Blood Glucose-attenuating Effects of Pea-derived Fractions: Exploration of Mechanisms of Action in a Rat Model of Glucose Intolerance

by

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Abstract

Pulses, including dried peas, are nutrient-dense foods rich in fibre that have shown efficacy in improving glucose control in diabetic subjects. The seed coats, in spite of being the highly fibrous part, are sometimes discarded as a food-processing by-product. We hypothesized that supplementing high fat diets (HFD) with pea seed coats (PSC) would improve glucose tolerance mainly by modifying gut responses to glucose and reducing HFD-induced stress on pancreatic islets. A secondary hypothesis was that the glucose-lowering effects of these fractions would be retained following cooking. HFD-induced glucose intolerant Sprague Dawley rats were fed a HFD supplemented with raw or cooked PSC for 4 weeks. HFD and low fat diet with inclusion of cellulose as the fibre source were used as control diets. The results showed that, compared to HFD, cooked PSC diet improved glucose response, decreased postprandial insulin secretion and enhanced fasting GLP-1 and GIP response to glucose. Cooked PSC feeding also decreased fasting glucagon, which was associated with reduced alpha-cell mass. Microbial analysis revealed that PSC diets significantly altered the overall composition of gut microbiota and resulted in increased population of *Lachnospiraceae*, a butyrate-producing family of bacteria. Additionally, cooked PSC was found to increase the expression of mRNA encoding mucin proteins in the ileum and induce a trend toward decreased expression of ileal TLR2. The effect of pea/bean consumption on the B vitamin status was examined in normoglycemic humans, showing that blood concentrations of thiamine and folate were not affected by pulse-containing diets. Overall, our results demonstrated that cooked PSC feeding can reverse adverse effects of HFD

on glucose homeostasis via gut- and islet-mediated mechanisms. In particular, cooked PSC feeding is associated with enhanced incretin secretion, reduced alpha-cell abundance and glucagon concentrations, beneficial alterations in the gut microbiota and finally up-regulated expression of protective genes involved in gut barrier function. The higher effectiveness of the cooked PSC as compared to raw PSC showed that cooking treatment enhanced beneficial impacts of these components on glucose homeostasis.

Preface

Chapter 3. A version of this material has been published in *Applied Physiology, Nutrition and Metabolism* [*40*(4), 323-333] by Zohre Hashemi, Kaiyuan Yang, Han Yang, Alena Jin, Jocelyn Ozga, Catherine B. Chan. *Cooking enhances beneficial effects of pea seed coat consumption on glucose tolerance, incretin and pancreatic hormones in high fat diet-fed rats.* I was responsible for leading the study, preparing study diets, occasionally feeding animals, conducting in vivo studies, tissue collection, serum analyses, immunohistochemistry, morphometry and quantitative PCR. I also performed the statistical analyses and drafted the manuscript. Kaiyuan Yang from our lab also prepared diets, assisted with in vivo studies and tissue collection. Han Yang, Alena Jin and Dr. Jocelyn Ozga prepared and analyzed the pea seed coats. The methodology and results of fibre analysis were provided by the Ozga lab.

Chapter 4. This chapter is presented in manuscript format and will be prepared for submission to a relevant journal. I was responsible for performing the gene expression experiments, analyzing the data and drafting the manuscript. Dr. Benjamin Willing's lab and his collaborators performed the analysis of microbial composition. The methodology for that section was also provided by the Willing lab.

Chapter 5. This chapter is based on collaboration with the laboratory of Dr. Rhonda Bell. I conducted the B vitamin analysis, performed the statistical analysis of all the

presented data and drafted the chapter. The Bell lab recruited participants and carried out sample collection.

Chapter 6. I performed the immunoblotting and PCR experiments discussed in this chapter and analyzed the data. Some of the mechanistic pathways proposed are based on previous work in our lab (Chan et al., 2014) to which I am a coauthor.

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Table of Contents

Title Page

Abstract

Preface

Acknowledgements

Table of Contents

List of Tables

List of Figures

List of Abbreviations

Chapter 1: Introduction and Literature Review

1.1 Introduction to Diabetes Mellitus 1

1.2 Type 2 Diabetes Overview 2

1.3 Prediabetes 3

1.4 Pathophysiology of Prediabetes 4

1.4.1 Glucose Metabolism and Homeostasis 5

1.4.2 Insulin Resistance 8

1.4.2.1 Free Fatty Acids and Insulin Resistance 9

1.4.3 Impaired Insulin Secretion 13

1.5 Interventional Treatments to Prevent T2D 14

1.5.1 Lifestyle Interventions 14

1.5.2 Pharmacologic Interventions 15

1.6 Nutrition Therapy for the Prevention and Management of Diabetes 17

1.6.1 Health Benefits of Peas (*Pisum Sativum*) 18

1.6.1.1 Health Outcomes in Clinical Studies 19

1.6.1.2 Health Outcomes in Animal Studies 27

1.7 Dietary Fibre 28

1.7.1 Dietary Fibre and Glycemic Control 29

1.7.2 Proposed Mechanisms of Action 31

1.8 The Gastrointestinal Microbiota and Insulin Resistance 34

1.8.1 Intestinal Barrier Function 34

1.8.2 Gut Microbiota 36

1.8.3 Metabolic Endotoxemia 39

1.8.3.1 The Linkage Between Metabolic Endotoxemia and T2D 42

1.9 Dietary Strategies for Gut Microbiota Modulation 44

1.10 Peas as Sources of Vitamins 45

1.11 Summary 48

Chapter 2: Research Plan

2.1 Rationale 50

2.2 Hypotheses and Objectives 52

Chapter 3: Cooking Enhances Beneficial Effects of PSC Consumption on Glucose Tolerance, Incretin and Pancreatic Hormones in High Fat Diet-fed Rats

3.1 Introduction 55

3.2 Methods and Materials 57

3.2.1 Animals and Diets 57

3.2.2 PSC Preparation and Analysis 62

3.2.2.1 Seed Coat Preparation 62

3.2.2.2 Starch and Protein Analysis 62

3.2.2.3 Non-starch polysaccharides analysis 63

3.2.3 Glucose and Insulin Tolerance Tests 64

3.2.4 Body Weight, Food Intake and Measurement of Body Composition 65

3.2.5 Tissue Collection 66

3.2.6 Assays of Serum 66

3.2.7 Immunohistochemistry and Morphometric Tissue Analysis 66

3.2.8 Quantification of Glucose Transporters Gene Expression 68

3.2.9 Statistical Analysis 70

3.3 Results 70

3.3.1 Fibre Analysis 70

3.3.2 Body Weight and Body Composition Analysis 73

3.3.3 Serum Biochemistry 73

3.3.4 Glucose Tolerance Tests and Insulin Tolerance Test 76

3.3.5 Pancreatic Beta- and Alpha-cell Mass Analysis 81

3.3.6 K- and L-cell Quantification 81

3.3.7 mRNA Expression of Glucose Transporters 85

3.4 Discussion 85

3.5 Conclusion 92

Chapter 4: Assessment of a Potential Role of the Gut Microbiome in the Beneficial Effects of Pea Seed Coat-supplementation on Glucose Homeostasis

4.1 Introduction 94

4.2 Methods 97

4.2.1 Animals, Experimental Diets and Tissue Collection 97

4.2.2 DNA Extraction and Microbial Profiling 98

4.2.2.1 Bioinformatics 98

4.2.3 RNA Extraction and Real-time Polymerase Chain Reaction 99

4.2.4 Statistical Analysis 100

4.3 Results 102

4.3.1 Microbial Community Structure 102

4.3.2 Gene Expression of TLRs, Tight Junction Proteins and Mucins 106

4.4 Discussion 109

4.5 Conclusion 117

Chapter 5: The Effect of Regular Consumption of Pulse-containing Meals on Parameters of Glucose Control, Thiamine and Folate Status in Hypercholesterolaemic Adults

5.1 Introduction 118

5.2 Subjects And Methods 120

5.2.1 Study Design and Overall Protocol 120

5.2.2 Subjects 123

5.2.3 Study Foods 125

5.2.4 Diet and Treatment Protocol 125

5.2.5 Anthropometrics, Food Record and Blood Collection 126

5.2.6 Biochemical Analysis of Serum 127

5.2.7 Statistical Analysis 127

5.3 Results 128

5.3.1 Subjects' Characteristics 128

5.3.2 Fasting Glucose, Insulin, HbA1c and HOMA-IR 129

5.3.3 Dietary Intakes of Thiamine and Folate 135

5.4 Discussion 135

5.5 Conclusion 141

Chapter 6: Final Discussion

6.1 Influence of PSC on Rats' Metabolic Profile 142

6.2 Influence of PSC on HFD-induced Stress in Pancreatic Islets 147

6.3 Influence of PSC on Insulin Resistance Features in the Gut 148

6.3.1 PSC and Incretin Secretion 148

6.3.2 PSC and Nutrient Absorption 151

6.3.3 PSC and Intestinal Microbiota and Permeability 152

6.4 Considerations for Future Directions 154

6.5 Implications of Thesis Findings 156

6.6 Conclusion 158

References 161

Appendix

Appendix A: Applied Physiology, Nutrition and Metabolism manuscript

Appendix B: Study information sheet and consent

Appendix C: Inclusion/exclusion criteria (case report form)

Appendix D: Study foods information

Appendix E: Three-day food record sample

Appendix F: Tracking document

List of Tables

Table 1-1. Summary of clinical trials on the effects of consumption of peas or pea components on markers of glycemic control 23

Table 1-2. Summary of animal trials on the effects of consumption of peas or pea components on markers of glycemic control 25

Table 3-1. Diet composition (g/kg) 61

Table 3-2. Primer sequences for RT-PCR 69

Table 3-3. Sugar components of raw and cooked seed coats of 'Canstar' by GC analysis (mg/100 mg dwt (%)) 71

Table 3-4. Protein and total starch components of raw and cooked PSCs of 'Canstar' 72

Table 3-5. Metabolic profile of rats fed diets containing pea fractions 74

Table 4-1. Primer sequences for RT-PCR 101

Table 5-1. Baseline lipid profile of study participants 124

Table 5-2. Baseline characteristics of study participants 130

Table 5-3. Effects of diet treatments on biomarkers of glucose control and B vitamin status 131

List of Figures

Figure 1-1. Mechanisms of fatty acid-induced inhibition of glucose utilization 12

Figure 1-2. A schematic representation of junctional complexes and the main structural components of the tight junctions 37

Figure 1-3. A simplified model of LPS signaling pathway in endothelial cells 41

Figure 3-1. Schematic diagram showing the experimental outline of the study 59

Figure 3-2. Effect of feeding HFD on oral glucose tolerance 60

Figure 3-3. Plasma GIP concentrations 75

Figure 3-4. Effect of feeding PSC on oral glucose tolerance 77

Figure 3-5. Effect of feeding PSC on intraperitoneal glucose tolerance 78

Figure 3-6. Effect of 4 weeks of feeding PSC on insulin tolerance 80

Figure 3-7. Effect of PSC feeding on pancreatic beta- and alpha-cell mass 82

Figure 3-8. Effect of PSC feeding on the incretin-secreting K- and L-cells 83

Figure 3-9. mRNA Expression of Glucose Transporters 84

Figure 4-1. Effect of feeding PSC on faecal microbial composition 104

Figure 4-2. Microbial taxonomy of faecal samples classified at the family level 105

Figure 4-3. mRNA expression of TLRs and tight Junction proteins 107

Figure 4-4. mRNA expression of mucins 108

Figure 5-1. Flow diagram of the participants through the study 122

Figure 5-2. Correlation of changes in thiamine with changes in glucose, insulin and HbA1c following 6 weeks of intervention 133

Figure 5-3. Changes in dietary intakes of thiamine and folate at baseline and final 134

Figure 6-1. Effects of feeding PSC on hepatic PEPCK levels measured by immunoblotting 146

Figure 6-2. Effects of feeding PSC on mRNA expression of proglucagon and PC1/3 in the ileal mucosa 150

Figure 6-3. Schematic representation of plausible mechanisms of actions through which PSC influences pathways involved in glucose homeostasis in gastrointestinal tract and pancreas 160

List of Abbreviations

ADA	American Diabetes Association
AGEs	advanced glycation end products
AMOVA	analysis of molecular variance
ANOVA	analysis of variance
AP-1	activating protein-1
AUC	area under the curve
BMI	body mass index
CDA	Canadian Diabetes Association
CHO	carbohydrate
CONSORT	consolidated standards of reporting trials
CP	cooked pea seed coat
DAG	diacylglycerol
DNA	deoxyribonucleic acid
DPP	Diabetes Prevention Program
DPP-IV	dipeptidyl peptidase
ELIZA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FFA	free fatty acid
FFAR	free fatty acid receptor
FID	flame ionization detector
G-6-P	glucose-6-phosphate
GAPDH	glyceraldehyde-3-phosphate dehydrogenase

GC	gas-liquid chromatography
GDM	gestational diabetes mellitus
GIP	gastric inhibitory polypeptide
GLP	glucagon-like peptide
GLUT/Glut	glucose transporter
GSIS	glucose-stimulated insulin secretion
HbA1c	hemoglobin A1c
HK	hexokinase
HDL-C	high-density lipoprotein-cholesterol
HFD	high fat diet
HOMA-IR	homeostatic model assessment of insulin resistance
IAUC	incremental area under the curve
IDF	International Diabetes Federation
IDF	insoluble dietary fibre
IFG	impaired fasting glucose
IGT	impaired glucose tolerance
IKK	IκB kinase
IL	interleukin
ipGTT	intraperitoneal glucose tolerance test
IRAK	interleukin-1 receptor-associated kinase
IRS	insulin receptor substrate
ITT	insulin tolerance test
JAM	junctional adhesion molecule

LAM	lipoarabinomannan
LDL	low-density lipoprotein
LDL-C	low-density lipoprotein-cholesterol
LBP	lipopolysaccharide-binding protein
LFD	low fat diet
LPS	lipopolysaccharide
Mal	myeloid differentiation factor 88 adaptor-like protein
MAPK	mitogen-activated protein kinase
MDA	malondialdehyde
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
Muc	mucin
MyD88	myeloid differentiation factor 88
NADPH	nicotinamide adenine dinucleotide phosphate
NCEP	National Cholesterol Education Program
NEFA	non-esterified fatty acid
NF-κB	nuclear factor-κB
NSP	non-starch polysaccharides
oGTT	oral glucose tolerance test
OUT	operational taxonomic units
PAMPs	pathogen-associated molecular patterns
PANDA	Physical Activity and Nutrition for Diabetes in Alberta
PBS	phosphate buffered saline

PC	prohormone convertase
PCoA	principal coordinate analysis
PDH	pyruvate dehydrogenase
PEPCK	phosphoenolpyruvate carboxykinase
PFK	phosphofructokinase
PGN	peptidoglycan
PI 3	phosphoinositide 3
PKC	protein kinase C
PPP	pentose phosphate pathway
PSC	pea seed coat
PYY	peptide YY
RDA	Recommended Daily Allowance
RDP	Ribosomal Database Project
RNA	ribonucleic acid
ROS	reactive oxygen species
RP	raw seed coat
rRNA	ribosomal ribonucleic acid
SCFA	short chain fatty acid
SDF	soluble dietary fibre
SEM	standard error of mean
SGLT	sodium-dependent glucose transporter-1
T1D	type 1 diabetes
T2D	type 2 diabetes

TC	total cholesterol
TG	triglyceride
TLR	toll-like receptor
TNFα	tumor necrosis factor α
TDP	thiamine diphosphate
TRAF	tumor necrosis factor receptor-associated factor
TZD	thiazolidinediones
ZO	zonula occludens
WC	waist circumference
WHO	World Health Organization

Chapter 1: Introduction and Literature Review

1.1 Introduction to Diabetes Mellitus

Diabetes mellitus is recognized as a group of metabolic disorders marked by chronic hyperglycemia and glucose intolerance. It develops mainly as a result of insufficient amounts of insulin, impaired insulin action, or the combination of both (American Diabetes Association [ADA], 2014). Diabetes is categorized into four major types, type 1 diabetes (T1D), type 2 diabetes (T2D), gestational diabetes mellitus (GDM), and other types (International Diabetes Federation [IDF], 2013); however, the majority of diabetic cases can be classified as either T1D or T2D, with T2D accounting for approximately 90-95% of people with diabetes (ADA, 2014). While T1D is characterized by the absence of sufficient insulin secretion, T2D develops as a result of a defect in the ability of cells to respond to insulin, accompanied by a deficient compensatory secretion of insulin by pancreatic islet cells (ADA, 2014; IDF, 2013).

Diabetes is currently one of the fastest growing chronic diseases in the world. In 2013, the prevalence of diabetes worldwide was estimated to be 382 million, and is expected to rise to 592 million by 2035 (IDF, 2013). A similar pattern is emerging in Canada with diabetes affecting 2.4 million Canadians in 2009, which corresponds to 6.8% of the total Canadian population and the prevalence is estimated to increase to 3.7 million by 2019 (Canadian Diabetes Association [CDA], 2013).

In a similar fashion, the global economic burden of diabetes is on the rise; in 2013, IDF estimated that the total health care expenditures attributable to diabetes worldwide were at the very least $548 billion USD. In Canada, the economic burden of diabetes was estimated at $12.2 billion CAD in 2010. By 2020, that number is projected to grow by $4.7 billion CAD (CDA, 2013). The total cost imposed on the health care system is associated with diabetes itself and diabetes-related complications. The most prevalent long-term health complications of diabetes are macrovascular and microvascular manifestations caused by chronic hyperglycemia of diabetes. Macrovascular complications include atherosclerotic cardiovascular, peripheral vascular and cerebrovascular disease. Microvascular complications on the other hand include diabetic retinopathy, nephropathy and neuropathy, which, over time, can lead to serious health conditions such as loss of vision, renal failure and foot amputations (ADA, 2014; CDA, 2013).

1.2 Type 2 Diabetes Overview

T2D, previously known as non-insulin-dependent diabetes, is the most common form of diabetes mellitus mainly witnessed in individuals with either overall obesity or abdominal adiposity (ADA, 2014; Scheen, 2003). As previously mentioned, this type of diabetes is caused by resistance to insulin action, along with insufficient compensatory insulin secretion due to loss of pancreatic beta-cell function. As a multifactorial disease, T2D results from an interaction between environmental factors such as obesity and sedentary lifestyle, and a definite genetic component (CDA, 2013; Scheen, 2003). Due to differences in genetic

predisposition, some people are more susceptible to developing diabetes than others even in the presence of identical environmental elements.

T2D is a progressive disease that can remain undiagnosed for a very long period of time as a result of the asymptomatic nature of the early phase of sustained hyperglycemia (ADA, 2014; CDA, 2013). As a consequence of this delay, approximately 50% of beta-cell function is already lost in T2D patients by the time they are diagnosed with their condition (Fonseca, 2007). This highlights the important role of early screening and diagnosis in the treatment of T2D. As hyperglycemia develops over the years, the symptoms and complications of diabetes progress, calling for more intense treatment requirements.

1.3 Prediabetes

It is firmly established that the ultimate manifestation of T2D is typically preceded by prediabetes, a condition in which blood glucose concentrations are elevated to higher than normal without reaching the required cutoff values for diagnosis of T2D (Fonseca, 2007). Prediabetes is characterized by impaired fasting glucose (IFG), impaired glucose tolerance (IGT), or the combination of both conditions (Bergman, 2013; Fonseca, 2007). IFG refers to fasting plasma glucose levels of 6.1 to 6.9 mmol/L, while IGT is defined by a 2-hour plasma glucose (after a 75 g oral glucose load) values of 7.8 to 11 mmol/L (CDA, 2013). According to CDA guidelines (2013), prediabetes is also diagnosed by hemoglobin A1c (HbA1c) levels of 6.0% to 6.4%. ADA, however, uses slightly different cutoff

values and defines prediabetes with any of the following criteria: a fasting plasma glucose value of 5.6 to 6.9 mmol/L, a 2-hour plasma glucose of 7.8 to 11 mmol/L after ingestion of 75 g of oral glucose load, or an HbA1c between 5.7% and 6.4% (ADA, 2014). Despite different underlying pathophysiological mechanisms, both IGT and IFG represent dysregulated glucose metabolism and incidence of insulin resistance (Fonseca, 2007).

The proportion of the world's population being affected by prediabetes is almost comparable to the increasing prevalence of T2D. According to IDF (2013), the total number of adults with IGT is estimated to reach 472 million by 2030. It is believed that up to 70% of prediabetic individuals will ultimately develop diabetes (Bergman, 2013), pointing out the importance of intervention at the stage of prediabetes to reverse the condition and prevent T2D.

1.4 Pathophysiology of Prediabetes

According to Weir's multistage model of diabetes development, prediabetes occurs during the second stage known as "stable adaptation" and extends to early third stage or "unstable early decompensation" (Weir & Bonner-Weir, 2004). The stable adaptation period is defined by presence of insulin resistance, while beta cells fail to completely compensate for it. It is also accompanied by changes in beta cell function and differentiation. Consequently, fasting and postprandial glucose levels are elevated to higher than normal and similar to those defined in IFG and IGT. This period is stable in the sense that individuals in this stage could steer clear

of developing diabetes for years. As transition to level three happens, beta cell dysfunction reaches a critical stage; thus, blood glucose levels start to rise at a relatively fast pace from prediabetic ranges to diabetic ranges seen in stage four or "unstable early decompensation". In summary, the core mechanisms underlying dysregulated glucose metabolism manifested in prediabetes can be narrowed down to insulin resistance and impaired insulin secretion due to beta cell dysfunction.

1.4.1 Glucose Metabolism and Homeostasis

Glucose homeostasis is a complex process through which the body maintains plasma glucose concentrations in a balanced range. Under normal physiological conditions, circulating glucose concentration is tightly regulated through interplay between pathways involved in glucose appearance and glucose clearance. Glucose appearance in the circulation relies upon exogenous glucose from food intake and endogenous glucose production in the liver.

In the fed state, blood glucose rises following ingestion of food, which results in stimulation of insulin secretion from pancreatic beta cells. Once released, insulin initially acts on skeletal muscle and adipose tissue to promote peripheral uptake of glucose from the blood. In the liver, insulin suppresses hepatic glucose output via inhibition of glycogenolysis and gluconeogenesis, as well as the promotion of glycogenesis (Aronoff et al., 2004, Kruger et al., 2006). While insulin is the primary hormone involved in glucose clearance, there are other glucoregulatory hormones that affect this process. In response to dietary nutrients,

mainly carbohydrates and lipids, intestinal enteroendocrine cells produce and release two incretin hormones, gastric inhibitory polypeptide (GIP), also called glucose-dependent insulinotropic polypeptide, and glucagon-like peptide-1 (GLP-1), into the blood stream (Kim & Egan, 2008; Seino et al., 2010). Both GIP and GLP-1 affect the glucose responsiveness of pancreatic beta cells by promoting insulin release (Aronoff et al., 2004; Kim & Egan, 2008), which is known as the "incretin effect". GLP-1, but not GIP, also suppresses postprandial glucagon secretion from pancreatic alpha cells, which further enhances the glucose-lowering potency of this incretin hormone (Kim & Egan, 2008; Kruger et al., 2006). Postprandial glucagon secretion can also be inhibited by amylin, which is another anabolic hormone secreted along with insulin from pancreatic beta cells (Aronoff et al., 2004; Kruger et al., 2006).

On the contrary, glucose control in the fasting state is not affected by incretins or amylin, and is mainly regulated by glucagon. Glucagon, which is the counter-regulatory hormone of insulin action, is secreted from the alpha cells of the pancreas and acts on the liver to increase hepatic glucose production, hence leading to increases in circulating blood glucose (Cherrington, 1999). During the first few hours of fasting, enhanced glucose output in the liver is the result of increased glycogenolysis, which is replaced by gluconeogenesis during longer periods of fasting. At the same time, glucagon also inhibits glycogen synthesis in the liver and affects glucose metabolism through inhibition of glycolysis (Jiang & Zhang, 2003).

Glucose uptake, transport and disposal are facilitated by membrane transporter proteins, which vary depending on the tissue type. There are two main classes of glucose transporters, Na^+-coupled glucose transports (sodium-dependent glucose transporters, SGLTs) and the facilitative glucose transporters (GLUTs) (Bell et al., 1990; Thorens & Mueckler, 2010). SGLTs transport glucose against the concentration gradient by using energy generated from coupling glucose transport with Na^+ downgradient movement (Wright et al., 2011). SGLT proteins are expressed in a variety of tissues; SGLT1 is abundantly expressed in the epithelial cells of small intestine, while SGLT2 is exclusively present in kidney (Kanai et al., 1994; Wright et al., 2007). To date, the GLUT family of glucose transporters consists of 14 members with GLUT1 and being the most widely expressed transporters across intestinal epithelia. In the intestinal lumen, glucose uptake is mediated by SGLT1 in the brush border membrane, which in turn promotes the recruitment of GLUT2 into this membrane, allowing for high capacity absorption of glucose from the lumen. Once inside the enterocytes, glucose is transported into the blood through facilitated diffusion by GLUT2 in the basolateral membrane (Kellet et al., 2008; Roder et al., 2014). Both SGLT1 and GLUT2 are also important for glucose sensing by the enteroendocrine cells and consequent secretion of incretins (Gorboulev et al., 2012; Mace et al., 2012). In the liver, there is evidence of expression of several GLUTs such as GLUT1, GLUT2, GLUT9 and GLUT10 with GLUT2 being the primary glucose transporter (Karim et al., 2012; Thorens et al., 1990). GLUT2 is also highly expressed in the pancreatic beta cells (Orci et al., 1990; Thorens et al., 2000). GLUT4, on the other hand, is the main

glucose transporter in the insulin sensitive tissues muscle and adipose tissue; in response to insulin, GLUT4 translocates from intracellular stores to the cell membrane in order to facilitate glucose uptake, thus having an important rate-limiting role in insulin-stimulated glucose disposal (Fukuda et al., 2009; Hashiramoto & James, 1998; Ishiki & Klip, 2005).

Homeostatic control of blood glucose ensures that circulating glucose concentrations are maintained in a relatively small range. According to WHO guidelines on diabetes (2006), normal glucose levels cannot be precisely defined due to lack of sufficient data; it is however advised that normoglycemia should be used to refer to glucose levels below those corresponding to IFG and IGT. The balanced homeostasis of blood glucose occurs as a result of the intricate interaction between the organs, hormones and feedback systems involved in glucose homeostasis. Any alteration of the normal glucose regulation can lead to metabolic disturbances and eventually diabetes mellitus, among other chronic diseases.

1.4.2 Insulin Resistance

Insulin plays a critical role in whole body glucose turnover; it is released from the beta cells in response to changes in blood glucose concentration, which then communicates with various effector tissues to regulate glucose clearance. The diminished ability of these target cells to respond to insulin leads to the physiological condition known as insulin resistance (Lebovitz, 2001). In general, insulin resistance is defined as impaired glucose tolerance in the presence of

normal or elevated circulating levels of glucose and insulin (Ferrannini, 2006). It is a complex disorder, which has been linked to multiple etiological pathways in several tissues, which will be discussed in the following sections.

1.4.2.1 Free Fatty Acids and Insulin Resistance

Excess calorie intake beyond energy requirements results in the expansion of adipose tissue, which plays an integral role in the pathophysiology of insulin resistance. As the adipose tissue mass expands, free fatty acid (FFA) release from the adipocytes increases, which in turn leads to elevated concentrations of FFA in the plasma (Qatanani & Lazar, 2007). Naturally, normal fatty acid homeostasis occurs as a result of a balance between lipolytic activity of adipose tissue and FFA uptake by peripheral tissues. When normal fatty acid metabolism is dysregulated, it induces insulin resistance in insulin-sensitive non-adipose tissues through generation and accumulation of unfavorable fat metabolites such as diacylglycerides and ceramides (Lewis et al., 2002; Raz et al., 2005).

Several mechanisms have been proposed to explain the detrimental effects of increased availability of FFA on glucose metabolism. It was first proposed by Randle et al. (1963) that elevated levels of circulating fatty acid lead to increased lipid oxidation and abnormalities of carbohydrate metabolism similar to those reflected in insulin resistance. According to the Randle cycle, elevated fatty acid oxidation, secondary to increased FFA flux, results in the elevation of acetyl-CoA production. This in turn inhibits the activity of pyruvate dehydrogenase (PDH) and

subsequently increases intracellular content of citrate. Increased levels of citrate then inhibit phosphofructokinase (PFK), leading to accumulation of glucose-6-phosphate (G-6-P). Increased intracellular G-6-P concentration results in inhibition of hexokinase (HK) activity, subsequent elevation of intracellular glucose concentrations and eventually a decrease in glucose uptake in the peripheral tissues (Figure 1-1A).

In a more recent hypothesis, Shulman (2000) suggested that intermediate FA metabolites play a more crucial role in explaining the FFA-induced insulin resistance. He proposed that increased FFA delivery to insulin responsive cells results in the elevation of intracellular levels of fatty acid metabolites such as acyl CoAs, diacylglycerol (DAG), and ceramides. These fatty acid derivatives activate a serine/threonine kinase cascade, which impairs the binding capacity of insulin receptor substrates IRS-1 and IRS-3, resulting in a marked decrease in phosphoinositide 3 (PI 3)-kinase activity. Disruption of PI 3-kinase pathway, as a key mediator of insulin signaling, leads to inhibition of insulin metabolic actions. In the muscle, intramyocellular lipid accumulation impairs insulin-mediated glucose uptake via impaired translocation of GLUT4, which subsequently results in decreased insulin-dependent glycogen synthesis. As a result of a decrease in glucose utilization in skeletal muscle, excess glucose is diverted to liver, where insulin actions on gluconeogenesis and glycogen synthesis are impaired by increased intrahepatic lipid content. This results in increased hepatic glucose output due to increased gluconeogenesis and decreased glycogen synthesis. The end result

is a decrease in glucose utilization in skeletal muscle and increased glucose output in the liver (Figure 1-1B) (Petersen & Shulman, 2002; Samuel & Shulman, 2012; Shulman, 2000).

In addition to liver and muscle, impaired insulin action in the adipose tissue also results in elevated lipolysis in adipocytes. This further promotes re-esterification of FFA in other tissues such as liver, which consequently worsens the existing hepatic lipid accumulation and insulin resistance. Overall, several physiological pathways involved in the pathogenesis of insulin resistance include: altered fat distribution (ectopic to visceral), activation of inflammatory responses via increased secretion of cytokines such as tumor necrosis factor α (TNFα) and interleukin-6 (IL-6), and Toll-like receptors, reduced insulin-stimulated glycogen synthesis, decreased mitochondrial function, and endoplasmic reticulum (ER) stress (Bergman, 2013; Martins et al., 2012, Petersen & Shulman, 2002; Qatanani & Lazar, 2007; Samuel & Shulman, 2012; Shulman, 2000).

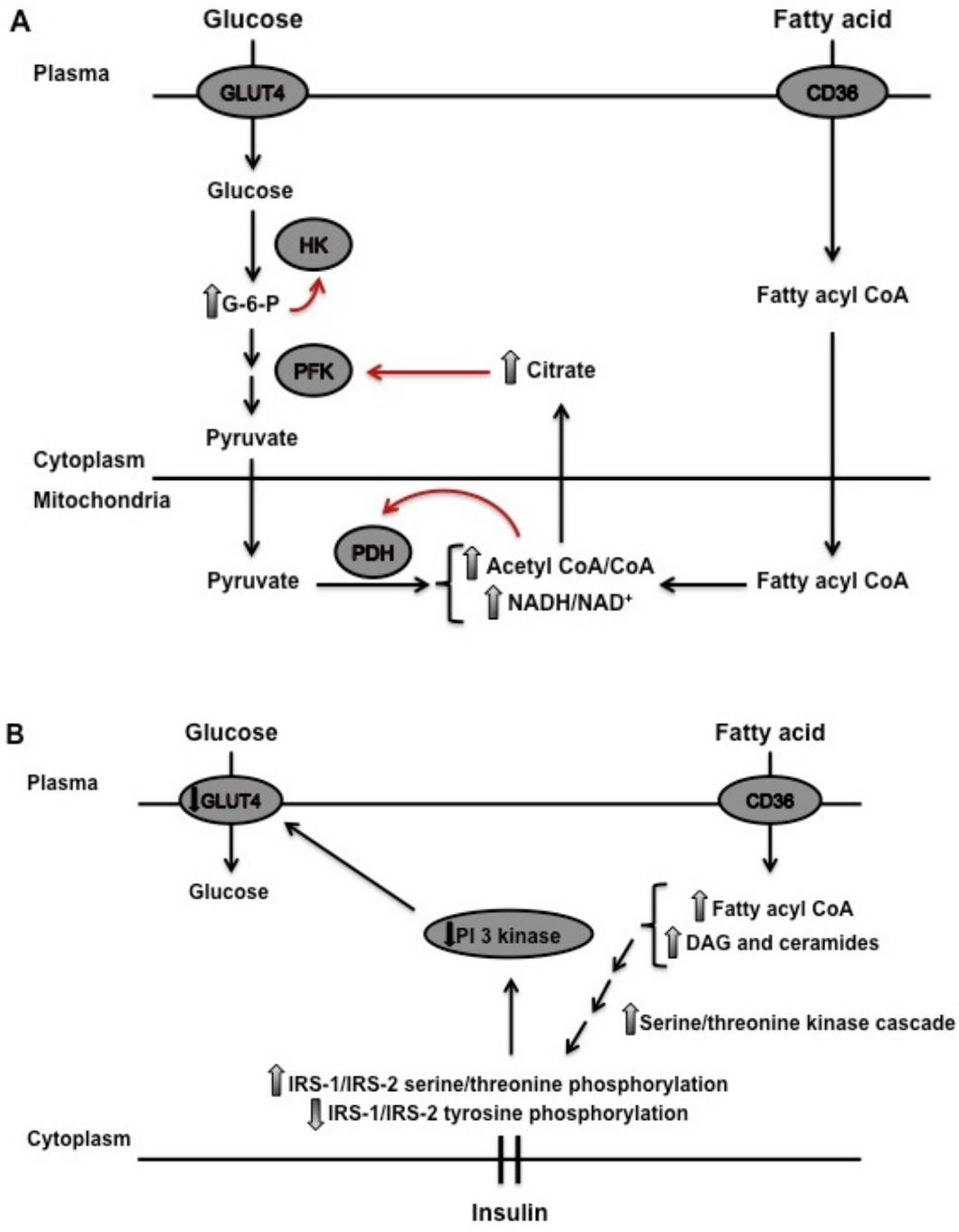

Figure 1-1. Mechanisms of fatty acid-induced inhibition of glucose utilization. (A) Mechanism of inhibition of glucose utilization by fatty acid oxidation known as the Randle cycle. (B) Mechanism of fatty acid-induced insulin resistance as proposed by Shulman. See text for details (adapted from Randle, 1998 and Shulman, 2000).

