanding of the potential roles of blueberries in controlling body weight, regulating blood glucose, and attenuating dyslipidaemia and related chronic inflammation will guide further rigorous investigations on the underlying mechanisms of their beneficial effects on health.

Conflict of interest

The authors declare that there is no conflict of interest.

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APPENDIX 2: Effects of fermentation conditions on the potential antihypertensive peptides released from yoghurt fermented by *Lactobacillus helveticus* and Flavourzyme®

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APPENDIX 3: The effect of cyanidin-3-O- β -glucoside and peptides extracted from yoghurt on glucose uptake and gene expression in human primary skeletal muscle myotubes from obese and obese diabetic participants



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The effect of cyanidin-3-O- β -glucoside and peptides extracted from yoghurt on glucose uptake and gene expression in human primary skeletal muscle myotubes from obese and obese diabetic participants

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ABSTRACT

Incidence of type II diabetes mellitus (T2DM) is rapidly increasing worldwide. This study aimed to investigate whether cyanidin-3-O- β -glucoside (C3G), or peptides with angiotensin converting enzyme (ACE) inhibitory activity, alone or in combination, alter glucose regulation in human primary myotubes derived from obese and obese T2DM participants. In the obese group, both low and high concentration of peptides and the combination of these peptides with high C3G concentration significantly enhanced glucose uptake in the presence or absence of insulin, and high concentration of peptide alone and its combination with low C3G down-regulated the mRNA expression of angiotensin II receptor, type 1 (AGTR-1), and up-regulated the mRNA expression of insulin receptor substrate 1 (IRS-1), and glucose transporter 4 (GLUT4). However, only high peptide concentration increased glucose uptake in the absence of insulin in the obese T2DM group, and the expression of AGTR-1 was decreased with high peptide and its combinations of C3G.

1. Introduction

Skeletal muscle is a key site for insulin mediated glucose disposal in mammals and is therefore an important regulator of whole body glucose homeostasis. In this tissue, insulin binds to the insulin receptor (IR) and activates IR substrates (IRS-1 and IRS-2) (Krishnapuram et al., 2013). Activated IR substrates trigger downstream activation of the lipid kinase phosphatidylinositol-3 kinase (PI3K), and finally stimulate the translocation of glucose transporter 4 (GLUT4) to facilitate the transport of glucose into cells (Guo et al., 2008; Johnston, Pirola and Van Obberghen, 2003). Additionally GLUT4 translocation can also be activated through the cellular energy sensor 5'adenosine monophosphate-activated protein kinase (AMPK) which regulates cell metabolism and integrates nutritional and hormonal signals (Alkhatieb and Qnais, 2017; Naimi, Vlavcheski, Murphy, Hudlicky and Tsiani, 2017). Activation of AMPK has been viewed as a targeted approach to increase glucose uptake by the skeletal muscle and therefore improve blood glucose homeostasis. Impaired glucose uptake into skeletal muscle is

attributable to insulin resistance, which is associated with several defects within the insulin signalling cascade, namely reduced IR substrates and PI3K response to insulin stimulation (Dos Santos, Moreli, Tewari and Benite-Ribeiro, 2015). Insulin resistance can be associated with obesity and plays a key role in the development of type 2 diabetes mellitus (T2DM) (Barazzoni, Gortan Cappellari, Ragni and Nisoli, 2018; Bouzakri, Koistinen and Zierath, 2005; Deshmukh, 2016).

Cyanidin-3-O- β -glucoside (C3G) is a predominant bioactive anthocyanin compound found in many edible plants and has been reported to be protective against T2DM by attenuating multiple disorders *in vivo* and *in vitro* (Guo, Guo, Jiang, Li and Ling, 2012; Guo et al., 2012; Scaccocchio, et al., 2011; Wang, Zhao, Wang, Huo and Ji, 2016). It has been found that C3G increased glucose uptake in human skeletal muscle cells due to its strong antioxidant activity (Ho, Kase, Wangenstein and Barsett, 2017). C3G derived from black soybeans ameliorated T2DM in db/db mice through the induction of differentiation of 3T3-L1 pre-adipocytes into smaller and insulin-sensitive adipocytes, which subsequently resulted in activation of insulin signalling and increased

Abbreviations: ACE, angiotensin converting enzyme; AGTR-1, angiotensin II receptor, type 1; AMPK, 5'adenosine monophosphate-activated protein kinase; Ang II, angiotensin-II; BCA, bichononic acid; C3G, cyanidin-3-O- β -glucoside; DEPC, diethylpyrocarbonate; DOG, deoxyglucose; FOXO1, forkhead box protein O1; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; GLUT1, glucose transporter 1; GLUT4, glucose transporter 4; IR, insulin receptor; IRS-1, insulin receptor substrate-1; IRS-2, insulin receptor substrate-2; PI3K, phosphatidylinositol-3 kinase; PPAR- α , peroxisome proliferator-activated receptor-alpha

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glucose uptake (Matsukawa, Inaguma, Han, Villareal and Isoda, 2015). Another *in vitro* study using L6 myotubes showed that C3G significantly induced AMPK activation and enhanced glucose uptake into the cells (Kurimoto et al., 2013). However, no information is available on the effect of C3G on glucose metabolism in human skeletal muscle cells, and the molecular mechanism/s underlying this action also remains unclear.

Angiotensin-converting enzyme (ACE) is a primary enzyme in the regulation of blood pressure through converting angiotensin-I into a potent vasoconstrictor, angiotensin-II (Ang II). Subsequently, angiotensin-II induces the release of aldosterone and therefore increases the sodium retention and blood pressure (Muro Urista, Alvarez Fernandez, Riera Rodriguez, Arana Cuenca and Tellez Jurado, 2011). The blood pressure regulator ACE has also been shown to influence glucose uptake. Angiotensin- II (Ang II) has been shown to antagonize insulin-independent contraction-induced glucose uptake in skeletal muscle tissue (Rao, 1994) via the Ang II type 1-receptor (AGTR-1) (Jones and Woods, 2003). The chronic effects of Ang II on myocytes are associated with a reduced protein expression of GLUT4, suggesting limited availability of the glucose transporter to be partially responsible for the restricted glucose uptake (Shiuchi et al., 2004). In addition, Ang II inhibits insulin-mediated actions at multiple levels of the insulin signalling pathway including IR, IR substrates and PI3K (Igarashi, Hirata, Nozaki, Kadomoto-Antsuiki and Tominaga, 2007; Shiuchi et al., 2004). Thus ACE inhibitors or AGTR-1 blockers could enhance whole-body and skeletal muscle insulin sensitivity, which is associated with increased expression of GLUT4 in skeletal muscle (Henriksen, Jacob, Kinnick, Teachey and Krekler, 2001). It has previously been determined that the ACE inhibitor temocapril improves insulin resistance and glucose intolerance by increasing glucose uptake in skeletal muscle at least in part through GLUT4 translocation (Shiuchi et al., 2002). AGTR-1 blockade also increases insulin sensitivity and glucose uptake in skeletal muscle of KK-Ay mice, via stimulation of the insulin signalling cascade and increasing GLUT4 translocation to the plasma membrane (Shiuchi et al., 2004). However, no studies have elucidated the underlying mechanism of yoghurt peptides with high inhibition of ACE activity extracted from yoghurt in improving glucose uptake in human skeletal muscle cells.

We therefore aimed to investigate whether, in isolation and/ or in combination, C3G and peptides with ACE inhibitory activity enhanced glucose uptake, and to understand the related molecular mechanisms of the action. We hypothesized that the combinations of C3G and peptides with ACE inhibitory activity would improve insulin sensitivity by regulating glucose disposal in human primary skeletal muscle myotubes derived from obese or obese T2DM individuals.

2. Material and methods

2.1. Material and reagents

HPLC grade C3G was provided by Polyphenols AS (Sandnes, Norway). Pierce™ bicinchoninic acid (BCA) Protein Assay Kit, Diethylpyrocarbonate (DEPC) water, α -MEM, amphotericin B, horse serum, fetal bovine serum (FBS), and trypsin/EDTA were obtained from Life Technologies Australia Pty Ltd (Mulgrave, VIC, Australia). iQ™ SYBR Green and iScript™ cDNA Synthesis Kit were purchased from Bio-Rad Laboratories Pty Ltd (Gladesville, NSW, Australia). Liquid scintillation cocktail and deoxy-D-glucose, 2-[1,2-³H (N)]- were purchased from Perkin-Elmer, Inc. (Glen Waverley, VIC, Australia). All other chemicals, unless otherwise specified, were obtained from Sigma-Aldrich Pty. Ltd. (Sunshine, VIC, Australia).

Peptides were extracted from yoghurt fermented by *Lactobacillus helveticus* 881315 in the presence of Flavourzyme for 12h. ACE inhibitory activity of peptides was determined using a reversed-phase-HPLC system (RP-HPLC, from Varian Analytical Instruments, Santa Clara, CA, USA) and the IC₅₀ value of peptides was 1.47 ± 0.04 mg/mL. The details on the process of fermentation, peptide extraction and

Table 1
Participant characteristics.

	Participants	
	Obese	Obese Type 2 Diabetes Mellitus
n (F/M)	8/2	8/2
Age (years)	49 ± 4	53 ± 2
BMI (kg/m ²)	40.42 ± 1.64	42.80 ± 2.38
Fasting plasma glucose (mmol/L)	5.02 ± 0.13	8.61 ± 0.76*
HbA1c (%)	5.59 ± 0.09	7.82 ± 0.47*
Plasma insulin (μ U/L)	10.17 ± 1.64	21.62 ± 2.53*
Cholesterol (mmol/L)	5.74 ± 0.52	5.01 ± 0.37
Fasting Triglyceride (mmol/L)	1.75 ± 0.31	2.36 ± 0.46

Values are expressed as means ± SEM.

* Indicated a significant difference between obese and obese T2DM groups ($p < 0.05$).

determination of ACE inhibitory activity have previously been reported (Min, Fatah, Michael, Andrew and Xiao, 2017). Subsequently, peptides derived from the same batch (with an IC₅₀ value of 1.47 ± 0.04 mg/mL) as we have previously reported were used in the present cell culture study (Min, et al., 2017).

2.2. Participants

10 obese (eight female and two male), and 10 obese T2DM (eight female and two male) participants undergoing abdominal surgery were recruited for additional tissue collection (Table 1). After fasting for 12–18 h, general anesthesia was induced with a short-acting propofol and maintained by a fentanyl and rocuronium volatile anesthetic mixture, and a biopsy of rectus abdominus muscle was removed as previously described (Chen et al., 2005). This research was approved by the Human Ethics Research committee of Victoria University (HRE08-158), and all participants gave written informed consent.

2.3. Human primary skeletal muscle myotubes

Human primary skeletal muscle cell culture was established as previously described (Blau and Webster, 1981; Gaster, Beck-Nielsen and Schroder, 2001). Cells were thawed at passage four and grown to 70% confluence in growth media [α -MEM supplemented with 10% FBS (vol/vol), 0.5% penicillin (vol/vol) and 0.5% amphotericin B (vol/vol)]. Cells were then detached and resuspended in the growth medium and seeded on six-well and twelve-well plates (Greiner, Longwood, FL) coated with essential coating matrix (ECM) gel. Once at 80% confluence, the growth medium was replaced by the differentiation media [α -MEM, 2% horse serum, 0.5% penicillin (vol/vol) and 0.5% amphotericin B (vol/vol)] for 6 days for subsequent experimentation.

2.4. Glucose uptake bioassay

Glucose uptake assay in human primary skeletal muscle myotubes was established according to previously described methods (Boue, Daigle, Chen, Cao and Heiman, 2016) with minor modification. Following differentiation cells were treated with C3G (10 and 100 μ M) and peptides with ACE inhibitory activity (150 μ g/mL and 1500 μ g/mL) in isolation and combination in the presence of serum-free medium for 24 h. At the end of the treatment period the cells were treated in the presence or absence of insulin 100 nM at 37 °C for 30 min and supplemented with 2-deoxyglucose (DOG)/2-[³H]DOG (10 μ M final concentration, 1 μ Ci/well) for 15 min at 37 °C. The reactions were stopped by washing cells with cold PBS. Cells were then solubilized by the addition of 0.5 mL of 0.3 M NaOH. An aliquot of 400 μ L was utilized for the determination of glucose transport activity by scintillation counter

(Tri-Carb 3180TR/SL; Perkin-Elmer, Inc.) and expressed as picomoles of DOG taken up per minute per milligram of total protein. The remaining solution was utilized for determination of protein concentration via BCA assay using a Pierce™ BCA Protein Assay Kit based on the previous method (Bainor, Chang, Mcquade, Webb and Gestwicki, 2011). Absorbance was read at 562 nm with a Varioskan™ LUX Multimode Microplate Reader (Thermo Fisher Scientific Laboratory Equipment, Hudson, United States). A standard curve was prepared and protein content for each sample was calculated.