1.4.3 Impaired Insulin Secretion

Impairment of insulin secretion results from pancreatic beta cell dysfunction caused by several underlying mechanisms including insulin resistance, chronic hyperglycemia and impaired incretin action. Chronic hyperglycemia *per se* is the major factor resulting in impaired beta cell function and insulin secretion through mechanisms involved in glucose desensitization, beta cell exhaustion and glucotoxicity. Both glucose desensitization and beta cell exhaustion are considered as acute reversible effects of hyperglycemia, and respectively refer to impaired insulin exocytosis in response to prolonged hyperglycemia in desensitized islets, and depletion of intracellular stores of insulin (Bergman, 2013; Cernea & Dobreanu, 2013). Glucotoxicity, however, pertains to irreversible effects of sustained hyperglycemia on production and secretion of insulin in glucotoxic islets. In other words, defective insulin secretion can only be reversed up to a certain point, implying that beta cell exhaustion is a progressive condition and the detrimental effect of glucotoxicity on beta cell function creates a vicious cycle that ultimately results in beta cell apoptosis. Some of the mechanisms that mediate this process are: dysregulation and loss of insulin gene expression, ER stress, reactive oxygen species (ROS)-induced oxidative stress and mitochondrial dysfunction (Cernea & Dobreanu, 2013).

1.5 Interventional Treatments to Prevent T2D

1.5.1 Lifestyle Interventions

Lifestyle modifications refer to interventions aimed at two key modifiable risk factors for the development of T2D, namely obesity and physical inactivity. They are accepted as powerful management tools to reverse prediabetes and prevent or delay its transition to diabetes (Tabak et al., 2012). Lifestyle modifications have been shown to be effective in terms of improving insulin sensitivity and recovering beta cell function in prediabetic patients.

Several clinical trials have reported considerable reductions in the number of new cases of diabetes in IGT subjects following diet and/or exercise interventional programs. The Da Qing IGT and Diabetes Study was one of the very first studies that examined the impact of diet only, exercise only and diet plus exercise on diabetes prevention in IGT patients. They showed that these interventions were respectively associated with 31%, 46% and 42% reductions in the incidence of diabetes compared to a control group (Pan et al., 1997). The Diabetes Prevention Program (DPP) is another randomized controlled clinical trial that investigated the impact of intensive lifestyle intervention on the incidence of diabetes. The intensive lifestyle intervention was planned to ensure participants achieved and maintained a weight loss of ≥7% of initial body weight and 150 minutes/week of moderate intensity physical activity. Patients in this group received 16 individualized sessions focused on lifestyle modification strategies followed by additional group and individual sessions. Meanwhile, a standard

lifestyle intervention group, with placebo or metformin, received written recommendations and one annual individual session on the benefits of a healthy lifestyle in the prevention of diabetes. Compared with standard lifestyle modification plus placebo or metformin, the diabetes incidence was reduced by 58% and 39% in the intensive lifestyle intervention group (Knowler et al., 2002). In both studies, the participants were followed up for several years, confirming that the reduction in the incidence of diabetes was sustained even in the long term, although at a lower rate (DPP Research Group, 2009; Li et al., 2008). Lifestyle medications also have the advantage of being cost-effective compared to other common types of modifications (Bergman, 2013; Fonseca, 2007). The optimal modifications are defined as achieving a 7% weight loss, mainly through reducing caloric intake and other dietary changes, and completing 150 min/week of moderate intensity physical activity (DPP Research Group, 2009; Knowler et al., 2002).

1.5.2 Pharmacologic Interventions

While healthy lifestyle modifications need to be practiced as the primary treatment option for prevention of diabetes in prediabetic population, pharmacologic treatment should be considered as a complementary approach if lifestyle interventions fail to be effective. It is suggested that a combination of diet/exercise and pharmacologic treatment could also prevent or delay the onset of diabetes through preservation of beta cell function and improving insulin sensitivity (Bergman, 2013; Fonseca, 2007).

Several oral agents have been used to reverse prediabetes and prevent diabetes in prediabetic patients. Metformin, which is a biguanide, is one of the most studied antidiabetic agents. There are reports of beneficial effects of metformin on reducing body mass index (BMI) and improving lipid profile (Tabak et. al., 2012). It reduces fasting plasma glucose and HbA1c, but fails to act on insulin secretion or preserve beta cell function (Bergman, 2013). Metformin appears to exert its antidiabetic effect via several mechanisms including reduction of hepatic glucose production through inhibition of gluconeogenesis, improvement in peripheral insulin sensitivity and modulation of the incretin effect; however, the primary function of metformin contributing to its beneficial effects on glucose tolerance is considered to be decreasing hepatic glucose production (Hundal et al., 2000; Maida et al., 2011; Viollet et al., 2012). In the DPP study, adherence to a metformin regimen reduced the rate of conversion to T2D by 31%, although it is noteworthy to mention that metformin success rate was still lower than the results seen in the lifestyle modification groups (Knowler et al., 2002). Similar to lifestyle interventions, metformin therapy is also claimed to be cost-effective, especially if used in its generic forms (Bergman, 2013).

Other forms of oral agents used to target diabetes prevention include: thiazolidinediones (TZD), acarbose, orlistat, GLP-1 analogs and dipeptidyl peptidase (DPP)-IV inhibitors. While all of these medications have been shown to be effective, albeit at dissimilar rates, in terms of preventing against progression to diabetes, some of them are accompanied by severe side effects. Therefore, it is

important to take into account all aspects of a treatment option before using it as a therapy (Bergman, 2013; Fonseca, 2007).

1.6 Nutrition Therapy for the Prevention and Management of Diabetes

Nutrition therapy plays an integral role in the management of diabetes and prediabetes. While the dietary modifications for T2D patients have several components, the recommendations for prediabetic individuals are mainly focused on modest weight loss management (7% body weight) through healthy and reduced calorie diets (ADA, 2014; CDA, 2013). CDA guidelines recommend that T2D patients should receive nutrition counseling and education and follow *Eating Well with Canada's Food Guide* for a nutritionally balanced diet. Patients who are obese and overweight are encouraged to achieve moderate and sustainable weight loss using healthy, balanced and calorie-conscious diets. The recommended macronutrient distribution of the diet is the same as the recommended ranges for general population (45-60% of total energy from carbohydrates, 15-20% from protein and 20-35% from fat). There are also some restrictions noted regarding energy from saturated fat (<7% of total daily energy intake) and added sucrose and fructose (<10% of total daily energy). Following a regular pattern in terms of timing and spacing of meals, opting for carbohydrate sources with a low glycemic index and using alternative dietary patterns that ameliorate glycemic control are some of the other highlights of these guidelines. A new addition to the latest CDA guidelines is recommendations on dietary patterns. Interestingly, one of the dietary patterns recommended (Grade B, Level 2 recommendation based on evidence from

randomized control trials and systematic reviews) is incorporation of dietary pulses including beans, peas, chickpeas and lentils into the diets of T2D patients (CDA, 2013).

1.6.1 Health Benefits of Peas (*Pisum Sativum*)

Pulses are dried seeds of non-oil-seed legumes that include dried beans, dried peas, lentils and chickpeas. Pulse crops are characterized as rich sources of fibre, protein and antioxidants, while being low in fat and energy (Marinangeli & Jones, 2011a). They also contain several vitamins and minerals such as iron, zinc, magnesium, folate and thiamine (Dahl et al., 2011; Pulse Canada, 2014). These characteristics along with the fact that pulses are low-cost and readily available food commodities have resulted in widespread interest in studying their health benefits both in animals and humans.

Dried peas are seeds of pea plant, scientifically known as *Pisum sativum L.*. Pea seeds are composed of two main structures including an embryo and a seed coat (hull). The embryo is mostly composed of starch and protein, while the seed coat is largely soluble and insoluble fibre (about 70% dry weight), with a small amount of protein (Duenas et al. 2004; Guillon & Champ, 2002; Whitlock et al., 2012). Both embryo and seed coat components contain minerals and vitamins (Guillon & Champ, 2002).

Evidence from multiple epidemiological and interventional studies in humans and animals suggest that peas and their components can favorably modify glycemic response and insulin resistance as seen in prediabetes and T2D (Dahl et al., 2012; Guillon & Champ, 2002). Most of the beneficial effects of peas in regard to glycemic tolerance have been attributed to their dietary fibre content. While there are several mechanisms that have been proposed to underlie these positive effects, the exact processes through which peas exert their effects remain to be fully understood. However, there are indications that pulse-derived fibre has benefits on glucose lowering distinct from other fibres (Eslinger et al., 2014; Marinangeli & Jones, 2011b; Marinangeli et al., 2011; Whitlock et al., 2012) and, further, that fibres from different pulses may also have differential benefits (Schafer et al., 2003; Shams et al., 2010; Sievenpiper et al., 2009; Thompson et al., 2012).

1.6.1.1 Health Outcomes in Clinical Studies

In one of the very first studies to investigate the effect of pea fibre on glycemic control, Hamberg and colleagues (1989) reported that incorporating 15 g of pea fibre into a control meal resulted in reduced glucose response in healthy subjects. The amount of the pea fibre, however, did not match the fibre dose of the control group, and the study was rather small with only eight participants. Another study examined the effect of pea fibre added to a balanced diet on glycemic control (Sandström et al., 1994). They reported reduced postprandial insulin concentrations in healthy subjects after consuming 16.7 g/day of pea fibre preparation for two

days, whereas the glycemic response was not affected by the diet. One major limitation of this study was using a low fibre diet as control, which makes it impossible to elucidate if pea fibre was more beneficial than the unspecified dietary fibre included in the control group. In another randomized control study by Seewi et al. (1999), pea starch, when compared with maze starch as control, was shown to significantly reduce post meal glucose and insulin concentrations.

In a more recent study consumption of a meal containing whole dried peas, alone or mixed with potatoes, both delayed and reduced the increase in postprandial glucose and insulin excursions when compared with potato-based meals (Schafer et al., 2003). Despite being isocaloric, treatment diets did not contain similar amounts of dietary fibre. Marinangeli et al. (2009) also investigated the benefits of incorporating whole yellow pea flour into select foods (banana bread, biscotti and pasta) by comparing them to boiled yellow peas and white bread as controls. The authors concluded that foods prepared with whole pea flour as the main source of carbohydrate resulted in reduced postprandial glucose responses in healthy individuals. While the pea-containing foods were matched for carbohydrate content, the negative control, white bread, most probably had less dietary fibre, although it is not discussed in the paper. It is important to control for fibre content since it alters the overall glycemic index of the diet independent from the type of fibre, which could as a result affect the primary outcomes.

There are also a few studies that have assessed the impact of different fractions of pea on glycemic and insulin response in humans. Lunde and colleagues (2011) investigated the effect of pea fractions and showed that consumption of pea-fibre-enriched breads resulted in improved post-prandial glucose tolerance in human subjects with a high risk of developing T2D. The pea-containing breads, however, also contained higher amounts of protein and were less calorie-dense compared to the control low fibre bread. Additionally, pea fibre was not the sole source of dietary fibre in the breads, making it unlikely to draw firm conclusions. A study by Marinangeli & Jones (2011b) compared the effect of whole pea flour and fractioned pea flour from pea hulls on markers of insulin resistance. Their results demonstrated that whole pea flour resulted in a larger reduction in fasting insulin concentrations (-13.5%) compared to the fractioned pea flour (-9.8%). Degree of insulin resistance measured by homeostatic model assessment of insulin resistance (HOMA-IR) was reduced to the same extent in both experimental groups, while glucose concentrations remained unchanged. The background diet consumed by participants was closely controlled to ensure weight fluctuations were not affecting the results. In another study, comparison of two different doses (10 g versus 20 g) of pea fibre and pea protein consumption in healthy adults revealed that pea protein was the beneficial fraction contributing to improved 2-h glucose control; the authors reported that this effect of the pea protein fraction on postprandial glycemia was dose-independent (Smith et al., 2012).

In a series of studies (Mollard et al., 2012; Mollard et al., 2014b) consumption of whole yellow peas incorporated in a control meal (tomato soup or pasta) resulted in lower postprandial glycemia in healthy men when compared with a control meal with no pulses. These studies also examined the effect of chickpeas, lentils and navy beans on blood glucose response and showed that the effect of yellow peas on postprandial glycemia was less pronounced than the other pulses. The yellow peas treatment, however, contained the least amount of fibre amongst the pulse treatments (9.1 g fibre) in the latter study (Mollard et al., 2014b). In addition, food consumption was *ad libitum* in the first study, which resulted in different food intakes and consequent varying fibre intake (Mollard et al., 2012). Another primary objective of these studies was to investigate the impact of pulses on blood glucose response following consumption of a second meal 2 h or 4 h post pulse consumption. They found that the primary effect of yellow peas on glycemia was not sustained after the second meal (referred to as the "second-meal effect").

Table 1-1. Summary of clinical trials on the effects of consumption of peas or pea components on markers of glycemic control

Study	Design	Participants/duration	Intervention diet(s)	Control diet	Outcomes
Hamberg et al. (1989)	Randomized, controlled cross-over clinical study	Eight healthy subjects/1-day treatments separated by minimum 3-day washout periods	30 g pea fibre (15 g pure fibre) added to bread served with a ground beef- based control meal	Similar control meal with no added fibre (white flour as carbohydrate source)	Improved postprandial glucose control (AUC glucose), no change in insulin response
Dubois et al. (1993)	Randomized, controlled crossover clinical study	Six healthy participants	10 g pea fibre added to a low fibre control meal	Low fibre control meal (2.8 g fibre)	No changes in post meal glucose and insulin responses
Sandström et al. (1994)	Randomized, controlled crossover clinical study	Eight healthy subjects/2-day treatments with 2-week washout periods	16.7 g pea fibre preparation added to bread served with breakfast and lunch	Low fibre diet matched for macronutrients distribution and energy	Enhanced postprandial insulin response, no change in glucose concentrations
Seewi et al. (1999)	Randomized, controlled clinical study	Ten healthy individuals/1-day treatments	30 g pea starch dissolved in 500 ml tap water	30 g maize starch preparation dissolved in 500 ml tap water	Reduced postprandial insulin and glucose concentrations
Schafer et al. (2003)	Randomized, controlled cross-over clinical study	Nine patients with T2D/1-day treatments with 1-week washout intervals	Mixed meals with whole dried peas (15 g fibre) or whole dried peas (10 g fibre) and potatoes (2:1) as CHO source	Isocaloric test meal with potatoes as CHO source	Delayed and reduced postprandial glucose and insulin excursions
Marinangeli et al. (2009)	Randomized, controlled, crossover clinical study	Nineteen healthy participants/1-day treatments with 2-day washout periods	Whole yellow pea flour (50 g CHO) in pasta (30%), banana bread and biscotti (100%)	Boiled yellow peas and white bread	Reduced glycemic responses in 100% pea flour groups (IAUC glucose)
Lunde et al. (2011)	Randomized, controlled cross-over clinical study	Ten participants with central obesity (WC≥80 cm)/1-day with 1-week washout periods	Pea hull fibre added to low fat and high fat bread (20 & 22 g total dietary fibre)	Low fibre bread (2.8 g total dietary fibre)	Improved postprandial glucose control (IAUC) in both pea groups

Marinangeli & Jones (2011b)	Randomized, controlled, single-blind crossover clinical study	Twenty-three hypercholesterolaemic overweight patients/4-week with 4-week washout periods	50 g CHO from whole pea flour or fractioned (pea hulls) pea fibre added to muffins in a NCEP-Step 1 diet	50 g CHO from white wheat flour	Reduced fasting insulin and improved insulin resistance (HOMA-IR) in both pea groups
Smith et al. (2012)	Single-blind, randomized, repeated-measures clinical study	Nineteen or tewnty healthy participants in experiments 1 & 2/1-day followed by 1-week washout periods	10 or 20 g pea fibre or pea protein added to a tomato soup meal	Tomato soup (2 g protein, 2g dietary fibre)	Improved postprandial glucose control in pea protein groups in experiment 1
Mollard et al. (2012)	Within-subject, repeated-measures clinical study	Twenty four healthy participants /1-day followed by 1-week washout periods	*ad libitum* pulse meal: yellow peas (2 g/100g fibre), chickpeas (2.1 g/100g fibre), lentils (2.5 g/100g fibre) or navy beans (3.2 g/100g fibre) added to a pasta meal	Pasta meal	Reduced blood glucose response following yellow pea consumption, no change in AUC and no second meal effect on blood glucose
Mollard et al. (2014a)	Randomized, single-blind crossover clinical study	Fifteen healthy participants/1-day treatments followed by 1-week washout periods	Pea hull fibre (7 g), pea protein (10 g), pea hull fibre + pea protein (7 g fibre) added to noodles and tomato sauce; or whole peas (406) served with tomato sauce	Noodles and tomato sauce	Lower AUC blood glucose in protein+fibre and whole peas, second-meal effect on blood glucose only seen with whole peas
Mollard et al. (2014b)	Randomized, crossover clinical study	Fifteen healthy participants/1-day treatments followed by 1-week washout periods	375.6 g whole yellow peas with 9.1 g fibre added to a tomato soup meal. Other pulses tested included: chickpeas (11.3 g fibre), lentils (13.5 g fibre) or navy beans (16.6 g fibre) added to tomato soup	White bread (2.8 g fibre) dipped in tomato soup	Reduced postprandial glucose response in the yellow pea group (2 h response)

AUC, area under the curve; CHO, carbohydrate; HOMA-IR, homeostatic model assessment of insulin resistance; IAUC, incremental area under the curve; NCEP, National Cholesterol Education Program; WC, waist circumference.

Table 1-2. Summary of animal trials on the effects of consumption of peas or pea components on markers of glycemic control

Study	Design	Intervention diets	Control diet	Outcomes
Marinangeli et al. (2011)	Forty-five male golden hamsters at 4 weeks of age were fed a hypercholesterolemic diet for 4 weeks	10 g of whole pea flour and fractioned pea flour (pea hulls) added to 100 g control diet (replacing 10% of corn strach). 4 weeks of treatment diets.	Control diet (10.5 g cellulose)	Fractioned pea flour reduced fasting insulin and glucose. Whole pea flour reduced circulating insulin.
Whitlock et al. (2012)	Male Sprague Dawley rats were fed a HFD (20% w/w) for three weeks	100 g of pea embryo, 56.5 of non-coloured see coat and 75 g of coloured seed coat were added to HFD (8% total fietary fibre). 4 weeks of treatment diets.	HFD (8% cellulose)	Pea seed coats diets improved glycemic control. Non-coloured seed coat reduced fasting insulin and improved GSIS.
Eslinger et al. (2014)	Fifty male Sprague Dawley rats made obese following 5 weeks of high fat/high sucrose diet.	Yellow pea fibre, yellow pea flour and yellow pea starch were added to AIN-93 M control diet (30% w/w)	Control diet and control diet with oligofructose (13% w/w)	Pea fibre diet resulted in lower fasting glucose and postpradial glucose control (AUC)

AUC, area under the curve; GSIS, glucose-stimulated insulin secretion; HFD, high fat diet.

The same research group (Mollard et al., 2014a) also investigated the effect of consumption of different components of peas (protein or pea hull fibre) versus whole peas on blood glucose response and food intake. They reported that a combination of pea protein and pea hull fibre (10 g pea protein plus 7 g pea fibre) was more effective in reducing postprandial blood glucose than each fraction alone. This effect was also comparable to that seen with whole peas. The study however used two different control meals with the pulse fractions, which were not matched for calorie or nutrient content. The authors concluded that incorporation of pea fractions in a meal with high glycemic carbohydrate reduced the glycemic response in healthy participants, but did not affect appetite, food intake or post second-meal glycemia.

In one of the early investigational trials, Dubois and colleagues (1993) failed to show any beneficial effects of pea hull consumption on glucose and insulin response in healthy subjects with incorporation of 10 g of dietary fibre from pea hulls into a complex meal. They had a very small sample size (six participants) and only used 10 g of pea fibre in their test meal, which could explain the lack of significant effect on glucose and insulin concentrations. A summary of clinical trials investigating the effects of consumption of peas or pea components on markers of glycemic control is provided in Table 1-1.

1.6.1.2 Health Outcomes in Animal Studies

Potential effects of dietary interventions of pea and pea fractions on markers of glycemic control and insulin resistance have been studied in limited number of animal trials (Table 1-2). In one recent study, hamsters were fed a hypercholesterolemic diet with a partial substitution of cornstarch with either untreated whole pea or pea hull flour as treatment diets. The authors reported that both experimental diets resulted in significant decreases in circulating glucose and insulin levels when compared to control group (Marinangeli et al., 2011b). It is worth noting that the control group contained less amount of total dietary fiber and the effect of pea feeding was only assessed in fasted state.

Whitlock et al. (2012) compared the use of uncooked pea preparations made from embryo and seed coats on markers of glucose homeostasis in insulin resistant rats. They found that diets supplemented with pea seed coat fractions resulted in improved glucose tolerance, and reduced fasting and post-glucose insulin levels. In another study, Eslinger et al. (2014) examined the impact of untreated yellow pea fibre, pea flour and pea starch on glycemic control. They concluded that yellow pea fibre was the most effective fraction responsible for lowering blood glucose.

Collectively, these studies (Tables 1-1 and 1-2) provide strong evidence for beneficial effects of diets/foods containing sources of pea and pea fractions on markers of glycemic control and insulin resistance. While most of the early studies completely lack mechanistic analyses, some of the more recent ones have assessed

a number of underlying mechanisms such as altered energy expenditure and incretin secretion (Elinger et al., 2014; Marinangeli & Jones, 2011b). There are, however, some aspects that have hardly been addressed, including the effects of pea fractions on intestinal microbiota, intestinal barrier function and pancreatic islet function. A general overview of the health benefits of dietary fibre and its putative mechanisms follows.

1.7 Dietary Fibre

Dietary fibre refers to a complex of non-digestible carbohydrates and lignin that is derived from plant material (Tosh & Yada, 2010). The carbohydrates include polysaccharides such as cellulose, hemicellulose and pectin, as well as oligosaccharides such as inulin and resistant starches. They are resistant to digestion and absorption in the small intestine, but undergo complete or partial fermentation in the large intestine (Babio et al., 2010; Tosh & Yada, 2010). Some natural sources of dietary fibre include whole grains, legumes, pulses, fruits and vegetables (Tosh & Yada, 2010).

Generally, dietary fibre is classified into two types based on solubility in water: soluble dietary fibre (SDF) and insoluble dietary fibre (IDF). SDF includes polysaccharides, oligosaccharides, pectins, β-glucans and gums, and IDF includes cellulose, hemicellulose and lignin (Kaczmarczyk et al., 2012). These two groups have different physical properties that lead to their specific physiological and nutritional benefits. Some of these characteristics include viscosity, bulk/volume,

water-holding capacity, binding ability and fermentability (Kaczmarczyk et al., 2012; Tosh & Yada, 2010).

Dietary fibre is viewed as an important component of healthy diets. Dietary fibre consumption has been vastly studied in terms of its substantial health benefits related to cardiovascular diseases, diabetes, obesity and some gastrointestinal disorders (Anderson 2009; Kaczmarczyk et al., 2012). Although the majority of health benefits of dietary fiber intake have been attributed to its unique physical and chemical properties, not all the underlying mechanisms are completely understood.

1.7.1 Dietary Fibre and Glycemic Control

Several groups have reviewed the relationship between dietary fibre consumption and glycemic control. Anderson et al. (2009) reviewed epidemiological and randomized control trials to elucidate the effect of diets high in dietary fibre on glucose control and insulin sensitivity. They reported that increasing dietary fibre intake as part of a balanced diet, and without any changes in the macronutrient distribution, ameliorates markers of glucose control in diabetic patients. Some of the significant improvements included lower postprandial glucose concentrations and reduced need for diabetes medication. They further summarized the effects of inclusion of dietary fibre from food sources such as rye and whole grains in diets of nondiabetic subjects. They indicated that incorporating a moderate to high dose of dietary fibre (6-20 g/day) was accompanied by

improved insulin sensitivity and reduced levels of fasting plasma glucose and insulin (Anderson et al., 2009).

Based on a systematic review focused on both experimental and observational studies, Babio and colleagues (2010) postulated that moderate to high consumption of dietary fibre had beneficial effects on markers of glycemic control; however, they reported the effect of dietary fibre intake on insulin sensitivity was rather controversial, with some studies showing a positive effects, while others failed to prove similar results.

In a more recent review study, Kaczmarczyk et al. (2012) analyzed several trials that investigated the impact of dietary fibre intake on markers of glycemic control in animal and human populations. They deduced that in animals, supplementing high fat diets (HFD) with SDF such as psyllium, sugarcane and β-glucans resulted in enhanced glucose tolerance and reduced plasma insulin concentrations. They reported similar improvements in terms of postprandial glucose control and insulin levels with consumption of SDF in humans.

In summary, favorable effects of dietary fibre consumption on markers of glycemic control emerges as a well-documented health claim in both animal and human studies, despite the fact that it is very challenging to pinpoint underlying mechanisms for different sources of dietary fibre.

1.7.2 Proposed Mechanisms of Action

Dietary fibre appears to act through several different pathways that contribute to improved glucose and insulin tolerance. The fermentative dietary process, as an important quality of dietary fibre, has been linked with most of its favorable physiological effects (Kaczmarczyk et al., 2012; Tosh & Yada, 2010). Dietary fibres pass through gastrointestinal tract without being digested and absorbed, while a fraction of them are completely or partially fermented in the large intestine to form short chain fatty acids (SCFA). The main SCFAs include acetate, propionate, and butyrate. Depending on the type of the dietary fibre, the degree of fermentation and the forms of SCFA produced are different. For example, pectin fermentation mainly favors acetate production, when the fermentation of gum Arabic yields propionate as the main SCFA (Kaczmarczyk et al., 2012).

SCFAs are shown to promote the release of GLP-1 both in vitro and in vivo (Freeland & Wolever, 2010; Yadav et al., 2013), however, the intracellular mechanisms for this effect are not completely known. Receptors for SCFAs, G-protein couple receptors FFAR2 (free fatty acid receptor2) and FFAR3, are expressed in GLP-1 producing L-cells (Karaki et al., 2006; Tazoe et al., 2009); hence might impact the cells function upon activation by SCFAs as shown by decreased GLP-1 in FFAR2-dificient mice (Tolhurst et al., 2012).

SCFAs were originally proposed to affect glucose metabolism through suppression of hepatic glucose output (Thorburn et al., 1993), however, other studies failed to show similar effects (Boillot et al., 1995; Cameron-Smith et al., 1994). Recently, it has been proposed that butyrate increases hepatic glycogen storage through competing with glucose for oxidation, and increasing the rate of glucose used for glycogen synthesis (Beauvieux et al., 2008). Our group also demonstrated that pea-derived dietary fibre resulted in reduced fatty acid oxidation in the liver, which was associated with increased levels of gut-derived 3-hydroxybutyrate (Chan et al., 2014).

The fermentation capacity of dietary fibre depends on the species of the bacteria that populate the gut, meaning that diets containing different types and doses of dietary fibre result in varying microbial profiles in the intestine. Furthermore, fermentable dietary fibres alter the microbial composition of the gut by feeding the flora with substrates for fermentation (Kaczmarczyk et al., 2012; Kuo, 2013). In general, the human gut microbiota is mainly composed of two dominant phyla: *Bacteroidetes* and *Firmicutes*. *Firmicutes* to *Bacteriodetes* ratio has been used to explain the microbial profiles related to health and disease, as well as to explain the effect of diets on composition of gut microflora. It is now well established that obesity and HFD feeding is associated with an increase in *Firmicutes* to *Bacteroidetes* (Burcelin et al., 2011; Kuo, 2013). In contrast, certain types of dietary fibre have been shown to favor the growth of beneficial species such as *Bifidobacterium spp*. (Kaczmarczyk et al., 2012; Kuo, 2013). Although the

bifidogenic property of certain dietary fibers has been confirmed by several studies, the mechanistic basis for positive effect of these bacteria on insulin resistance is not yet clearly understood. One main pathway is reduced production of lipopolysaccharide (LPS) as a key initiator of inflammation. Another proposed mechanism is the effect of specific microbial profiles on white adipose tissue plasticity via modifying fat storage regulation, energy harvesting and adiposity (Burcelin et al., 2011). Moreover, microbial modifications caused by dietary fibers with prebiotic properties have been linked with improved intestinal permeability, which will be discussed in detail in following sections (Cani et al., 2009; Neyrinck et al., 2012). Glucagon-like peptide-2 (GLP-2) has recently been proposed as a potential mediator of these enhancing effects of prebiotics on gut barrier function and microbiota, based on evidence that showed treatment with GLP-2 antagonist reversed these effects (Cani et al., 2009). GLP-2 belongs to the family of glucagon-derived peptides and is co-secreted along with GLP-1 from the enteroendocrine L-cells located in distal intestine in response to nutrient ingestion (Hartmann et al., 2000). GLP-2 has intestinotrophic actions and is known to enhance nutrient absorption, promote mucosal proliferation, suppress intestinal mucosal apoptosis, reduce gut permeability and bacterial translocation and decrease expression of proinflammatory cytokines (Burrin et al., 2001; Drucker 2002; Meier et al., 2006).

Furthermore, dietary fibre consumption, independent from its role in glycemic control, is linked with reductions in body weight in both animal and human studies (Du et al., 2010; Jimenez-Escrig et al., 2008; Reimer et al., 2011);

however this effect varies depending on the type of dietary fibre. Dietary fibre may promote satiety through modulation of appetite and satiety hormones such as peptide YY (PYY), ghrelin, GIP and GLP-1 (Cani et al., 2004; Cani et al., 2009; Everard & Cani, 2013). It also enhances satiety as a result of increasing bulk and transition time of the intestinal contents (Slavin, 2005).

1.8 The Gastrointestinal Microbiota and Insulin Resistance

1.8.1 Intestinal Barrier Function

Intestinal homeostasis is maintained through the interplay between gut microbiota, the intestinal epithelium, and the host immune system. The intestinal epithelial monolayer is an important mediator of this process that regulates the mucosal immune responses to resident intestinal bacteria (Peterson & Artis, 2014). It constitutes a biochemical and physical barrier that separates luminal contents of the intestine from the underlying lamina propria (Salles Teixeira et al., 2014), and selectively promotes fluxes of nutrients and ions while hindering the passage of toxins and pathogens from dietary and microbial sources (Ménard et al., 2010).

The intestinal epithelium deploys a plethora of defense mechanisms against the pathogenic stimuli, which starts at the mucus layer as the first line of defense. Mucus is continuously secreted from secretary goblet cells of the epithelial monolayer to protect the epithelium from pathogenic bacteria, bacterial and other toxic components (de Kivit et al., 2014; Johansson et al., 2011). The major component of the mucus layer is mucins, which are epithelial glycoproteins with

high molecular weight (Montagne et al., 2004). Although several mucin proteins are secreted from the intestinal epithelium, the mucus layer is primarily composed of mucin2 (Muc2) (Linden et al., 2008). Mucin secretion, and hence the integrity of the mucus layer, can be altered in disease states (Kim & Ho, 2010).

Generally, transport of molecules across the intestinal epithelium occurs through two pathways: transcellular and paracellular (Arrieta et al., 2006; Bjarnason et al., 1995). The transcellular route refers to the transport of molecules across the epithelial cells via carrier-mediated and passive processes, whereas the paracellular route is the major route for molecules permeation taking place between the adjacent epithelial cells through passive diffusion (Arrieta et al., 2006; Salles Teixeira et al., 2014). Intestinal permeability refers to the property of the intestine through which the intestinal epithelium allows passage of molecules by means of non-mediated passive diffusion (Montalto et al., 1997). Increased intestinal permeability has been associated with several intestinal and non-intestinal human diseases including diabetes, Crohn's disease, coeliac disease and multiple sclerosis (Arrieta et al., 2006). While this phenomenon is believed to result from alterations of both transcellular and paracellular pathways (Ahrne & Hagslatt, 2011), the latter plays a more important role in its etiology. Paracellular permeability, in particular, is the transport that happens through the space between the adjacent epithelial cells via paracellular pathway. Each epithelial cell is linked to its immediate neighboring cells via intercellular junctional structures. They consist of four major complexes: tight junctions, adherens junctions, desmosomes and gap junctions. Tight junctions,

however, are the major regulators of the paracellular permeability (Barreau & Hugot, 2013).