2.5. RNA extraction

Following treatments, human primary myotubes were lysed using TRIzol and then stored at -80°C prior to total cellular RNA extraction according to previously established methods (Mcainch et al., 2006). Total RNA was quantified using NanoDrop spectrometry (NanoDrop 2000, Thermo Fisher Scientific, Wilmington, DE) at 260 nm. $0.5\ \mu\text{g}$ of total RNA was reverse transcribed into cDNA using iScript™ cDNA Synthesis Kit according to the manufactures instructions using a MyCycler™ Thermo Cycler (Bio-rad Laboratories, Hercules, CA).

2.6. Real-time polymerase chain reaction (PCR) analysis

'Real-time' PCR was conducted using MyiQ™ multiplex 'real-time' PCR detection system (Bio-Rad Laboratories, Hercules, CA) with iQ™ SYBR Green Supermix. Forward and reverse oligonucleotide primers (Table 2) for the gene of interest were designed using OligoPerfect™ Suite (Invitrogen, Carlsbad, CA). Selective gene homology for genes of interest was confirmed with BLAST (Basic Local Alignment Search Tool, National Centre for Biotechnology Information, Bethesda, MD). To compensate for variations in RNA input amounts and reverse transcriptase efficiency, mRNA abundance of the housekeeping genes, cyclophilin and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) were also measured. The 'real-time' PCR cycling parameters were as follows: initial denaturation and enzyme activation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing and extension at 60°C for 45 s. Relative changes in mRNA abundance was normalised to the average of two housekeeping genes (cyclophilin and GAPDH) and then quantified using the $2^{-\Delta\Delta\text{CT}}$ method. Treatment groups were expressed relative to control treatment and all experiments were repeated at least three times.

2.7. Statistical analysis

All results were expressed as mean \pm standard error of the mean (SEM) for each measurement ($n = 8-10$). Two-way ANOVA was performed to determine the differences of glucose uptake rate between the

presence and absence of insulin in all treatments using GraphPad Prism 7 (GraphPad Software, Inc, La Jolla, CA, USA). Student *t*-test was used to analyse the significant difference in characteristics of participants between obese and obese T2DM groups. One-way ANOVA was performed to analyse the significant differences in the mRNA expression of genes between control and treatments. Fisher's (least significant difference; LSD) test was used to differentiate significant differences amongst the treatment groups. $P < 0.05$ was considered significant.

3. Results

3.1. Effect of C3G and peptides with ACE inhibitory activity on glucose uptake in human primary skeletal muscle myotubes of obese and obese T2DM participants

As shown in Fig. 1A, both low and high C3G concentrations (10 and $100\ \mu\text{M}$), and the combinations of low C3G with peptides (both low and high concentrations) did not alter glucose uptake in the absence of insulin in human skeletal muscle myotubes from obese participants. However, glucose uptake in human myotubes from obese participants treated with high C3G ($100\ \mu\text{M}$) with insulin stimulation was significantly increased 1.41 fold ($P = 0.044$), compared to cells treated with insulin alone. Furthermore, high C3G concentration ($100\ \mu\text{M}$) combined with peptides at the concentrations of $150\ \mu\text{g}/\text{mL}$ and $1500\ \mu\text{g}/\text{mL}$ in the presence or absence of insulin significantly enhanced glucose uptake ($P < 0.001$), compared with their respective controls. Furthermore, compared with high C3G concentration alone, the combinations of C3G and peptides significantly increased glucose uptake ($P < 0.001$, Fig. 1A) in the absence of insulin. The high peptide concentration ($1500\ \mu\text{g}/\text{mL}$) alone resulted in the highest glucose uptake with 1.87 ± 0.59 fold being recorded in the absence of insulin, compared with the control (Fig. 1A). Similarly, neither low C3G concentration alone nor its combination with low peptide altered glucose uptake in the presence of insulin in the obese group, compared with the control (Fig. 1A). In contrast, glucose uptake was improved when treated with low C3G concentration combined with high peptide concentration ($P = 0.011$, Fig. 1A) in the presence of insulin, compared to control. In addition, the other treatments including high C3G concentration, low and high peptide concentrations alone and their combinations resulted in a significant increase in glucose uptake in the presence of insulin, compared with the control in the obese group.

Fig. 1B illustrates that, only high peptide ($1500\ \mu\text{g}/\text{mL}$) significantly enhanced glucose uptake (by 1.39 ± 0.23 fold) in the absence of insulin in the obese T2DM group, compared with the control group ($P = 0.002$). Glucose uptake was not altered by any of the treatments in the presence of insulin in the obese T2DM group.

Table 2
Human primer sequences used for 'Real-time' PCR analysis.

Gene	Forward Primer (5' – 3')	Reverse Primer (5' – 3')
AGTR-1	CTGATGCCATCCGAAAAGT	CTCCAGCTTTGGGACAATC
AMPK- α	AACTGCAGAGAGCCATTCACTTT	GGTGAAGCTGAAGACAATGTGCTT
Cyclophilin	CATCTGCACTGCCAAGACTGA	TTTCATGCCTCTTTCACCTTTGC
FOXO1	TCATGGATGGAGATACATTTGGATT	TCCTGCTGTGAGACAATCTGAAG
GAPDH	CAA CGA CCA CTT TGT CAA GC	TTA CTC CTT GGA GGC CAT GT
GLUT1	GGGCCAAGAGTGTGCTAAAG	TGCCGACTCTCTTCCTTCAT
GLUT4	CACCCTCACCACCCTCTG	CTTTCCGAAGCCACTG
IRS-1	GTTTCCAGAAGCAGCCAGAG	TGAAATGGATGCATCGTACC
IRS-2	ACGCCAGCATTGACTTCTTGT	TGACATGTGACATCCTGGTGATAA
PI3K	GGAAGCAGCAACCGAAACAA	TTCCCGCTCCCACTACA
PPAR- α	GAAGCTGTCTGGCTCAGAT	GGGGACCACAGGATAAGTCA

AGTR-1, angiotensin II receptor type 1; AMPK- α , 5' AMP-activated protein kinase alpha; FOXO1, Forkhead box protein O1; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; GLUT1, Glucose transporter 1; GLUT4, Glucose transporter 4; IRS-1, Insulin receptor substrate 1; IRS-2, Insulin receptor substrate 2; PI3K, Phosphoinositide 3-kinase; PPAR- α , Peroxisome proliferator-activated receptor alpha.

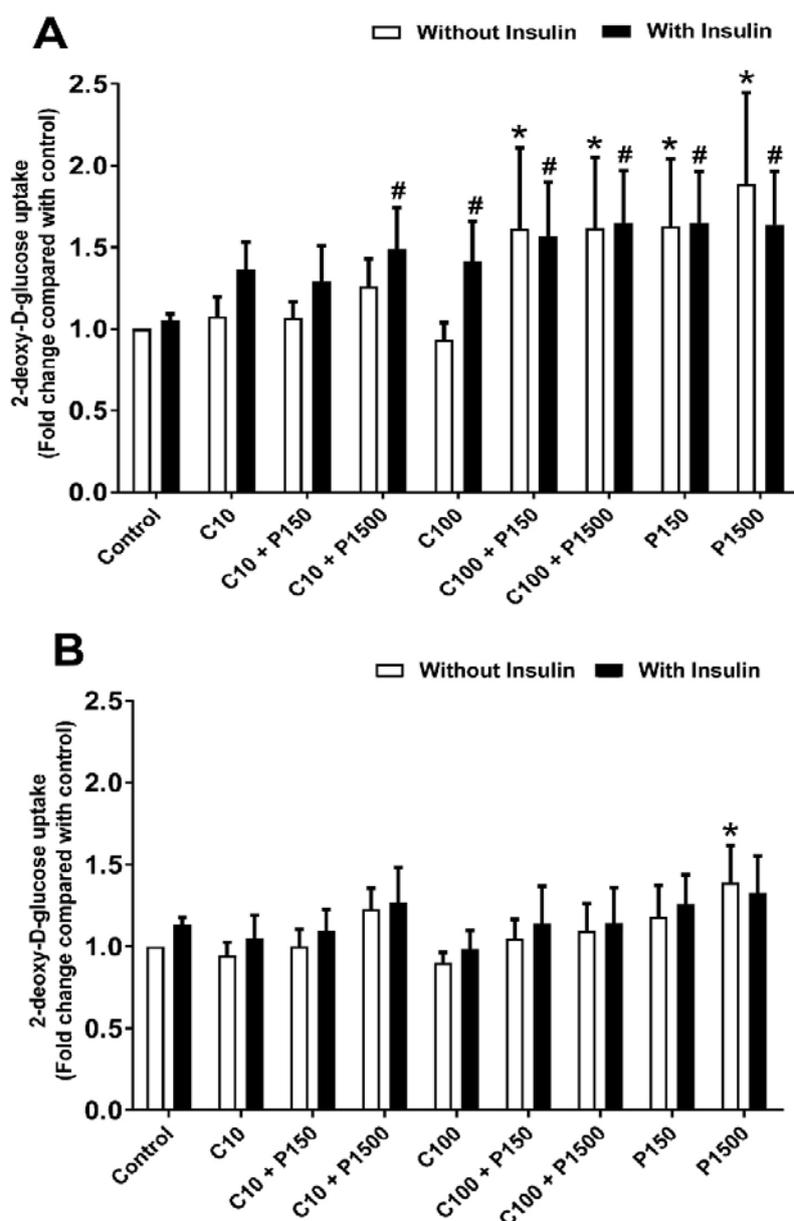


Fig. 1. The effect of C3G and peptides on glucose uptake in human primary skeletal muscle myotubes derived from obese participants (A) and obese T2DM participants (B). Cells were treated with BSA (as a vehicle control), 10 μ M or 100 μ M of C3G, 150 μ g/mL or 1500 μ g/mL of peptides and combinations of C3G/peptides in the presence or absence of insulin for 24 h. Data was normalised to control with or without insulin, respectively. Data was expressed as mean \pm SEM (n = 10). * P < 0.05 compared with control without insulin stimulation; # P < 0.05 compared control with insulin stimulation.

3.2. Expression of AGTR-1, GLUT4, IRS-1 and forkhead box protein O1 (FOXO1) in primary human skeletal muscle myotubes of obese and obese T2DM participants

In this study, we investigated the effects of C3G and peptides in isolation and in combination on the expression of multiple genes associated with obesity and T2DM including AGTR-1, GLUT4, IRS-1 and FOXO1 by 'Real-Time' PCR analysis. As shown in Fig. 2A, human primary skeletal muscle myotubes from obese participants treated with high C3G (100 μ M), low and high peptide concentrations as well as

their combinations exhibited significantly lower AGTR-1 expression compared to control treatment (P < 0.05, n = 8). The similar results were also observed in the obese T2DM group. AGTR-1 expression was significantly reduced (P < 0.05), in myotubes from obese T2DM participants following the treatment with both low and high peptide concentrations and the combination of high peptide (1500 μ g/mL) with low C3G concentration (10 μ M) for 24 h (n = 9, Fig. 2B). However, high C3G concentration alone and its combination with peptides did not alter AGTR-1 expression in human primary myotubes obtained from obese T2DM participants, compared with control treatment (Fig. 2B).