Epithelial tight junctions are composed of transmembrane proteins belonging to families of claudin, occludin and junctional adhesion molecules (JAM), which interact with the zonula occludens (ZO) family of proteins on the intracellular side of the membrane. Zonula occludens are scaffolding proteins that anchor the cytoplasmic part of the tight junction structure to the cell's actin-myosin cytoskeleton (Catalioto et al., 2011). This structure forms a dynamic barrier whose function depends on the expression and localization of its constituent proteins (Figure 1-2) (Chiba et al., 2008).

1.8.2 Gut Microbiota

The human body harbours an enormous collection of millions of microbes, which play an important role in human health and well-being (Sekirov et al., 2010). It is famously known that the total number of bacterial cells hosted by our body is ten times greater than the number of cells in human itself (Savage, 1977). These microorganisms colonize all surfaces of the body that come into contact with the external environment, in particular the skin, and the urogenital, respiratory and gastrointestinal tracts (Gerritsen et al., 2011). The gastrointestinal tract has the highest density of microbial colonization among these sites. This is not surprising considering the fact that this organ has an extensive surface area and is a great source of readily available nutrients for microbial use (Sekirov et al., 2010).

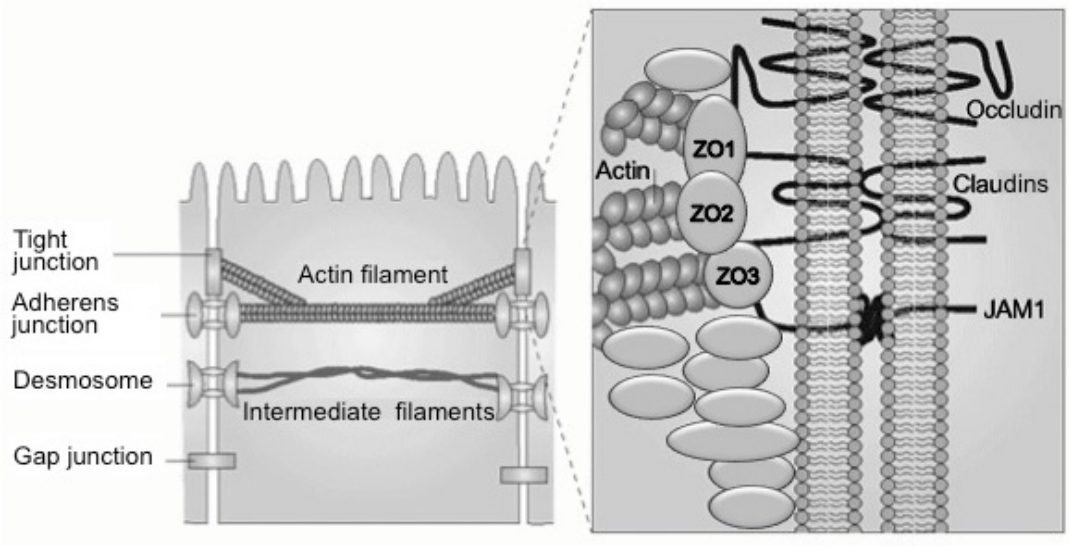

Figure 1-2. A schematic representation of junctional complexes and the main structural components of the tight junctions. This figure is reproduced from Aktories and Barbieri, 2005.

The microbial community that inhabits the human intestinal tract is referred to as the gut microbiota (Shen et al., 2013), a great majority of which are anaerobic bacterial species (Gerritsen et al., 2011). The human gut microbiota, despite having a diverse composition of bacteria, is dominated by two major phyla: the Bacteroidetes and the Firmicutes. Small quantities of other bacterial phyla including Proteobacteria, Actino-bacteria, Fusobacteria, and Verrucomicrobia are also present (Eckburg et al., 2005). These bacteria, however, are not equally scattered inside the gastrointestinal tract. In fact, gut microbiota has a heterogeneous nature, and the number of bacterial cells per gram of content increase along the axis of the gastrointestinal tract; it reaches maximum density of 10^{11}-10^{12} bacteria per gram of colonic content in the large intestine (O'Hara & Shanahan, 2006). Additionally, the intestinal microbiota shows a significant latitudinal variation in terms of the composition. The microbiota is present in the intestinal lumen as well as the mucosal epithelial layer, however, the microbial composition of these sites vary significantly (Sekirov et al., 2010).

In addition to being site-specific, the composition of the gut microbiota is also host-specific and varies considerably between different individuals (Adlerberth & Wold, 2009; Nam et al., 2011). Microbial colonization of the human intestine begins immediately after birth, and changes substantially during the first two years to develop a relatively stable bacterial profile similar to that of adults (Fouhy et al., 2012). However, the gut microbiota continues to mature during adulthood and changes further in old age (Mariat et al., 2009). The initial

microbiota composition is affected by several factors including prenatal maternal exposures to environmental stimuli, mode of birth delivery and infant diet (Azad et al., 2013; Fujimura et al., 2010; Huurre et al., 2008). Gut microbiota exerts a variety of functional properties, to the extent that some refer to it as a "hidden organ" (O'Hara & Shanahan, 2006). It contributes to metabolic, nutritional, immunological and physiological functions of the host through several key activities such as degradation of non-digestible polysaccharides, production of SCFAs and select vitamins (vitamin K, vitamin B12 and folic acid), generation of antimicrobial substances and promotion of structural properties of the gastrointestinal tract (Gerritsen et al., 2011; Sekirov et al., 2010).

1.8.3 Metabolic Endotoxemia

Metabolic endotoxemia is defined as subclinically elevated levels of Gram-negative bacteria-derived LPS, which is referred to as endotoxin, in the bloodstream. The concept was first expounded by Cani and colleagues (2007) in a study that examined the effect of HFD feeding on bacterial LPS in mice. They reported that following a HFD for 4 weeks raised plasma LPS levels two to three times higher than the normal level. They further assessed the physiological relevance of this threshold by studying the impact of chronic administration of LPS, where they showed that it creates similar disturbances in terms of fasted glycemia and insulinemia as seen in HFD feeding. The authors defined the presence of the observed level of plasma LPS as metabolic endotoxemia. This was additionally confirmed by showing that mice lacking the ability to respond to LPS

through toll-like receptor 4 (TLR4) signaling were protected against HFD-induced insulin resistance (Cani et al., 2008).

LPS, as a major component of the outer membrane of the Gram-negative bacterial cell wall, can be recognized by host immune cells, activating a signaling cascade that ultimately leads to production of critical proinflammatory cytokines such as TNFα and IL-6 (Takeuchi et al., 1999). The LPS transduction pathway starts with the recognition of LPS by LPS-binding protein (LBP), which then forms a complex with the surface molecule CD14. This in turn enables LPS to bind to its receptor complex composed of TLR4 and MD-2. TLR4 is the main part of this complex with MD-2, acting as a cofactor that facilitates LPS binding. At this stage TLR signaling can act through two different pathways. The first pathway leads to the recruitment of myeloid differentiation factor 88 (MyD88) and MyD88 adaptor-like protein (Mal) to the receptor complex and is naturally called the MyD88-dependent pathway. Upon MyD88 recruitment, it activates downstream proteins from IL-1 receptor-associated kinase (IRAK) family, which in turn activates another adaptor protein named TRAF6 (TNF receptor-associated factor 6). This protein forms a complex with multiple other downstream enzymes that eventually activate downstream IKK (IκB kinase) and MAPK (mitogen-activated protein kinase) pathways. The end result is activation of the transcription factors NF-κB (nuclear factor- κB) and AP-1 (activating protein-1) and subsequent expression of the proinflammatory cytokines such as TNFα and IL-1β (Lu et al., 2008; Pålsson-McDermott & O'Neill, 2004).

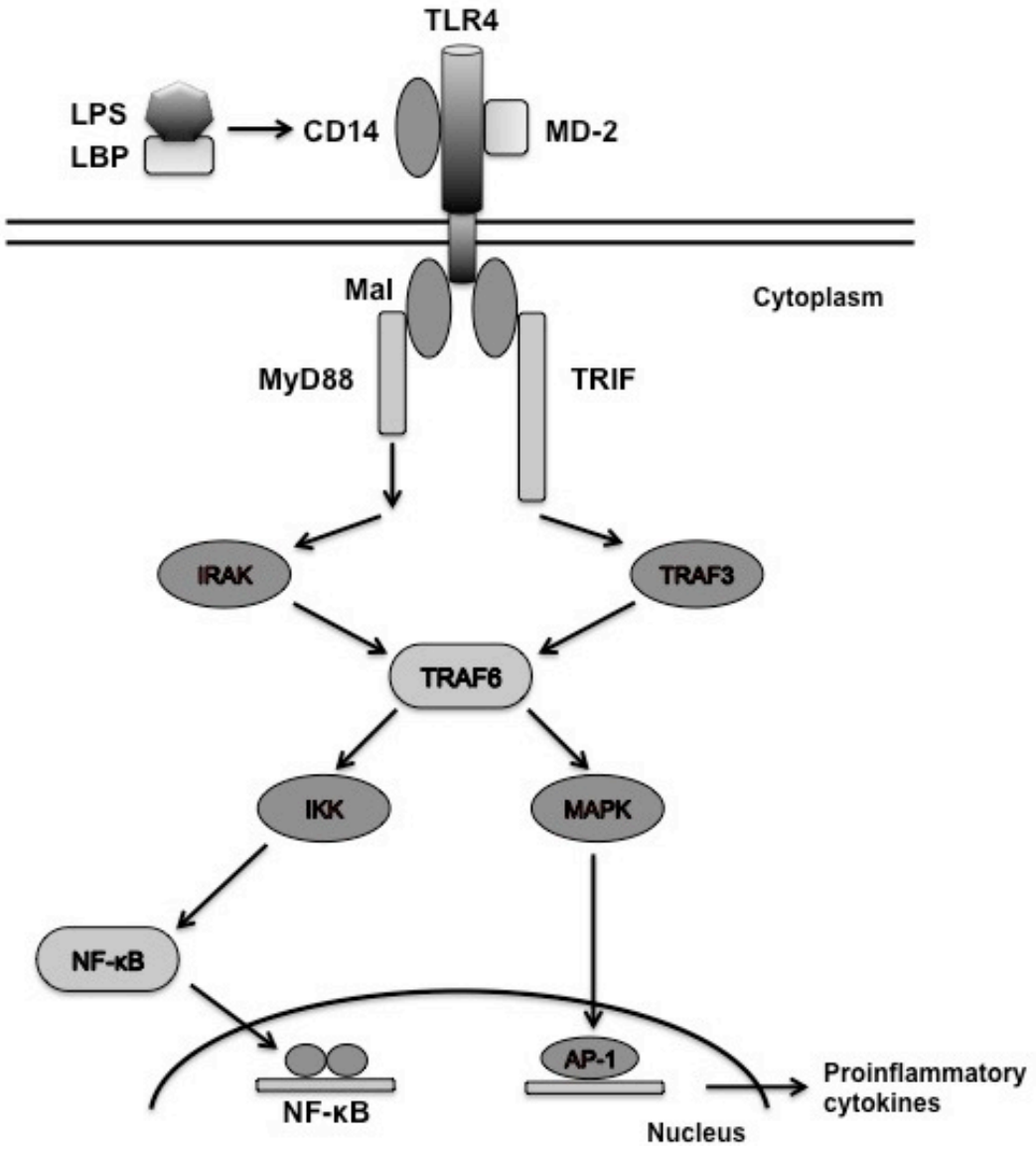

Figure 1-3. A simplified model of LPS signaling pathway in endothelial cells. See text for details. Adapted from Dauphinee and Karsan, 2006 and Lu et al., 2008.

While the MyD88-independent pathway works via different adaptor proteins than MyD88 (Figure 1-3), it eventually results in very comparable end products (Lu et al., 2008)

1.8.3.1 The Linkage Between Metabolic Endotoxemia and T2D

Obesity and T2D are closely linked to a state of low-grade inflammation (Donath & Shoelson, 2011; Lee et al., 2013). Recently, metabolic endotoxemia has been suggested as an underlying mechanism contributing to this chronic inflammation. The physiological role of LPS in obesity and T2D has been confirmed in several rodent studies (Cani et al., 2008; Cani et al., 2009; Muccioli et al., 2010; Shan et al., 2013), as well as human studies (Al-Attas et al., 2009; Dixon et al., 2008; Jayashree et al., 2014; Pussinen et al., 2011).

The origin of metabolic endotoxemia has been mainly attributed to changes in gut microbiota and intestinal barrier function. A growing body of evidence suggests that HFD-induced metabolic disorders such as obesity are associated with gut microbiota alterations in animal models, which can be specifically characterized by a decrease in *Bacteroidetes* and an increase in *Firmicutes* (Cani et al., 2007a; Hildebrandt et al., 2009; Murphy et al., 2010; Turnbaugh et al., 2006). Human studies, however, provide a somewhat controversial body of evidence related to alterations of microbial profile in obesity and T2D. Following a calorie-restricted diet resulted in decreased abundance of *Firmicutes* and increased abundance of *Bacteroidetes*, which was correlated with body weight loss (Ley et

al., 2006). While this observation was in line with existing evidence from animal models, other human studies failed to produce similar results in obese or diabetic subjects (Larsen et al., 2010; Schwiertz et al., 2010; Turnbaugh et al., 2009; Wu et al., 2010). In spite of no definite changes in bacterial composition at the level of phyla, obese and diabetic subjects tend to have lower abundance of the *Bifidobacterium* genus (Murri et al., 2013; Schwiertz et al., 2010; Wu et al., 2010), which is believed to have anti-inflammatory properties in vivo (O'Mahony et al., 2010).

Another important factor contributing to the pathology of metabolic endotoxemia-induced low-grade inflammation is intestinal barrier dysfunction. The intestinal epithelium is responsible for maintaining a balanced relationship between gut microbiota and host immune system (Peterson & Artis, 2014). Under normal physiological conditions, intestinal barrier protects the internal environment from invasion of gut microbes and their components (Salles Teixeira et al., 2014). This is achieved mostly through maintaining a normal intestinal permeability. Accordingly, disruption of intestinal barrier function results in leakage of microbial molecules into the host tissues and circulation. This has been supported by several studies showing an association between metabolic endotoxemia and increased intestinal permeability in different mouse models of obesity (Cani et al., 2008; Cani et al., 2009; De La Serre et al., 2010; Lam et al., 2012). Increased intestinal permeability has in turn been linked with decreased expression and altered distribution of tight junction proteins occludin and ZO-1 in obese mice (Everard et

al., 2011; Cani et al., 2009). In addition, long term (25 weeks) HFD feeding in mice resulted in alterations of mucin composition, which were proposed to make the mucus layer more susceptible to bacterial degradation (Mastrodonato et al., 2014).

1.9 Dietary Strategies for Gut Microbiota Modulation

It is well documented that intestinal barrier function is either directly or indirectly affected by host microbiota and the composition of diet (Guzman et al., 2013). Additionally, gut microbiota itself can be largely manipulated by host diet (Voreades et al., 2014). One major group of dietary components that has been studied in terms of its microbial modifying properties is prebiotics. Prebiotics are classified as non-digestible yet fermentable oligosaccharides that stimulate the growth and activity of certain health-promoting bacteria in the host intestine (Gibson et al., 2004). Two of the most well studied prebiotics are inulin and oligofructose (fructooligosaccharides).

In obese mice, oligofructose induced changes in gut microbiota composition, which was accompanied by improved intestinal permeability and reduced metabolic endotoxemia (Cani et al., 2007b; Cani et al., 2009). Oligofructose was also shown to increase bifidobacterial population in obese rats (Bomhof et al., 2014). The use of a combination of inulin and oligofructose as the prebiotic fibre yielded similar results in terms of increase in *Bifidobacteria* (Parnell & Reimer, 2012). The bifidogenic properties of inulin and oligofructose are also

well documented in human studies (Bouhnik et al., 2006; Kolida et al., 2007; Ramirez-Farias et al., 2009).

In spite of the fact that not all types of fermentable dietary fibre can be categorized as traditional prebiotics, some have been demonstrated to possess prebiotic properties. Corn-based soluble fibre (Boler et al., 2011), resistant starch (Martinez et al., 2010) and wheat dextrin (Lefranc-Millot et al., 2012) are among those shown to have bifidogenic effects on gut microbiota. Moreover, some studies have examined the bifidogenic activities of different pulses and pulse-derived components. Comparison of diets containing different types of pulses such as pea, chickpea, common bean and lentil revealed that peas were the most effective source of dietary fibre in term of the ability to increase *Bifidobacterium* population (Queiroz-Monici et al., 2005). Eslinger et al. (2014) examined different components of pea for their microbial modifying properties. Decreases in *Firmicutes* were reported following consumption of pea flour and pea fibre fractions in diet-induced obese rats. None of these two studies, however, assessed the impact of peas or their fractions on markers of intestinal permeability.

1.10 Peas as Sources of Vitamins

Pulses including dried peas are very good sources of several vitamins and minerals such as thiamine, niacin, folate and riboflavin (Pulse Canada, 2014). Thiamine and folate sources are particularly relevant in the context of diabetes and its complications.

Thiamine is an essential nutrient that is present in dietary sources in the form of thiamine diphosphate (TDP). It is originally hydrolyzed to free thiamine in the proximal part of the small intestine and absorbed into the enterocytes via simple diffusion or carrier-mediated active transport. It becomes phosphorylated back to thiamine diphosphate upon absorption (Page et al., 2011). Thiamine can also be synthetized by intestinal microbiota in both free and phosphorylated forms (Nabokina & Said, 2012). TDP acts as an integral cofactor for several enzymes involved in carbohydrate and energy metabolism such as transketolase and PDH (Castiglioni et al., 2015; Naito et al., 2002; Page et al., 201; Thornalley, 2005).

In terms of diabetes and glucose homeostasis, thiamine is specifically important for transketolase activity. Hyperglycemia, as a key hallmark of diabetes, is accompanied by activation of several major cellular pathways such as increased polyol pathway flux, overactivity of hexoseamine pathway, activation of protein kinase C (PKC) and increased non-enzymatic glycation, which are the underlying mechanisms responsible for overall hyperglycemia-induced cell damage in diabetic complications (Giacco & Brownlee, 2010). In fact, under hyperglycemic conditions in the fasting and/or postprandial states, an elevated rate of glycolysis results in the over-production of mitochondrial superoxide and loss of antioxidant equivalents such as nicotinamide adenine dinucleotide phosphate (NADPH) and gluthatione. This ultimately leads to over-production of ROS as the unifying link between hyperglycemia and intracellular pathways of hyperglycemia-induced cell damage (Brownlee, 2005; Folli et al., 2011; Rolo & Palmeira, 2006). On the other hand, a

number of intercellular pathways including pentose phosphate pathway (PPP) play potential protective roles against hyperglycemia-induced accumulation of ROS. PPP is as an alternative glucose oxidation pathway that has two different phases: oxidative and non-oxidative. Through its non-oxidative branch, PPP metabolizes intermediate glycolytic metabolites such as fructose-6-phosphate and glyceraldehyde 3-phosphate into harmless metabolites, thus blocking their processing in damaging metabolic pathways such as hexosamine and PKC pathways (Brownlee, 2005; Pacal et al., 2011; Stincone et al., 2014). The two major enzymes involved in the non-oxidative branch of PPP are transketolase and transaldolase (Stincone et al., 2014). TDP acts as an essential co-factor for the enzyme transketolase. (Brownlee, 2005; Page et al., 2011; Pacal et al., 2014). It is suggested that the protective role of TPP in glucose metabolism can be interrupted due to inhibition of transketolase activity in the presence of relative or absolute thiamine deficiency (Page et al., 2011; Pacal et al., 2014). Thiamine deficiency happens due to inadequate dietary intake and/or increased demands by the body among other factors. Low concentrations of thiamine in plasma are prevalent in patients with T1D and T2D (Page et al., 2011; Thornalley et al., 2007).

Supplementation with thiamine or its derivatives has been effective in restoring the impaired glucose metabolism and attenuating hyperglycemic damage in vitro (Hammes et al., 2003) and decreasing fasting glucose and HbA1c levels in diabetic animal models (Karachalias et al., 2010). In humans, daily thiamine

administration was shown to improve fasting glycemia (Gonzalez-Ortis et al., 2011), although some discrepancies are noted (Rabbani et al., 2009).

Folate is another B vitamin whose status has been associated with risk of developing cardiovascular disease, a major complication of diabetes. Folate is essential for normal homocysteine metabolism, acting as a cofactor for some of its key enzymes. Folate deficiency causes impairment in homocysteine cycling, resulting in elevated homocysteine concentrations, a known risk factor for cardiovascular disease (Cacciapuoti, 2012). Folate deficiency due to reduced dietary intake has been tremendously reduced since the introduction of folate food fortification in North America (Antoniades et al., 2009), however, deficiency could still occur as a result of reduced gastrointestinal absorption in certain conditions. Diabetic patients can suffer from folate deficiency due to administration of diabetic medications such as metformin (Fisman et al., 2008; Sahin et al., 2007). In an attempt to correct for folate deficiency, supplementation has been used in diabetic patients, which resulted in lower homocysteine levels in plasma (Hunter-Lavin 2004).

1.11 Summary

In summary, this review provides evidence for the favourable effects of peas and pea components on glucose control; however, some existing gaps in the literature are noted. There is a lack of mechanistic studies exploring the influence of pea and pea-derived fractions on the gut microbiota. Other areas of interest such

as regulatory mechanisms in the pancreatic islets have not been addressed. Furthermore, in spite of the fact that pulse consumption in humans requires cooking processes, the animal trials investigating the health benefits of peas in terms of glycemia have used untreated components; hence, the effect of heating treatments such as cooking on the proposed beneficial properties of peas is yet to be addressed.

Chapter 2: Research Plan

2.1 Rationale

Diabetes mellitus is arguably one of the most prevalent and challenging chronic diseases, affecting over 382 million individuals worldwide (IDF, 2013). In 2009, 2.4 million Canadians were estimated to have diabetes and the disease caused a huge economic burden of $12.2 billion CAD in 2010 (CDA, 2013). T2D accounts for 90-95% of cases of diabetes, highlighting the importance of prevention and management of this disease. Dietary modifications and nutrition therapy are established as critical components of T2D and prediabetes management. While nutrition therapy aims to target several aspects of the patients' diets, incorporation of healthy foods with beneficial impacts on glucose homeostasis has provided promising results during the recent couple of decades.

Pulses are among those foods whose consumption beneficially affects markers of glycemia and insulin resistance (Mudryj et al., 2014; Sievenpiper et al., 2009). In addition to containing several healthful components, studying health benefits of pulses are of great interest in Canada due to their high availability in this country. Canada is one of the largest producers of pulses in the world, with overall Canadian production of peas and lentils respectively accounting for 32% and 38.5% of the world production. In addition, Canada is the largest exporter of peas in the world (Pulse Canada, 2015). Despite these facts, consumption of pulses in Canada is very low and only 13.1% of Canadian adults consume pulses on any given day (Mudryj et al., 2012). Since the high production of pulses in Canada is

not reflected in the consumption rate, implementing practical strategies to increase consumption of pulses in Canadians is deemed important. One strategy is developing functional foods using pulses and their components, especially parts such as the seed coats that are removed during processes such as pulse splitting. This, however, requires for pulse components to have shown promising health claims.

While there are numerous studies that have investigated the beneficial effects of different types of pulses on glycemia, only a few studies have so far assessed the potential mechanisms exerting these effects. Furthermore, comparisons of different pulse fractions and the effects of different preparation methods are rarely investigated. Dried peas are one of the most abundantly grown pulses in Canada and have been studied for their health benefits in terms of improving glucose control and insulin resistance. As an abundant crop in Alberta, our laboratory initially became interested in studying peas as part of a project named Physical Activity and Nutrition for Diabetes in Alberta (PANDA). This project was a multidisciplinary project that aimed to develop a nutritional and physical activity guideline for people with diabetes in Alberta. One focus of this provincial project was to assess locally grown crops for potential their effects on diabetes. Our group previously examined the effects of different fractions of dried peas on glucose intolerance in rats. Pea seed coats were shown to have greater benefits than the pea embryo (Whitlock et al., 2012). We aimed to study the

impact of different methods of preparation of the seed coat fraction of the peas on glucose homeostasis with a focus on the role of the gut in the underlying pathways.

2.2 Hypotheses and Objectives

Chapter 3 overall hypothesis: Supplementing high fat diets with pea seed coat fractions ameliorates glucose tolerance in glucose-intolerant rats via modification of insulin and incretin secretion and potency of this favourable effect of seed coats is retained following cooking.

To examine this hypothesis, I will investigate the following objectives:

1. To assess the effect of pea seed coat-supplemented diets on glucose tolerance and insulin sensitivity in vivo in HFD-fed Sprague Dawley rats. The hypothesis is that rats fed pea seed coat-supplemented diets will show improvements in glucose control measured during glucose tolerance tests. I also hypothesize that insulin sensitivity will be improved in these rats, as measured by improved glucose response during an insulin tolerance test.
2. To compare the effect of preparation method of pea seed coat containing diets on glucose tolerance. It is hypothesized that cooking of pea seed coats followed by freeze-drying will not reduce the glucose lowering effects of the seed coats.
3. To assess the effect of preparation method of pea seed coats on their composition. It was hypothesized that any possible differences in the effects of raw versus cooked seed coats might be due to changes in the dietary fibre and/or

carbohydrate composition of the pea seed coats following cooking and freeze-drying.

4. To determine the effect of pea seed coat supplementation on incretin secretion and glucose absorptive capacity in the gut. I hypothesize that GLP-1 and GIP secretion will be increased as a result of pea seed coat-supplementation. Another hypothesis is that pea seed coats will suppress glucose absorption in the gut through down-regulation of intestinal glucose transporters expression.

5. To identify the impact of pea seed coat supplementation on HFD-induced stress on pancreatic islets. It is hypothesized that pea seed coat supplementation will attenuate HFD-induced expansion of beta-cell mass.

Chapter 4 hypothesis: Dietary supplementation with pea seed coat fractions modifies intestinal microbiota and markers of intestinal permeability in HFD-fed glucose intolerant rats.

The following objectives will be examined to test this hypothesis:

1. To measure the effect of pea seed coat supplementation on micorobial composition in the intestine. It is hypothesized that pea seed coat-containing diets will change the overall microbial composition of the intestine compared to control diets and is associated with the growth of beneficial bacteria such as *Bifidobacterium*.

2. To determine the impact of pea seed coat-containing diets on markers of intestinal barrier function. I hypothesize that pea seed coat will partially revert the

HFD-induced changes in intestinal barrier through normalizing the expression of tight junction proteins ZO1 and occludin, toll-like receptors and mucin proteins.

Chapter 5 hypothesis: Incorporating foods containing peas or beans into the diets of hypercholesterolemic adults will increase serum thiamine and folate and this will be associated with improved markers of glucose control.

The objective is to determine the effect of feeding meals containing peas or beans on whole blood thiamine and serum folate concentrations and to correlate these with serum glucose, insulin and hemoglobin A1c.

Chapter 3: Cooking Enhances Beneficial Effects of Pea Seed Coat Consumption on Glucose Tolerance, Incretin and Pancreatic Hormones in High Fat Diet-fed Rats[1]

3.1 Introduction

Pulse grains, including dried peas, are rich sources of fibre with low glycemic indices. Their unique nutritional profile has led to many investigations of the health benefits of different varieties of pulses. In a meta-analysis of randomized control trials, Sievenpiper et al. (2009) found that consumption of non-oil-seed pulses was associated with enhanced long-term glycemic control. Consumption of dried peas has specifically been linked with enhanced glycemic control in several human intervention studies. Patients with T2D consuming a mixed meal containing whole dried peas had a delayed increase in postprandial plasma glucose and insulin concentration compared with controls eating potato-based meals (Schafer et al., 2003) and whole yellow pea flour-containing foods, when compared to the same foods made from whole-wheat flour, improved postprandial glucose response in healthy subjects (Marinangeli et al., 2009). Most studies identifying beneficial effects of pulses on glycemia have used the whole grain (Sievenpiper et al., 2009) but some studies of pulse fractions are emerging. In hamsters, feeding a hypercholesterolemic diet with partial substitution of cornstarch with pea hull flour resulted in significant decreases in circulating glucose and insulin levels (Marinangeli et al., 2011).

[1] A version of this chapter has been published in the journal of *Applied Physiology, Nutrition and Metabolism*. A copy of this paper is attached in Appendix A.

Lunde and colleagues (2011) also showed that consumption of pea fibre-enriched breads resulted in improved post-prandial glucose tolerance and increased satiety in human subjects with a high risk of developing T2D. In humans, whole or fractionated pea flour (pea hulls) muffins ameliorated insulin resistance in overweight subjects compared with wheat flour muffins (Marinangeli & Jones, 2011b). Another study reported yellow pea fibre as the most beneficial fraction, compared with pea flour or starch, in terms of lowering blood glucose (Eslinger et al., 2014). One study, however, suggests that pea protein is the fraction accountable for improved acute glucose control following consumption of pea containing meals in healthy adults (Smith et al., 2012).

The pea seed (*Pisum sativum* L.) consists of an embryo (cotyledons and an embryo axis), which is enclosed in a seed coat (hull). The nutrient components of the embryo are mostly starch and protein, while the seed coats are largely soluble and insoluble fibre (Duenas et al., 2004; Guillon & Champ 2002; Whitlock et al. 2012). Many studies have shown that dietary fibre, particularly soluble fibre has positive effects on postprandial glucose control (reviewed by Babio et al. 2010). However, deeper insights into the mechanisms by which different sources of fibre affect glucose metabolism are yet to be elucidated. It is known that dietary fibre is fermented by colon microflora, producing SCFAs like acetate, propionate and butyrate (Jenkins et al. 2000). SCFAs have been linked with enhanced secretion of incretins and increased abundance of beneficial microbes in the gut (Freeland & Wolever, 2010; Lecerf et al., 2012). SDF also dissolves in water to form a viscous

slow-moving solution that results in slowed gastric emptying; however, the effect of this increased transit time on digestion and absorption is controversial (Lattimer & Haub, 2010).

We previously showed that insulin-resistant rats fed a raw pea seed coat (PSC)-supplemented diet had better glucose homeostasis compared to embryo-supplemented diet fed rats, suggesting that the beneficial effects are associated with the seed coat fraction (Whitlock et al., 2012). One limitation of that study was that raw PSCs incorporated into the diet were not suitable for human consumption. Some studies have suggested that processing reduces the effectiveness of pulses in improving glycemia (Jenkins et al., 1982). Therefore, this study was undertaken to examine the effects of grinding and cooking followed by freeze-drying on the ability of PSC fractions to improve glucose control and to identify potential physiological mechanisms. We hypothesized that supplementing diets with PSC fractions would ameliorate glucose tolerance by modulating glucose handling by the gut and reducing high fat diet-induced stress on pancreatic islets. We further hypothesized that the beneficial effects of PSC fibre consumption would not be lost following cooking.

3.2 Methods and Materials

3.2.1 Animals and Diets

Our animal care protocols were all in accordance with the guidelines of the Canadian Council on Animal Care. They were reviewed and approved by the

Health Sciences Animal Care and Use Committee at the University of Alberta. Eight-week old male Sprague Dawley rats were purchased from the Department of Biology, University of Alberta or Charles River Canada (St. Constant, QC, Canada). They were housed two per cage with *ad libitum* access to normal chow and water for one week. After acclimatization, rats received 6 weeks of HFD (20% w/w) to induce glucose intolerance, except for the low fat diet (LFD) control group, which remained on chow (Figure 3-1). Interim oral glucose tolerance test (oGTT) showed that HFD-fed rats had an overall significantly higher glucose excursion compared to normal chow-fed rats (Figure 3-2). The HFD-fed rats were then randomly assigned to the following 3 diets: HFD, raw PSC (RP, HFD supplemented with raw seed coats), cooked PSC (CP, HFD supplemented with cooked seed coats). All these diet groups were isocaloric and maintained a macronutrient ratio of 42:33:25 for fat, carbohydrate and protein. The chow fed rats were put on LFD (6% w/w), in which carbohydrate replaced the fat resulting in a macronutrient ratio of 15:55:30 for fat, carbohydrate and protein. As noted in Table 3-1, the fibre source in the treatment groups, which was 10% w/w cellulose in HFD and LFD, was replaced by prepared PSC fractions so that the total fibre weight per gram of chow was identical. The protein was adjusted as necessary to ensure the diets were isonitrogenous. The animals were on the PSC diets for four weeks with *ad libitum* access to food and water. A total of four animal trials were conducted to fulfill the purpose of the study. This chapter includes the outcomes of at least one of the four trials, which is reflected in the varying number of samples described in each graph's legend.

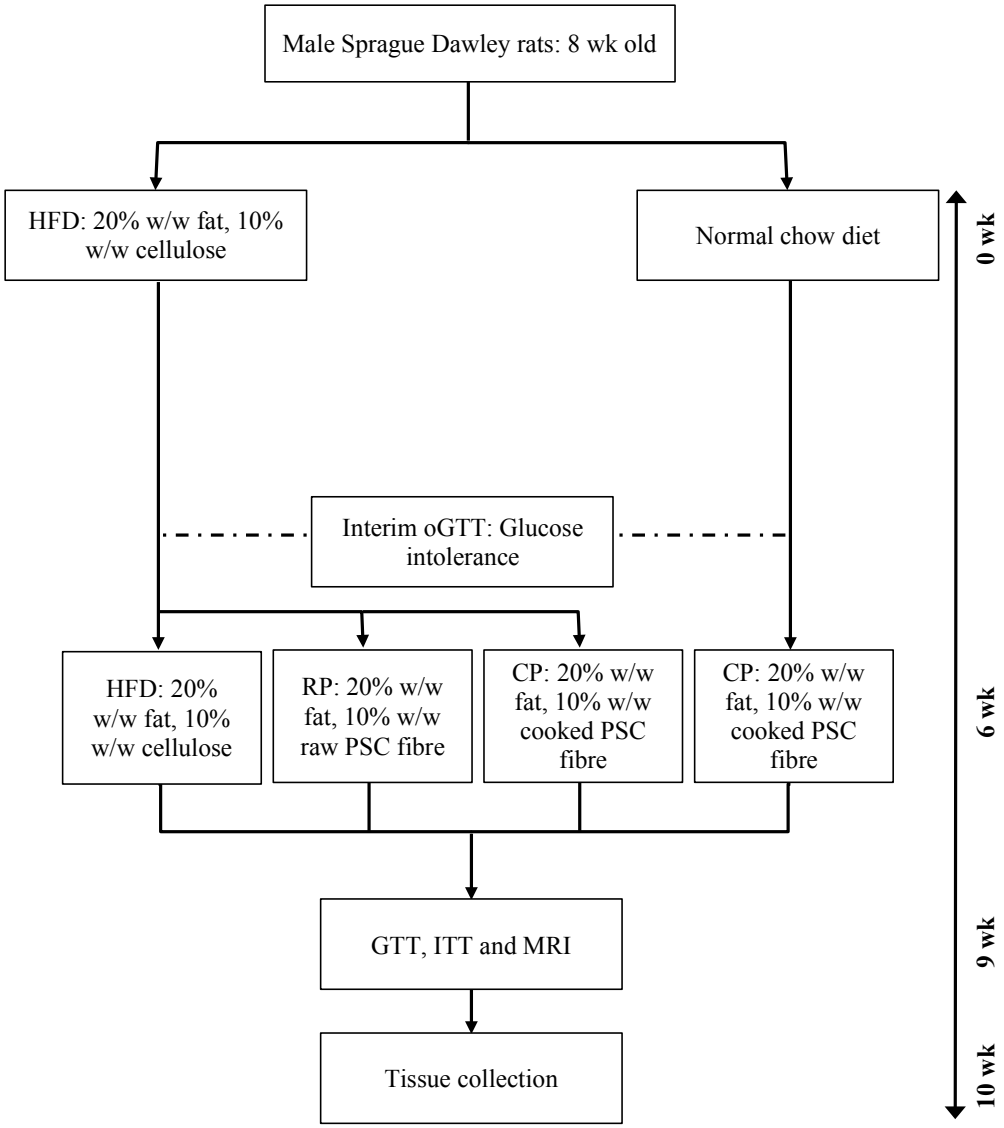

Figure 3-1. Schematic diagram showing the experimental outline of the study.

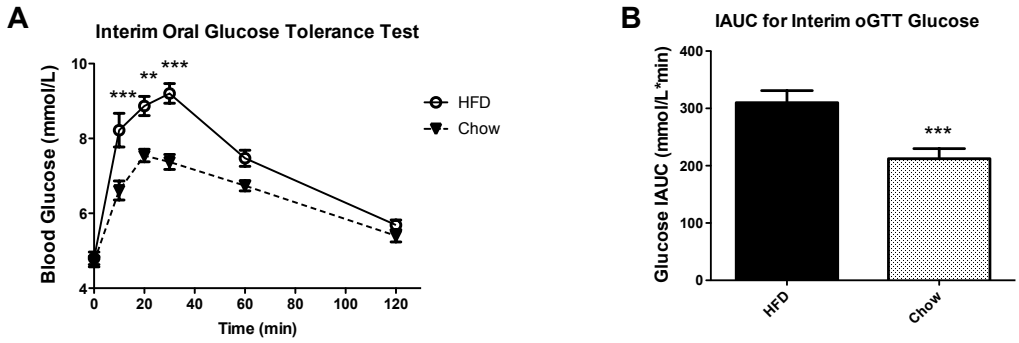

Figure 3-2. Effect of feeding HFD on oral glucose tolerance. (A) Effect of 6 weeks of feeding a high fat diet (HFD, 20% w/w) versus normal chow on blood glucose levels measured basally and following oral administration of 1g/kg glucose. (B) Incremental area under the curve (IAUC) was calculated for glucose during oral glucose tolerance test (oGTT). The data are means ± SEM, n = 22 (HFD) and n = 16 (Chow). Asterisks show significant difference compared to HFD by one-way ANOVA followed by Bonferroni's post-hoc comparison test in graph A, and significant difference between the two groups by student's *t* test in graph B (***P<0.001, **P<0.01).

Table 3-1. Diet composition (g/kg)

	HFD	HFD+PSC	LFD
Canola sterine	99.5	99.5	29.85
Flaxseed oil	6	6	1.8
Sunflower oil	94.5	94.5	28.35
Casein	270	263	270
Dextrose	189	189	255
Corn starch	169	169	245
Cellulose	100	0	100
Pea seed coat (dry weight)*	0	107	0
L-methionine[§]	2.5	2.5	2.5
Mineral mix[†]	51	51	51
Vitamin mix[†]	10	10	7.6
Inositol[§]	6.3	6.3	6.3
Choline chloride[§]	2.8	2.8	2.8
Total weight (g)	1000.6	1000.6	1000.2
Carbohydrate %	36	36	51
Fat %	20	20	6
Protein %	27	27	27

* The composition of Canstar pea seed coats has been reported previously (Whitlock et al., 2012); per 107 g dry weight it includes 100 g fibre and 7 g protein. Therefore, the amount of PSC was adjusted to provide 100 g fibre per kg diet, thereby matching the amount of cellulose added to the HFD and LFD. The amount of casein was decreased in the PSC diets by the amount of protein in the PSC.
[§] MP Biomedicals.
[†] Mineral mix (Bernhart & Tomarelli) and vitamin mix (AIN-93-VX) from Harlan.

3.2.2 PSC Preparation and Analysis

3.2.2.1 Seed Coat Preparation

The PSC fractions used in this study were produced from the seeds of the pea (*Pisum sativum* L.) cultivar Canstar that were grown in Alberta, Canada. 'Canstar' is a yellow-seeded field pea cultivar with little to no proanthocyanidins present in its seed coats. Whole peas were dehulled, and the hulls (seed coats) screened and ground as described in Whitlock et al. (2012). A portion of the ground samples were used unprocessed (raw seed coat material) and a portion was subjected to a cooking treatment (cooked seed coat material), which consisted of boiling the samples at 100 ^{o}C in deionized water (approximately 10 volumes of water to 1 volume of seed sample) for 30 min. After 30 min of cooking, the samples were cooled down to room temperature and stored at -20 °C until lyophilization of samples (using a freeze dryer; Virtis Ultra 35L Freeze Dryer, Stone Ridge, NY, USA) for 7 days. For starch, protein and fibre analyses, both raw and cooked seed coat material were lyophilized for 7 days and further ground using a Retsch, ZM 200 (PA, USA) mill to produce finely ground powder that passed through a 0.5 mm screen.

3.2.2.2 Starch and Protein Analysis

The ground lyophilized samples were assayed for total starch content using the Total Starch Assay Procedure AA/AMG 11/01 (Megazyme International Ireland Ltd., Bray, Ireland; AOAC Method 996.11). A nitrogen analyzer (LECO TruSpec CN Carbon/Nitrogen Determinator; Leco Corporation, St. Joseph, MI,

USA) was used to estimate the total protein content, which was calculated by multiplying the nitrogen content with a conversion factor of 6.25 (AOAC method 968.06). Caffeine (150 mg) and ethylenediaminetetraacetic acid (100 mg) were used as standards for instrument calibration.

3.2.2.3 Non-starch polysaccharides (NSP) analysis (fibre)

The total, water insoluble and soluble non-starch polysaccharide (fibre) components of seed coats were determined using the methods described in Englyst and Hudson (1987) and Englyst (1989) using 45 to 50 mg of ground seed coat per sample. The resulting starch-free sample residues were processed for total and insoluble NSP determination in independent samples (two replicates per sample). After processing, myo-inositol (20 mg/mL; 0.1 mL) was added as an external standard to the total NSP and insoluble NSP residue samples. For conversion of the hydrolyzed sugars to their alditol acetates, the hydrolysate was vortexed and centrifuged at 2000 *g* for 5 min. NH_4OH (12 M, 0.2 mL) was added to a 1 mL aliquot of the hydrolysate and the mixture was vortexed, then freshly prepared $NaBH_4$ solution (0.1 mL; 100 mg $NaBH_4$ per mL of 3 M aqueous NH_4OH solution) was added and the solution was incubated for 1 h in a 40 ^oC water bath. Subsequently, glacial acetic acid (0.1 mL) was added to the solution, followed by vortexing. A 0.2 mL aliquot of the acidified solution was added to 0.3 mL 1-methylimidazole. Acetic anhydride (2 mL) was then added to this solution and vortexed continuously for 10 min. Distilled water (5 mL) was subsequently added to the solution to decompose excess acetic anhydride and aid in phase separation.

After the solution was cooled to room temperature, dichloromethane (4 mL) was added and mixed for 15 sec. After centrifugation at 700 *g* for 5 min, the top layer was aspirated off and distilled water (5 mL) was added. The solution was again centrifuged at 700 *g* for 5 minutes, the top layer was aspirated off, and the bottom layer was dried in a 50 ^{o}C evaporator. Dichloromethane (1 mL) was added to the residue and a 0.5 µL aliquot of the derivatized sample was injected onto a DB-17 fused silica capillary column (0.25 mm i.d. x 30m; J&W Scientific, Folsom, CA, USA) connected to a Varian 3400 gas chromatograph equipped with a cool-on-column injector. Helium was used as the carrier gas with a flow rate of 1.5 mL/min. The injector temperature was increased from 60^{o}C to 270^{o}C at the rate of 150 ^{o}C/min and maintained for 20 min. Oven temperature was raised from 50^{o}C to 190^{o}C at a rate of 30 ^{o}C/min, and maintained for 3 min, then increased to 270^{o}C at the rate of 5^{o}C/min, and maintained for 5 min. The flame ionization detector (FID) temperature was set at 270^{o}C. Peak area integration for carbohydrate analyses was according to a Shimadzu Ezchrom Data System (Shimadzu Scientific Instruments Inc., Columbia, MD, USA). The soluble NSP values were estimated by subtracting the insoluble NSP value from the total NSP value for a given sample.

3.2.3 Glucose and Insulin Tolerance Tests

After 3 weeks of experimental diets (9 weeks in total on HFD), rats were appointed to either an oral oGTT or an intraperitoneal glucose tolerance test (ipGTT). The tests were performed following an overnight fast. Fasting blood glucose was measured and blood was collected from a tail vein for insulin

determination. Then, each rat received 1g of glucose per kg of body weight via oral administration or intraperitoneal injections. Blood glucose values were obtained at 10, 20, 30, 60, and 120 minutes, using a glucometer (Accu-Check Compact Plus, Roche Diagnostics, Laval, QC, Canada). About 50 μl of blood was taken at each time point during ipGTT and centrifuged to obtain serum, which was stored at -20^{o}C. DPP-IV inhibitor (Millipore, Billerica, MA, USA) was added to aliquots obtained at baseline and 30 minutes in order to assay GIP. Insulin tolerance test (ITT) was conducted at the end of the fourth week, during which animal received an intraperitoneal injection of 20 μg/kg dose of insulin, and blood glucose was determined at 0, 15, 30, 60, 90 & 120 minutes. Area under the curve (AUC) and incremental area under the curve (IAUC) were calculated in accordance with established methods (Wolever, 2004). Homeostatic model assessment insulin resistance (HOMA-IR) was generated based on the following equation: fasting glucose (mmol/L) × fasting insulin (μU/mL) /22.5 (Matthews et al., 1985; Tran et al., 2003).

3.2.4 Body Weight, Food Intake and Measurement of Body Composition

Body weights were measured on a weekly basis. After introduction of the supplemented diets, food intake was measured for 24 hours twice during the 4-week period. In addition, one day prior to tissue collection, magnetic resonance imaging (MRI) technique was applied to specify lean and fat mass body composition using an EchoMRI Whole Body Composition Analyzer (Echo Medical Systems LLC, Houston, TX, USA).

3.2.5 Tissue Collection

At the end of the 10th week, animals were fasted overnight and then euthanized by an overdose of xylazine/ketamine via ip injection. A 3-5 mL blood sample was obtained by cardiac puncture and serum obtained following centrifugation, which was then stored at -80 ^{o}C. Intestinal segments and pancreatic tissue were collected and fixed in buffered formalin, dehydrated in graded ethanol and embedded in paraffin. They were then cut to generate 5 μm cross sections using a microtome and adhered to glass slides.

3.2.6 Assays of Serum

Samples from the ipGTT were assayed for insulin using an ELISA kit (Alpco Diagnostics, Salem, NH, USA). GIP was assayed by Meso Scale Discovery human total GIP kit (validated for use with rat samples). Serum obtained at euthanasia was assayed for triglyceride (Sigma-Aldrich, St. Louis, MO, USA) and free fatty acids (Waco Diagnostics, Richmond, VA, USA) by colourimetric assays, glucagon (Sceti K.K., Tokyo, Japan) and active GLP-1 (Millipore, Billerica, MA, USA) by ELISA according to manufacturers' instructions. All samples were run in duplicates and controls were included for each separate plate to ensure accuracy of procedures. The r^2 for all standard curves was >0.99.

3.2.7 Immunohistochemistry and Morphometric Tissue Analysis

Tissue slides were rehydrated, endogenous peroxidases quenched and non-specific binding reduced using techniques described previously (Whitlock et al.

2012). Non-specific binding was reduced by blocking with appropriate non-immune sera (1:20 dilution in phosphate buffered saline (PBS)) for twenty minutes at room temperature. For pancreas, rabbit anti-glucagon (Linco) and guinea pig anti-insulin primary antibodies (Dako) were diluted 1:100 in PBS, applied to the tissue sections and incubated overnight at 4^{o}C. For jejunum and ileum, mouse anti-GIP (generously provided by Dr. Timothy Kieffer, University of British Columbia) and rabbit anti-GLP-1 (Epitomics, Burlingame, CA, USA) were respectively diluted 1:1000 and 1:250 in PBS, then applied and incubated under the same conditions. Following washes, appropriate peroxidase-coupled secondary antibodies (1:200) were applied to the sections and the slides were incubated for 1 h at room temperature. Positive reactions were identified by peroxidation of diaminobenzidene in the presence of H_2O_2. Imaging was performed using an Axiovert microscope connected to an AxioCam MRm digital camera (Carl Zeiss, Toronto, ON, Canada), and controlled with AxioVision 4.6 software.

For pancreas, each section of the tissue was photographed under ten times magnification and then total pancreatic tissue area as well as alpha- and beta-cell areas were quantified by ImageJ software (U.S. National Institutes of Health, Bethesda, MA, USA). The ratios of the alpha-cell and beta-cell to total pancreatic area were calculated for each rat. For jejunum and ileum, random sections of each tissue were selected and photographed under twenty times magnification, and total number of GIP-positive and GLP-1-positive cells were calculated. The number of positive cells was then normalized to the number of villi.

3.2.8 Quantification of Glucose Transporters Gene Expression (Glut2, Glut5, SGLT)

Total RNA was isolated from ileal tissue using Trizol reagent and purified with an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) per manufacturer's instructions. The complementary DNA (cDNA) was generated from RNA samples using a cloned AMV first-strand cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA). The cDNA samples were amplified using primers synthesized by the IBD core at the University of Alberta and analyzed by quantitative reverse transcription polymerase chain reaction (qRT-PCR). The sequences of primers are in presented in Table 3-2. The housekeeping gene 18S ribosomal RNA (rRNA) was used for normalization of the target genes expression. All sample reactions were prepared using Evolution Eva Green qPCR mastermix (Montreal Biotech, Montreal, Canada) and run in duplicate on a Corbett Rotor-Gene 6000 cycler.

Table 3-2. Primer sequences for RT-PCR

Gene (Accession Number)	Sequence (5'-3')
Glut2 (NM_012879)	
Forward	GACACCCCACTCATAGTCACAC
Reverse	CAGCAATGATGAGAGCATGTG
Glut5 (NM_031741)	
Forward	AACTTTCCTAGCTGCCTTTGGCTC
Reverse	TAGCAGGTGGGAGGTCATTAAGCT
SGLT1(NM_013033)	
Forward	ATGGTGTGGTGGCCGATTGGGTGTAG
Reverse	ATGTCCATGGTGAAGAG
18S rRNA	
Forward	AGCGATTTGTCTGGTTAATTCCGATA
Reverse	CTAAGGGCATCACAGACCTGTTATTG

3.2.9 Statistical Analysis

Statistical analyses were conducted using GraphPad Prism 5 (Graphpad Software Inc., La Jolla, CA, USA). Prior to analyses, data were tested for normality of distribution by the Shapiro-Wilk test. Two-way repeated measures ANOVA was performed on the oGTT, ipGTT, and insulin ELISA data. One-way ANOVA, Kruskal-Wallis test and student's *t* test were used to compare the other data, as appropriate. All data are expressed as means ± SEM; Bonferroni and Dunn's post-hoc comparison tests were performed to assess differences between diet groups and a p-value <0.05 was considered to be significant. Post-hoc tests were corrected for multiple comparisons by the software.

3.3 Results

3.3.1 Fibre Analysis

Analysis of the fibre constituents from raw and cooked PSCs is reported in Table 3-3. The total fibre (NSP) content of the raw seed coat fraction was 68% w/w, with 64-65% composed of insoluble fibre and 3-4% soluble fibre. The total fibre fraction was composed mainly of glucose moieties (52%), while the total and insoluble NSF fibre fraction was also rich in xylose. Arabinose (4%) and mannose (0.2%) were also present in the total fibre of PSCs, but at low levels. Consistently, the amount of rhamnose was enriched in the soluble fraction compared to the total and insoluble fibre fractions. Galactose, xylose and a small amount of fucose were also detected in the soluble fibre fraction. The cooking treatment did not affect the fibre classes of the PSCs (P>0.05).

Table 3-3. Sugar components of raw and cooked seed coats of 'Canstar' by GC analysis (mg/100 mg dwt (%))

Fibre class	Rhamnose	Ribose	Fucose	Arabinose	Xylose	Mannose	Glucose	Galactose	Total
Raw seed coat fraction									
Total	0.73±0.01	0.05±0.01	0.27±0.02	3.73±0.33	10.59±0.87	0.19±0.01	51.81±1.11	0.77±0.03	68.13±2.04
Insoluble	0.43±0.05	0.02±0.01	0.14±0.00	2.02±0.12	9.99±0.56	0.17±0.01	52.07±0.65	0.43±0.02	65.28±0.17
Soluble	0.30±0.07	0.03±0.01	0.12±0.02	1.71±0.21	0.60±0.31	0.02±0.00	1.00±1.00	0.34±0.02	4.11±1.50
Cooked seed coat fraction									
Total	0.62±0.01	0.06±0.01	0.25±0.01	3.71±0.36	10.64±0.91	0.20±0.01	51.50±0.56	0.86±0.05	67.84±1.84
Insoluble	0.34±0.01	0.01±0.00	0.14±0.01	1.76±0.16	9.50±0.76	0.17±0.00	52.64±1.15	0.40±0.03	64.96±1.97
Soluble	0.28±0.02	0.04±0.01	0.11±0.01	1.95±0.21	1.14±0.15	0.03±0.00	0.30 ±0.30	0.46±0.07	4.33±0.31

Data are means ± SEM, n = 3. The total % (sum of sugar components) of the raw seed coat fraction was compared to the cooked seed coat fraction within each fibre class (total, insoluble and soluble fibre) using a one-way ANOVA analysis, and no significant treatment effects were detected (P>0.05).

Table 3-4. Protein and total starch components of raw and cooked PSCs of 'Canstar'

PSC Fraction	Protein (%)[†]	Total starch (%)[†]
Raw	6.65 ± 0.05	0.16 ± 0.01
Cooked[§]	6.91 ± 0.03*	0.59 ± 0.02*

Data are means ± SEM, n = 3. The raw seed coat fraction was compared to the cooked seed coat fraction for percent protein and percent total starch using a student's *t* test analysis.

* Significantly different ($P<0.05$) from the raw seed coats fractions within each nutrient component.

[§] Placed in boiling water for 30 minutes.

[†] % = mg/100 mg dry weight of sample.

The total protein content of the raw PSC fraction was 6-7% by weight, and the total starch content was less than 1% (Table 3-4). Cooking treatment significantly increased the total protein and starch content of the PSC fractions ($P<0.05$).

3.3.2 Body Weight and Body Composition Analysis

Rats in all groups gained the same amount of weight, calculated as % of baseline, at the end of the study (Table 3-5, $P>0.05$). Food intake data were also comparable between groups indicating that the palatability of the diets did not affect the results. MRI data revealed no differences in body composition, in the form of lean and fat mass as % of body weight, between the groups (Table 3-5).

3.3.3 Serum Biochemistry

These data are reported in Table 3-5. Fasting blood glucose was significantly higher in RP than LFD ($P<0.05$). Fasting serum insulin and glucagon were significantly lower in CP than HFD ($P<0.05$). HOMA-IR index, calculated from fasting glucose and insulin values as a marker of insulin resistance, was similar between the treatment diets. Serum triglyceride concentrations were significantly higher ($P<0.05$) in HFD than CP or LFD, but no differences in serum NEFA were detected (Table 3-5). Serum GLP-1 was significantly higher only in CP compared with HFD (Table 3-5, $P<0.05$).

Table 3-5. Metabolic profile of rats fed diets containing pea fractions

Diet group	HFD	RP	CP	LFD
	Mean ± SEM			
BW (g), baseline	401±11.0	402±10.0	394±14.1	397±13.3
BW (g), final	627±12.3	649±13.9	660±16.1	608±14.1
Change (% of baseline BW)	65.8±3.80	67.6±3.13	74.5±3.34	62.5±3.91
Fat mass (% of final BW)	18.9±1.21	19.1 ±0.97	18.1±0.92	16.2±1.08
Lean mass (% of final BW)	67.5±0.99	66.4±1.02	68.2±1.20	69.3±0.68
Food intake (kcal/day)	134±4.91	138±8.36	143±14.5	153±10.1
Fasting blood glucose (mmol/L)[§]	5.30±0.18	5.50±0.14[a]	4.90±0.28	4.40±0.08
Fasting serum insulin (ng/mL)[§]	1.04±0.13	1.24±0.19	0.49±0.08*	0.72±0.15
HOMA-IR (mmol/L×μU/mL)	7.32±0.89	7.50±1.06	4.51±0.72	5.84±1.09
Serum TG (mg/dL)[†]	52.2±2.29	45.3±8.69	30.4±4.37*	32.6±4.00
Serum NEFA (mmol/L)[†]	0.50±0.10	0.49±0.07	0.37±0.09	0.37±0.08
Fasting serum glucagon (pg/mL)[†]	308±32.3	287±12.3	167±26.9*	247±17.6
Fasting serum GLP-1 (pg/mL)[†]	18.3±0.67	23.1±2.45	27.9±1.57*	23.4±1.46

BW, Body weight; HFD, high fat diet; RP, raw pea seed coat (HFD supplemented with raw seed coats); CP, cooked pea seed coat (HFD supplemented with cooked seed coats); LFD, low fat diet; HOMA-IR, homeostatic model assessment insulin resistance (fasting glucose (mmol/L) × fasting insulin (μU/mL) /22.5. Data are means ± standard error of the mean (SEM) analyzed by one-way ANOVA followed by Bonferroni's post-hoc comparison test, n = 4-25. Asterisks show significant difference compared to HFD (*P<0.05). Superscript letter indicates significant difference compared to LFD ([a]P<0.05).

[§] Blood sampling was done at the end of the feeding trial during oral glucose tolerance test.

[†] Serum for TG, NEFA, glucagon and GLP-1 assessment was obtained from blood samples collected from fasted rats by cardiac puncture at the time of tissue collection.

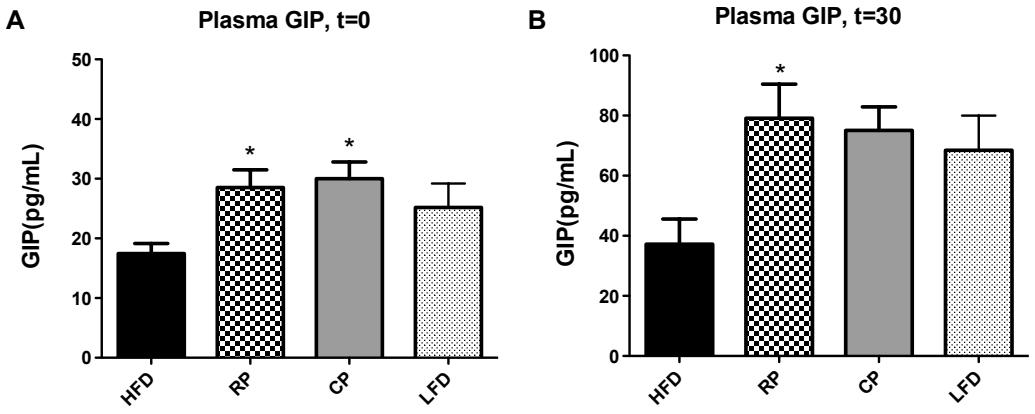

Figure 3-3. Plasma GIP concentrations. Plasma concentrations of GIP measured during oGTT at fasting (t = 0 min), and following administration of 1g/kg glucose (t = 30 min). The bars are the mean ± SEM, n = 4-7. * indicates significant difference compared to HFD by one-way ANOVA followed by Bonferroni's post-hoc comparison test (*P<0.05).

As shown in Figure 3-3, in fasted rats, fasting serum GIP concentrations were 50% higher in RP and CP than HFD ($P<0.05$ for both). A similar trend was observed with GIP measured 30 min after glucose administration in the oGTT, in which RP was 2-fold higher than HFD (Figure 3-3A and B, $P<0.05$).

3.3.4 Glucose Tolerance Tests and Insulin Tolerance Test

oGTT and ipGTT results are shown as responses over 120 minutes (Figures 3-4A and 3-5A), and as incremental area under the curve (IAUC, Figures 3-4B and 3-5B). As expected, during oGTT, LFD had lower glucose response compared to HFD at t = 10 min ($P<0.05$), and t = 20, 30, and 60 min ($P<0.001$). CP but not RP rats had lower glucose response compared to the HFD group at t=10 ($P<0.05$), t = 20 ($P<0.001$), and t = 30 ($P<0.01$) min. IAUC during oGTT showed that both CP and LFD groups had glucose values that were significantly lower than HFD (Figure 3-4B, $P<0.05$ and $P<0.001$ respectively). Although neither CP nor RP had different ipGTT from HFD group (Figure 3-5A), LFD had improved response at t = 20 ($P<0.001$) and t = 30 ($P<0.01$) min. Trends for LFD and CP to lower IAUC during ipGTT were attenuated and not statistically significant (Figure 3-5B).

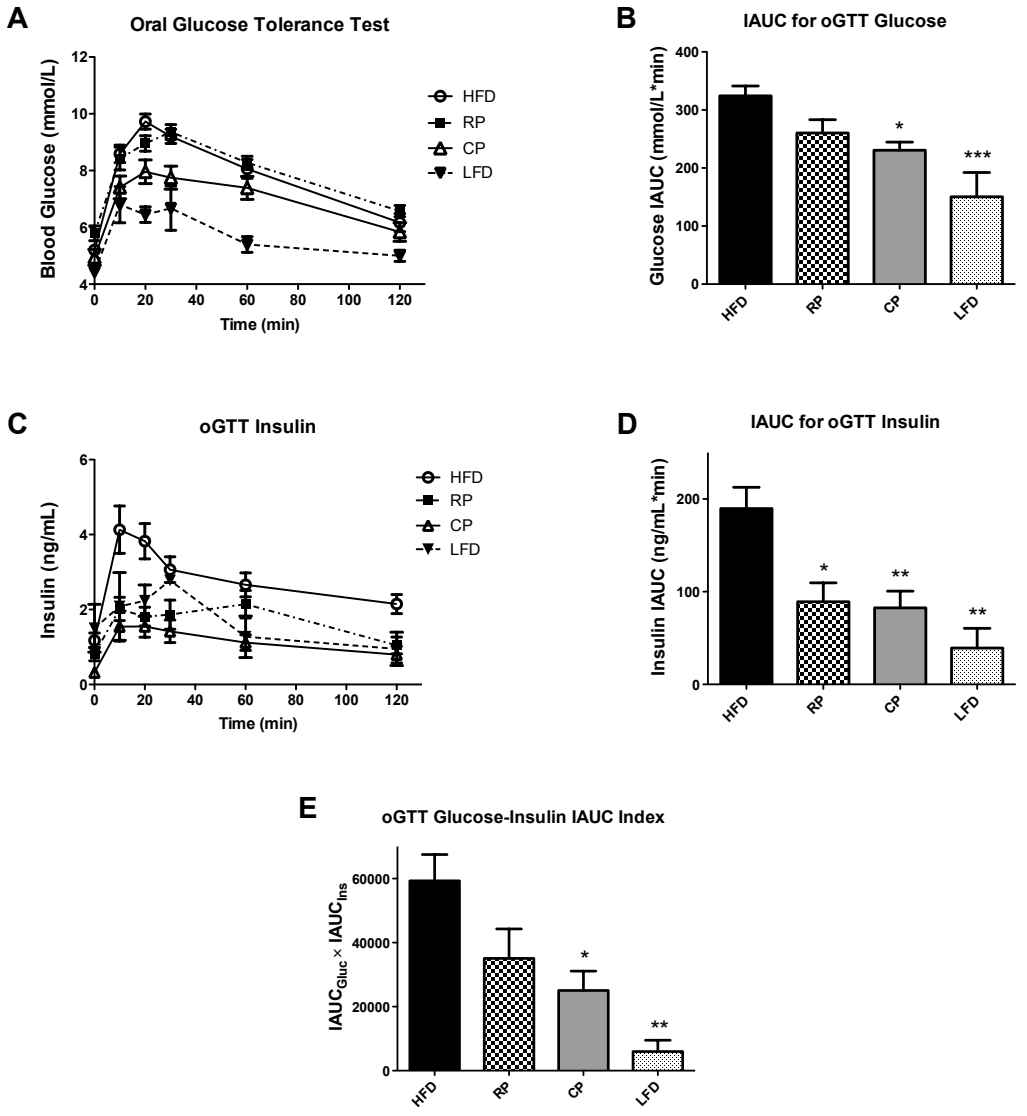

Figure 3-4. Effect of feeding PSC on oral glucose tolerance. (A) Effect of 3 weeks of feeding a high fat diet (HFD, 20% w/w) supplemented with raw (RP) or cooked (CP) pea seed coats on blood glucose levels measured basally and following oral administration of 1g/kg glucose. (B) Incremental area under the curve (IAUC) was calculated for glucose during oral glucose tolerance test (oGTT). **(**C) Plasma insulin levels measured using the blood samples collected during oGTT. (D) IAUC for insulin during oGTT. (E) Glucose-insulin IAUC index calculated from IAUC values for glucose and insulin during oGTT. The data are means ± SEM, n = 4-14. Significant differences seen at different time points are explained in the text, while differences between IAUC are depicted here. Asterisks show significant difference compared to HFD by one-way ANOVA followed by Bonferroni's post-hoc comparison test (***P*<0.01, **P*< 0.05).