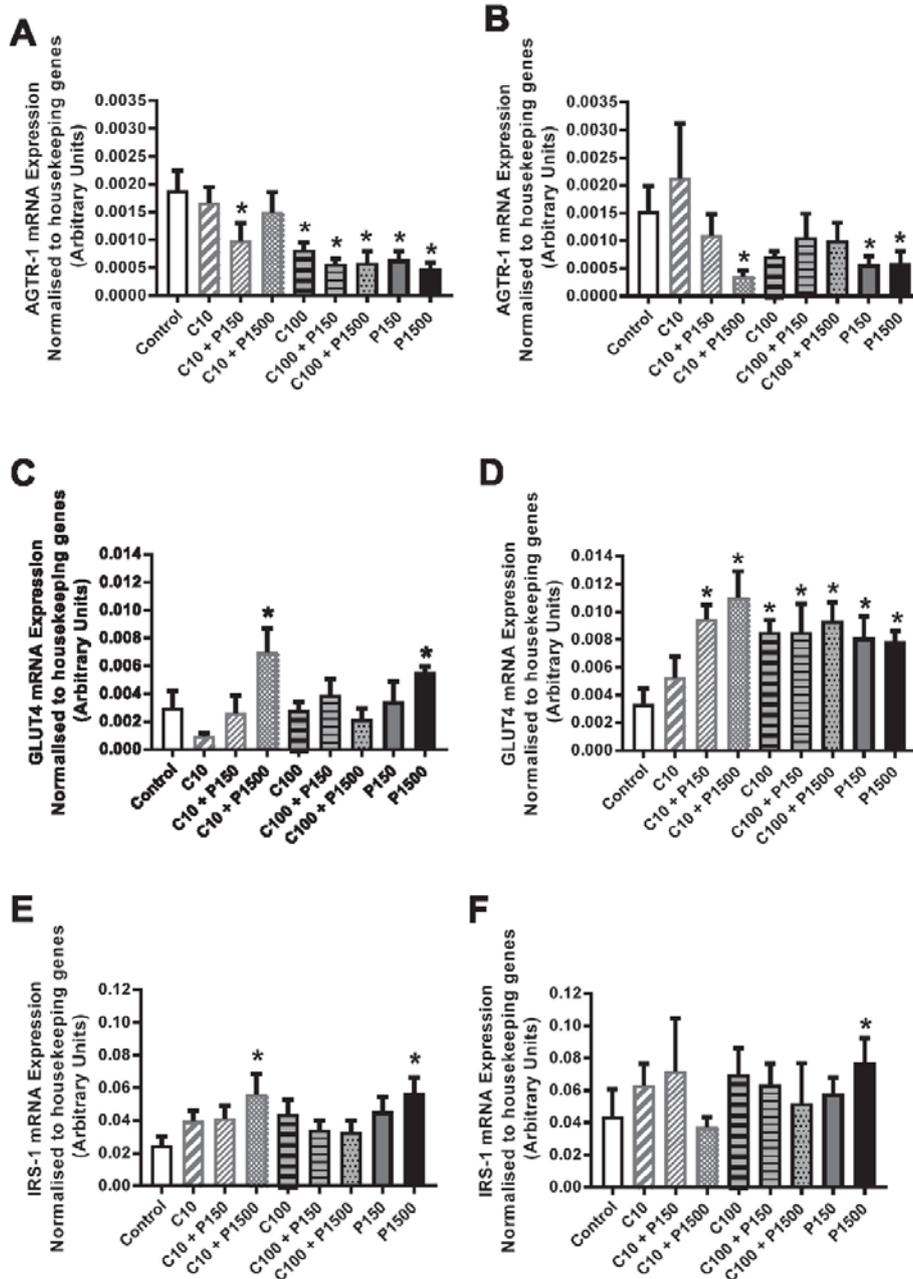


Fig. 2. The expression of AGTR-1, GLUT4, IRS-1 and FOXO1 in human primary skeletal muscle myotubes derived from obese and obese T2DM participants. Cells were treated with 10 μ M and 100 μ M of C3G, 150 μ g/mL and 1500 μ g/mL of peptides, and combinations of C3G/peptides for 24 h. All genes were normalised to the average of two housekeeping genes, GAPDH and cyclophilin. Data was expressed as mean \pm SEM (n = 8–10). Significance is indicated by * $P < 0.05$. The expression of (A) AGTR-1, (C) GLUT4, (E) IRS-1 and (G) FOXO1 in human primary skeletal muscle myotubes derived from obese participants. The expression of (B) AGTR-1, (D) GLUT4, (F) IRS-1 and (H) FOXO1 in human primary skeletal muscle myotubes derived from obese T2DM participants.

1500 μ g/mL of peptide combined with 10 μ M of C3G up-regulated the mRNA expression of GLUT4 in the obese group (Fig. 2C). For the obese T2DM group, high C3G, high and low peptide concentrations alone, as well as their combinations significantly increased the mRNA expression of GLUT4. However, low C3G concentration (10 μ M) did not change the expression of this gene (Fig. 2D). GLUT4 mRNA was

significantly up-regulated when 10 μ M of C3G was combined with 150 μ g/mL ($P = 0.011$, n = 10) and 1500 μ g/mL of peptide ($P = 0.008$, n = 10), compared with low C3G concentration (10 μ M) alone in the obese T2DM group (Fig. 2D).

The mRNA expression of IRS-1 was significantly increased in myotubes derived from obese participants following treatments with high

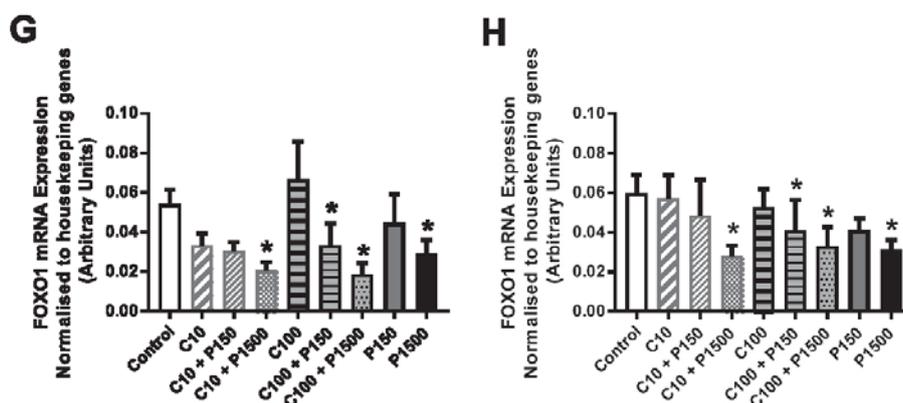


Fig. 2. (continued)

peptide concentration alone as well as combined with low C3G concentration ($P < 0.05$, $n = 10$, Fig. 2E). However, in the obese T2DM group, only high peptide treatment increased the expression of IRS-1 compared with control treatment ($P = 0.016$, $n = 10$, Fig. 2F).

Low C3G and peptide concentrations alone as well as in combination did not alter FOXO1 mRNA expression significantly in human skeletal muscle myotubes. A similar result was also observed following the treatment of high C3G concentration (Fig. 2G, H). In contrast, high peptide concentration alone or in combination with both low and high C3G concentrations ($P < 0.05$, $n = 10$) resulted in a significant reduction in the mRNA expression of FOXO1 in both obese and obese T2DM groups, compared with the control treatment.

3.3. 'Real-time' PCR analysis of the expression of others genes related to glucose metabolism in human primary skeletal muscle myotubes obtained from obese and obese T2DM participants

Fig. 3A showed that GLUT1 mRNA expression was up-regulated by the high peptide concentration (1500 $\mu\text{g}/\text{mL}$) in the obese group, compared with the control treatment. Treatments with C3G or peptides alone did not alter GLUT1 mRNA expression significantly in human skeletal muscle myotubes obtained from obese T2DM participants (Fig. 3B). In contrast, the combination of 100 μM of C3G and 150 $\mu\text{g}/\text{mL}$ of peptide ($P = 0.012$, $n = 8$, Fig. 3B) significantly increased the expression of GLUT1 mRNA in myotubes from T2DM participants, compared with the control treatment.

C3G or peptides alone did not alter IRS-2 mRNA expression in the obese group (Fig. 3C) or obese T2DM group (Fig. 3D), however it was down-regulated in both groups by the combinations of high C3G concentration with both low and high doses of peptides (150 $\mu\text{g}/\text{mL}$ and 15000 $\mu\text{g}/\text{mL}$; $P < 0.05$, Fig. 3C, D).

All treatments including low and high C3G concentrations and their combinations with low and high peptide concentrations ($P < 0.05$, Fig. 3E), but not high peptide concentration alone, significantly reduced the mRNA expression of PI3K in the obese group. High C3G concentration and its combinations with peptides (both of low and high concentrations) resulted in a significant reduction in mRNA expression of PI3K in the obese T2DM group, and the combination of low C3G (10 μM) and high peptide concentration (1500 $\mu\text{g}/\text{mL}$) ($P < 0.05$, Fig. 3F). The other treatments did not alter mRNA expression of PI3K in the obese T2DM group.

There was no change in the mRNA expression of AMPK following the treatments of C3G and peptides alone, as well as the combinations of low C3G concentration with peptides in the human primary skeletal muscle myotubes from obese participants. But peptides combined with high C3G concentration resulted in a down-regulation in the mRNA expression of AMPK in the same group (Fig. 3G). On the other hand, no

treatment caused significant changes in the mRNA expression of AMPK, compared with control in the obese T2DM group (Fig. 3H).

C3G and peptide treatments alone and their combinations did not alter the mRNA expression of PPAR- α in the obese group (Fig. 3I). However, high C3G concentration (100 μM) alone and its combination with peptides (both low and high concentrations) ($P < 0.05$, Fig. 3I) down-regulated the mRNA expression of PPAR- α , compared with the control treatment. In the obese T2DM group, the mRNA of PPAR- α was not significantly different following the treatments of low C3G alone or peptides alone (both concentrations, Fig. 3J). In contrast, the expression of PPAR- α was decreased with the treatments of high peptide concentration combined with both concentrations of C3G, as well as high C3G alone ($P < 0.05$, Fig. 3J).

4. Discussion

Anthocyanins extracted from plants have previously been shown to increase glucose uptake in both L6 and C₂C₁₂ myotubes as well as in 3T3-L1 adipocytes (Harbilas et al., 2009; Martineau et al., 2006; Rojo et al., 2012). ACE inhibitors have also been found to enhance the whole-body glucose disposal and glucose transport activity in the skeletal muscle, in a variety of insulin-resistant animal models (Henriksen, et al., 2001; Sugiishi et al., 2013) and insulin-resistant humans with essential hypertension (Kinoshita et al., 2002). The present study is the first to look at the synergistic effects of C3G and peptides with ACE inhibitory activity on glucose uptake in human primary myotubes derived from obese and obese T2DM participants.

In this study, high concentration of C3G in the presence of insulin significantly increased glucose uptake, compared with insulin treatment alone in the obese group, but low C3G concentration in the presence or absence of insulin did not change glucose uptake in either the obese or obese T2DM groups. In agreement with our result, it has previously been demonstrated that an increase in glucose uptake of L6 myotubes was obtained with higher concentrations of anthocyanin combined with insulin (Rojo et al., 2012). It is well known that human primary myotubes express obese or diabetic phenotypes when they are established from participants with obesity or diabetes (Berggren, Tanner and Houmar, 2007; Thingholm, Bak, Beck-Nielsen, Jensen and Gaster, 2011). A previous study showed that a reduction of glucose transport stimulated by insulin was observed in myotubes only from obese T2DM participants, not from nondiabetic obese participants, indicating that differentiated myotubes raised from patients with T2DM remain insulin resistant *in vitro* (Henry, Abrams, Nikoulina and Ciaraldi, 1995). In this current study, only donors who had diabetes demonstrated the characteristics of diabetes with elevated plasma insulin levels and high fasting blood glucose, while donors who were obese but not diabetic did not display any evidence of hyperglycaemia (Table 1).

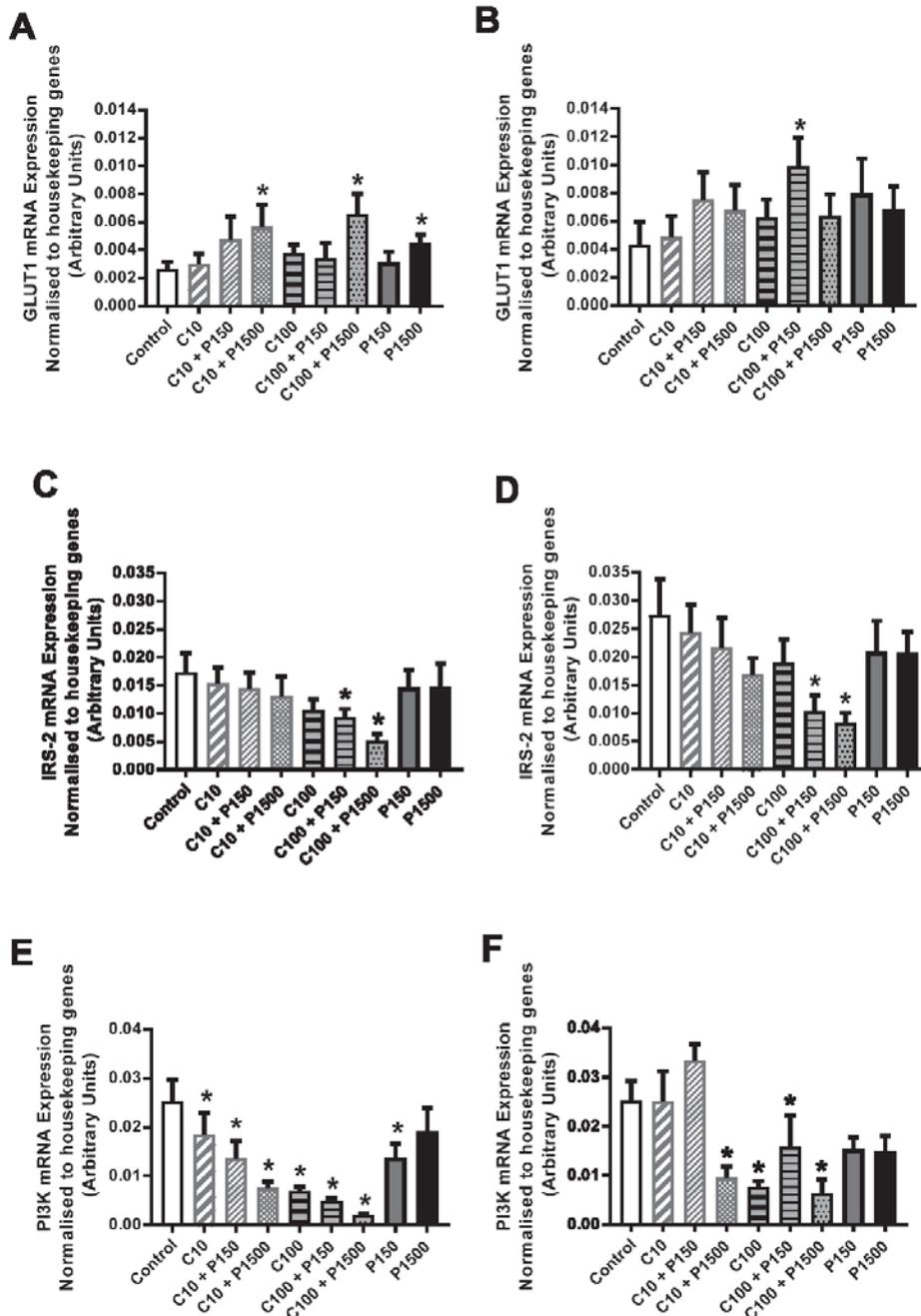


Fig. 3. The expression of GLUT1, IRS-2, PI3K, AMPK and PPAR- α in human primary skeletal muscle myotubes derived from obese and obese T2DM participants. Cells were treated with 10 μ M and 100 μ M of C3G, 150 μ g/mL and 1500 μ g/mL of peptides, and combinations of C3G/ peptides for 24 h. All genes were normalised to the average of two housekeeping genes, GAPDH and cyclophilin. Data are expressed as mean \pm SEM. Significance is indicated by * $P < 0.05$ compared to control treatment (n = 8–10). The expression of (A) GLUT1, (C) IRS-2, (E) PI3K, (G) AMPK and (I) PPAR- α in human primary skeletal muscle myotubes derived from obese participants. The expression of (B) GLUT1, (D) IRS-2, (F) PI3K, (H) AMPK and (J) PPAR- α in human primary skeletal muscle myotubes derived from obese T2DM participants.

Therefore, differential effects of the treatments on glucose uptake between obese and obese T2DM groups were possibly associated with human primary myotubes preserving their phenotype. In addition, the variation of fibre types, mitochondrial function and mitochondrial

capacity in myotubes from different groups may have also exacerbated the differences following the treatments (Minet and Gaster, 2010; Thingholm, et al., 2011). The differences observed in the current study between obese and T2DM cell lines are however consistent with

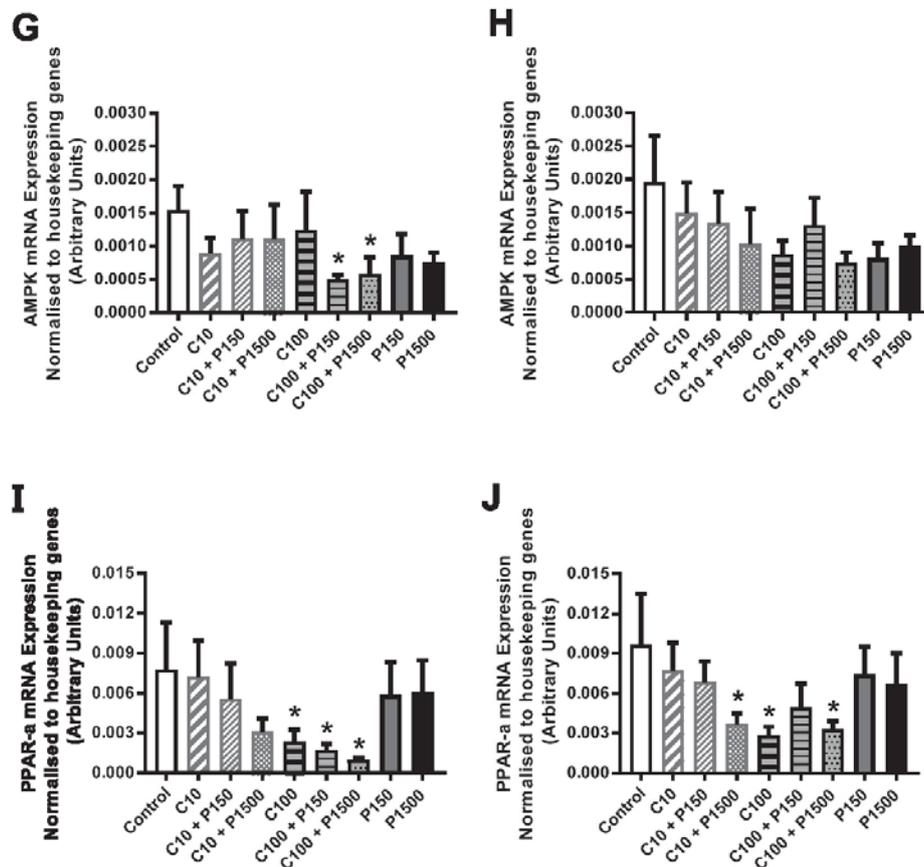


Fig. 3. (continued)

previous studies, such as those reported with globular adiponectin with variations in a dose effect on AMPK activity in human myotubes obtained from both obese and obese T2DM participants (Chen et al., 2005). This finding also suggests that peptides with ACE inhibitory activity have a potential to improve insulin sensitivity and attenuate insulin resistance in both obese and obese T2DM patients, however this may be dose dependent in the obese T2DM state. It has been reported that ACE inhibitors on skeletal muscle glucose uptake were associated with insulin signalling up-regulation, including enhanced IRS-1 tyrosine phosphorylation and PI3K activity (Dal Ponte, Fogt, Jacob and Henriksen, 1998; Henriksen and Jacob, 2003), which was consistent with our findings that high peptide concentration improved the expression of IRS-1 in human primary myotubes in both obese and obese T2DM groups. *In vivo* studies have reported that the ACE inhibitors captopril (Dal Ponte et al., 1998) trandolapril (Steen et al., 1999) or imidapril (Nawano, et al., 1999) could improve oral glucose tolerance and whole-body insulin sensitivity in obese Zucker rats. In this study, peptides with ACE inhibitory activity were extracted from yoghurt fermented by *L. helveticus*. It has been reported that *L. helveticus*, one of the most efficient lactic acid bacteria, possess an efficient proteolytic system capable of producing short peptides from the casein matrix (Sadat-Mekmene et al., 2011). Furthermore, the small size peptides that were produced, contained proline and hydroxyproline residues, and can usually resist breakdown by digestive enzymes, thus they can arrive to intestine due to detection in faecal samples after ingestion (Saito, Sakamoto, Takizawa and Benno, 2004; Seppo, Jauhainen, Poussa and Korpela, 2003). Therefore, these small peptides that exerted the regulatory function observed on glucose metabolism in the current study

may be able to exert their function *in vivo* due to their bioavailability.

To further explore the mechanisms underlying the efficacy of C3G and peptides with ACE inhibitory activity in promoting glucose uptake in human primary myotubes, multiple genes involved in glucose metabolism were assessed. To the best of our knowledge, this is the first study to explore the mRNA expression of AGTR-1 in human primary myotubes following the treatments of C3G and/or peptides that have ACE inhibitory activity. Based on our findings that C3G and/or peptide treatments significantly down-regulated mRNA expression of AGTR-1, and significantly increased the mRNA expression of IRS-1, in both obese and obese T2DM groups, may suggest a possible crosstalk between Ang II and insulin-signalling pathways. Ang II induces insulin resistance via tyrosine phosphorylation of IRS-1 associated with the AGTR-1, thereby attenuating insulin-induced activation of PI3K associated with IRS-1 (Dal Ponte et al., 1998; Folli, Kahn, Hansen, Bouchie, & Feener, 1997; Fujimoto et al., 2004; Ogihara et al., 2002). ACE inhibitors also up-regulate the mRNA expression of GLUT4 in skeletal muscle (Carnagarin, Dharmarajan and Dass, 2015). Consistent with these observations our results also revealed that the expression of GLUT4 was significantly increased by all treatments except low C3G concentration alone in the obese T2DM group, although the increase in mRNA expression, was not translated into an increase in glucose uptake with the exception of the high peptide concentration in isolation. The mechanisms by which C3G and/or peptides improved glucose uptake in human primary myotubes remains unclear, however a down-regulation of AGTR-1 mRNA and an up-regulation of GLUT4 mRNA expression may indicate that these bioactive components have a role in glucose metabolism, but further analysis is required.

FOXO1, which belongs to the Forkhead family of transcription factors, has a role in skeletal muscle insulin and glucose homeostasis. The over expression of FOXO1 has been shown to impair glycaemic control as a result of reduced skeletal muscle mass (Sugiishi et al., 2013). The activated FOXO1, as a negative regulator of insulin sensitivity, plays a critical role in muscle insulin resistance (Wang et al., 2017). Previous studies have reported that insulin dramatically reduced the FOXO1 nuclear level in insulin-stimulated vehicle-treated myotubes (Southgate et al., 2005), significantly decreasing the stability of FOXO1 protein (Matsuzaki, Daitoku, Hatta, Tanaka and Fukamizu, 2003), as well as suppressing FOXO1 transcriptional activity. A recent study has shown that the reduction in FOXO1 could increase GLUT4 expression in transfected C₂C₁₂ cells with siRNA-FOXO1 (Wang et al., 2017). In the present study, mRNA expression of FOXO1 was suppressed by high peptide concentration and its combination with C3G in both obese and obese T2DM groups, suggesting that this inhibition may be attributed to, at least partially, to peptides and C3G having insulin-like properties. Further analysis however, is required to elucidate the mechanisms that caused this observation in human primary myotubes.

It has been established that activation of AMPK increases glucose uptake in skeletal muscle and helps regulate blood glucose homeostasis (Naimi et al., 2017). In the current study, the expression of AMPK was decreased with the treatments of high C3G combined with both low and high peptide concentrations in the obese group, while no change was observed in the obese T2DM group. These results are in conflict with previous published findings in which fermented blueberry juice (that had *Serratia vaccinii* bacterium added to it) increased glucose uptake by 48% in C₂C₁₂ myotubes as a result of AMPK activation (Vuong, Martineau, Ramassamy, Matar and Haddad, 2007). It has also been demonstrated that dietary bilberry extract significantly activated AMPK in skeletal muscle, and enhanced glucose uptake into skeletal muscle tissue (Takikawa, Inoue, Horio and Tsuda, 2010). The different experimental design, such as species (human primary vs mouse immortalised cell-line), phenotype of myotubes (human primary myotubes obtained from obese and T2DM participants vs C₂C₁₂ myotubes), the treatments (single compound vs mixture including various anthocyanins and experimental system (*in vivo* vs *in vitro*) could account for the differences observed between previously reported data and the current study.

In this study, only mRNA of AMPK and GLUT4 were measured, but future determination of phosphorylation of AMPK and the translocation of GLUT4 would be of value to ensure the changes in mRNA were translated into functional changes within the cell. Numerous studies however, have shown that mRNA of AMPK and GLUT4 are correlated with phosphorylation of AMPK and the translocation of GLUT4 (Chen, Lin and Shih, 2014; Dhanya, Arya, Nisha and Jayamurthy, 2017; Han et al., 2015; Huang et al., 2010). Thus mRNA expression of AMPK and GLUT4 still can be recognized as suitable markers to assess glucose metabolism in human primary myotubes.

5. Conclusions

In summary, the results presented here demonstrate that high C3G concentration in combination with peptides with high ACE inhibitory activity extracted from yoghurt (both low and high concentrations) can enhance glucose uptake with or without insulin stimulation in human primary myotubes from obese participants. Only high peptide concentration however, increased glucose uptake in myotubes derived from obese T2DM participants in the absence of insulin. It is possible that the peptides extracted from yoghurt with high ACE inhibitory activity increased glucose uptake through the inhibition of AGTR-1 mRNA expression, and subsequently the increase of IRS-1 and GLUT4 mRNA expression. C3G also demonstrated its potentially anti-diabetic activity, at least in part, in an insulin-dependent signalling pathway, associated with the increase of IRS-1 mRNA expression and GLUT4 mRNA expression. However, the combinations of C3G and peptides appear to

have not had an additive effect on either glucose uptake or the regulation of multiple genes. The results suggest that C3G and peptides with ACE inhibitory activity provide a novel insight into the potential implications of natural anti-diabetic compounds for the regulation of glucose metabolism in obese and obese T2DM participants. Further research is required to elucidate the mechanisms of these active compounds involved in the signalling pathways via regulation of the related protein expression and translocation of GLUT4 to the cell membrane. Moreover, the efficacy of C3G and peptides on glucose metabolism will need to be validated through human clinical trials.

6. Ethics statement

This original manuscript obtained ethical approval for this study from The Human Ethics Research Committee of Victoria University, Melbourne Australia (HRE08-158) and all participants gave written informed consent.

7. Author contributions

MS, XQS and AJM conceived and designed the experiments. MS conducted experiment, collected data and drafted the manuscript. XQS, AJM, LOK and AS critically reviewed manuscript. AJM, LOK and AS collected and established the cell lines. All authors read and approved the manuscript.