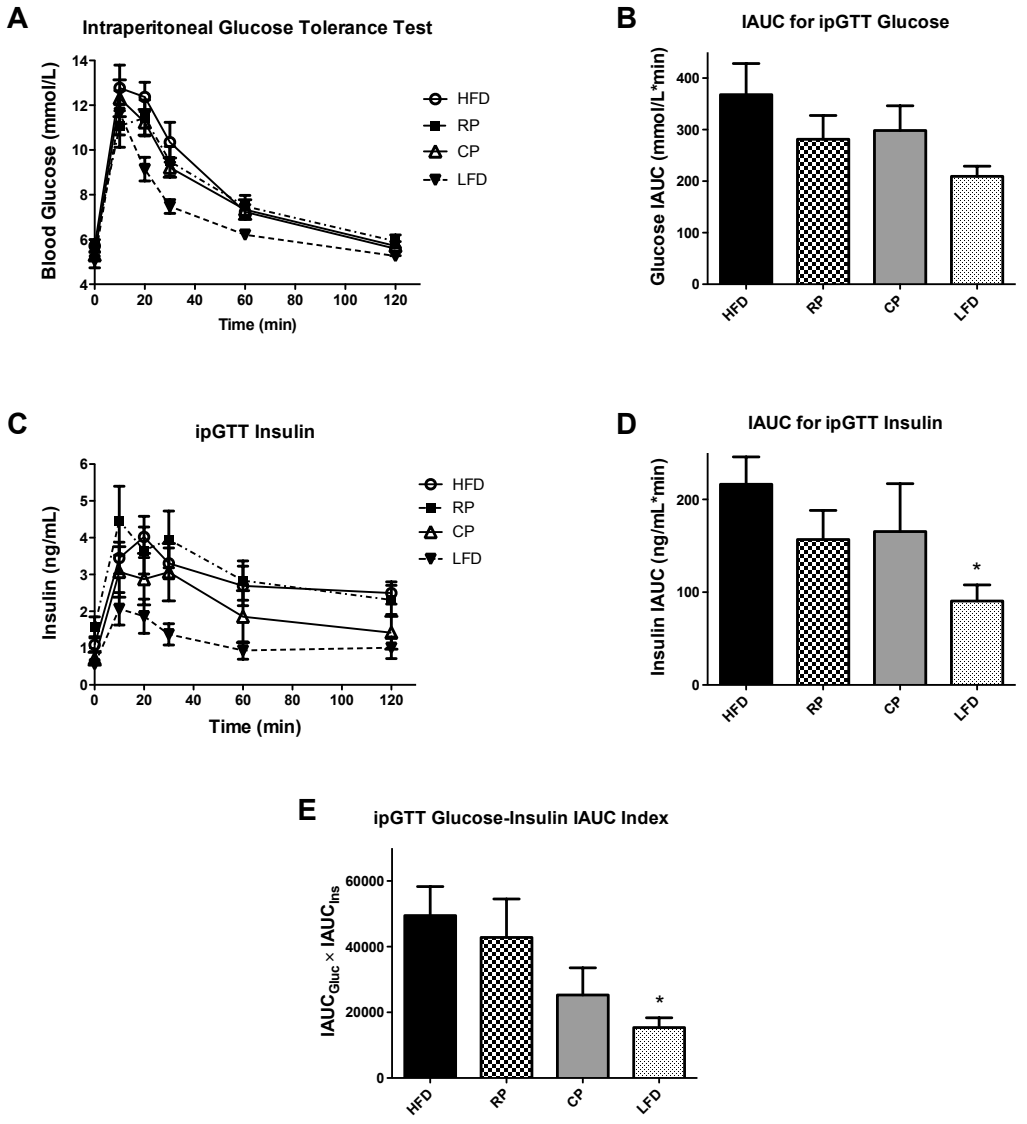

Figure 3-5. Effect of feeding PSC on intraperitoneal glucose tolerance. (A) Effect of 3 weeks of feeding a high fat diet (HFD, 20% w/w) supplemented with raw (RP) or cooked (CP) pea seed coats on blood glucose levels measured basally and following intraperitoneal administration of 1g/kg glucose. (B) Incremental area under the curve (IAUC) was calculated for glucose during intraperitoneal glucose tolerance test (ipGTT). **(**C) Plasma insulin levels measured using the blood samples collected during ipGTT. (D) IAUC for insulin during ipGTT. (E) Glucose-insulin IAUC index calculated from IAUC values for glucose and insulin during ipGTT. The data are means ± SEM, n = 8-11. Significant differences seen at different time points are explained in the text, while differences between IAUC are depicted here. Asterisks show significant difference compared to HFD by one-way ANOVA followed by Bonferroni's post-hoc comparison test (*P< 0.05).

During oGTT, RP rats had lower insulin concentrations than HFD rats at t=10 and 20 min (Figure 3-4C, $P<0.001$). CP group had decreased insulin concentrations compared to the HFD group at t = 10 ($P<0.001$), t = 20 ($P<0.001$), t = 30 ($P<0.01$), t = 60 ($P<0.01$) and t = 120 ($P<0.05$) min. The LFD group had lower serum insulin than the HFD group at t = 10 ($P<0.01$) min. Both RP and CP groups had smaller IAUC values when compared to HFD (Figure 3-4D, $P<0.05$ and $P<0.01$ respectively); LFD rats also had significantly lower IAUC ($P<0.01$). Insulin concentrations of CP and RP groups during ipGTT were not different than those of HFD (Figure 3-5C). However, LFD had decreased concentrations at t = 20 and 30 min ($P<0.05$). IAUC data also only revealed a difference between HFD and LFD (Figure 3-5D, $P<0.05$). The glucose-insulin IAUC index, calculated from IAUC values for glucose and insulin during GTTs, was used as a marker of whole body insulin action, where a lower value represents indirect evidence for improved insulin action (Sutherland et al., 2008; Yang et al., 2015). Compared with the HFD, both CP and LFD had lower values of glucose-insulin IAUC index during oGTT (Figure 3-4E, $P<0.05$ and $P<0.01$ respectively). The ipGTT glucose-insulin IAUC index was significantly reduced in LFD (Figure 3-5E, $P<0.05$).

Blood glucose levels during the glucose disappearance phase (0-30 min) of the ITT were comparable among the groups (Figure 3-6A, B). During the recovery phase (60-120 min), HFD rats rebounded most quickly and this was significantly faster than for RP rats (Figure 3-6C, $P<0.05$).

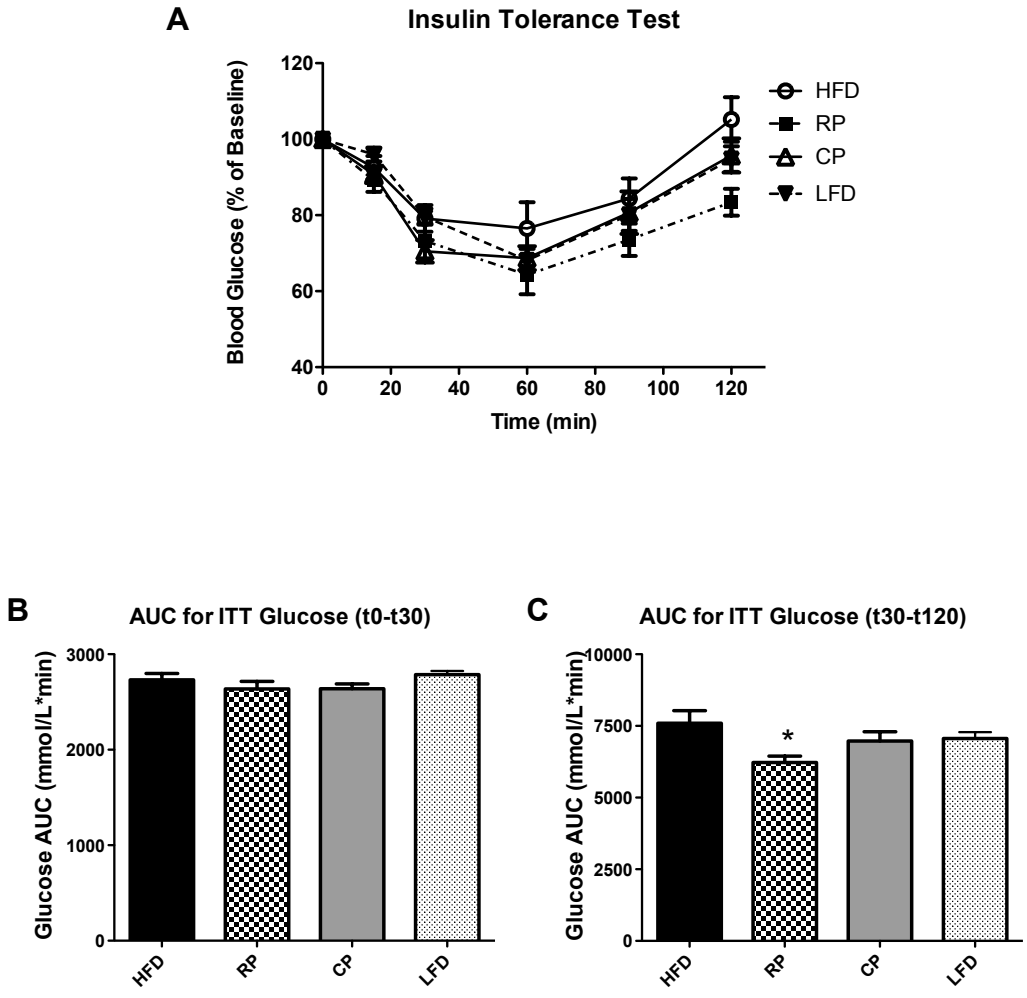

Figure 3-6. Effect of 4 weeks of feeding PSC on insulin tolerance. Insulin tolerance test (ITT) was performed on rats after a 4 hour fast. Blood glucose levels are shown as (A) % of basal glucose, and Area Under the Curve (AUC) from (B) t = 0 to t = 30 min and (C) t = 30 to t = 120 min for blood glucose. A significant decrease in glucose for RP compared to HFD was observed at t = 30-120 min (**P*<0.05). Data are means ± SEM analyzed by one-way ANOVA followed by Bonferroni's post-hoc comparison test, n = 8.

3.3.5 Pancreatic Beta- and Alpha-cell Mass Analysis

Pancreatic beta- and alpha-cell area at the end of the study are shown in Figure 3-7A and B. After four weeks of PSC intervention, no significant difference in beta-cell area between diet groups was observed (Figure 3-7A, $P>0.05$). As shown in Figure 3-4B, alpha-cell area in the four diet groups followed a similar pattern with beta-cell area; however, significant differences were found between diet groups ($P<0.05$), with CP fed rats having a significantly smaller alpha-cell area compared with the RP fed rats ($P<0.05$). Total islet area was estimated by adding alpha- and beta-cell areas and are presented in Figure 3-7C ($P = 0.16$; denoting trend to increased islet area in the RP group). Representative micrographs depicted in Figure 3-7D suggest that the increase in islet area of the RP group is due to an increase in the number of islets, rather than the size of individual islets.

3.3.6 K- and L-cell Quantification

There was no significant difference in the number of K-cells expressing GIP in the jejunum shown in Figure 3-8A ($P>0.05$). Similarly, the number of GLP-1 positive L-cells in the ileum was comparable between all the groups (Figure 3-8B, $P>0.05$).

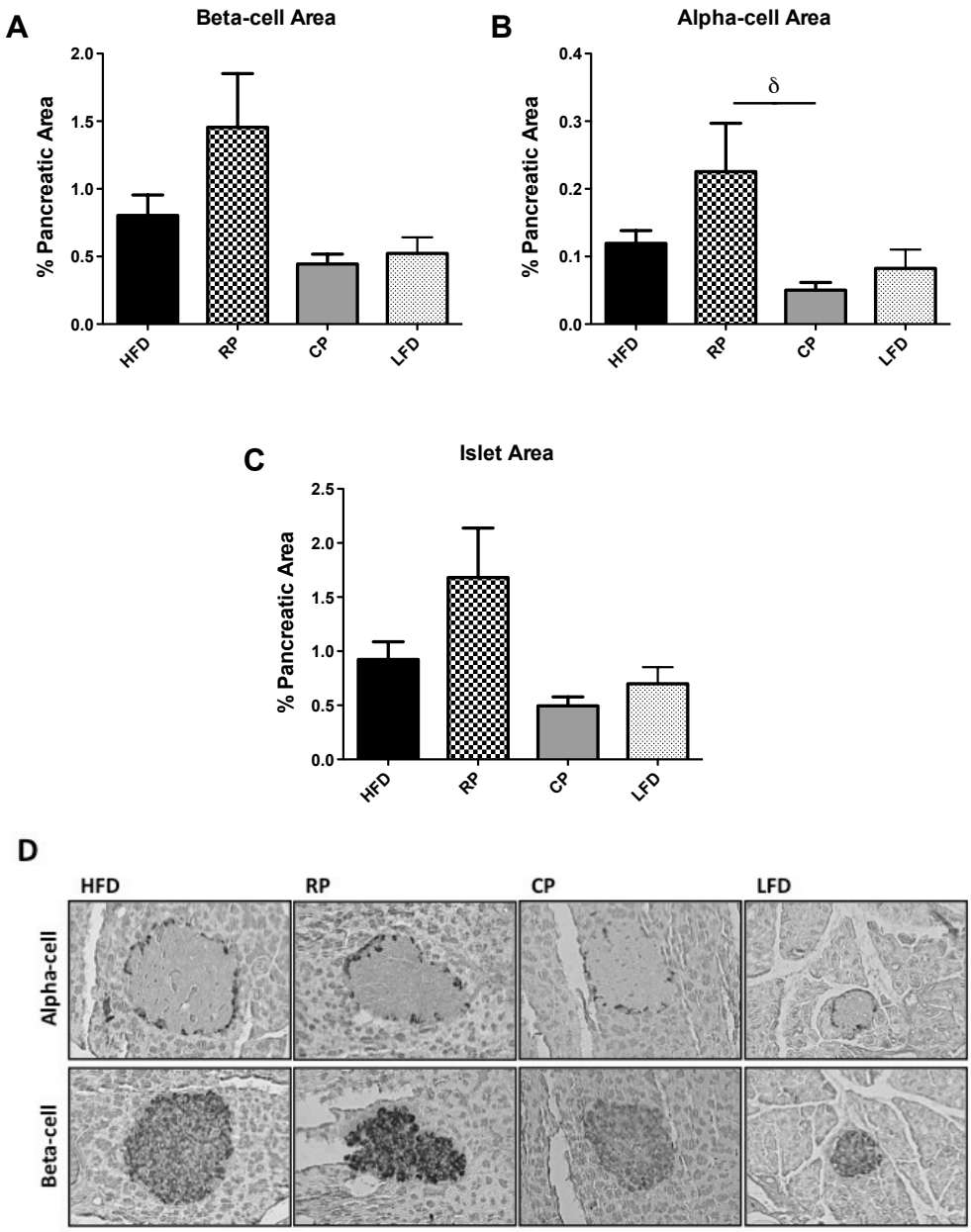

Figure 3-7. Effect of PSC feeding on pancreatic beta- and alpha-cell mass. (A) Beta-cell and (B) alpha-cell areas of rats fed a high fat diet (HFD), raw PSC (RP), cooked PSC (CP) and low fat diet (LFD) for 4 weeks, presented as percentage of pancreatic area (C) Estimated total islet area. Alpha-cell area was significantly different between the groups ($P<0.05$), where CP rats revealed a smaller alpha-cell area ($^{\delta}P<0.05$) when compared to RP. Data are means ± SEM analyzed by one-way ANOVA or Kruskal-Wallis test followed by Bonferroni and Dunn's post-hoc comparison tests as approriate, n= 6-8. (D) Representative insulin- and glucagon-stained islets of all the groups.

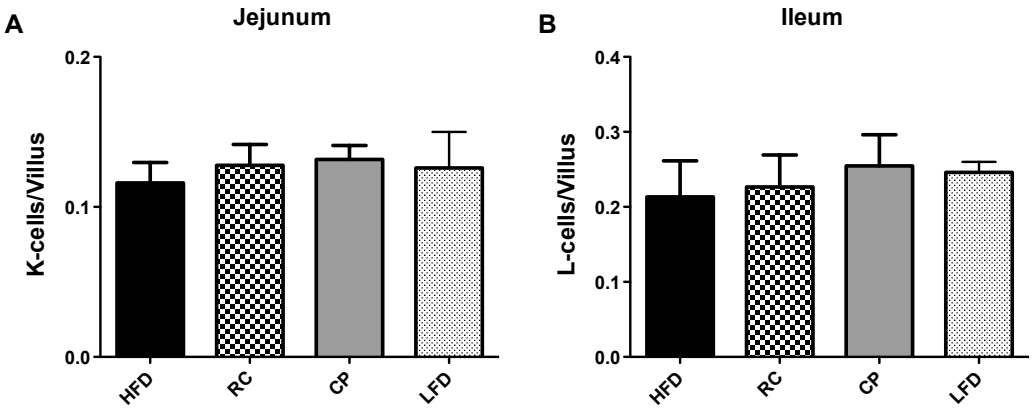

Figure 3-8. Effect of PSC feeding on the incretin-secreting K- and L-cells. (A) Number of K-cells and (B) L-cells as detected using GIP and GLP-1 immunoreactivity in jejunum and ileum, presented as number of positive cells per villus. Data are means ± SEM analyzed by one-way ANOVA followed by Bonferroni's post-hoc comparison test, n = 4. No significant differences were observed.

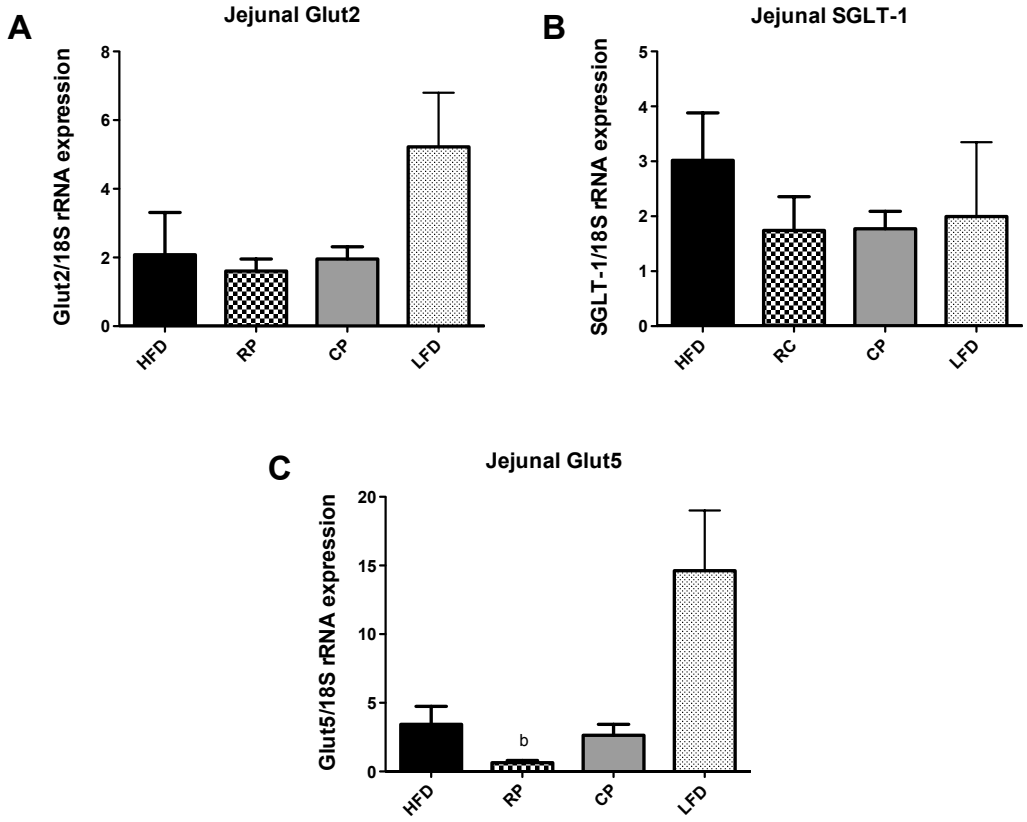

Figure 3-9. mRNA Expression of Glucose Transporters**.** (A) mRNA expression of Glut2, (B) SGLT1 and (C) Glut5 in jejunum of the rats normalized to 18S rRNA expression. Data are means ± SEM analyzed by one-way ANOVA or Kruskal-Wallis test followed by Bonferroni and Dunn's post-hoc comparison tests as appropriate, n= 5-12. Letter ^{b} indicates significant difference compared to LFD ($^{b}P<0.01$).

3.3.7 mRNA Expression of Glucose Transporters

Jejunal mRNA expression of Glut2 and SGLT1 were similar between the groups (Figure 3-9A and B); however, Glut5 expression was significantly different between diet groups (P=0.005), with a lower expression in RP when compared to LFD (Figure 3-9C, P<0.01).

3.4 Discussion

The present study demonstrated that supplementing a HFD with cooked PSCs improved glucose tolerance, whereas raw seed coat supplementation was not as beneficial. We also observed that the effect of the PSC fibre on postprandial glucose excursions was only detectable when glucose was administered orally and not intraperitoneally. In other words, bypassing the gastrointestinal tract during ipGTT diminished the improved glycemic excursions in the pea fibre groups to a high degree. These divergent outcomes on oral versus ip glucose tolerance led us to consider mechanisms of action involving the gastrointestinal tract.

The total fibre fraction of PSCs was mainly composed of the monosaccharide glucose (Table 3-3), indicating that the most abundant polysaccharide present was cellulose (made up of linear chains of glucose). Given that cellulose is a water-insoluble polysaccharide, the insoluble fibre component was also mainly made up of cellulose. The glucose content determined in the total NSP fibre of PSCs in this study is consistent with that of 58% reported by Weightman et al. (1994). The higher percentage of xylose (also consistent with

Weightman et al., 1994), along with the occurrence of fucose, galactose and glucose in the total and insoluble NSF fibre fraction indicates the presence of the cell wall cellulose microfibril cross-linking polysaccharide, fucogalacto-xyloglucan, commonly found in legume family members (Carpita and McCann, 2002). The presence of arabinose in the total fibre of PSCs (Weightman et al., 1994 reported 3.9% arabinose in this fraction) suggests the presence of glucuronoarabinoxylans and/or pectins (Carpita & McCann, 2002). Very low levels of mannose indicate minimal presence of glucomannans, galactoglucomannans, or galactomannans in interlocking microfibrils of the cell wall (Carpita & McCann, 2002). The major non-cellulosic neutral sugars, arabinose and xylose, detected in the soluble fibre PSC fraction indicate the presence of pectin (Carpita & McCann, 2002; Weightman et al., 1994). Rhamnose, which is another constituent of pectins, was also enriched in the soluble fraction of PSCs. Galactose, xylose and the small amount of fucose also indicate the presence of fucogalacto-xyloglucans in the soluble fibre fraction.

Cooking treatment improved glucose tolerance but did not alter the fibre or sugar classes of the pea seed coat fraction. One explanation is that the boiling process may have caused separation and/or hydration of the fibre components that were stabilized by the subsequent lyophilization. Our results are consistent with previous studies showing that cooking procedures did not affect the total dietary fibre (Goodlad & Mathers, 1992; Marconi et al., 2000). In another study, however, it was reported that cooking followed by freeze-drying resulted in increased

insoluble dietary fibre (IDF) in whole legume seeds (Almeida Costa et al., 2006). Conversely, Kutosˇ et al. (2003), found decreased IDF content when examining the effect of thermal processing on whole beans. Other thermal procedures such as autoclaving have also been shown to result in changes in the physical properties of wheat bran fibre including increased aqueous recovery of specific NSP such as inulin and resistant oligosaccharides (Harding et al., 2014). In addition, boiling may lead to partial solubilization and depolymerization of hemicelluloses and insoluble pectic substances (Marconi et al., 2000), which may change the properties of the fibre with respect to gut fermentation. Altered microstructures of pea flour, as a result of thermal treatments in general, can promote its nutritional and functional characteristics, among which are increased fat and water absorption capacity, and emulsifying and gelling activity (Ma et al., 2011). In addition to higher nutritional value, the thermal processing-derived characteristics of pea flour have been suggested to improve its practicality for food application (Almeida Costa et al., 2006; Ma et al., 2011). We also observed that cooking increased the protein component of the PSC fraction; however, considering this amount accounted for only 2.5% of the total protein content of the diets, it is unlikely this change was accountable for any secondary outcomes. The same could be applied to the increased amount of starch following cooking.

In the current study, we reported enhanced glucose control in the CP group during oGTT versus no improvement during ipGTT. This effect could be explained by several different mechanisms. SDF in general has been proposed to improve

glycemic control and insulin sensitivity through mechanisms such as delayed gastric emptying and glucose absorption by increasing gastrointestinal viscosity (Galisteo et al., 2008). In our study however, insulin sensitivity did not appear to be affected by pea seed coat supplementation. SDF can also be fermented to SCFA in the colon, which are absorbed into the blood and are reported to promote GLP-1 secretion (Freeland & Wolever, 2010;Yadav et al., 2013) and stimulate skeletal muscle uptake of glucose (Lu et al., 2004). A previous study by our group showed increased circulating 3-hydroxybutyrate believed to be derived from butyrate of dietary origin because butyrate dehydrogenase expression in the liver was suppressed (Chan et al., 2014). On the other hand, IDF, in spite of lacking effects on viscosity, has also been shown to have a role in regulating glucose homeostasis through potential mechanisms such as increasing the passage rate of nutrients, decreasing their absorption and increasing GIP secretion (Schenk et al., 2003; Weickert et al., 2005; Weickert et al., 2006). We speculated that CP might improve oGTT by down-regulating the expression of intestinal glucose transporters. However, HFD appeared to be the main negative driver of glucose transporter expression, and there was no subsequent modulation upon addition of RP or CP. Contrary to our results, a study performed on dogs showed that a diet containing high fermentable dietary fiber resulted in increased jejunal SGLT1 and Glut2 mRNA abundance (Massimino et al., 1998), but that would not explain enhanced glucose tolerance.

PSC-supplemented diets significantly enhanced plasma incretin concentrations. GLP-1 has insulinotropic effects and acts directly on pancreatic islets to stimulate insulin secretion from beta-cells, promote beta-cell proliferation and suppress apoptosis (Seino et al., 2010) as well as noninsulinotropic effects such as inhibiting gastric emptying. GLP-1 also inhibits glucagon secretion, and decelerates endogenous production of glucose (Seino et al., 2010). In several animal studies, consumption of fermentable dietary fibres has been linked with elevated plasma GLP-1 (Grover et al., 2011; Massimino et al., 1998; Wang et al., 2007). In addition, in both healthy (Johansson et al., 2013; Tarini & Wolever, 2010) and hyperinsulinemic (Freeland et al., 2010) human subjects, diets high in SDF increased GLP-1 in plasma. In our study, we observed 50% higher fasting GLP-1 in CP group relative to HFD. This could be positive given that it has previously been reported that diabetic patients had significantly lower fasting serum GLP-1 when compared with non-diabetic overweight subjects (Legakis et al., 2003); however, the physiological significance of fasting GLP-1 levels remains elusive. Pannacciulli et al. (2006) examined the association between fasting plasma GLP-1 concentration and energy expenditure and fat oxidation, and reported a positive association between them. In another study, both in-vitro and in-vivo results showed that GLP-1 increased basal uptake of glucose in the muscle through a nitric oxide-dependent pathway, although the concentration of the GLP-1 used was higher than the fasting levels seen in our rats (Chai et al., 2012).

GIP is another incretin that is secreted in response to nutrient ingestion resulting in many similar actions as GLP-1 in the pancreas; however, outside the pancreas, GIP and GLP-1 seem to function differently from one another. GIP secretion has been reported by many studies to be normal or sometimes increased in the state of impaired glucose tolerance and T2D, whereas its insulinotropic effect is diminished in T2D (Kim & Egan, 2008). Studies of the effects of dietary fibre intake on circulating GIP have produced diverse results, with SDF suppressing and IDF augmenting GIP in diabetic and healthy human subjects (Weickert & Pfeiffer, 2008). In healthy adults, a whole barley kernel meal resulted in higher postprandial GIP in plasma (Johansson et al., 2013). In another human study, healthy subjects had lower GIP responses following a whole-kernel rye bread when compared to a white bread meal (Juntunen et al., 2002). In our study, we observed higher GIP responses in the pea fibre-fed rats before and post-glucose ingestion, independent of changes in K-cell number, suggesting increased sensitivity to stimulation. However, because GIP secretion changes were similar in RP and CP, this could not account for the differential effects on oGTT between the groups. Furthermore, improved glucose tolerance could not be accounted for by differences in body weight gain or body fat amongst the groups. In contrast, male Wistar rats, following a high fibre diet (21% wt/wt), composed of inulin and oligufructose, had a lower percentage of body fat (Reimer et al., 2012). In our study, we failed to detect a significant change in body composition, which may be related to the short length of our study (10 weeks) versus that of (21 weeks) Reimer et al. (2012).

Finally, the current study demonstrated that HFD supplementation with CP for 4 weeks resulted in almost 50% decrease in beta-cell area in glucose intolerant rats (non-significant). This observation was expected based on the oGTT results suggesting CP-fed rats had an improved glucose tolerance compared with those on the diets supplemented with either RP or cellulose. Given the well-documented beta-cell mass expansion as a major adaptation to insulin resistance (Ahren et al., 2010), the marginal decline in beta-cell mass shown here, in the absence of further elevation in plasma glucose concentrations, could be an indicator of a reversed progression of insulin resistance. A novel finding upon dietary intervention with PSC was the significant difference in alpha-cell mass between groups. Specifically, supplementation with CP decreased alpha-cell mass in glucose intolerant rats to the level comparable to that seen in the LFD group. While it has been widely asserted that HFD-induced insulin resistance results in expansion in beta-cell area (Ahren et al., 2010; De Almeida Costa et al., 2006; Goodlad & Mathers, 1992; Hull et al., 2005; Marconi et al., 2000; Pick et al., 1998), it is not very clear if it has the same impact on alpha-cells. Dysregulated glucagon secretion has been proposed as an early hallmark of type 2 diabetes (D'Alessio, 2011; Liu et al., 2011; Weiss et al., 2011). In diabetic mice increased number of alpha-cells and alpha-cell mass was reported as diabetes developed over time (Liu et al., 2011). In general, it has been suggested that alpha-cell proliferation is regulated by both insulin and glucagon. In response to insulin resistance, elevated intra-islet insulin concentration can originally inhibit glucagon secretion; however, consequently, as alpha-cells develop resistance to insulin, the regulation of glucagon secretion will be impaired.

Elevated circulating glucagon then, independent of intra-islet insulin secretion, leads to excessive hepatic glucose production and aggravating hyperglycemia (Liu et al., 2011). In our study, increased alpha-cell area in HFD rats may be due to elevated insulin and glucagon levels in the plasma. At the same time, rats in the CP group had significantly lower plasma insulin concentrations compared to those in the RP and HFD groups, which could explain the smaller alpha-cell area and lower fasting glucagon in that group. In addition, lower glucagon secretion could result in downregulation of hepatic gluconeogenesis and hence reduced fasting plasma glucose concentration, although this did not appear to be the case in our animals. A smaller beta-cell area observed in rats fed CP as compared to those fed RP or cellulose may thus be a result of reduced stress on pancreatic beta-cells.

3.5 Conclusion

These data show that daily intake of cooked PSCs improves glucose tolerance and decreases postprandial insulin in glucose intolerant rats. Moreover, feeding cooked PSCs decreases fasting glucagon levels likely as a result of smaller alpha-cell area. The same diet also enhances plasma incretin concentrations. The most likely component of the PSCs exerting these effects was the fibre, because the amount of protein contributed to the overall diet was only 0.7% of the diet (w/w) and this variety of pea did not contain appreciable amounts of tannins (Whitlock et al. 2012). Current Canadian guidelines (2013) recommend that the diabetic population increase its dietary fibre intake to 15-25 g per 1000 kcal. In the present study, rats received 3.3 g of pea fibre for every 140 calories of diet consumed daily,

which corresponds to a daily intake of 23 g fibre per 1000 kcal. Given that this amount is still within the range of the current recommendations of CDA for dietary fibre intake in diabetic adults, incorporating a similar amount of pea fibre into diets seems feasible, from a practical perspective, when translating our results to human interventions.

Chapter 4: Assessment of a Potential Role of the Gut Microbiome in the Beneficial Effects of Pea Seed Coat-supplementation on Glucose Homeostasis

4.1 Introduction

Obesity and T2D are among several abnormalities arising in the context of low-grade inflammation (Lee et al., 2013). The gut microbiota is now well established as a modulator of low-grade inflammation caused by elevated circulating levels of LPS, known as metabolic endotoxemia (Cani et al., 2007a; Cani et al., 2009; Muccioli et al., 2010; Shan et al., 2013). Cani and colleagues (2007) were the first to report that exposure to HFD led to elevated levels of LPS (2-3 times higher than normal); following infusion of similar levels of LPS, mice displayed raised glucose and insulin concentrations comparable to those of HFD-fed mice (Cani et al., 2007a). Suppression of TLR-4 signaling, the main LPS detection pathway, has been shown to improve insulin sensitivity and glucose tolerance in rat models of diet-induced obesity (Liu et al., 2013; Oliveira et al., 2011). In addition, TLR-4 loss-of-function mutant mice demonstrated protection against diet-induced insulin resistance and obesity (Tsukumo et al., 2007). Human studies have also confirmed these results showing that high-fat feeding is associated with increased endotoxin in plasma (Erridge et al., 2007; Pendyala et al., 2012).

Aside from involvement of gut microbiota in metabolic abnormalities due to LPS, further evidence implicating important roles for microbiota in obesity and its related metabolic disorders has been reported. One instance is the report of

germ-free mice being protected against diet-induced obesity (Backhed et al., 2007), which was in accordance with a previous study showing that germ-free mice conventionalized with normal microbiota developed features of metabolic diseases including increased fat mass and insulin resistance (Backhed et al., 2004).

In addition, diet-induced obesity is strongly associated with altered gut microbiota composition (Cani et al., 2007; Ley, 2010; Turnbaugh et al., 2006) and impaired gut barrier function recognized by increased intestinal permeability (Cani et al., 2008; De La Serre et al., 2010; Lam et al., 2012). Increased intestinal permeability itself is believed to result from reduced expression of tight junction proteins such as occludin and ZO-1 and their altered pattern of distribution (Everard et al., 2011; Cani et al., 2009).