Conflict of interest

The authors have no conflict of interest to declare.

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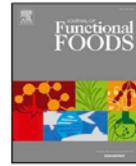
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APPENDIX 4: The effects of supplementation with blueberry, cyanidin-3-O- β -glucoside, yoghurt and its peptides on obesity and related comorbidities in a diet-induced obese mouse model



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The effects of supplementation with blueberry, cyanidin-3-O- β -glucoside, yoghurt and its peptides on obesity and related comorbidities in a diet-induced obese mouse model

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ABSTRACT

It is widely acknowledged that type 2 diabetes mellitus (T2DM) is associated with obesity, insulin resistance and hypertension. Cyanidin-3-O- β -glucoside (C3G), an anthocyanin in blueberry, and peptides with angiotensin converting enzyme (ACE) inhibitory activity derived from yoghurt are potentially beneficial for numerous health conditions including improving insulin resistance and glucose intolerance. In this study, the synergistic/additive effects of combined supplementations with blueberry and yoghurt, and C3G and peptides were determined. Blueberry and yoghurt alone, and the combination of C3G and peptides significantly reduced both systolic and diastolic blood pressure in diet-induced obese mice. Yoghurt supplementation significantly reduced body weight, percentage body fat and improved intraperitoneal glucose tolerance. Furthermore, peptides and the combination of peptides and C3G resulted in a significant reduction of percentage body fat and improved intraperitoneal glucose tolerance. As widely available, safe and nutritious foods, blueberry and yoghurt showed therapeutic potential in the treatment of obesity, diabetes and hypertension.

1. Introduction

Obesity is a serious problem in industrialized and developing countries worldwide. As the prevalence of obesity has increased, so has the prevalence of obesity-related comorbidities, including type 2 diabetes mellitus (T2DM), cardiovascular diseases, hypertension, stroke, certain forms of cancer and chronic inflammation (Chan et al., 2002; Crujeiras et al., 2012; Landsberg, 2000). In recent years much attention has been focused on food components that may have beneficial effects against dysfunction of obesity and its related metabolic diseases (Basu et al., 2010; Stull, Cash, Johnson, Champagne, & Cefalu, 2010).

Consumption of blueberries has been shown to prevent obesity and its related comorbidities in several animal models, such as Obese Zucker rats, KKAy mice, C57BL/6J mouse and Sprague-Dawley rats (Elks, Terrebonne, Ingram, & Stephens, 2015; Seymour et al., 2011; Vuong et al., 2009). Specifically, long-term feeding of blueberry-enriched diet lowered blood pressure (BP) and improved redox status in

kidneys of hypertensive rats and concomitantly demonstrated the potential to delay or attenuate development of hypertension-induced renal injury (Elks et al., 2011). Another study on the obesity-prone Zucker rats, fed a high-fat (45% fat) diet found that supplementation with whole blueberry powder (2% w:w) appeared to reduce fat accumulation and improve insulin sensitivity (Seymour et al., 2009). On the contrary, other studies indicated that administering purified anthocyanins from blueberry via drinking water prevented the development of dyslipidemia and obesity in mice, but feeding diets containing whole berries did not alter the development of obesity (Prior et al., 2008, 2009). However, it is not clear from these studies whether the anti-obesity and anti-diabetic effects of blueberries were due to the anthocyanins or the whole fruits of blueberry.

Cyanidin-3-O- β -glucoside (C3G), one of the most widely distributed anthocyanins in fruits and vegetables, is an important component in blueberries (Olivas-Aguirre et al., 2016; Wang, Zhao, Wang, Huo, & Ji, 2016). It has been reported that C3G ameliorates insulin resistance,

Abbreviations: ACE, angiotensin converting enzyme; AUC, the area under the curve; BP, blood pressure; BW, body weight; C3G, cyanidin-3-O- β -glucoside; HFHC, high-fat-high-carbohydrate-diet; ipGTT, intraperitoneal glucose tolerance test; ipITT, intraperitoneal insulin tolerance test; SHR, spontaneously hypertensive rat; T2DM, type 2 diabetes mellitus

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hyperglycaemia and obesity-associated inflammation with potent anti-obesity and anti-diabetes properties (Guo et al., 2012; Liu, Li, Zhang, Sun, & Xia, 2014; Sasaki et al., 2007). Furthermore, *in vitro* studies have also shown that C3G protected 3T3-L1 adipocytes against tumor necrosis factor- α induced insulin resistance (Guo et al., 2008), and eliminated the impacts of high-glucose on the induction of lipolysis in 3T3-L1 adipocytes (Guo, Guo, Jiang, Li, & Ling, 2012), although the potential mechanisms are still unclear.

Yoghurt, a fermented milk product by lactic acid bacteria (LAB), not only provides energy and nutrients but has also been demonstrated to be beneficial in obesity and diabetes (Eviwie, Huo, Igene, & Bian, 2017; Honda, Moto, Uchida, He, & Hashizume, 2012; Mekkes, Weenen, Brummer, & Claassen, 2013; Tsai, Cheng, & Pan, 2014). The diet supplemented with yoghurt fermented by probiotic *Lactobacillus acidophilus* and *Lactobacillus casei* significantly delayed the onset of glucose intolerance, hyperglycemia, hyperinsulinemia, dyslipidemia, and oxidative stress in high fructose-induced diabetic rats, indicating a lower risk of diabetes and its complications (Yadav, Jain, & Sinha, 2007). Furthermore, yoghurt is an excellent source of bioactive peptides, including the antihypertensive peptides or angiotensin-converting enzyme (ACE) inhibitors (Kajimoto et al., 2002; Papadimitriou et al., 2007). Many studies confirmed the importance of ACE inhibition in relation to the prevention of obesity and obesity-related diseases (Manikkam, Vasiljevic, Donkor, & Mathai, 2016). Carter et al. (2004) suggested that ACE inhibition in aged rats improved their body composition and physical performance compared to control rats (Carter et al., 2004). Another study has shown that peptides with ACE inhibitory activity (derived from bovine α -lactalbumin) significantly reduced body weight, blood glucose and insulin levels, and downregulated inflammation-related gene expression in adipose tissues of high-fat diet (HFD)-fed C57BL/6J mice (Gao, Song, Du, & Mao, 2018). Furthermore, our previous study has shown that peptides with ACE inhibitory activity can be extracted from yoghurt fermented by *Lactobacillus helveticus* 881315 in the presence of Flavourzyme (Shi, Ahtesh, Mathai, McAinch, & Su, 2017). However, there is no data available on the effect of combining C3G and antihypertensive peptides for the prevention of obesity and its related disorders.

In this study, we determined the effects of supplementation with blueberry, yoghurt, C3G and yoghurt peptides in isolation and combination on obesity related comorbidities. Following the eight-week supplementation period, changes in the body weight, body fat mass, energy expenditure, glucose tolerance and blood pressure in the diet-induced obese C57BL/6 mice were assessed. We hypothesised that combinations of blueberries and yoghurt, as well as peptides and C3G would have synergistic/additive effects on obesity and its related comorbidities.

2. Materials and methods

2.1. Chemicals and reagents

C3G (purity > 97%) was provided by Polyphenols AS (Sandnes, Norway). Fresh blueberries (Rabbiteye blueberry) were obtained from Bhatti and Manj Australian Blueberries (Woolgoolga, NSW, Australia). Fructose was purchased from Consolidated Chemical Company (Dandenong, South VIC, Australia). 36% Fat Modified Rodent Diet (59% of total energy from fat) was obtained from Specialty Feeds (Glen Forrest, WA, Australia). All other chemicals, unless otherwise specified, were obtained from Sigma-Aldrich Pty. Ltd. (Sunshine, VIC, Australia).

Skim milk was fermented by *Streptococcus thermophilus* ASCC 1275 and *Lactobacillus delbrueckii* subsp. *bulgaricus* 1466 to produce the yoghurt. The yoghurt was further fermented by *Lactobacillus helveticus* 881315 in the presence of Flavourzyme and the end product was used in the animal study. Peptides with ACE inhibitory activity were extracted from the yoghurt and the IC_{50} value was 1.47 ± 0.04 mg/mL. The details on the process of fermentation, peptide extraction and

determination of ACE inhibitory activity have previously been reported (Shi et al., 2017). Subsequently, peptides derived from the same batch (with an IC_{50} value for ACE inhibition of 1.47 ± 0.04 mg/mL) as we have previously reported (Shi et al., 2017) were used in the present animal study.

2.2. Animal feeding

Six-week-old male C57BL/6 mice were obtained from Animal Resources Centre (Perth, WA, Australia). As male mice are more likely to develop diabetes than females (Wang & Liao, 2012), and also considering the fluctuation of female hormones associated with the menstrual cycle, male mice were used in this study. All animals were housed in a stable environment maintained at 22 ± 1 °C with a 12 h light/dark cycle (7:00–19:00). The mice had *ad libitum* access to food (standard chow) and water for four days prior to commencement of the experimental procedures. Mice were separated into eight groups ($n = 10$ for each group) and fed different diets: standard chow with 5% fat (SCD), high-fat high-carbohydrate (59% of total energy from fat plus 30% fructose water, diet alone (HFHC)) and HFHC with six dietary supplements including blueberry (HFHC + B), C3G (HFHC + C), yoghurt (HFHC + Y), peptides extracted from yoghurt (HFHC + P), combined blueberry and yoghurt (HFHC + B + Y) and combined C3G and peptides (HFHC + C + P). The supplements were added to the mixture of gelatine and water at a temperature just below 40 °C. The set supplemented jelly was then fed to mice separately to their HFHC diet. The dose of supplementations were 6.4 g/kg body weight (BW)/ day of blueberries, 0.02 g/kg BW/ day of C3G, 3 g/kg BW/ day of yoghurt and 0.2 g/kg BW/ day of peptides. The amount of blueberries and peptides were chosen based on the previous studies (Mykkanen et al., 2014; Nakashima et al., 2002). The supplemented dosage of C3G was matched with its content in blueberries (0.31% of cyanidin in blueberry powder). Furthermore, the dosage of yoghurt was matched with the amount of peptides it contained (7% in yoghurt powder). Mice were housed individually in divided cages and provided a separate diet with gelatine during the 8-week treatment period. To keep consistency, mice in SCD and HFHC groups were also fed gelatine but without supplementation. Diet supplements were prepared fresh and stored at 4 °C for a maximum of 3–6 days following preparation. The standard chow and high fat diets were purchased from Speciality Feeds (Glen Forrest, WA, Australia). Food intake, water consumption, body weight and total energy intake were monitored twice per week and a weekly average for both measurements was calculated. All animal experiments were carried out in accordance with Australian Animal Welfare Act 1992. Experimental procedures were approved by the Animal Ethics Committee of Victoria University (AEC NO: 16/005).

2.3. Echo magnetic resonance imaging (MRI)

Echo MRI™ system (Echo-MRI™ 900, Houston, TX, United States of America) was utilised to determine the body composition. Calibration of the machine was undertaken using the canola oil before measurement according to the manufacturer instructions. Animals were scanned three times in the Echo MRI to determine body composition. Mice were acclimatised to this procedure at week one and week five, and then measurements were taken at the 9th week (baseline), 13th week (mid-treatment) and then at the 17th week (end of the treatment).

2.4. Blood pressure measurement

Systolic and diastolic blood pressure (BP) were measured every four weeks using a volumetric tail-cuff blood pressure analyser (CODA 8-Channel, Kent Scientific Corporation, CT, USA). Specifically, each mouse was placed into a holder; the holder was then placed into a warming chamber for 5 min at 32 °C for acclimation. The mouse tail was threaded through the sensor. Both systolic and diastolic BP were

measured for twenty times and data were analysed using the CODA software. Upon completion of the procedure, mice were immediately removed from the cuffs and holder, and returned to their home cage.

2.5. Measurement of metabolic variables

Metabolic variables including oxygen consumption (VO₂), carbon dioxide consumption (VCO₂), energy expenditure, respiratory quotient (VO₂/VCO₂), movement and rest time were monitored using the Promethion system (Sable Systems International, North Las Vegas, NV, United States of America) once every four weeks. Data were recorded for 24 h continuously.

2.6. Intraperitoneal glucose tolerance test (ipGTT)

Intraperitoneal glucose tolerance test (ipGTT) was conducted at week nine (pre-supplementation baseline) and week seventeen (end of treatment). Fructose water was replaced by normal water in the HFHC control and all the treatment groups and mice were fasted overnight for approximately 12–13 h, with free access to water. A tail snip was performed and a drop of blood (~50 µL) was gently obtained and placed onto a blood glucose strip (FreeStyle Optium Neo H, Abbott, USA) for the measurement of the baseline blood glucose concentration using a glucometer (FreeStyle Optium Neo H, Abbott, USA). Following this, the mouse was injected i.p. with a dose of 1.5 g/kg body weight of glucose/0.9% sterile saline solution with 150 mg/mL of glucose (Mcfarlane Medical, Surry Hills, Victoria, Australia). The blood glucose concentration was measured at 15, 30, 45, 60, 90, 120 mins following glucose injection. Once this procedure was completed the mice were given access to food and water again.