Given the documented association between gut microbiota and host diet, recent studies have used a dietary approach to induce beneficial changes in the composition of gut microbiota. Dietary probiotics such as inulin and oligofructose are amongst two of the most studied dietary compounds linked with favorable microbial modifying qualities (Bomhof et al., 2014; Bouhnik et al., 2006; Kolida et al., 2007; Parnell & Reimer, 2012). Furthermore, recent evidence suggests that lack of soluble fibre in general contributes to HFD-induced obesity in mice; when compared to HFD with cellulose, inclusion of inulin was shown to protect mice against HFD-induced obesity. It was also reported that this effect was associated with increased fecal SCFA production, suggesting the protective effect of inulin

was mediated by gut microbiota (Chassaing et al., 2015). Recently, other types of fermentable dietary fibres including resistant starch, corn-based soluble fibre, wheat dextrin and pea fibre have also been of interest (Boler et al., 2011; Eslinger et al., 2014; Lefranc- Martinez et al., 2010; Millot et al., 2012; Queiroz-Monici et al., 2005). In healthy Wistar rats, peas were shown to possess the strongest bifidogenic properties when compared to other types of pulses including chickpea, bean and lentil (Queiroz-Monici et al., 2005). Another study reported decreased *Firmicutes* in pea flour- and pea fibre-fed obese rats (Eslinger et al., 2014).

Considering the potential of peas to alter gut microbiota due to their fermentable dietary fibre content, the objective of the present study was to elucidate the effects of feeding glucose intolerant rats PSC fractions on the microbial composition of the gut and select features of gut barrier function, particularly the abundance of TLRs, tight junction and mucin proteins. Since HFD feeding has been associated with adverse microbial modifications in the microbiota, we hypothesized that dietary supplementation with PSC fractions would alter the overall microbial composition of the gut compared to control diets and it would be associated with the growth of beneficial bacteria such as *Bifidobacterium*. A secondary hypothesis was that PSC-containing diets would partially reverse the HFD-induced changes in intestinal barrier through normalizing the expression of tight junction proteins ZO1 and occludin, toll-like receptors and mucin proteins.

4.2 Methods

4.2.1 Animals, Experimental Diets and Tissue Collection

Male Sprague Dawley rats (n=32) were obtained from the Department of Biology, University of Alberta at age eight weeks. During one week of acclimatization, animals were housed two per cage under controlled conditions of temperature and humidity, on a 12-hour light/dark cycle with free access to normal chow and water. The complete composition of the diets and their effects on glucose homeostasis, body weight, body composition and food intake were described in chapter 3. In brief, rats were fed HFD for 6 weeks to induce glucose intolerance. They were then randomly assigned to HFD supplemented with either raw (RP) or cooked (CP) pea seed coats. The two control groups were HFD and low fat diet (LFD) with cellulose as the source of dietary fibre, whereas in the RP and CP groups, cellulose was substituted with PSC preparations. Also of note is that all the experimental diets had the same total dietary fibre content. Rats were fed the treatment diets for 4 weeks. They were then anesthetized with ketamine and xylazine and euthanized by exsanguination. Segments of ileum and colon were removed and scrapings were collected along with faecal samples. The animal protocols were approved by the Health Sciences Animal Care and Use Committee at the University of Alberta and conformed to the guidelines of the Canadian Council on Animal Care.

4.2.2 DNA Extraction and Microbial Profiling

Stool pellets from animals were collected for microbial composition analysis at the time of tissue collection. Total DNA was extracted according to manufacture's instructions (QIAamp DNA Stool Mini kit, Qiagen, Valencia, CA, USA) with the addition of a 60s homogenization step (FastPrep instrument, MP Biomedicals, Solon, OH, USA). 16S rRNA gene fragments were amplified using a set of 33 nucleotide-bar-coded primer pairs (27F; 5′-AGAGTTTGATCMTGGCTCAG-3′, 519R; 5′-GWATTACCGCGGCKGCTG-3′) in triplicate. PCR products were then gel-purified with a QIAquick gel extraction kit (Qiagen, Valencia, CA, USA). The resultant PCR amplicons (100 ng each) were pooled and pyrosequenced with a 454 Titanium platform (Roche, Branford, CT, USA).

4.2.2.1 Bioinformatics

Sequences were processed using MOTHUR according to the standard operating procedure, accessed on July 10, 2013 (Schloss et al., 2011). Quality sequences were obtained by removing sequences with ambiguous bases or quality read length less than 200 bases and chimeras identified using chimera.uchime. Quality sequences were aligned to the silva bacterial reference alignment and operational taxonomic units (OTU) were generated using a dissimilarity cutoff of 0.03. Sequences were classified using the classify.seqs command with Ribosomal Database Project (RDP) as reference. Inverse Simpson's diversity index was used to calculate diversity. Differences in microbial communities between groups were

investigated using the phylogeny-based weighted UniFrac distance metric. Significant differences in community structure were determined by analysis of molecular variance (AMOVA). Diversity, similarity and abundance of bacterial OTUs and families were compared using the Mann-Whitney *U*-test or student's *t* test for nonparametric and parametric data respectively. Bonferroni correction was applied in cases of multiple comparisons.

4.2.3 RNA Extraction and Real-time Polymerase Chain Reaction

Total RNA was extracted from ileal and colonic scrapings using Trizol reagent (Invitrogen, Carlsbad, CA, USA) followed by column-based purification with an RNeasy mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Reverse transcription was performed on 1 μg of total RNA using a cloned AMV first-strand cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA). Primers generated by the IBD core at the University of Alberta were used for cDNA amplification by real-time PCR. The sequences of primers are in Table 4-1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene for normalization of the target genes expression. PCR reactions were performed using Perfecta SYBR green supermix (Quanta BioSciences, Gaithersburg, MD, USA). All assays were run in duplicate on a ViiA^{tm} 7 PCR cycler (Applied Biosystems, Grand Island, NY, USA).

4.2.4 Statistical Analysis

Statistical analyses were conducted using GraphPad Prism 5 (Graphpad Software Inc., La Jolla, CA, USA). Prior to analyses, data were tested for normality of distribution by the Shapiro-Wilk test. For gene expression data, ΔΔCT analysis was used and statistically significant differences were determined by using one-way ANOVA for parametric and Kruskal-Wallis test for nonparametric data. Bonferroni and Dunn's post-hoc comparison tests were performed as appropriate to assess differences between individual diet groups. Post-hoc tests were corrected for multiple comparisons by the software. All data are expressed as means ± SEM and a p-value <0.05 was considered to be significant.

Table 4-1. Primer sequences for RT-PCR

Gene	Sequence (5'-3')	Reference
TLR2 (*tlr2*, ID 310553)		
Forward	GTACGCAGTGAGTGGTGCAAGT	Le Mandat Schultz et al. (2007)
Reverse	GGCCGCGTCATTGTTCTC	
TLR4 (*tlr4*, ID 29260)		
Forward	AATCCCTGCATAGAGGTACTTCCTAAT	Le Mandat Schultz et al. (2007)
Reverse	CTCAGATCTAGGTTCTTGGTTGAATAAG	
Occludin (*ocln*, ID 83497)		
Forward	ATCTAGAGCCTGGAGCAACG	___
Reverse	GTCAAGGCTCCCAAGACAAG	
ZO-1 (*tjp1*, ID 292994)		
Forward	GCATGTAGACCCAGCAAAGG	___
Reverse	GGTTTTGTCTCATCATTTCCTCA	
Muc1 (*muc1*, ID 24571)		
Forward	TCGACAGGCAATGGCAGTAG	Montoya et al. (2010)
Reverse)	TCTGAGAGCCACCACTACCC	
Muc2 (*muc2*, ID 24572)		
Forward	GCACCTTCTTCAGCTGCATG	___
Reverse	GCGCAGCCATTGTAGGAAAT	
Muc3 (*muc3*, ID 687030)		
Forward	CTTGAGGAGGTGTGCAAGAAA	Montoya et al. (2010)
Reverse	CCCCAGGGTGACATACTTTG	
Muc4 (*muc4*, ID 303887)		
Forward	GCTTGGACATTTGGTGATCC	Montoya et al. (2010)
Reverse	GCCCGTTGAAGGTGTATTTG	
GAPDH (*gapdh*, ID 24383)		
Forward	GTGGCAGTGATGGCATGGAC	___
Reverse	CAGCACCAGTGGATGCAGGG	

4.3 Results

4.3.1 Microbial Community Structure

After quality filtering a mean of 1275±36 sequences were obtained per sample. Addition of RP or CP to the HFD induced a substantial shift in the composition and structure of the faecal microbial community. Grouping of microbial composition by dietary treatment is reflected in the principal coordinate analysis (PCoA) plot in Figure 4-1A. Pairwise comparisons of microbial composition by analysis of molecular variance (AMOVA indicated that both CP and RP treatments differed from both HFD and LFD treatment groups ($P<0.005$), whereas the two PSC treatments did not differ from each other ($P=0.179$). Unifrac distances of microbial community compared to HFD were significantly different between groups (Figure 4-1B, $P<0.05$). The effects of both RP and CP were very similar, however, only CP revealed a higher mean weighted unifrac distance compared to HFD ($P<0.05$). While RP and CP differed from HFD, there was no indication that overall community structure became more similar to LFD. As well as showing a shift away from HFD, there was an increase in diversity in response to both RP and CP fractions as indicated by inverse Simpson diversity index (Figure 4-1C, $P<0.05$ and $P<0.01$ respectively). Again, this did not make CP and RP more similar to LFD as bacterial diversity in LFD and HFD was similar.

At the phylum level, there was an overall increase in the proportion of *Firmicutes* ($P<0.05$) and a decrease in the proportion of *Bacteroidetes* ($P<0.05$) in CP as compared to HFD. Similar overall patterns were seen with RP, however they

did not reach statistical significance (P=0.06 for *Bacteroidetes* and P=0.12 for *Firmicutes*). Mean proportion of *Firmicutes* was 63.9± 4.3% in HFD, 71.3±2.2% in RP, 77.4±3.5% in CP and 65.6±3.5% in LFD group and proportion of *Bacteroidetes* was 34.2±4.2% in HFD, 25.4±1.7% in RP, 19.5±3.2% in CP and 29.6±2.2% in LFD rats.

The effect of dietary treatments on faecal microbial composition at the family level is depicted in Figure 4-2. The effects of PSC for the most part did not return the microbial population to that seen in LFD rats. The one exception to that was the population of *Porphyromonadaceae*. This is the only bacterial family that was affected by PSC the same as LFD, and was the only bacterial family that differed between HFD and LFD. The separation of PSC groups by multivariate analysis was largely associated with the relative proportion of bacteria from the *Lachnospiraceae* family. *Lachnospiraceae* was also the most abundant bacterial family in all treatment groups. There was also an increase in *Prevotellaceae* in RP and CP groups (Figure 4-2). The pattern of alterations in bacterial populations at all taxonomic levels was very consistent between the two PSC groups, however the CP had a slightly stronger effect on microbial populations, shifting further away from HFD microbiota.

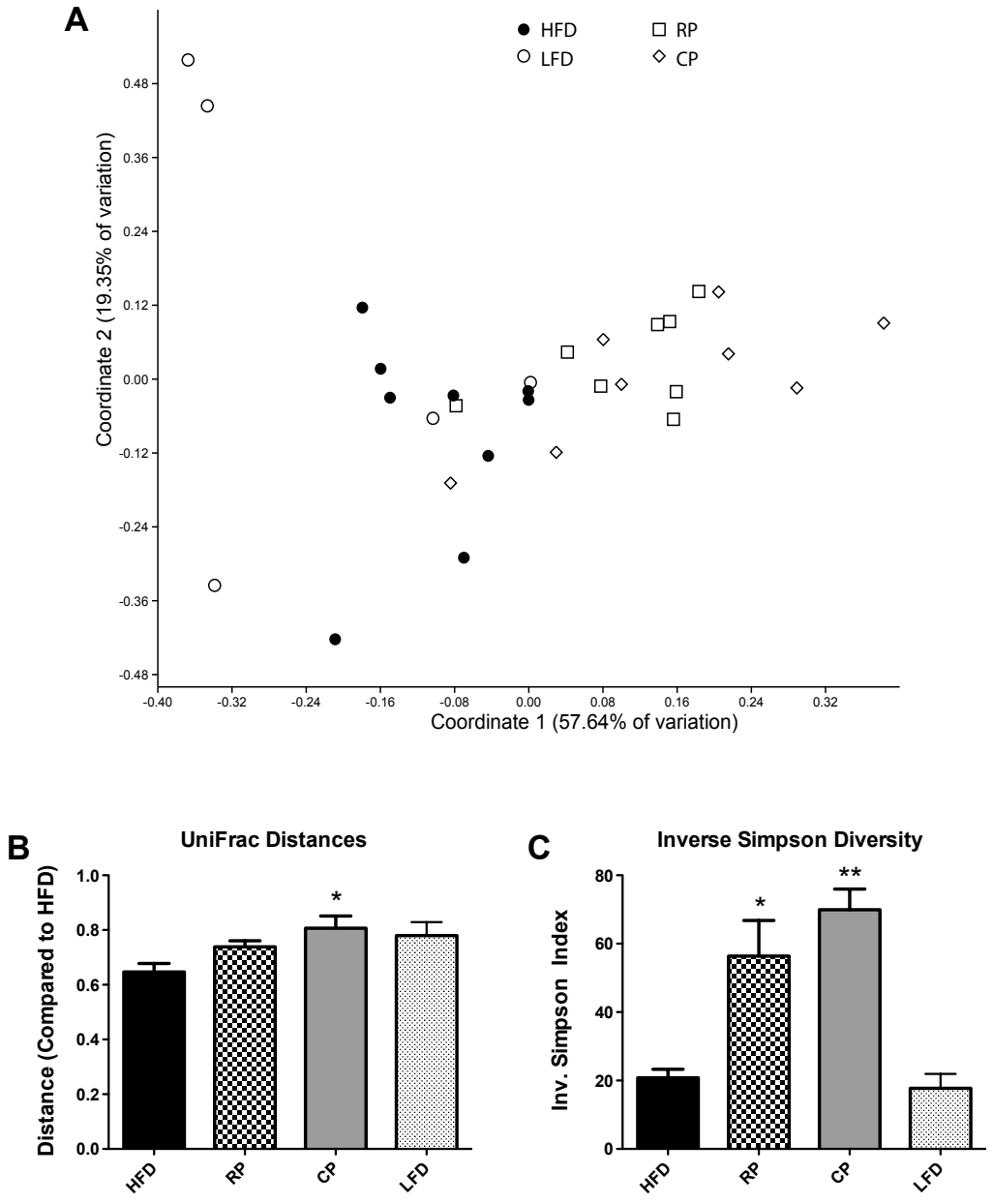

Figure 4-1. Effect of feeding PSC on faecal microbial composition. (A) Fecal bacterial communities clustered using PCoA analysis of weighted UniFrac distances, analyzed by AMOVA. The percentage of variation explained by each coordinate is shown in parentheses. (B) Weighted UniFrac distances compared to HFD. (C) Inverse Simpson diversity index as a measure of diversity within each sample. Both UniFrac distances and inverse Simpson diversity indices differed significantly among the groups ($P<0.05$ and $P<0.05$). Bars are means ± SEM analyzed by one-way ANOVA (B) and Kruskal-Wallis test (C) followed by Bonferroni and Dunn's post-hoc comparison tests (n= 6-8); asterisks show significant difference compared to HFD (**$P<0.01$, *$P< 0.05$).

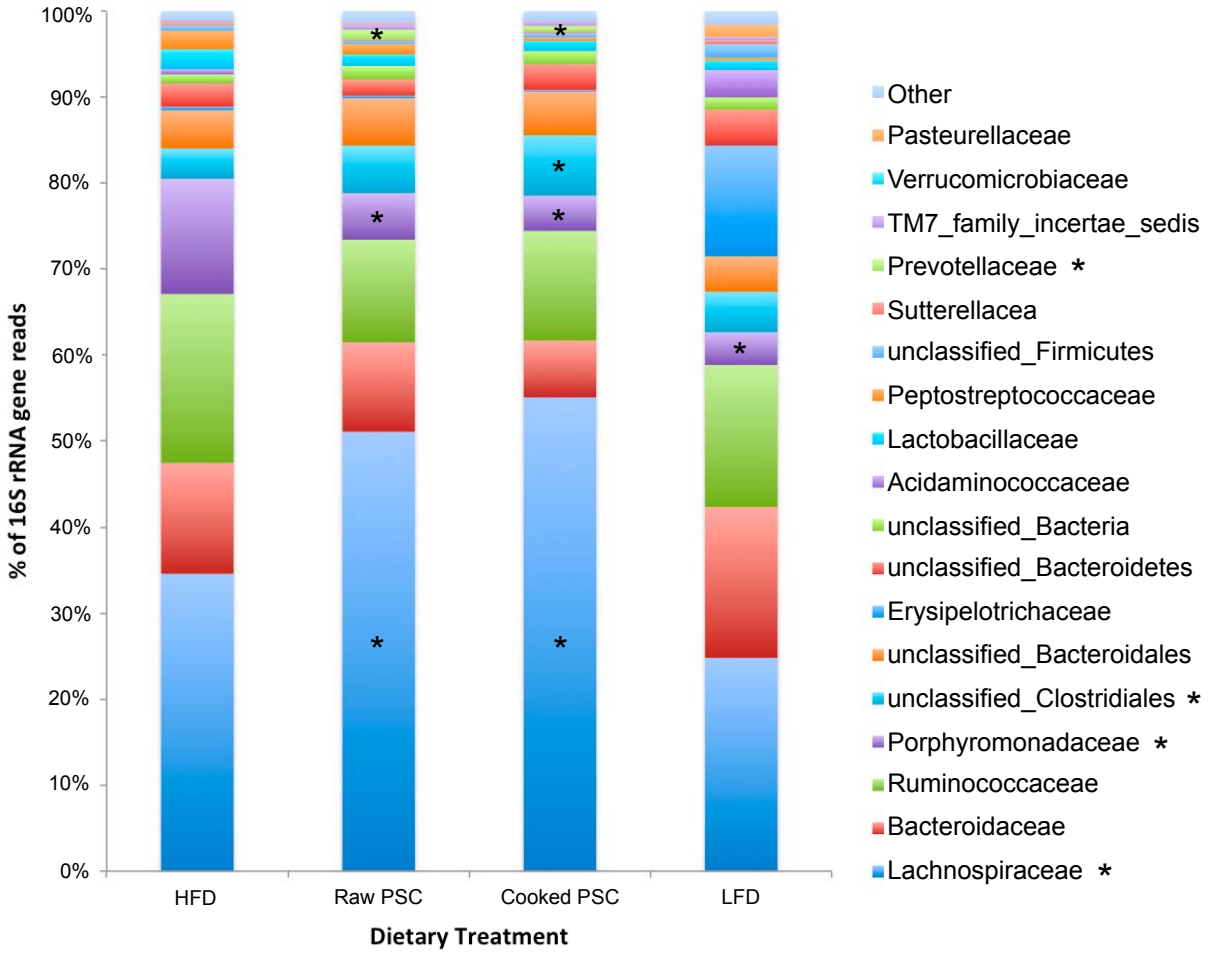

Figure 4-2. Microbial taxonomy of faecal samples classified at the family level. Asterisks indicate bacterial families whose population differed significantly compared to HFD (P<0.05 with Bonferonni correction for multiple comparisons), n=6-8.

4.3.2 Gene Expression of TLRs, Tight Junction Proteins and Mucins

Following 4 weeks of feeding experimental diets, ileal expression of TLR2 showed a pattern of elevated expression in HFD relative to other treatments (Figure 4-3A, (P=0.09). TLR4 expression did not show the same trend (Figure 4-3B, P=0.19). Although not statistically significant, compared to HFD relative expression of TLR2 was decreased by 61% and 63% respectively in the RP and CP rats, which was similar to the LFD rats at 68%. Relative mRNA expression of occludin (84% increase compared to HFD) and ZO-1 in the ileum was numerically highest in CP but did not show significance (Figure 4-3C and D, P=0.16 and P=0.86 respectively).

Relative expressions of mucin genes (Muc1, Muc2, Muc3 and Muc4) in the ileum are shown in Figure 4-4. After 4 weeks of PSC supplementation, Muc1, Muc2 and Muc4 mRNA expression levels differed between the diet groups (P<0.05). In particular, CP rats showed elevated expression of Muc2 and Muc4 mRNA compared to the HFD group (P<0.05). No significant differences were observed in the ileal expression of Muc3 (Figure 4-4C).

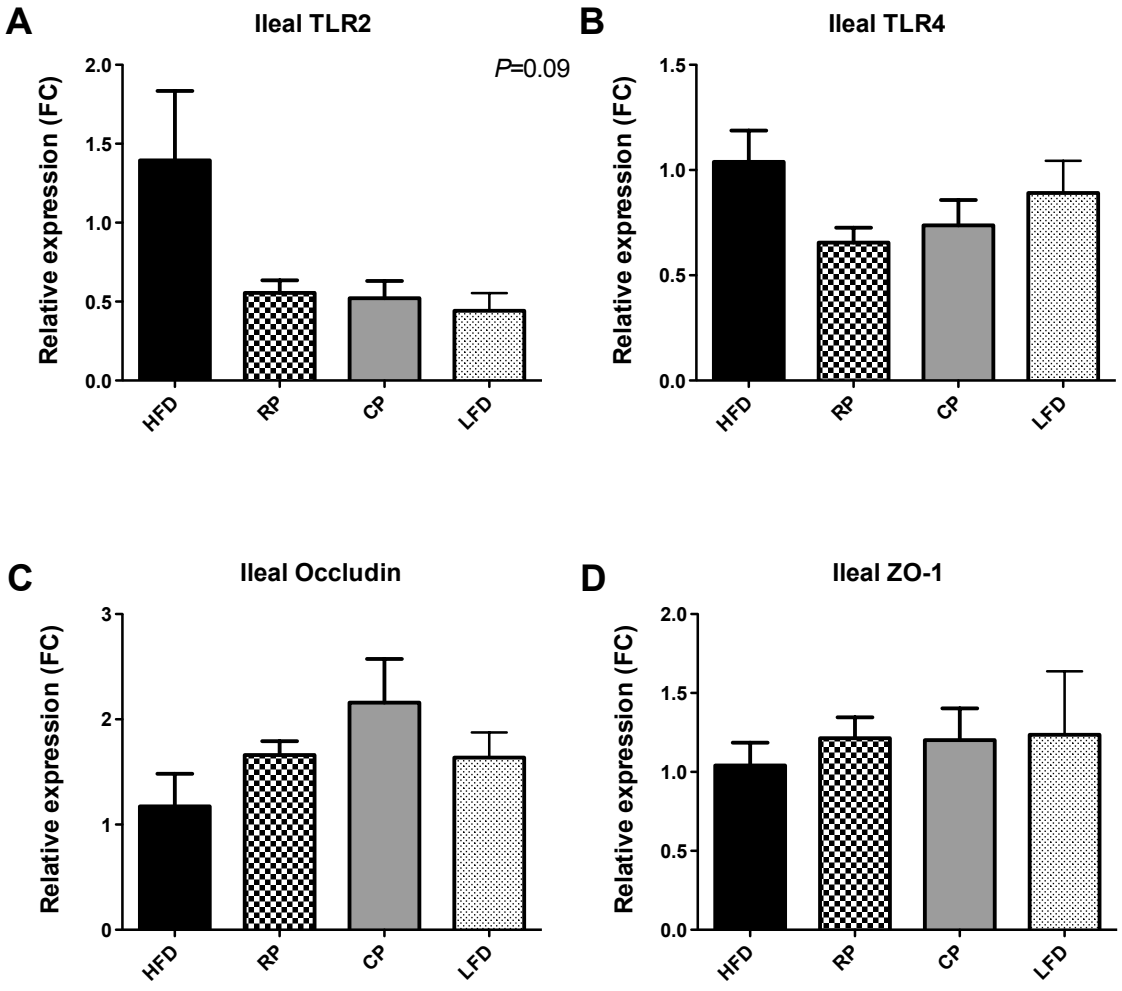

Figure 4-3. mRNA expression of TLRs and tight Junction proteins. Mean relative mRNA expression (FC, fold change) of (A) TLR2, (B) TLR4, (C) occludin and (D) ZO-1 in ileum of the rats normalized to GAPDH expression. Data are means ± SEM analyzed by one-way ANOVA or Kruskal-Wallis test followed by Bonferroni and Dunn's post-hoc comparison tests as appropriate, n= 5-8. No significant differences were found between groups.

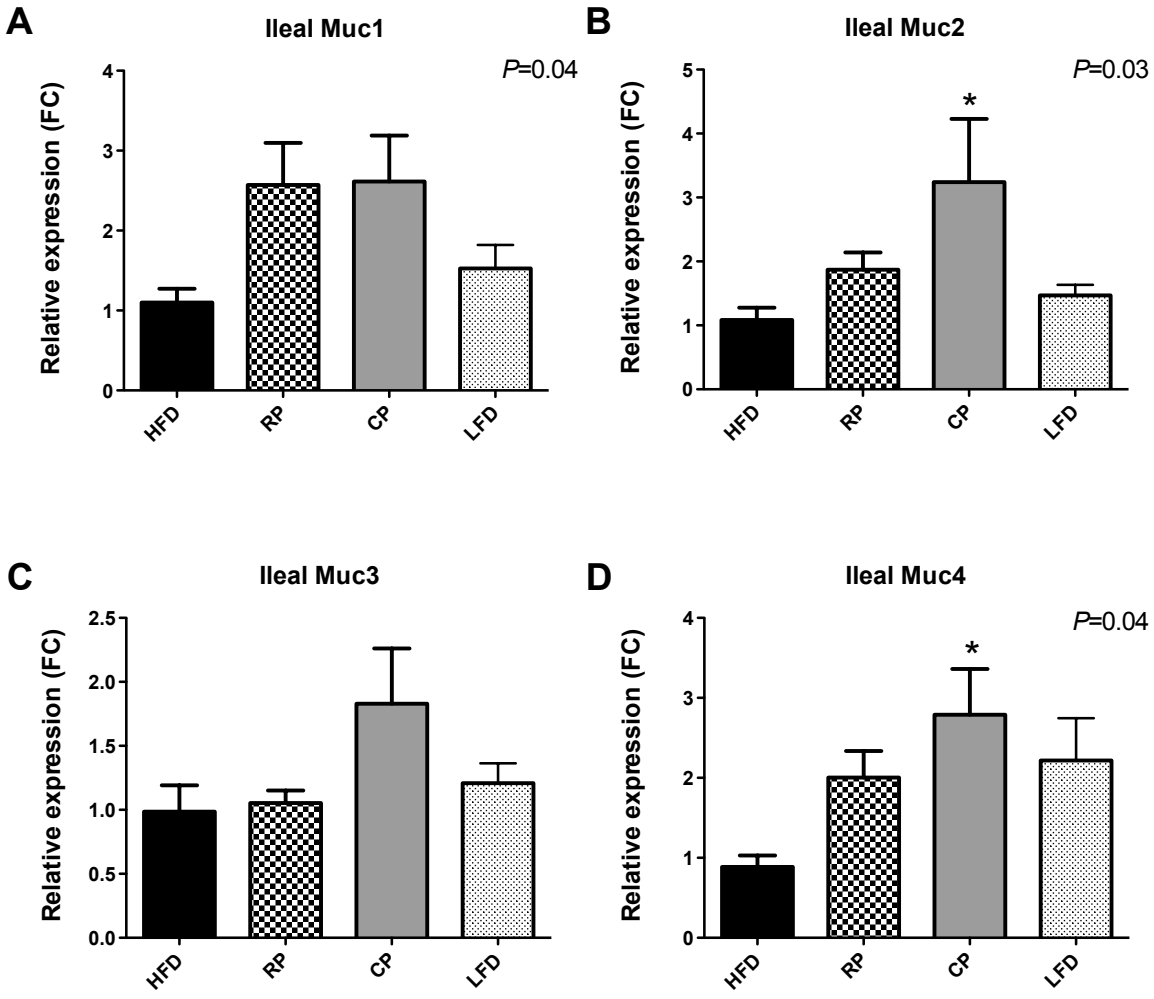

Figure 4-4. mRNA expression of mucins. Relative mRNA expression (FC, fold change) of (A) Muc1, (B) Muc2, (C) Muc3 and (D) Muc4 in the ileum. Gene expression data was normalized to GAPDH as the house-keeping gene and presented as means ± SEM. One-way ANOVA or Kruskal-Wallis test followed by Bonferroni and Dunn's post-hoc comparison tests as appropriate was used for data analysis, n=6-8. Relative expression of Muc1, Muc2 and Muc4 were significantly different between the groups ($P<0.05$). CP group showed increased expression of Muc2 and Muc4 genes when compared to HFD group (*$P<0.05$).

4.4 Discussion

The present study evaluated the effects of PSC feeding on the composition of gut microbiota and markers of intestinal barrier function in HFD-induced glucose intolerant rats. We previously showed that rats fed with PSC-supplemented diets had improved glucose homeostasis compared to a control group fed with HFD containing cellulose (Hashemi et al., 2014). We also observed that enhanced glucose tolerance in the same groups was blunted when the gastrointestinal tract was bypassed during glucose tolerance test (presented in Chapter 3). Based on these results, we hypothesized that the mechanisms responsible for the improvement in glucose tolerance in our animal model were, at least in part, mediated by the gut. Specifically, our hypothesis was that PSC supplementation effects on glucose tolerance and insulin resistance would be associated with modified gut microbial composition and enhanced intestinal barrier function.

Firstly, we investigated the effects of RP and CP diets on the overall composition of gut microbiota. Adding PSC to the diet of glucose intolerant rats had the same effect on overall microbial composition whether it was cooked or not. Both RP and CP increased the abundance of *Lachnospiraceae*, a butyrate-producing family that belongs to the phylum *Firmicutes* (Vital et al., 2014). This finding is consistent with a previous study by our group that indicated an increase in gut-derived 3-hydroxybutyrate with RP inclusion (Chan et al., 2014). Butyrate is one of the three predominant SCFAs generated in the gut, which constitutes the major source of energy for the colonocytes (Bergman, 1990). In addition to serving

as an energy substrate, butyrate also acts as a signaling molecule and plays regulatory roles in host metabolism via activation of SCFA receptor FFAR3 (Donohoe et al., 2011; Hara et al., 2014; Lin et al., 2012; Tazoe et al., 2008). Butyrate administration in mice was shown to improve glucose control, increase postprandial levels of GLP-1 and GIP and overall resulted in protection against HFD-induced obesity (Lin et al., 2012). This effect of butyrate on incretin release is consistent with increased basal GLP-1 and GIP secretion in CP rats and enhanced basal and glucose-stimulated GIP secretion in RP group in our study (Chapter 3). In addition, there was an increase in *Prevotellaceae* in both PSC groups, which is in line with a previous animal study showing that inclusion of pectin as the source of fermentable dietary fibre resulted in a 3-23 times increase in the abundance of *Prevotellaceae* when compared to diets containing arabinoxylan (Ivarsson et al., 2014). In mice, consumption of a diet containing whole grain oats increased the abundance of *Prevotellaceae* family by 175.5% compared to a low bran oat diet, a change that was also associated with improved insulin sensitivity (Zhou et al., 2015). Furthermore, African children, who consumed diets high in dietary fibre, were found to harbor a large population of the genus *Prevotella*, one of the four genera belonging to the family *Prevotellaceae,* compared to European children who lacked this bacteria and consumed a typical low-fibre western diet (De Filippo et al., 2010). Likewise, Wu et al. (2011) reported that in adults, dietary fibre intake was associated with a microbiota dominated by *Prevotella. Prevotella* species contain bacterial genes that enable them to utilize polysaccharides such as water-soluble xylans (Flint et al., 2012). This was in keeping with our fibre

analysis data showing that xylose, the primary building block for xylan, was present in both raw and cooked PSC preparations.

The few previous studies that have examined the impact of peas or pea-derived components on the microbial profile of the gut have reported increased *Bifidobacterium* population (Queiroz-Monici et al., 2005), reductions in the abundance of *Firmicutes* (Eslinger et al., 2014) and substantial changes in the structure of this phylum (Marinangeli et al., 2011); however, these studies were performed under considerably different conditions than ours. Increased *Bifidobacterium*, for instance, was found following consumption of whole peas supplemented to a balanced diet based on AIN-93G diet. Additionally, those experimental diets were not matched for total dietary fibre and pea diet contained more than double the amount of dietary fibre in the control group (Queiroz-Monici et al., 2005). Eslinger et al. (2014), used commercially available yellow pea-derived components (fibre, starch and flour) as a part of basal diet formulated according to AIN-93M diet with a slightly higher proportion of total dietary fibre (13% wt/wt); the duration of their study (5 weeks of intervention with treatment diets) was also longer than that of ours. Changes in the composition of *Firmicutes* were reported in hamsters fed untreated commercially available pea flours from whole seeds and seed coats; differences were compared to a control diet containing a lower amount of dietary fibre (Marinangeli et al., 2011). Given that thermal procedures such as boiling, as conducted in our study, can result in partial solubilization and depolymerization of dietary fibre components and alter their

functional properties with respect to fermentation, presence or lack of treatment could be partially responsible for differences seen between these studies in terms of microbiota composition. Consistent with our results (presented in Chapter 3), in the two latter studies, pea fibre-containing diets resulted in lower circulating glucose levels (Eslinger et al., 2014; Marinangeli et al., 2011).