2.7. Anaesthesia and tissue collection

After the 8-week supplementation period, mice were deeply anaesthetised using isoflurane. They were then sacrificed via a cardiac puncture. Sequentially, heart, epididymal fat, mesenteric fat, liver and kidney were collected and weighed.

2.8. Statistical analysis

All results were expressed as mean ± standard error of the mean (SEM, n = 9–10). Two-way ANOVA was performed to determine the differences in cumulative food intake, blood pressure, body composition, and blood glucose level between the treatment groups at various time points using GraphPad Prism 7 (GraphPad Software, Inc, La Jolla, CA, United States of America). Post-Hoc analysis was conducted using Tukey HSD test for multiple comparisons. p < 0.05 was considered significant.

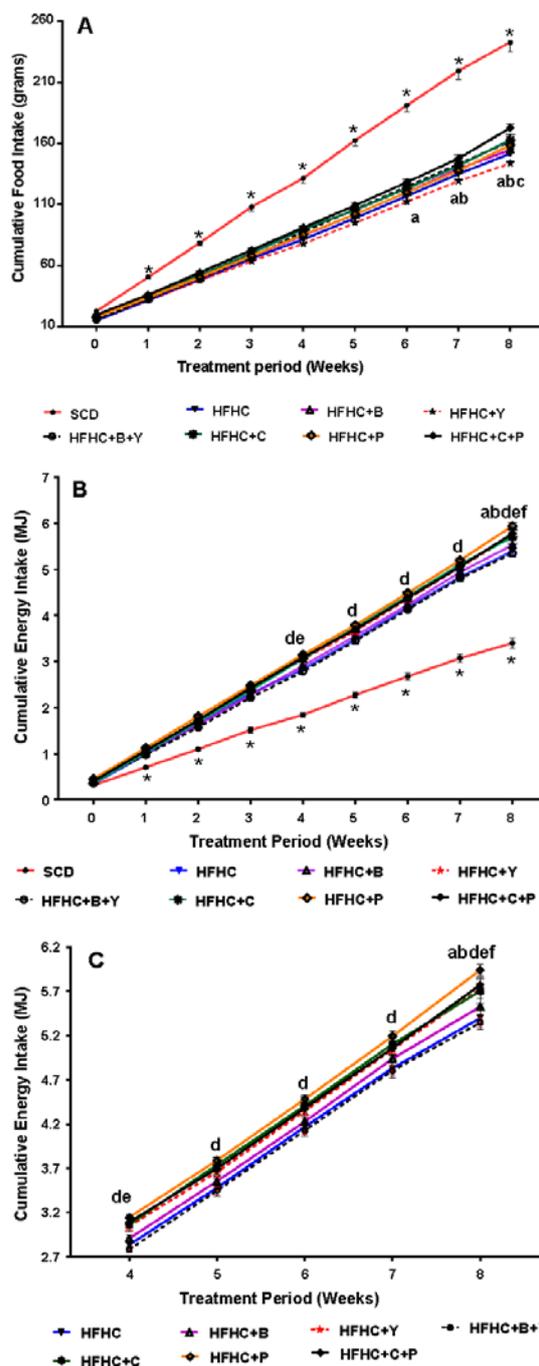
3. Results

3.1. Cumulative food and energy intakes

Cumulative weekly food intake (in grams) was significantly higher in the control group (SCD diet) than in other groups at the beginning of dietary supplementation (Fig. 1A). The group supplemented with the combination of C3G and peptides (HFHC + C + P) showed a significantly lower cumulative food intake in the last two weeks of treatment compared to the obese control (HFHC) group, while other treatment groups did not show significant differences in food intake from to the HFHC control group. Furthermore, at the end of treatment, the HFHC + Y group showed lower cumulative food intake compared to the HFHC + P group and the HFHC + B + Y group, respectively.

The mice fed the SCD had the least cumulative energy intake (p < 0.05) compared with other groups (including the HFHC group) from week one onwards (Fig. 1B). The animals supplemented with the

peptides (HFHC + P) showed a significantly higher cumulative energy intake from week four onwards compared to the obese control group (HFHC, Fig. 1C). At the end of treatment, the effects of blueberries or blueberries plus yoghurt on cumulative energy intake (HFHC + B and



(caption on next page)

Fig. 1. Cumulative food intake and energy intake during the treatment period. Food intake and energy intake were measured every week during the eight-week treatment period, (A) Cumulative food intake (g), (B) cumulative energy intake (MJ) during the whole treatment period and (C) cumulative energy intake (MJ) excluding SCD group from week 4 to week 8 of the treatment. Data were reported as mean ± SEM. Significant differences ($p < 0.05$) between groups are indicated as follows: * HFHC vs SCD control; ^arepresents HFHC + Y vs HFHC + B + Y; ^brepresents HFHC vs HFHC + C + P, and ^crepresents HFHC + Y vs HFHC + P; ^drepresents HFHC vs HFHC + P, HFHC + B vs HFHC + P, HFHC + B + Y vs HFHC + C, HFHC + B + Y vs HFHC + P; ^e represents HFHC + B + Y vs HFHC + C + P; ^frepresents HFHC vs HFHC + Y, HFHC vs HFHC + C.

HFHC + B + Y) were not significantly different to the obese control group (HFHC), while the other supplemented groups showed an increased cumulative energy intake compared to the obese control group (HFHC, Fig. 1C).

3.2. Changes in the body weight

As shown in Fig. 2A, the HFHC group had a higher body weight than the SCD group from week five. Following the treatment of yoghurt for six weeks, body weight was significantly reduced compared to the HFHC group with no significant difference from the SCD group. However, supplementation with blueberries (HFHC + B) did not seem to protect against the diet-induced weight gain. At the end of the 8-week treatment period, obese mice supplemented with blueberries (HFHC + B) showed a significant increase in the body weight compared with HFHC control mice (Fig. 2B). In addition, mice supplemented with blueberries (HFHC + B) were heavier compared to mice supplemented with C3G (HFHC + C, $p < 0.05$, Fig. 2B).

3.3. Body composition

At the end of the 8-week treatment, both the weight of body fat and the percentage body fat in the HFHC group were higher than those in the SCD group (Table 1). Peptide treatment has reduced both the weight and percentage of body fat significantly compared to the HFHC group, and resulted in a comparable outcome to the SCD group. However, other treatment groups showed no significant differences from the HFHC group.

The weight of body fat in all groups was significantly increased throughout the supplementation period, with the exception of HFHC + Y group in which no significant change was found (Table 1). When the whole body weight was considered, the percentage body fat in the HFHC + Y group did not significantly alter in the first four weeks, while a significant increase was recorded in the following four weeks compared to baseline and week four. Based on the data of percentage changes in body composition from baseline, it was noticed that blueberry supplementation resulted in a significant increase in body fat compared with the HFHC group in weeks 4 and 8 (data not shown). In addition, the combination of blueberry and yoghurt supplementation (HFHC + B + Y) for 8 weeks also significantly increased body fat compared to the HFHC group while no other groups showed significant differences (data not shown).

3.4. Tissue weights

At the end of the 8-week treatment period, the percentage of heart weight was significantly higher in the SCD, HFHC + Y and HFHC + P groups compared to all other groups with the exception of the HFHC + C + P group (Table 1).

As expected, the epididymal fat was higher in the obese HFHC mice compared to the SCD group (Table 1). The supplementation of yoghurt (HFHC + Y) significantly decreased epididymal fat compared with the HFHC group, and no alterations compared to the SCD group.

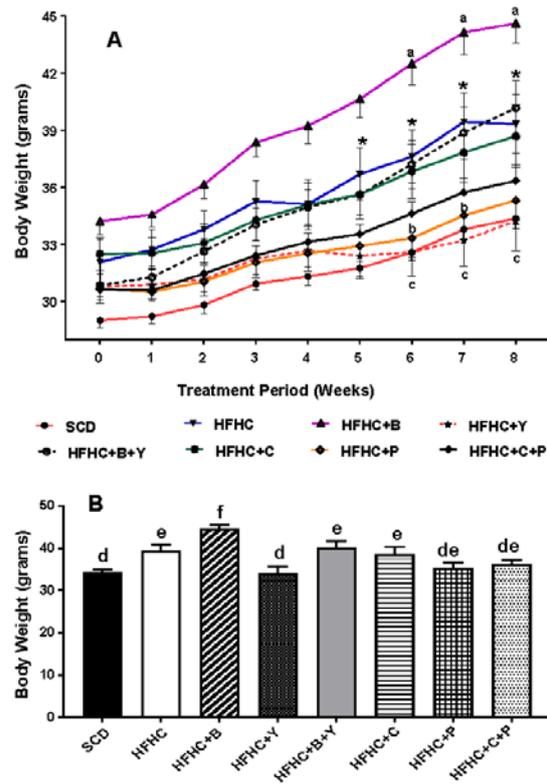


Fig. 2. The changes of body weight during the treatment period. Body weight of each group was measured every week during the eight-week treatment period, (A) body weight (g) during the treatment period and (B) body weight (g) at the end of treatment. Data were reported as mean ± standard error of mean (SEM). Significant differences ($p < 0.05$) between groups are indicated as follows: * HFHC vs SCD control; ^a represents HFHC vs HFHC + B; ^b represents HFHC vs HFHC + P and ^c represents HFHC vs HFHC + Y in Fig. 2A. The asterisk denotes the significant difference between the SCD and HFHC group from week 5. Fig. 2B different letters indicate significant differences between groups ($p < 0.05$).

The percentage of mesenteric fat in the HFHC + B group was greater than in all other groups except the HFHC and HFHC + B + Y groups (Table 1). Mesenteric fat in mice with the other supplements did not differ from both the SCD and the HFHC groups.

The percentage of liver weight was unchanged in all groups (Table 1). No significant differences in the percentage of kidney weight were observed in all treatment groups compared to both the SCD and the HFHC groups (Table 1). However it was significantly decreased in the HFHC + B group compared with the HFHC + Y, HFHC + P and HFHC + C + P groups (Table 1).

3.5. Intraperitoneal glucose tolerance test (ipGTT)

As expected, HFHC diet impaired glucose tolerance in obese mice compared to SCD after eight weeks feeding (Fig. 3A, B). There was no difference in glucose clearance in all groups prior to introduction of the intervention diets except the SCD group (Fig. 3B). Following the 8-week supplementation, mice supplemented with yoghurt (HFHC + Y), peptides (HFHC + P) and the combination of C3G and peptides (HFHC + C + P) showed a significant improvement in the intraperitoneal glucose tolerance compared to the HFHC group, and the outcomes are comparable with that in the SCD group (Fig. 3C, D). In

Table 1
Effects of dietary supplementation on body composition, tissue index and metabolic variables of C57BL/6 mice.

	SCD	HFHC	HFHC + B	HFHC + Y	HFHC + B + Y	HFHC + C	HFHC + P	HFHC + C + P
<i>Body composition</i>								
Baseline								
Body fat (g)	4.07 ± 0.39 ^a	8.32 ± 0.57 ^b	8.30 ± 0.63 ^b	5.17 ± 0.93 ^{ab}	6.01 ± 0.70 ^{ab}	7.23 ± 0.76 ^{ab}	4.26 ± 0.48 ^a	5.67 ± 0.49 ^{ab}
Body fat (%)	11.48 ± 0.14 ^a	24.76 ± 1.05 ^b	24.63 ± 1.50 ^b	17.45 ± 1.99 ^{ab}	18.98 ± 2.00 ^{ab}	22.20 ± 2.01 ^b	13.95 ± 1.35 ^a	18.42 ± 1.54 ^{ab}
Week Four								
Body fat (g)	4.42 ± 0.52 ^a	9.65 ± 0.55 ^{bc}	13.11 ± 0.74 ^d	5.75 ± 0.98 ^a	8.60 ± 0.93 ^c	8.66 ± 1.11 ^{cd}	4.84 ± 0.52 ^a	6.25 ± 0.68 ^{ad}
Body fat (%)	12.49 ± 0.59 ^a	26.56 ± 0.80 ^{bc}	33.35 ± 1.35 ^d	17.97 ± 2.17 ^{cd}	24.50 ± 2.24 ^{cd}	24.25 ± 2.55 ^{cd}	14.77 ± 1.26 ^a	18.79 ± 1.85 ^{ac}
Week Eight								
Body fat (g)	7.26 ± 0.58 ^d	13.71 ± 1.07 ^{cd}	17.57 ± 0.82 ^{de}	7.09 ± 1.43 ^{bc}	13.14 ± 1.26 ^{cd}	11.84 ± 1.44 ^{cd}	7.77 ± 1.05 ^{ad}	9.36 ± 1.06 ^{cd}
Body fat (%)	19.92 ± 1.09 ^{cd}	33.80 ± 1.44 ^{cd}	38.99 ± 1.12 ^{de}	21.07 ± 2.82 ^{cd}	32.52 ± 2.30 ^{de}	29.90 ± 2.59 ^{de}	21.51 ± 2.07 ^{cd}	25.44 ± 2.46 ^{cd}
<i>Tissue index (% BW)</i>								
Heart	0.43 ± 0.01 ^a	0.35 ± 0.01 ^{bc}	0.31 ± 0.01 ^b	0.43 ± 0.02 ^a	0.35 ± 0.01 ^b	0.36 ± 0.02 ^{bc}	0.43 ± 0.01 ^a	0.41 ± 0.02 ^{ac}
Epididymal fat	2.43 ± 0.16 ^a	3.88 ± 0.24 ^b	3.18 ± 0.20 ^{ab}	2.30 ± 0.37 ^a	3.35 ± 0.18 ^{ab}	3.22 ± 0.27 ^{ab}	2.64 ± 0.31 ^{ab}	3.14 ± 0.38 ^{ab}
Mesenteric fat	0.78 ± 0.07 ^a	1.23 ± 0.11 ^{bc}	1.61 ± 0.15 ^{bc}	0.79 ± 0.12 ^a	1.13 ± 0.11 ^{ab}	1.04 ± 0.09 ^a	0.92 ± 0.12 ^a	1.03 ± 0.13 ^a
Liver	4.55 ± 0.11 ^a	4.61 ± 0.12 ^a	4.92 ± 0.33 ^a	4.44 ± 0.19 ^a	4.44 ± 0.08 ^a	4.91 ± 0.16 ^a	4.40 ± 0.09 ^a	4.79 ± 0.23 ^a
Kidney	1.21 ± 0.03 ^{bc}	1.17 ± 0.03 ^{bc}	1.02 ± 0.03 ^a	1.35 ± 0.05 ^{bc}	1.19 ± 0.04 ^{abc}	1.17 ± 0.04 ^{ab}	1.37 ± 0.06 ^c	1.34 ± 0.07 ^{bc}
<i>Metabolic variables (Week 8)</i>								
VO ₂ (mL/min)	1.39 ± 0.03 ^a	1.77 ± 0.04 ^b	1.94 ± 0.04 ^b	1.77 ± 0.03 ^b	1.90 ± 0.05 ^b	1.84 ± 0.05 ^b	1.84 ± 0.04 ^b	1.80 ± 0.03 ^b
VCO ₂ (mL/min)	1.15 ± 0.03 ^a	1.51 ± 0.04 ^b	1.65 ± 0.04 ^b	1.51 ± 0.02 ^b	1.59 ± 0.04 ^b	1.59 ± 0.04 ^b	1.59 ± 0.03 ^b	1.57 ± 0.03 ^b
Energy expenditure (kcal/day)	9.73 ± 0.20 ^a	12.46 ± 0.29 ^b	13.65 ± 0.29 ^b	12.45 ± 0.20 ^b	13.33 ± 0.36 ^b	12.99 ± 0.32 ^b	13.00 ± 0.27 ^b	12.71 ± 0.23 ^b
Respiratory quotient (VO ₂ /VCO ₂)	0.82 ± 0.01 ^a	0.85 ± 0.01 ^{abc}	0.85 ± 0.01 ^{abc}	0.85 ± 0.01 ^{abc}	0.84 ± 0.01 ^{ab}	0.86 ± 0.01 ^{bc}	0.86 ± 0.01 ^{bc}	0.87 ± 0.01 ^c
Movement (Meters)	181.62 ± 11.97 ^a	205.47 ± 7.07 ^{ab}	252.94 ± 17.94 ^{bc}	230.72 ± 11.24 ^{bc}	247.24 ± 14.78 ^{bc}	229.80 ± 9.70 ^{bc}	246.72 ± 18.77 ^{bc}	218.94 ± 14.93 ^{bc}
Rest time (h)	17.82 ± 0.35 ^a	17.39 ± 0.21 ^{ab}	16.32 ± 0.33 ^{ab}	17.76 ± 0.48 ^{ab}	15.89 ± 0.27 ^{ab}	15.80 ± 0.22 ^b	17.55 ± 0.52 ^{ab}	16.46 ± 0.20 ^{ab}

Data are reported as mean ± SEM. * indicate a significant difference to baseline for the respective group (p < 0.05). # indicates a significant difference to week four data for the respective group (p < 0.05). Different letters indicate significant differences between groups for the corresponding time point (p < 0.05). BW, body weight; VO₂, oxygen consumption; VCO₂, carbon dioxide consumption. SCD, standard chow with 5% fat; HFHC, high-fat diet (59% of total energy from fat) plus 30% fructose water; HFHC + B, HFHC diet supplemented with blueberries; HFHC + Y, HFHC diet supplemented with yogurt; HFHC + B + Y, HFHC diet supplemented with blueberries and yogurt; HFHC + C, HFHC diet supplemented with cyanidin-3-O-β-glucoside; HFHC + P, HFHC diet supplemented with peptides; HFHC + C + P, HFHC diet supplemented with cyanidin-3-O-β-glucoside and peptides.

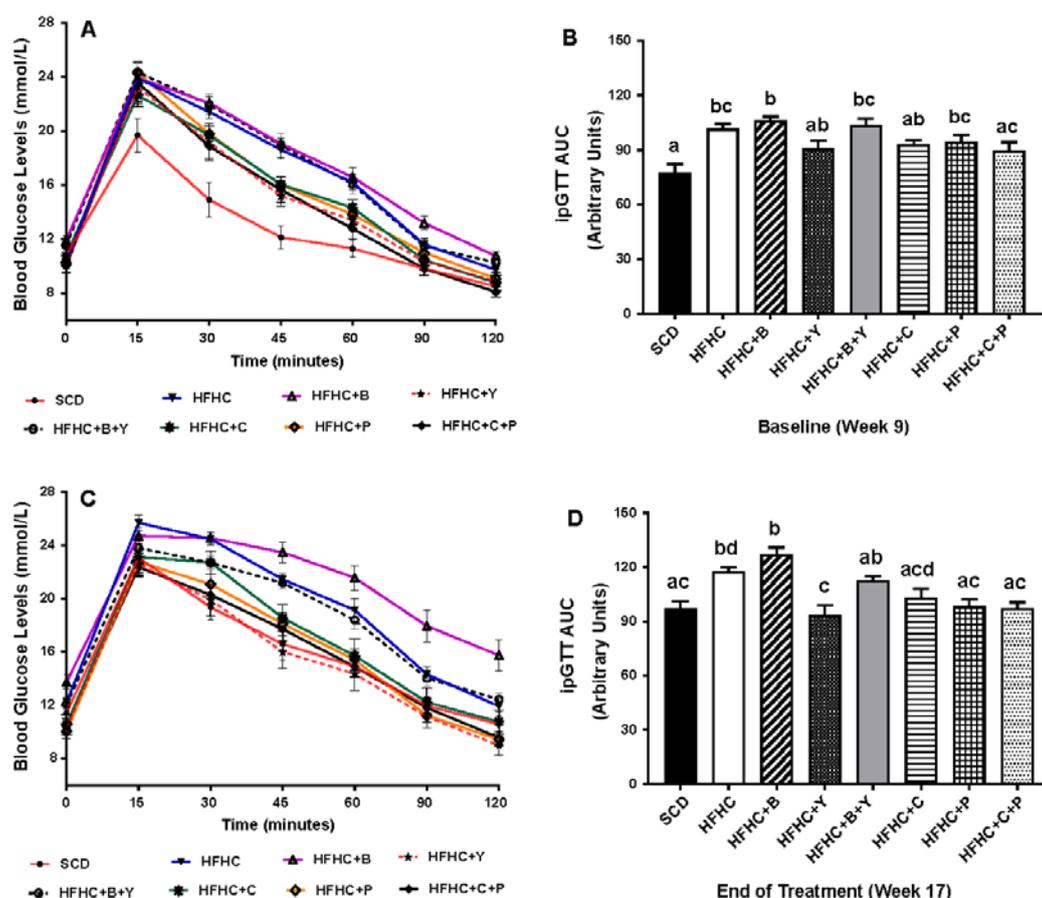


Fig. 3. Changes in response to blood glucose level as shown in the intraperitoneal glucose tolerance test (ipGTT). Blood glucose concentrations of mice were measured at pre- and post- treatment with different diets. (A) Pre-treatment ipGTT (intraperitoneal glucose tolerance test) blood glucose concentration (mmol/L) (week 9); (B) Pre-treatment ipGTT AUC (area under the curve, arbitrary units) (week 9); (C) ipGTT blood glucose concentrations (mmol/L) at the end of treatment (week 17); (D) Post-treatment ipGTT AUC (arbitrary units) at the end of treatment (week 17). Data were reported as mean \pm SEM. Different letters indicate a significant difference between groups ($p < 0.05$).

contrast, supplementation with blueberries (HFHC + B) resulted in the same level of glucose tolerance as in the HFHC group, suggesting that blueberries did not attenuate glucose tolerance, as shown in Fig. 3D.

3.6. Blood pressure

As shown in Fig. 4A, none of the supplements altered systolic BP in the first four weeks compared with the HFHC or SCD groups. A significantly lower systolic BP was however observed in all supplement groups except the C3G group (HFHC + C) by the end of the feeding period compared with the HFHC group.

Mice fed HFHC diet for eight weeks showed significantly higher diastolic BP, compared with the SCD group (Fig. 4B). Obese mice supplemented with blueberries (HFHC + B), yoghurt (HFHC + Y) and the combination of C3G and peptides (HFHC + C + P) had significantly decreased diastolic BP, compared to the HFHC group at the end of the supplement period.

3.7. Metabolic parameters

VO_2 , VCO_2 , and energy expenditure were significantly increased in

the HFHC group, compared to these in the SCD group. Furthermore, VO_2 , VCO_2 , and energy expenditure were higher in all supplemented groups than the SCD group, while there was no significant difference between all the supplemented groups and the HFHC group throughout the treatment period (Table 1).

No significant differences were observed in respiratory quotient between the SCD and the HFHC groups. There were also no differences between all treatment groups and the HFHC group (Table 1). Respiratory quotient in the groups supplemented with C3G (HFHC + C), peptides (HFHC + P) and the combination of C3G and peptides (HFHC + C + P) was higher than in the SCD group.

None of the treatments altered movement compared with the HFHC control group, while mice supplemented with blueberries (HFHC + B), peptides (HFHC + P) and the combination of blueberries and yoghurt (HFHC + B + Y) had increased movement compared with the SCD diet group (Table 1).

At the end of treatments, no significant difference in the resting time was observed between the SCD and the HFHC groups. The resting time in the HFHC + C group was significantly decreased compared to the SCD group. There were no significant differences observed between any supplementation group and the HFHC or the SCD groups (Table 1).

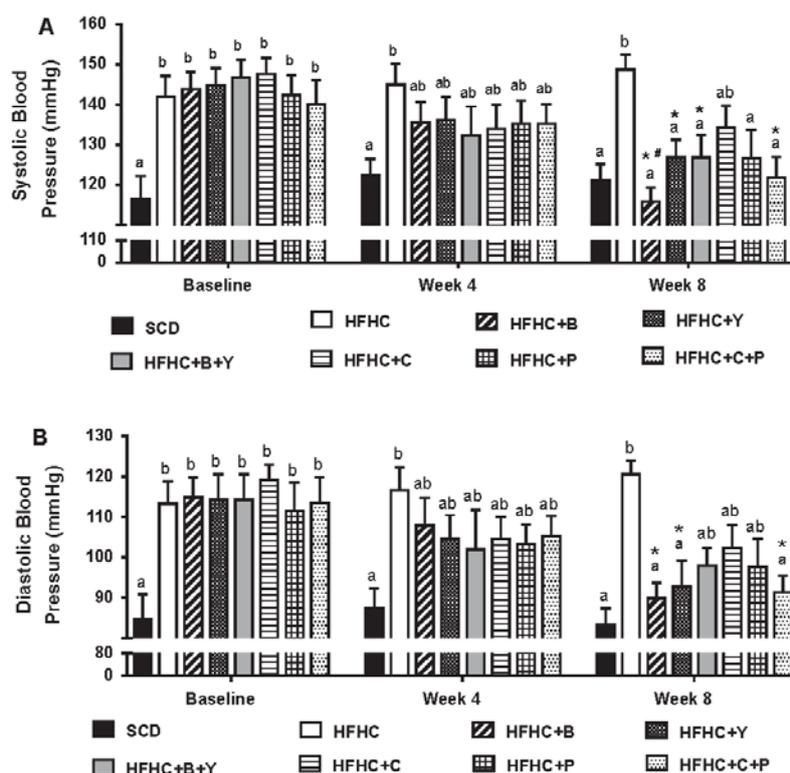


Fig. 4. Changes in blood pressure following dietary supplementations. (A) Systolic blood pressure and (B) Diastolic blood pressure of C57BL/6 mice were measured every four weeks during the treatment period. Data were reported as mean \pm SEM. ■ SCD (n = 10), □ HFHC (n = 9), ▨ HFHC + B (n = 10), ▩ HFHC + Y (n = 10), ▪ HFHC + B + Y (n = 10), ▫ HFHC + C (n = 10), ▬ HFHC + P (n = 10), ▭ HFHC + C + P (n = 10). * indicates a significant difference to baseline for the respective group ($p < 0.05$). # indicated a significant difference to week four data for the respective group ($p < 0.05$). Different letters indicate significant differences between groups ($p < 0.05$).