Prebiotics such as inulin and oligofructose have been widely examined for their ability to modulate gut microbiota. Changes in the abundance of *Bifidobacterium* are arguably the most reported microbial outcome at the genus level in prebiotic interventions, both in animals and humans (Bouhnik et al., 2006; Bomhof et al., 2014; Kolida et al., 2007; Parnell & Reimer, 2012; Ramirez-Farias et al., 2009). In the present study however, no *Bifidobacterium* was detected in feces; this might be reflective of housing conditions, a factor known to affect abundance of bifidobacterial populations in mice although at a small magnitude (Thoene-Reineke et al., 2014), or the specific primers used for 16S rRNA gene pyrosequencing. Specifically, some primers have been shown to have limitations in terms of amplification of *Bifidobacterium* species (Palmer et al., 2007). At the phylum level, HFD-induced metabolic disorders including obesity and insulin resistance are sometimes characterized by an increased ratio of *Firmicutes* to *Bacteroidetes* (Hildebrandt et al., 2009; Ley et al., 2006; Murphy et al., 2010; Turnbaugh et al., 2006), however, several other studies do not support this link (Duncan et al., 2008; Larsen et al., 2010; Schwiertz et al., 2010; Wu et al., 2010; Zhang et al., 2008). In our study, we observed an overall significant increase in the

proportion of *Firmicutes* and a reduction in the proportion of *Bacteroidetes* in CP compared to HFD, whereas RP displayed a similar non-significant pattern. These results are consistent with those of Wu et al. (2011) indicating that dietary fibre consumption was positively linked with increased abundance of *Firmicutes* in healthy individuals. Considering the inconsistency of reports regarding the *Firmicutes* to *Bacteroidetes* ratio, and the fact that these two phyla contain a variety of genera with distinct properties (Eckburg et al., 2005; Haakensen et al., 2008), it is safe to suggest that in order to be more conclusive, interpretation of differences at the level of phylum need to be approached at a more refined level.

We also examined the effect of PSC diets on the expression of TLR2 and TLR4 in the ileum. TLRs are essential for the recognition of microbial components known as pathogen-associated molecular patterns (PAMPs) including LPS, peptidoglycan (PGN), lipoarabinomannan (LAM) and zymosan. While TLR4 recognizes LPS originating from the outer membrane of Gram-negative bacteria, TLR2 is implicated in the recognition of Gram-positive bacterial components such as PGN (Takeuchi & Akira, 2001; McCucker & Kelley, 2013). Upon activation by corresponding PAMPs, TLR2 and TLR4 initiate signaling cascades that eventually result in the production of proinflammatory cytokines, e.g. TNFα and IL-1β (McCucker & Kelley, 2013). It has been shown that inability to respond to LPS through TLR4 protects mice from developing HFD-induced insulin resistance. In addition, HFD feeding resulted in increased expression of TLR2 in adipocytes of insulin resistant mice (Murakami et al., 2007), a finding that corresponds to our

observed trend toward an increased mRNA expression level of TLR2 in ileal tissue in HFD rats. Another study reported that mice with TLR2 deficiency were protected against HFD-induced insulin resistance (Ehses et al., 2010). Furthermore, patients with newly diagnosed T2D had increased monocyte TLR2 and TLR4 mRNA expression and protein content, a condition that was positively correlated with markers of glucose tolerance such as HOMA-IR, glucose and HbA1c (Dasu et al., 2010). In our study, both RP and CP groups showed more than 60% reductions in the expression of TLR2 and displayed a trend toward lower levels compared to HFD (P=0.09), suggesting that PSC supplementation was effective in protecting rats from the adverse effects of HFD on TLR2 expression and presumably activation of the downstream signaling pathways. However, a preliminary analysis of the potential effect of these diets on the production of proinflammatory cytokine IL-6 showed no differences in IL-6 gene expression in the ileal mucosa between the treatment groups (data not shown).

To assess evidence for effects of PSC on gut permeability, relative expression of tight junction proteins occludin and ZO-1 was assessed in the ileum. Although not significant, the highest expression levels of occludin (85% above that of HFD) were observed in CP rats, which also had the most enhanced glucose and insulin responses during the oGTT (Chapter 3). It is known that in obese mice, metabolic endotoxemia is associated with decreased expression and altered distribution of occludin and ZO-1 (Everard et al., 2011; Cani et al., 2009). To our knowledge, effects of prebiotics on tight junction proteins in models of obesity and

obesity-related metabolic disorders have not been reported. However, in a rat model of acute pancreatitis, supplementation with galactooligosaccharides was shown to increase intestinal mRNA levels of occludin, which was linked with improved intestinal barrier function (Zhong et al., 2009). Another study reported increased expression of occludin in healthy rats following supplementation with xylo-oligosaccharide, although the intervention did not result in enhanced intestinal integrity (Christensen et al., 2014). It is important to keep in mind that gene expression data is not an optimal predictor for the functions of tight junction proteins since their organization and distribution plays a more important role in their functionality.

Finally, mRNA levels of mucins in the ileum were examined and significant between-group differences were found for Muc1, Muc2 and Muc4, specifically that CP had higher relative expressions of Muc2 and Muc4 compared to HFD. This finding supports our hypothesis based on the fact that mucins are important for the integrity of mucus layer. In mice, HFD feeding has been associated with changes in the oligosaccharide chains of mucins and consequently their altered composition (Mastrodonato et al., 2014). Consistent with our results, another animal study also reported decreased expression of duodenal Muc2 following intake of HFD (Schulz et al., 2014). Muc2 is the main structural component of mucus layer, and hence important for the protective function of this layer (Linden et al., 2008). In addition, emerging evidence on the role of mucin-degrading bacteria *Akkermansia muciniphila* in gut barrier function further proves

the importance of mucins for the homeostatic actions of mucus layer. Everard et al. (2013) showed that the population of these bacteria was decreased following HFD feeding in mice; the authors continued to demonstrate that administration of oligofructose restored the abundance of *A. muciniphila* and corrected metabolic endotoxemia. Similarly, treatment with viable *A. muciniphila* alongside the HFD abolished metabolic endotoxemia and improved fasting glycemia. While these changes were independent of overall gut microbial composition, they were accompanied by an increase in the mucus layer thickness. The authors concluded that restoration of mucus layer and gut barrier function ameliorated metabolic endotoxemia and improved glycemic control (Everard et al., 2013). Our results on the expression of mucins suggest that PSC-containing diets, especially when cooked, were able to reverse the effect of HFD on mucin expression and potentially benefit the animals with regard to intestinal barrier integrity, which was not directly measured in this study.

There are a few limitations that should be kept in mind when interpreting the findings of this study. The specific microbiota composition derived from gene pyrosequencing is highly affected by the 16S rRNA regions and primers chosen for amplification (Claesson et al., 2010; Liu et al., 2008). We also recognize that gene expression analysis of tight junction proteins is not conclusive without considering the importance of their structure and distribution, which could be modified independently from the abundance of their proteins. Furthermore, it is impossible

to infer if changes observed with regard to gut microbial composition were primary or secondary to changes in glucose tolerance in the rats.

4.5 Conclusion

Overall, the present study demonstrates that inclusion of raw and cooked PSC fractions in diets of glucose intolerant rats alters the composition of gut microbiota, including an increase in one butyrate-producing family. This observation was accompanied by an increased expression of mRNA encoding mucin proteins in the ileum and a trend toward decreased expression of ileal TLR2. These effects, both on microbiota structure and protective gene expression, were consistently stronger in the CP group, which also benefitted the most from PSC supplementation in terms of glucose tolerance. Our findings suggest a potential protective role for PSC fractions against HFD-induced alterations in the microbial composition of the gut and elements of gut barrier function.

Chapter 5: The Effect of Regular Consumption of Pulse-containing Meals on Parameters of Glucose Control, Thiamine and Folate Status in Hypercholesterolemic Adults

5.1 Introduction

The importance of nutrition therapy as an essential element in a multifactorial approach to management of T2D is well recognized by both ADA and CDA (CDA, 2013; Evert et al., 2013). While consumption of low glycemic index foods and high intake of dietary fibre have long been recommended, incorporating pulse grains, as foods possessing these properties, into diets of diabetic patients has only recently been specified in the CDA guidelines (2013). Evidence from several clinical studies suggests that consumption of pulses is linked with enhanced markers of glycemic control (Jenkins et al., 2012; Lunde et al., 2011; Marinangeli & Jones, 2011b; Olmedilla-Alonso et al., 2012).

Pulse grains are the non-oil seeds of leguminous crops with the most common varieties grown in Canada being peas, beans, lentils and chickpeas (Pulse Canada, 2014). It is important to note that, despite dietary fibre being credited as the main component of pulses responsible for the majority of their health benefits, pulses are in fact valuable sources of protein, some polyphenols and several B vitamins and minerals as well. It has been reported that daily consumption of ½ cup of peas and beans leads to increased intakes of fibre, protein and folate, along with minerals such as zinc and iron (Mitchell et al., 2009). Among these vitamins and minerals, thiamine and folate are of particular importance to diabetic patients.

Thiamine in its phosphorylated form (TDP) plays a role in glucose metabolism as an essential cofactor for the enzyme transketolase in the PPP (Page et al., 2011; Pacal et al., 2014). This in turn suggests that low thiamine could disrupt glucose oxidation and lead to deterioration of blood glucose and HbA1c as markers of glucose control. Relative thiamine deficiency appears to be prevalent in diabetic patients (Page et al., 2011; Thornalley et al., 2007). Administration of thiamine supplements is reported to reduce glucose concentrations in patients with T2D who are not receiving any medications for diabetes (Gonzalez-Ortis et al., 2011).

Folate status is also associated with T2D and cardiovascular disease as its major macrovascular complication (Cacciapuoti, 2012). Folate acts as a cofactor for enzymes involved in homocysteine metabolism; folate deficiency as a result impairs normal homocysteine cycling, leading to elevated plasma homocysteine, an independent risk factor for cardiovascular disease (Cacciapuoti, 2012; Stanger et al., 2004). Supplementation with folate is effective in lowering plasma homocysteine levels in diabetic patients who are not taking insulin (Hunter-Lavin et al., 2004).

Despite the fact that pulses are very good sources of thiamine and folate, to our knowledge, none of the recent clinical trials focused on pulse consumption has addressed the effects of pulses on circulating thiamine and folate concentrations in the plasma. Therefore, the aim of this study is to evaluate the effect of feeding

meals containing peas and beans on thiamine and folate status. We hypothesize that pulse consumption will result in increased thiamine and folate concentrations in plasma and this will be associated with improved indices of glucose control.

5.2 Subjects And Methods

5.2.1 Study Design and Overall Protocol

The study followed an unblinded, randomized control design and consisted of three treatment arms with a duration of 6 weeks each. Participants were randomly assigned to consume foods containing peas, beans or rice 5 days per week for the duration of the study. The enrollment of participants started in May 2013 and is still ongoing to reach the goal of 75 participants. The recruitment and study procedures and protocols were approved by the ethics board at the University of Alberta, and written consents were obtained from all participants. The present study was registered at clinicaltrials.gov (NCT01661543). A copy of the study information sheet and consent form can be found in Appendix B.

Originally, all individuals who were interested in participating in the study were interviewed briefly over the telephone and those eligible after this initial step were invited to attend an in-person screening session. After providing a written consent, basic demographic information including age and sex, medical history and current intake of pulses was collected via questionnaire. Twelve-hour fasted blood samples were collected and analyzed for TG and LDL (low-density lipoprotein). Anthropometric measurements were taken twice using a 752KL digital scale

(Health o Meter Professional, Bridgeview, IL, USA) for weight and a digital stadiometer for height (QuickMedical Heightronic Digital Stadiometer, Issaquah, WA, USA). All participants who were deemed eligible for the intervention were notified and scheduled for the baseline visit. They were also required to fill out a set of additional questionnaires that were provided to them at the screening session. Block randomization was used to assign participants to one of three treatment groups. Participants attended a total of three sessions at baseline, week 3 and week 6 (final); however, the outcomes reported in this chapter are based on data collected at baseline and at the end of the study. The CONSORT (Consolidated Standards of Reporting Trials) flow diagram of the study protocol and participants is depicted in Figure 5-1.

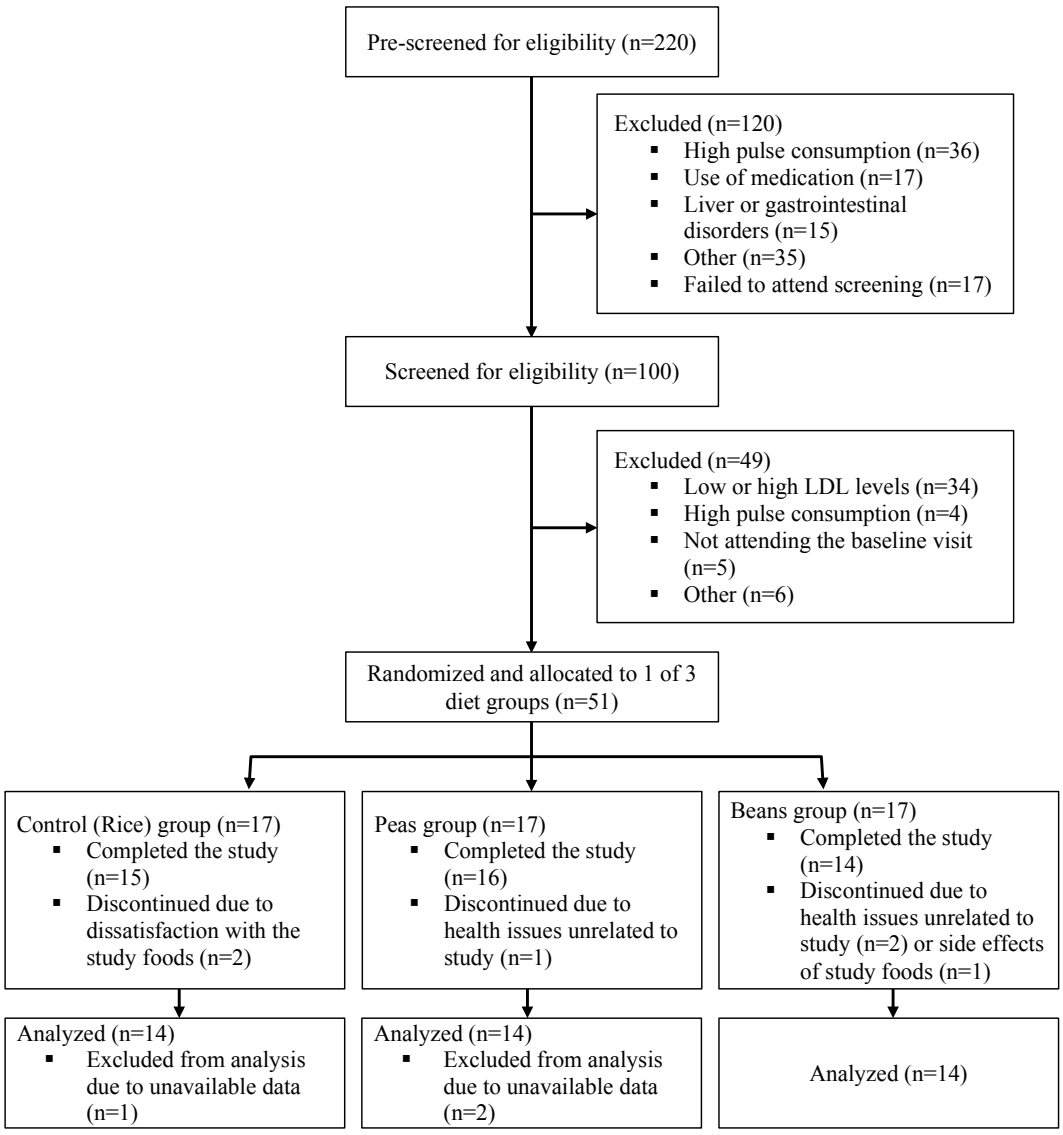

Figure 5-1. Flow diagram of the participants through the study.

5.2.2 Subjects

Participants were recruited from the Edmonton area using newspaper advertisements, newsletters and posters on campus of the University of Alberta and volunteer websites. A total of 51 participants have been recruited and randomized so far, however, only 42 participants were included in the analyses (Figure 5-1). Eligible participants were hypercholesteromic men and non-pregnant, non-lactating women aged 20 to 75 years, who had fasting serum LDL-C ≥ 3.00 mmol/L and ≤ 5.00 mmol/L and fasting serum triglycerides ≤ 4.00 mmol/L (Table 5-1), with stable body weight (±5%) for 3 months prior to the start of the study and a body mass index (BMI) of 20 – 40 kg/m^2. Those who were taking medications, vitamin, mineral and other dietary supplements also had to be on a stable regime for the previous 3 months. The ability to read, write and communicate in English, and willingness to comply with the study requirements such as consuming no other pulse-containing foods throughout the study period were required as well. On the other hand, exclusion criteria included regular high pulse consumption (>2 servings equal to >1.2 cups per week), the use of lipid-lowering or hypoglycemic medications, medical history of liver disease, renal insufficiency, inflammatory bowel disease or other gastrointestinal disorders, history of acute medical or surgical conditions within 3 months prior to the start of the study. Individuals with a history of gastrointestinal reactions or allergies to peas, beans or rice-based foods and those who consumed a vegan diet were also excluded from participation in the study. A complete list of the inclusion and exclusion criteria can be found in Appendix C.

Table 5-1. Baseline lipid profile of study participants

	Control (n=16)	Peas (n=16)	Beans (n=16)
TC (mmol/L)	6.1±0.2	5.9±0.2	6.3±0.2
TG (mmol/L)	1.8±0.2	1.7±0.2	1.7±0.2
HDL-C (mmol/L)	1.6±0.2	1.5±0.1	1.6±0.1
LDL-C (mmol/L)	3.7±0.2	3.6±0.1	4.0±0.2

TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.
Data are means ± SEM analyzed by one-way ANOVA followed by Bonferroni post-hoc comparison tests. No significant differences were found between groups.

5.2.3 Study Foods

Pulses used in the study consisted of yellow and green peas, black beans, pinto beans, navy beans and great northern beans. These were administered to participants with foods in the forms of soup and casserole. The recipes for study foods were developed at the University of Manitoba by a food scientist on the research team. Peas and beans were generously provided by the Alberta Pulse Growers and all foods were prepared from the recipes by trained staff at the Alberta Diabetes Institute's Clinical Research Unit. Briefly, pulses were soaked in water for 12-16 hours at 4 °C, which was followed by boiling for 35-55 and 120 min, respectively for beans and peas; rice was prepared in a conventional rice cooker. The background soup and casserole recipes were formed to contain similar ingredients in all the three groups. Upon preparing each treatment food item, it was portioned into individual servings and mixed with ¾ cup (120 g) cooked peas, beans or rice to form the final dish. Foods were then transferred to freezer–safe storage bags and stored at -20 °C until they were distributed among the participants. A full list of study foods and their recipes is included in Appendix D.

5.2.4 Diet and Treatment Protocol

Participants were provided with three-week supplies of study foods at baseline and week 3 visits. For the duration of the study, participants were allowed to maintain their regular habitual diet but were required to avoid consuming pulses besides the dose included in the study foods. They were instructed to store their supply in the freezer, take out one package each day and defrost the food in the

refrigerator prior to heating it up. All participants also had the option to consume the intervention foods anytime during the day and were asked to ensure that they consumed the entire content of the food package. In order to accommodate personal taste preferences, participants were free to add any additional ingredients including seasonings to the foods.

5.2.5 Anthropometrics, Food Record and Blood Collection

At each study visit, certified study personnel weighed the participants using a 752Kl digital scale (Health o meter Professional, Bridgeview, IL, USA). Participants were asked to remove their shoes before weight measurement. Waist circumference (WC) was measured at the minimum circumference between the rib cage and iliac crest by a heightronic digital stadiometer (QuickMedical, Issaquah, WA, USA). Height measurements were recorded once at the screening session and used for calculation of BMI throughout the study. Three-day food records were completed for analysis of energy and nutrient intakes at each visit. A sample of the three-day dietary intake record can be found in Appendix E.

Twelve-hour fasting serum, plasma and whole blood samples were collected for assessment of glucose, insulin, HbA1c, folate and thiamine. Samples were collected onsite using validated protocols and collection tubes, and stored at -80°C until analyzed.

5.2.6 Biochemical Analysis of Serum

Fasting glucose concentrations were determined using SYNCHRON glucose reagent in conjunction with the SYNCHRON LX System (Beckman Coulter, Fullerton, CA, USA). Serum insulin levels were measured using the Elecsys insulin reagent kit (Roche Diagnostics). HbA1c values were analyzed by standard laboratory methods at DynaLIFE$_{DX}$ laboratory (Edmonton, AB, Canada). For HOMA-IR calculations, insulin values were transformed from pmol/L to μU/mL by dividing values by a factor of 6 (Vølund, 1993). The resultant fasting insulin values and glucose concentrations were used to calculate HOMA-IR based on the following equation: fasting glucose (mmol/L) × fasting insulin (μU/mL) /22.5 (Matthews et al., 1985). Microbiological assay kits were used for determination of folate and thiamine in serum and whole blood samples according to manufacturer's protocols (ALPCO, Salem, NH, USA).

5.2.7 Statistical Analysis

Descriptive statistics and were used to describe the baseline characteristics of the study population. Endpoint differences between treatment groups for fasting glucose, insulin, HbA1c, HOMA-IR, thiamine and folate were compared using repeated measures (mixed model) ANOVA followed by Bonferroni's post hoc tests. Differences between baseline and final values within each treatment were analyzed by paired *t* test. Pearson's correlation was used to determine the relationship between changes in thiamine and folate with changes in markers of glucose homeostasis. For mixed model and Pearson's correlation analyses, we used

intention-to-treat approach for data analysis, which is a statistical concept to analyze data from all subjects who are randomized to intervention regardless of compliance. Multiple imputations were then used to generate data for missing values. The final reported values are the mean of five sets of randomly imputed values generated based on the existing dataset. Statistical analyses were conducted using GraphPad Prism 5 (Graphpad Software Inc., La Jolla, CA, USA) and IBM SPSS Statistics Version 22 (IBM Corp., Armonk, NY, USA). R version 3.1.1 (The R Foundation for Statistical Computing, Vienna, Austria) was used for multiple imputations. All data were tested for normality of distribution by the Shapiro-Wilk test prior to analyses. All results are presented as means ± SEM unless otherwise indicated. $P< 0.05$ was considered statistically significant.

5.3 Results

5.3.1 Subjects' Characteristics

Two hundred twenty subjects were screened for eligibility, 51 were eligible and randomized, and 42 were included in the analyses (Figure 5-1). Among the main reasons for exclusion from the study were high pulse consumption, too low or too high LDL-C levels and use of glucose- or lipid-lowering medications. Forty-two participants out of the original 51 participants were included in the analyses, while 9 were excluded due to failure to complete the study or unavailable data. Reasons for drop-out included dissatisfaction with study foods (n=2, rice group), health issues unrelated to study (n=3, peas and beans groups), and side effects of

the study foods (n=1, beans group). Baseline characteristics of the participants are summarized in Table 5-2.

Mean age of participants was 51.9 ± 1.9 years and it was comparable between treatment groups. The majority of participants were women (n=27, 64.3%) and did not take any supplements containing thiamine and/or folate (69%). Based on mean BMI for each group, participants were generally overweight and according to World Health Organization (WHO, 2008) cut-off points for WC in overweight population (>94 cm for men and >80 cm for women), female participants had increased risk of developing metabolic complications associated with obesity, while men in the pea group had substantially increased risk (>102 cm).

5.3.2 Fasting Glucose, Insulin, HbA1C and HOMA-IR

These data are reported in Table 5-3. Final fasting glucose and insulin concentrations and HbA1c levels were not affected by treatment diets (P>0.05). Paired *t* tests did not reveal any within-treatment differences between baseline and final values of these markers either. HOMA-IR was calculated as a measure of insulin resistance; no treatment differences were observed in final HOMA-IR between the groups. These results remained insignificant after adjusting for sex as a potential confounding factor.

Table 5-2. Baseline characteristics of study participants

	Control (n=14)	Peas (n=14)	Beans (n=14)
Age (mean ± SEM, years)	46.0±3.9	51.9±3.4	53.5±3.2
Range	22-68	25-68	30-70
Sex (n, %)			
Men	4, 28.5%	5, 36%	6, 43%
Women	10, 71.5%	9, 64%	8, 57%
Weight (mean ± SEM, kg)	71.1±2.5	82.0±5.0	80.4±4.3
BMI (mean ± SEM, kg/m^2)	25.8±1.2	28.2±1.2	26.8±1.0
WC (mean ± SEM, cm)			
Men	84.3±1.7	101.1±7.9	90.2±4.3
Women	84.5±3.9	87.0±3.5	88.2±3.8
Supplement intake (n, %)	3, 21%	3, 21%	7, 50%

BMI, body mass index; WC, waist circumference.
Data are means ± SEM. Age, weight, BMI and WC data were analyzed by one-way ANOVA followed by Bonferroni post-hoc comparison tests. No significant differences were found between groups.

Table 5-3. Effects of diet treatments on biomarkers of glucose control and B vitamin status

	Control (n=14)	Peas (n=14)	Beans (n=14)	*P* value Treatment	*P* value Interaction
	Mean ± SEM				
Glucose (mmol/L) §					
Baseline (W0)	5.3±0.2	5.1±0.1	5.0±0.2	0.49	0.67
Final (W6)	5.2±0.2	5.0±0.1	5.0±0.1		
Insulin (pmol/L) §					
Baseline	73.6±10.9	91.6±17.1	67.9±9.0	0.71	0.04*
Final	77.7±10.4	73.1±11.8	70.1±10.6		
HbA1c (%)					
Baseline	5.3±0.1	5.4±0.1	5.3±0.1	0.77	0.52
Final	5.3±0.1	5.4±0.1	5.3±0.1		
HOMA-IR (mmol/L×μU/mL)					
Baseline	2.9±0.5	3.6±0.8	2.5±0.4	0.67	0.04*
Final	3.1±0.5	2.8±0.5	2.6±0.4		
Thiamine (ng/mL) †					
Baseline	49.3±3.0	50.5±1.1^{aa}	48.6±1.2^{aa}	0.5	0.93
Final	54.0±1.6	55.0±0.9	52.4±1.2		
Folate (ng/mL) §					
Baseline	28.3±2.0	28.7±1.3	30.7±0.8	0.81	0.27
Final	28.7±1.7	28.5±1.8	28.8±1.3		

HOMA-IR, homeostatic model assessment insulin resistance (fasting glucose (mmol/L) × fasting insulin (μU/mL) /22.5; HbA1c, hemoglobin A1c. The 1st column of *P* values represents the significance of treatment effect between the groups and the 2nd column shows the significance of interaction effect (diet x time) as compared by mixed model ANOVA. There was a significant interaction of diet x time on insulin and HOMA-IR ($P<0.05$ for both).
Asterisks show significant difference between groups (*$P<0.05$). Superscript letter indicates significant difference within group analyzed by paired *t* test ($^{aa}P<0.01$).
§ Measured in fasting serum samples. † Measured in fasting whole blood samples.

Whole blood thiamine levels were comparable between the groups at the end of the study; however, final thiamine concentrations were increased in both peas and beans groups as compared to baseline ($P<0.01$ for both). Serum folate concentrations were unchanged following the treatment period ($P>0.05$). Likewise, within-treatment differences in serum folate concentrations were not significant in any of the groups.

Changes (final value-baseline value) in thiamine, glucose and insulin concentrations along with HbA1c were used for correlation analysis. Since within-treatment changes were significantly different in both peas and beans groups, values from these groups were pooled to increase the probability of detecting a significant correlation. Although no statistically significant correlations were observed between changes in thiamine, and changes in insulin and HbA1c in the pulse group (Figure 5-2), there was a trend toward a reverse correlation between changes in thiamine and changes in glucose (Figure 5-2B, $P=0.05$). In contrast, higher thiamine changes were positively correlated with increases in glucose concentrations in the control group (Figure 5-2A, $P<0.05$).

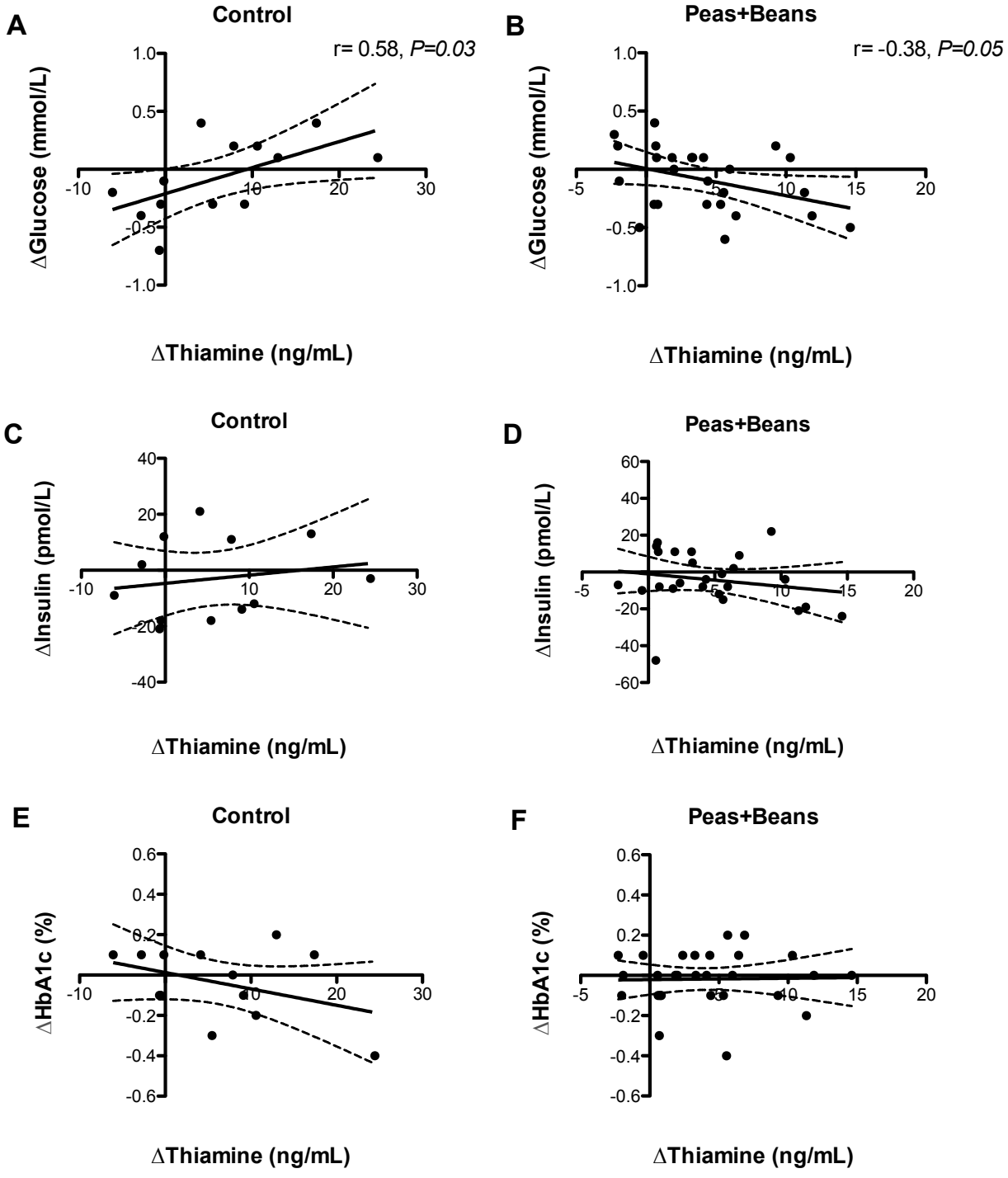

Figure 5-2. Correlation of changes in thiamine with changes in glucose (A, B), insulin (C, D) and HbA1c (E, F) following 6 weeks of intervention. Dashed lines indicate 95% confidence interval (CI) for the regression lines.