4. Discussion

Blueberry is known to be particularly rich in anthocyanins, which have been found to have beneficial effects for people with obesity and metabolic syndrome (Lehtonen et al., 2011; Shi, Loftus, McAinch, & Su, 2017; Wu et al., 2013). In addition, our previous study showed that bioactive peptides extracted from yoghurt fermented by *L. helveticus* had high ACE inhibition activity, which indicates its potential anti-hypertensive properties. The current study demonstrated that both systolic and diastolic BP were significantly reduced following supplementations of blueberry, yoghurt and the combination of peptides and C3G groups; a significant inhibition of body weight gain from the HFHC diet following yoghurt supplementation; and a significant reduction in body fat following peptide supplementation. In addition, an improvement in glucose tolerance was observed following supplementations of yoghurt, peptides and the combination of C3G and peptides.

In agreement with our results that eight weeks of blueberry supplementation reduced both systolic and diastolic BP in HFHC induced obese mice, diets enriched with blueberries have been reported to improve vascular tone and decreased BP in spontaneously hypertensive rats, compared with control animals (Kristo, Kalea, Schuschke, & Klimis-Zacas, 2010; Shaughnessy, Boswall, Scanlan, Gottschall-Pass, & Sweeney, 2009). Feeding a 3% blueberry diet for six weeks reduced plasma ACE activity in the spontaneously hypertensive stroke prone rats (Wiseman et al., 2011). A human trial also showed that consumption of 50 mL of 1.5 mM total polyphenols per day for two-weeks resulted in a 36% reduction in serum ACE activity and a 5% reduction in systolic BP of hypertensive patients (Aviram & Dornfeld, 2001). Therefore, the anti-hypertensive effect of blueberry was at least partially due to inhibition of ACE activity in the blood. Interestingly a pure anthocyanin, C3G, at the same dose as what was found in blueberries did not alter the BP in HFHC induced obese mice, suggesting that

positive outcomes of the whole blueberry may be associated with other compounds, instead of C3G. Further studies are required to determine all the active components in blueberries and the underlying mechanisms associated with their anti-hypertensive properties.

Yoghurt fermented milk by LAB has received much attention as a functional food and nutrient. Consumption of yoghurt has been demonstrated to reduce fasting and postprandial 2 h blood glucose levels in db/db mice (Yun, Park, & Kang, 2009), while consumption of a probiotic yoghurt also improved fasting blood glucose in type 2 diabetic patients (Ejtahed et al., 2012). In agreement with previous research, the current study showed that feeding yoghurt and its peptides with ACE inhibition for eight weeks improved glucose tolerance in obese mice compared with the HFHC control group. It has been suggested that yoghurt regulates blood glucose due to LAB, which possess anti-diabetic propensity on their hosts and thus can play a crucial role in human health care (Evvivie et al., 2017). The anti-diabetic activity of LAB differed depending on the bacterial strain and whether the bacteria are able to hydrolyse protein to produce short peptides with possible resistance to physiological digestion after ingestion (Honda et al., 2012; Matsui et al., 2002). It has been reported that *L. helveticus*, one of the most effective LAB, possess an efficient proteolytic system capable of producing short peptides from the casein matrix (Sadat-Mekmene et al., 2011). Furthermore, the small size peptides produced by *L. helveticus*, containing proline and hydroxyproline residues, can usually resist breakdown by digestive enzymes, thus they can reach the intestine and can be detected in faecal samples after ingestion (Saito, Sakamoto, Takizawa, & Benno, 2004; Seppo, Jauhainen, Poussa, & Korpela, 2003; Takano, 1998). It has been reported that peptides with ACE inhibitory activity containing proline and hydroxyproline residues are absorbed intact and present in the bloodstream (Segura-Campos, Chel-Guerrero, Betancur-Ancona, & Hernandez-Escalante, 2011). Taken together, in this study, the dietary supplementations with yoghurt fermented by *L.*

helveticus displayed their anti-diabetic effect, which was most likely related to its LAB, *L. helveticus*, and the bioavailability of these bioactive peptides.

Milk contains high quality proteins including whey protein and casein which can contribute to appetite control by increasing satiety and suppressing food intake (Akhavan et al., 2014; Akhavan, Luhovyy, Brown, Cho, & Anderson, 2010; Nabavi, Raftaf, Somi, Homayouni-Rad, & Asghari-Jafarabadi, 2015). The results from the present study are in agreement with the impact of dairy proteins on appetite as the cumulative food intake was reduced significantly following yoghurt supplementation, compared with peptides supplementation. Moreover, the body weight, the percentage of body fat and epididymal fat of obese mice were reduced significantly in the yoghurt supplemented group, compared with the HFHC control group, although there was no significant difference in cumulative food intake between these two groups. Other possible mechanisms underlying the role of yoghurt in attenuating obesity, as suggested by Tsai et al. (2014) and Evivie et al. (2017), include modulation enzymes involved in processing of dietary polysaccharides and the regulation of energy metabolism (Evivie et al., 2017; Tsai et al., 2014). It appears that peptides have no effect on obesity, and this may be partially due to increased energy intake compared with the HFHC group.

The proteolytic system of *L. helveticus* has been widely studied due to its ability to release peptides containing proline during milk fermentation with ACE inhibitory activity (Beganovic et al., 2013; Chen et al., 2014; Jauhainen et al., 2005; Seppo et al., 2003). However, the health benefits of fermented yoghurt by adding *L. helveticus* have not previously been investigated. Our study found that feeding fermented yoghurt for eight weeks significantly reduced both systolic and diastolic BP in HFHC induced obese mouse, which may be due to the ACE-inhibitory peptides extracted from yoghurt. In order to confirm the mechanisms of BP regulation following yoghurt supplementation, peptides with ACE inhibitory activity, extracted from yoghurt were also included in the animal study with the same dosage as in the yoghurt group. It is interesting to note that purified peptides significantly reduced only the systolic BP compared to the HFHC control group. The effect on hypertension in the peptide group was not fully matched with that of the yoghurt group, possibly due to other active components in yoghurt. In addition, peptides failed to reduce diastolic BP, and this may be due to the low dosage. Several reports have shown that the anti-hypertensive effect of milk peptides is dose-dependent (De Leeuw, Van der Zander, Kroon, Rennenberg, & Koning, 2009; Hirota et al., 2011). The present dose of peptides (0.2 g/kg BW/day) maybe insufficient to change the BP in HFHC-induced obese C57BL/6 mice by a single oral administration daily. These findings suggested that yoghurt might be more effective to regulate BP, which related to the synergistic action with other components, such as protein, amino acids and the minerals, in addition to the peptides with ACE inhibitory activity. Cell wall fragments from LAB in yoghurt may be another possibility for the better effect of yoghurt on hypertension as suggested by Sawada et al. (Sawada et al., 1990). It is unclear why alone both blueberry supplementation and yoghurt supplementation decreased diastolic BP, but when combined there was not an equal reduction or synergistic/additive effect observed. As both of these supplements contain a number of potential active components any number of these could have had accumulative or antagonistic effects on diastolic BP. Further research is warranted to uncover these complex interactions.

In this study, lower body weight and body fat mass were observed in the C3G group, compared to the blueberry group. This is consistent with the previous findings by Prior et al. (2009), which showed that consumption of purified anthocyanins from blueberries reduced obesity, but not the whole blueberry, which did not prevent and may have actually increased obesity in the C57BL/6J mouse model (Prior et al., 2008). DeFuria et al. (2009) also found that blueberry supplementation (4% w:w whole blueberry powder) did not affect HFD associated alterations

in energy intake, metabolic rate, body weight, or adiposity in C57BL/6J mice (DeFuria et al., 2009). In this study, C3G content supplemented to obese mice was matched between the blueberry group and C3G group. The discrepancy in the effects of blueberries and C3G on body weight and fat mass, thus implicates that blueberries are not as effective as the C3G in protecting against diet induced obesity. This is possibly due to the amount of simple sugars presented in the whole fruit as it is well known that simple sugars, e.g., fructose and sucrose are closely associated with body weight gain and obesity (Messier, Whately, Liang, Du, & Puissant, 2007; Ronn et al., 2013; Surwit et al., 1995).

Several studies have demonstrated that blueberries have anti-diabetic properties and supplementation or consumption of blueberry cause changes in glucose metabolism and improve insulin sensitivity (Seymour et al., 2009; Vuong et al., 2009). Supplementation of 2% (w:w) freeze dried blueberry powder improved glucose clearance, as measured by the glucose tolerance test in the obesity-prone Zucker fatty rat for 13 weeks (Seymour et al., 2009). Conversely, Prior et al. (2008) reported that long term supplementation with freeze-dried whole blueberry powder (10% diet) did not affect the results of a glucose tolerance test in C57BL/6J obese mice. A similar observation was found in our study. Blueberry supplementation did not affect glucose metabolism and showed no significant difference in glucose tolerance from the HFHC control animals. To eliminate the interference of sugar and other components in the whole blueberry, an extract of its active component, anthocyanin C3G (with the same dosage in blueberries) was also used in the current study. A previous study showed that blueberry polyphenols improved oral glucose tolerance in hyperglycemic C57BL/6 mice fed HFD for seven weeks (Roopchand, Kuhn, Rojo, Lila, & Raskin, 2013). Furthermore, dietary C3G (0.160–0.3 g/kg BW/day) reduced fasting glucose level and markedly improved the insulin sensitivity in HFD fed C57BL/6J mice, compared with the untreated control (Guo et al., 2012). However the present study found that glucose tolerance was significantly improved by pure anthocyanin, C3G, compared with the whole blueberry fruit, although there was no difference between C3G and HFHC groups. This may be due to the small amount of C3G (0.02 g/kg BW/day) which was administered. Moreover mouse strain maybe another factor that may have contributed to the discrepancies in response to glucose following the treatments. For example, for a genetic diabetic mouse (db/db mouse), treatment with low dose of C3G (0.01 g/kg BW/day) for 12 weeks improved the abnormalities of glucose metabolism as evidenced by a decrease in both glucose and insulin levels (Qin et al., 2018).

Based on the findings from this study, the equivalent human doses of treatments would be 0.52 g/kg BW/day for blueberry supplementation and 0.243 g (3/12.3)/kg BW/day for yoghurt supplementation (Nair & Jacob, 2016). This indicates that, for a young male adult with approximately 70 kg body weight, 36.4 g of dried blueberry powder and 17.1 g of dried yoghurt powder would be required daily, which equal to 182 g of fresh blueberries (20% of dry matter in the fresh blueberry) and 142.5 g of normal yoghurt (12% of dry matter in normal yoghurt). These doses are feasible for the general public (ie less than one small punnet of blueberries and one small tube of yoghurt), thus the study highlights the potential benefits of regular blueberry and yoghurt consumption, and the potential for future manufacturing/cultivation processes to enhance the effectiveness of these food products.

It is acknowledged that the study had several limitations which include that despite attempting to standardise several baseline measurements within the animals following the diet induced obese period, animals in different treatment groups began the treatment period with variations with baseline body composition. In addition, only one dose of C3G (0.02 g/kg BW/day) was included in the study. This low dose of C3G, despite being chronically administered may not have been high enough to alter a number of measurements undertaken in the current study. Further studies with higher doses of C3G are warranted to verify the health effects of C3G.

5. Conclusions

This study demonstrated that both systolic and diastolic BP were significantly reduced in obese mice supplemented with blueberries. However, the active component of blueberry, C3G did not reduce the blood pressure compared to the HFHC fed animals, although its content was fully matched with that in the blueberries. This implicates that the anti-hypertensive effect of blueberries may be associated with other active components or it may be related to the synergistic effect of multiple components in the whole fruit. In addition, yoghurt fermented by *L. helveticus* possesses protective effects against HFHC induced metabolic disturbances associated with developing obesity, diabetes and hypertension in C57BL/6 mice. Peptides extracted from yoghurt also showed anti-obesity effects with significant reduction in the percentage of body fat and improvement of glucose tolerance being observed in obese mice. Our findings attest that blueberry and yoghurt are promising candidates for the prevention of obesity related diabetes and hypertension, although the responsible mechanisms behind the beneficial effects require further investigation.

Ethics statements

All animal experiments were carried out in accordance with Australian Animal Welfare Act 1992. Experimental procedures were approved by the Animal Ethics Committee of Victoria University (AEC NO: 16/005).

Author contributions

MS, MM, AJM and XQS conceived and designed the experiments. MS conducted experiment, collected data and drafted the manuscript. MM, AJM, GX and XQS critically reviewed manuscript. All authors read and approved the manuscript.

Conflict of interest

The authors have declared no conflict of interest.

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