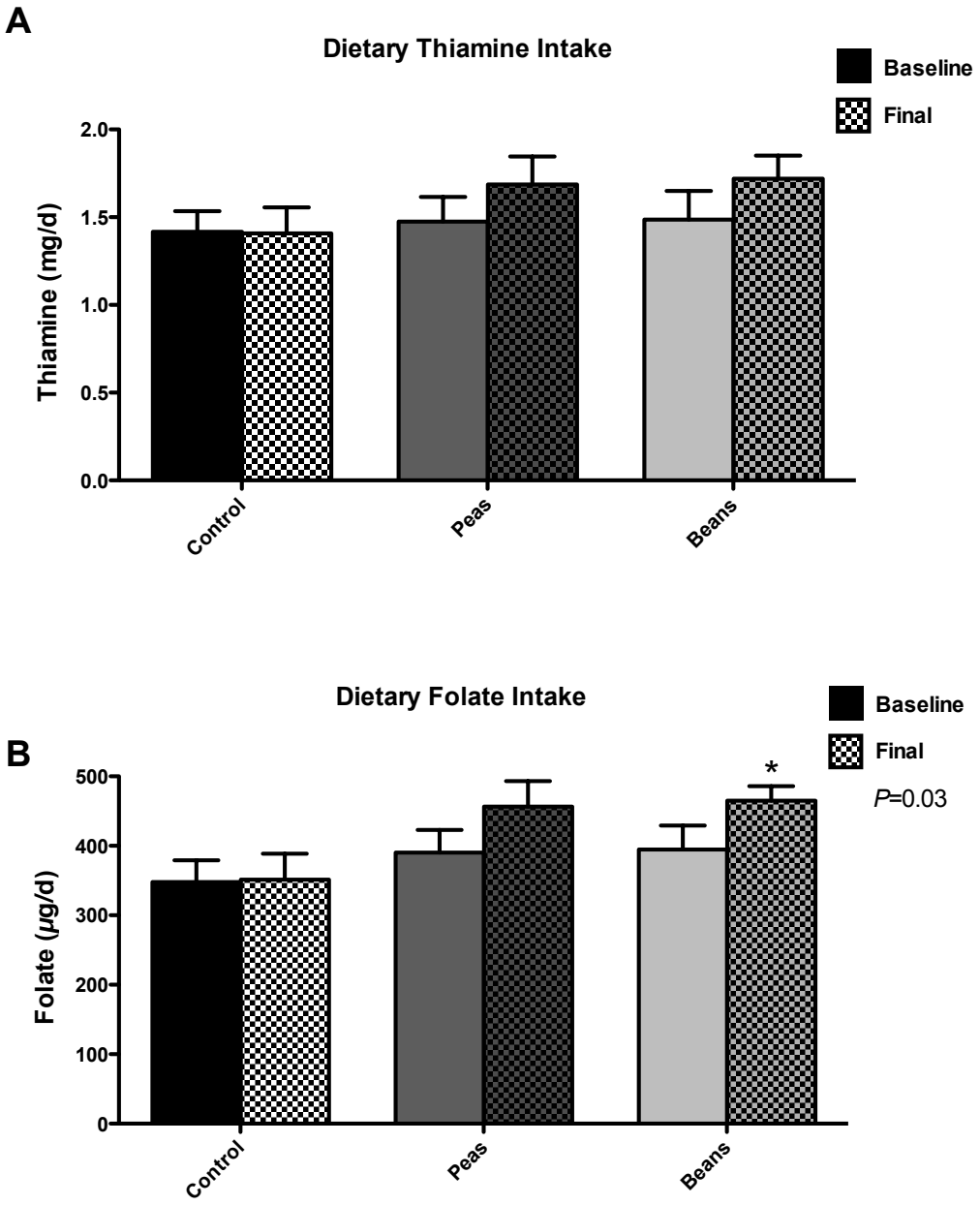

Figure 5-3. Changes in dietary intakes of thiamine and folate at baseline and final. The bars are mean ± SEM, n = 12-14. * indicates significant difference compared to control analyzed by mixed model ANOVA followed by Bonferroni's post-hoc comparison test (*P<0.05).

5.3.3 Dietary Intakes of Thiamine and Folate

These results are shown in Figure 5-3. The average absolute intakes of dietary thiamine and folate were comparable between groups at baseline (Figure 5-2, $P>0.05$). Following 6 weeks of intervention, however, there was a significant effect of diet on dietary intake of folate (Figure 5-3B, $P<0.05$). Post hoc comparison test revealed that folate intake in the beans group participants was significantly increased compared to the control group ($P<0.05$). Thiamine intake was not significantly increased by treatment diets (Figure 5-3A, $P>0.05$).

5.4 Discussion

This study attempted to define the effect of incorporating 120 g/day of peas or beans on thiamine and folate status along with markers of glycemic control in a group of hypercholesterolemic individuals. Contrary to our original hypothesis, pulse consumption at the dose used in this study did not increase whole blood thiamine and serum folate concentrations when compared to the rice group as control. This lack of effect was observed independent of supplement intake in participants. However, participants in peas and beans groups had significantly higher blood thiamine levels compared to baseline (8.9% and 7.8% respectively). Studies of healthy populations have reported blood thiamine levels at a range of 46-69 ng/mL measured in whole blood samples (Kawai et al., 1980; Kimura et al., 1982; Kuriyama et al., 1980), however, it is unclear if some of these measurements were done in fasting or fed states. Another study reported that total thiamine in erythrocytes from healthy adults ranged from 73-131 ng/mL in non-fasting state

(O'Rourke et al., 1990), which could be the reason for a higher range. In addition, in a study with an obese population, a range of 38-122 ng/mL was considered normal (Carrodeguas et al., 2005). Mean baseline concentration of fasting whole blood thiamine for our participants was 50 ng/mL, suggesting they had normal thiamine status and no deficiency when entering the intervention. Although the aim of our study was not to examine improvements in thiamine deficiency with pulse consumption, we think it is reasonable to assume that normal baseline levels of thiamine might have masked any possible effect of the intervention diets on thiamine concentrations.

Similarly, folate status remained unchanged following 6 weeks of pulse consumption. According to WHO, serum folate concentrations of 6-20 ng/mL are considered normal, while concentrations higher than 20 ng/mL are viewed as elevated levels (WHO, 2012). Given that participants' mean serum folate concentration was 29 ng/mL at baseline, they fall within the elevated category in terms of folate status. No participants had baseline or final folate concentrations that were lower than the normal range. This is not a surprising observation considering the folate status of the general Canadian population. Evidence from the Canadian Health Measures Survey (Cycle 1) suggests that almost 100% of Canadians have red blood cell folate concentrations that are equal or higher than the cut-off level of 135 ng/mL for folate deficiency (Colapinto et al., 2011). This cut-off is also comparable to that of WHO (<100 ng/mL in red blood cell and <3 ng/mL in serum). These statistics could be explained by the mandatory folic acid

fortification policy implemented in Canada since 1998 (Canada Gazette Part II, 1998). In North America, the prevalence of folate deficiency resulting from low dietary intake has been extremely decreased by the introduction of folic acid fortification policies (Antoniades et al., 2009). The fact that almost all participants had normal to elevated levels of folate at the beginning of the study could have masked any potential effects of dietary folate from pulse consumption.

In the Canadian population, pulse consumption at a dose of 294 g/d (approximately 2 cups) is associated with increased intake of folate among other micronutrients. Unlike folate, thiamine intake does not appear to be higher in pulse-consumers when compared with non-consumers (Mudryj et al., 2012). A high folate intake was reported as positively correlated with serum folate concentrations in elderly women (Wolters et al., 2003). Health Canada's Recommended Dietary Allowance (RDA) for folate is 400 μg/d and is similar for men and women within the age group of this study. RDA for thiamine is 1.2 mg/d for men and 1.1 mg/d for women. In this study, participants had comparable absolute intakes of dietary thiamine and folate at baseline. In response to 6 weeks of intervention, however, a significant effect of diet on dietary intake of folate was found ($P<0.05$). In addition, participants in the beans group increased their dietary folate intake by 18%, from an average of 395 μg/d to 465 μg/d, which was significantly different from that of control group ($P<0.05$). The peas group also had a similar magnitude of increase in folate intake (17%), although it was not statistically significant. Thiamine intake, on the other hand, was not significantly

increased by treatment diets. At the end of the study, the average dietary intake of thiamine by peas and beans groups was similar at 1.7 mg/d, which again was comparable to that of control group. It is important to note that these are absolute dietary intakes of thiamine and folate; given that the beans group also had increased daily intake of energy compared to the control (Liang, 2014), it is possible that higher intake of folate in this group is partially due to overall higher consumption of food sources of this vitamin rather than the bean-containing foods. In addition, lack of a significant effect of pulse consumption on thiamine intake could be secondary to the fact that participants consumed the study foods as a part of their habitual diets; it is likely that overall intake of thiamine from sources other than pulses were high in the control group, hence disguising any potential impact of pulse incorporation into the diet on the daily intake of thiamine. The same reasoning could be applicable to the inability of peas to induce changes in the dietary intake of folate. Given that energy intake was not changed in the peas group, it is also plausible that these participants decreased their intake of other food sources of thiamine and folate, thereby maintaining their overall intake of these vitamins at doses similar to pre-intervention.

Furthermore, while the average intake of thiamine at baseline (1.4±0.1 mg/d in control, 1.6±0.2 mg/d in peas and 1.7±0.3 mg/d in beans) was higher than the RDA for this vitamin (1.2 mg/d for men and 1.1 mg/d for women), not all the participants met the RDA. At the final visit, however, the proportion of the participants who met the RDA for thiamine increased from 86% to 92% in the peas

group and from 71% to 100% in the beans group. In contrast, this proportion decreased in the control group, whose participants consumed rice, from 71% to 66%. The final percentages are reflective of the participants who completed the study. Participants in the peas and beans groups also showed improvements in terms of meeting the RDA for folate; the percentage of those who had dietary folate intakes higher than RDA increased from 43% to 62% and 36% to 67%, respectively in the peas and beans groups, whereas the control group showed a decrease in the proportion of those who met the RDA for folate (36% to 25%). It is intriguing to see that although almost all the participants had normal or elevated levels of thiamine and folate in blood during the study, this was not accompanied by similar rates in terms of adhering to RDAs for these vitamins.

Finally, markers of glycemic control were examined in the participants, revealing no significant changes in fasting glucose, insulin and HbA1c following the intervention. Failure to reduce these markers could be secondary to the fact that almost all participants were already normoglycemic with mean average glucose concentrations of 5.07 mmol/L and HbA1c of 5.3%. Based on CDA's criteria for diagnosis of prediabetes (fasting plasma glucose= 6.1-6.9 mmol/L or HbA1c=6.0-6.4%) (CDA, 2013), only one person was prediabetic at the beginning of the trial. Similar to our observation, Hartman et al. (2010) failed to detect any effects of consumption of 250 g/d of beans on glucose and insulin concentrations in spite of using double the amount of pulses used in our study; however, the duration of their study was slightly shorter at 4 weeks. This study was conducted in a population at

risk of developing colorectal cancer who had normal glycemia. Another study, however, reported improved HbA1c levels in T2D patients following consumption of 190 g/d of mixed pulses. The length of their study was 3 months and pulses were consumed as part of a low glycemic index diet, which could be why they detected significant changes in term of HbA1c (Jenkins et al., 2012).

To our knowledge, this is the first study to assess the effect of incorporation of pulses on thiamine and folate status, respectively measured in whole blood and serum. Strengths of the study showed that overall compliance rate was very high at 98.3% (98.9% in control, 98.8% in peas and 96.9% in beans; Liang, 2014). Compliance rate was assessed based on a tracking document that was filled out by participants throughout the intervention (Appendix F). This suggests that the daily dose of pulses used in the study were well tolerated by participants and that incorporation of this amount of pulses into the diet of Canadians is feasible. Our limitations were the relatively short length of the study and the relatively low dose of pulses, the type of background diets consumed by participants and the fact that participants were already healthy and normoglycemic. This was inevitable since participants were recruited based on the inclusion criteria set for the main outcome of the study, which was cholesterol. In addition, it is important to note that this work was only a preliminary analysis of the original study designed to recruit 150 participants in order to have enough power to detect any expected effects on the primary outcome (LDL-C levels). While preliminary analysis of a randomized clinical trial is a common approach, it will most likely be underpowered to detect

any differences in terms of secondary outcomes when compared to a comprehensive analysis of the study. Our food preparation protocol could also be challenged since long term freezing has been shown to change the vitamin content of a different cultivar of peas (Korus et al., 2002). However, given the length of the study and the number of participants enrolled, it would not have been practical to provide them with fresh food on a weekly basis. The intention-to-treat method used for data analysis is commonly used in many randomized clinical trials, although some cons are noted as well (Gupta, 2011).

5.5 Conclusion

Overall, regular consumption of 120 g/day of peas or beans for 6 weeks did not affect thiamine and folate status. No beneficial effects were observed in terms of insulin, glucose and HbA1c. Future studies would benefit from examining the impact of pulse consumption in T2D patients who are more likely to suffer from thiamine and folate deficiencies. Furthermore, a more long-term trial would be useful to test the efficacy of pulses in improving B vitamin status.

Chapter 6: Final Discussion

The main purpose of the present research was to investigate the effects of dietary supplementation of HFD with PSC fractions on glucose homeostasis, address the impact of the preparation method on the outcomes and unravel possible mechanisms contributing to glucose-lowering actions of PSC fractions. A schematic illustration of our main findings and the suggested working mechanisms of PSC fractions is presented in Figure 6-3. Summaries are provided in the following sections.

6.1 Influence of PSC on Rats' Metabolic Profile

Chapter 3 experiments showed that PSC supplementation to HFD-induced glucose-intolerant rats resulted in improved glycemia as measured by oGTT. This effect appeared to be dependent on the method of preparation since raw PSC fractions were not as beneficial as cooked ones. Analysis of PCS fractions showed that both preparations were composed of similar proportions of dietary fibre and protein. Mean protein content was higher in the cooked fractions, although the difference was too small at less than 0.03% of total weight to be of any importance at physiological levels. On the other hand, neither the total amount of dietary fibre and dietary fibre classes, nor soluble versus insoluble, were altered due to cooking. Regardless, cooking treatment affected glucose tolerance; we speculate this resulted from separation and/or hydration of the fibre components along with partial solubilisation and depolymerisation of select dietary fibre substances upon cooking, as documented before (Harding et al., 2014, Marconi et al. 2000), and its

consequent impact on the interactions between dietary fibre and gut microbiota (as discussed below).

The majority of studies on the effects of peas or their fractions on metabolic outcomes have identified dietary fibre as the key active component in terms of health benefits. The protein component, on the other hand, has been suggested to be capable of accelerating energy expenditure via increasing the thermogenic effect of diet (Marinangeli and Jones, 2012). In our animal model, however, energy expenditure did not appear to be affected by PSC fractions since weight gain was similar between all the groups. In addition to overall energy expenditure, consumption of pea fractions has also been shown to induce changes in substrate oxidation (carbohydrate:fat ratio) in humans (Marinangeli and Jones, 2011a). Unfortunately, in this work we were not able to examine substrate oxidation rates since the available metabolic cages were deemed too small for the size of the rats. Body composition is another metabolic aspect that has been linked with consumption of peas. In particular, consumption of whole or fractioned pea flour was reported to reduce body fat in Golden Syrian Hamsters (Marinangeli and Jones, 2012) and whole pea flour intake lowered the android:gynoid fat ratio in female subjects (Marinangeli and Jones, 2011b). This was not the case in our study as we showed similar fat mass amongst the groups, which could be due to the short length of our study.

Consistent with improved glucose tolerance, we showed insulin secretion in response to glucose ingestion was lower in both PSC-supplemented diets. CP also led to reduced fasting insulin. In other words, PSC-fed rats required less insulin to maintain glucose at levels lower than those of HFD-fed rats, which suggested they might be more insulin sensitive. ITT, however, did not reveal any overall differences between the groups; compared to HFD, there was an increased glucose clearance in RP rats in the recovery phase, although it was not associated with improved glucose tolerance. A slower glucose rebound in the second phase of the ITT has been attributed to an enhanced ability of insulin to suppress gluconeogenesis (Alaya et al., 2010). Additionally, in the presence of hypoglycemia, as induced during ITT, other factors such as glucagon are also involved in the glucose counter-regulatory mechanisms (Pacini et al., 2013). Since HFD-fed rats displayed significantly higher concentrations of basal glucagon, it is possible that a faster rebound during ITT in this rats resulted from improved counter-regulatory actions of glucagon.

Given that the second phase of changes in glucose levels during an ITT is indicative of insulin actions on hepatic glucose production, we further investigated the effect of treatment diets on hepatic gluconeogenesis. Protein levels of phosphoenolpyruvate carboxykinase (PEPCK) in liver were measured by immunoblotting, as described elsewhere (Whitlock et al., 2012). PEPCK is a key enzyme in hepatic gluconeogenesis that is a target for regulatory actions of insulin in the liver at the transcriptional level (Quinn and Yeagley, 2005). Hence, if insulin

actions on hepatic gluconeogenesis were improved in the PSC groups, we would expect a larger inhibitory effect of insulin on PEPCK and a lower expression of this enzyme. However, no differences were detected in PEPCK expression (Figure 6-1), which was in keeping with similar levels of fasting plasma glucose between the groups. These results suggested that hepatic gluconeogenesis was not altered; hence it was not contributing to improved glucose tolerance seen in CP rats. In addition to hepatic gluconeogenesis, our group also previously assessed hepatic lipid metabolism in PSC-fed rats. We showed that PSC feeding reduced fatty acid oxidation in the liver and possibly resulted in increased carbohydrate utilization as a compensatory response and consequently reduced circulating levels of glucose (Chan et al., 2014).

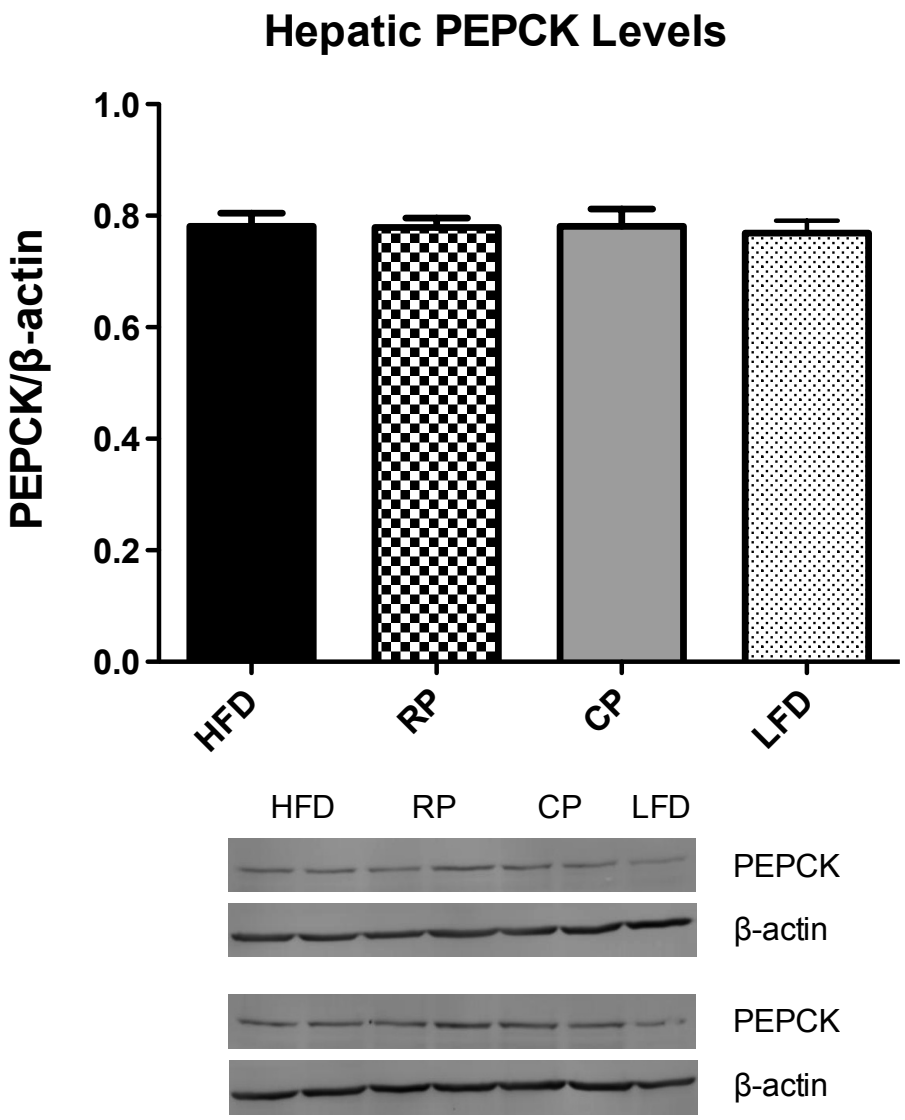

Figure 6-1. Effects of feeding PSC on hepatic PEPCK levels measured by immunoblotting. PEPCK expression in the liver normalized to that of β-actin as control. Data are means ± SEM analyzed by one-way ANOVA followed by Bonferroni post-hoc comparison test, n= 5. No significant differences were found between groups. The insets are representative blots for PEPCK and β-actin

6.2 Influence of PSC on HFD-induced Stress in Pancreatic Islets

Another key finding reported in Chapter 3 was the significant impact of PSC-supplemented diets on alpha-cell mass. Alpha-cell mass in CP rats was decreased to a level similar to that of LFD rats. The same rats also displayed lower glucagon concentrations in the plasma. Dysregulated glucagon secretion is now considered to play a role in the promotion of hyperglycemia as seen in insulin resistance and T2D (Liu et al., 2011; Weiss et al., 2011). Our results implied that supplementation with CP attenuated increased alpha-cell mass and glucagon secretion induced by HFD feeding. Moreover, HFD-induced insulin resistance is widely known to induce expansion of beta-cells. In our animal model, assessment of beta-cell mass revealed that it was not affected by PSC intervention, despite sharing a similar pattern of expansion with alpha-cell mass. The proliferative response of beta-cells is also dependent on the degree of beta-cell apoptosis (Butler et al., 2003), which in turn is caused by oxidative stress, among other factors (Anuradha et al., 2014). In our lab, it was previously shown that islets from the RP rats displayed reduced oxidative stress damage as measured by malondialdehyde (MDA) immunostaining (Whitlock et al., 2012). MDA is a byproduct of lipid peroxidation and a known marker of oxidative stress (Trevisan et al., 2001); therefore, reduced expression of MDA reflected decreased oxidative stress in the RP rats. In the present study however, preliminary analysis of immunostaining for MDA was not suggestive of any significant changes between the groups (data not shown).

6.3 Influence of PSC on Insulin Resistance Features in the Gut

6.3.1 PSC and Incretin Secretion

The effect of PSC supplementation on GLP-1 and GIP secretion was also examined. Fasting GLP-1 was increased in CP rats; both CP and RP groups had improved fasting concentrations of GIP, while RP rats also displayed enhanced GIP in the post-glucose state. GLP-1 exerts both insulinotropic and glucagonostatic actions on pancreatic islets to stimulate insulin secretion, promote beta-cell proliferation and suppress apoptosis and glucagon secretion (Seino et al. 2010). It is believed that GLP-1's effects on insulin secretion and glucagon inhibition play equal parts in the beneficial effects of GLP-1 on glucose homeostasis (Hare et al., 2010). One limitation present is that GLP-1 results in our study are only based on measurements in the fasting state due to technical issues. While the physiological importance of fasting GLP-1 levels is not quite clear, there are reports that diabetic patients have lower fasting GLP-1 concentrations compared to a non-diabetic population (Legakis et al., 2003). In addition, there is evidence on a positive association between fasting plasma GLP-1 concentration and energy expenditure and fat oxidation. Given the glucagonostatic actions of GLP-1, our results imply that increased fasting GLP-1 levels in CP rats were possibly accompanied by improved secretion of this incretin hormone in the fed state that induced a reduction in glucagon secretion. Although it might be tempting to suggest that secretory capacity of beta-cells is also likely enhanced by increased GIP and GLP-1 secretion, our present evidence is insufficient to confer a conclusion in this

regard. A test of in-vitro glucose-stimulated insulin secretion (GSIS) on pancreatic islets would be required to assess this postulation.

GIP, on the other hand, is known to display diminished insulinotropic effects in impaired glucose tolerance and T2D. As reported in Chapter 3, improved GIP responses in RP and enhanced fasting GIP concentrations in CP could not explain the differential effects of PSC fractions on glucose tolerance. The effects of PSC-supplemented diets on incretin secretion were independent of changes in L-cell and K-cell numbers, suggesting that endocrine cells had increased their capacity to secrete GLP-1 and GIP. To further assess these effects, proglucagon gene expression was measured in the ileum, yet no differences were found between the groups (Figure 6-2A). Proglucagon is the common precursor of multiple peptides including glucagon, GLP-1 and GLP-2, encoded by one single gene (Kieffer and Habener, 1999). Post-translational processing of the proglucagon precursor to tissue-specific peptides is mediated by prohormone convertase (PC) enzymes. In intestinal L-cells, PC 1/3 is the enzyme responsible for the production of GLP-1 (Holst, 2007). We suspected that PSC fractions might have affected GLP-1 secretion through increasing the expression of PC1/3 enzyme. To address this possibility, we measured gene expression of PC1/3 in ileum. Although CP appeared to increase mean relative expression of PC1/3 by 30%, it did not reach statistical significance (Figure 6-2B). These results show that increased fasting GLP-1 was not mediated by differences at the gene expression level.

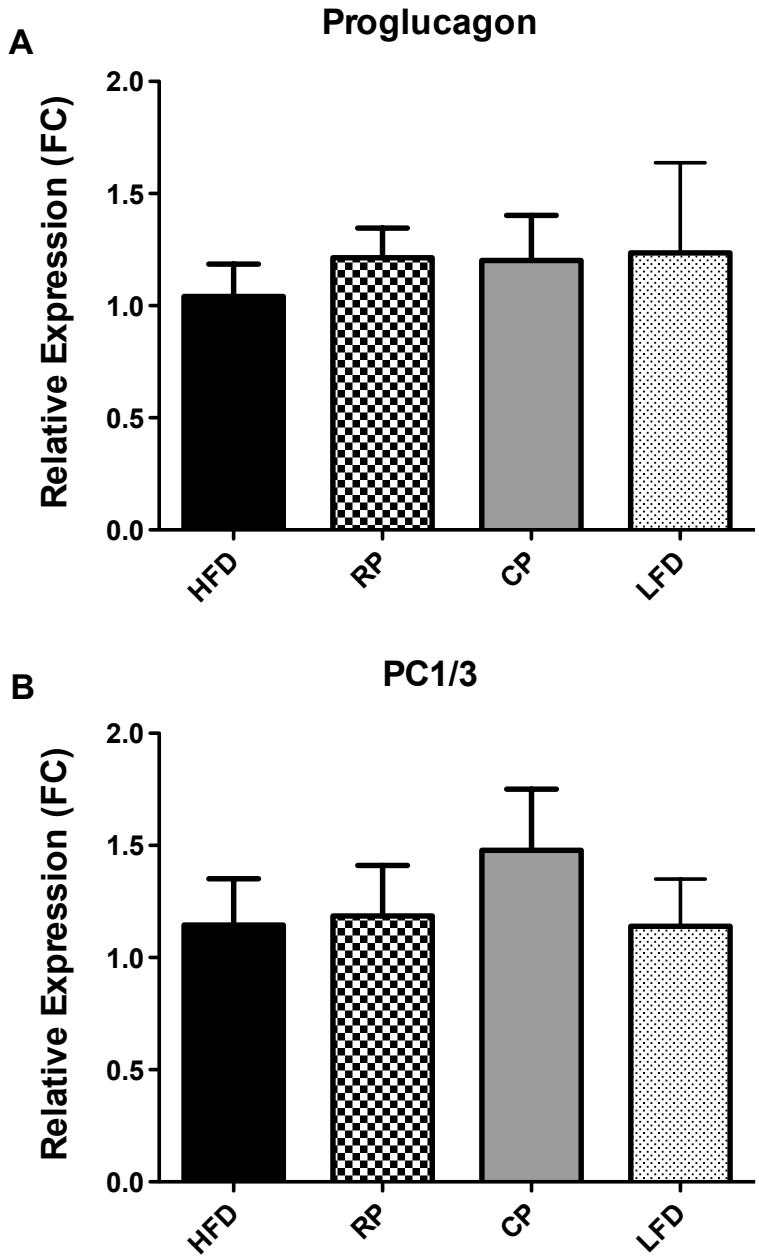

Figure 6-2. Effects of feeding PSC on mRNA expression of (A) proglucagon and (B) PC1/3 in the ileal mucosa. GAPDH was used as the control gene for data normalization. Bars are means ± SEM analyzed by Kruskal-Wallis test (A) and one-way ANOVA (B) followed by Bonferroni and Dunn's post-hoc comparison tests (n= 5-8); No significant differences were found between groups.

6.3.2 PSC and Nutrient Absorption

Also reported in Chapter 3 is the effect of PSC feeding on intestinal glucose transporters. We speculated that lower circulating glucose during oGTT may be due to reduced glucose absorption from the gastrointestinal tract. However, RP and CP did not affect glucose transporters expression differently from HFD; HFD appeared to be the main source of negative impact on glucose transporters expression. This is a curious finding and we think it could be suggestive of a counter-regulatory response to hyperglycemia.

In Chapter 5, we assessed the effect of incorporating 120 g/day of peas or beans on thiamine and folate status in hypercholesterolemic normoglycemic human subjects. Given that pulses are very good sources of thiamine and folate and the importance of thiamine and folate status in diabetes and its complications, we hypothesized that 6 weeks of pulse consumption would result in increased thiamine and folate concentrations. We found that pulse consumption, compared to consumption of rice as control, did not increase whole blood thiamine and serum folate concentrations. These negative results could be secondary to several factors, including the dose of pulses, the duration of the study, existing normal or elevated levels of thiamine and folate in participants and alterations in overall intake of non-pulse sources of these vitamins.

6.3.3 PSC and Intestinal Microbiota and Permeability

Findings from experiments in Chapter 4 demonstrated that supplementing HFD with PSC fractions resulted in significant changes in the overall microbial composition of the gut and these effects, for the most part, were not dependent on the preparation method. Specifically, RP and CP resulted in an increase in the abundance of *Lachnospiraceae*, a butyrate-producing family from the phylum *Firmicutes*. These results are in keeping with our previous finding where we showed feeding RP led to an increase in gut-derived 3-hydroxybutyrate (Chan et al., 2014). Considering the known effects of butyrate administration on incretin secretion (Lin et al., 2012), it is speculated that a higher population of *Lachnospiraceae* resulted in increased production of butyrate in PSC groups and positively altered basal GLP-1 and GIP secretion in CP rats and enhanced basal and glucose-stimulated GIP secretion in RP rats, as reported in Chapter 3. Another significant finding was increased population of *Prevotellaceae* in both PSC groups. This family of bacteria is composed of species that can utilize polysaccharides such as water-soluble xylans (Flint et al., 2012). This finding was consistent with our fibre analysis data showing that xylose, the primary building block for xylan, was present in both raw and cooked preparations. In a recent animal study by Huang et al. (2014), an insoluble fibre-rich fraction from pineapple peel that contained xylan and xyloglucan in the form of hemicellulose along with cellulose and pectic substances was compared to cellulose alone for its effects on intestinal functions. It was reported that hamsters receiving the diet supplemented with pineapple-derived fibre exhibited altered gut microbiota and enhanced growth of beneficial bacteria.

These changes were associated with an increase in the total pool of cecal SCFA. Similar to our results, this study suggests that not all sources of IDF have the ability to favourably change microbial composition of the gut and some, such as those made of xylans, could display stronger beneficial properties.

Another novel finding from Chapter 4 was the impact of PSC diets on the expression of TLR-2. RP and CP diets induced more than 60% reductions in the expression of TLR2 in the ileum, which appeared as a trend (P=0.09). HFD-induced promotion of TLR-2 expression has previously been reported in adipose tissue of insulin resistant mice (Murakami et al., 2007). We think our results are positive since they suggest that addition of PSC reversed the adverse effects of HFD on ileal TLR2 expression, although this was measured in ileum not adipose tissue. Further assessment of TLR-2 expression in adipose tissue could confirm this finding. Activation of the TLR2 signaling pathway results in the production of proinflammatory cytokines (McCucker & Kelley, 2013). Having this in mind, we further investigated the possible effect of PSC enrichment on the production of proinflammatory cytokine IL-6 and found that IL-6 gene expression in the ileal mucosa did not change with treatment diets (data not shown). Chapter 4 results also demonstrated that relative expressions of Muc2 and Muc4 were increased following CP feeding; Muc1 expression was also significantly affected by PSC-supplemented diets. These findings are indicative of a protective role for CP fractions against HFD-induced disruption of mucus layer composition; this effect would likely result in improved intestinal barrier integrity. Finally, assessment of

tight junction proteins expression did not reveal any significant effect of PSC diets, despite a considerable 85% increase in the expression of occludin in CP-fed rats.

6.4 Considerations for Future Directions

The studies conducted as part of this thesis support a beneficial role for cooked PSC fractions on glucose homeostasis in glucose intolerant rats. Moreover, the current findings provide molecular explanations for the effects exerted by these components. It is important to acknowledge that some of the mechanistic pathways we have identified here are speculative and further investigations would be required to confirm them.

Based on Chapter 3 results, I suggested that CP may enhance beta-cell secretory capacity, and this would be mediated by increased basal GLP-1 release. To confirm this effect, assessment of beta-cell function by in-vitro GSIS would be required. Another experiment that could be improved from a technical point of view is assessment of insulin sensitivity, which we assessed using ITT. While ITT provides a measure of insulin sensitivity by determining the magnitude of blood glucose changes in response to exogenous insulin, it does not effectively control for a couple of confounding factors. For instance, although insulin dose is normalized to the animal body weight, differences in body composition, specifically lean body mass reflecting the proportion of insulin-sensitive tissues, could influence the glucose response (Ayala et al., 2010). Hyperinsulinemic-euglycemic clamp is the most accurate alternative technique, which is widely

known as the gold-standard method for assessment of insulin sensitivity, although it has some limitations as well.

In Chapter 4, results regarding gene expression of tight junction proteins are not conclusive without considering the importance of their organization and distribution. There is a possibility that PSC supplementation affected the structure of tight junctions independent from the abundance of their constituent proteins. This could be further investigated by immunohistochemistry techniques. In addition, since TLR2 gene expression in the ileal mucosa indicated a near-significant difference between groups, examining the status of this receptor in other tissues such as fat and islets might shed some light on the effect of PSC feeding on TLR2 expression in these tissues. Recently, it was reported that TLR2 ligands cause an increase in cytokine gene expression in human islets and mediate reduction of insulin gene expression and insulin secretion (Nackiewicz et al., 2014). To even further dissect the cross talk between PSC fractions and TLR2 regulation, an in vitro time-course study of treatment of endothelial cell line with PSC components upon stimulation with TLR2-agonists could be conducted. TLR4 gene expression, on the other hand, did not appear to be affected by PSC fractions. Since TLR2 and TLR4 respond to different microbial components, this could suggest that failure to detect a change in TLR4 expression is secondary to the lack of altered LPS content, although it was not measured in the present study.

In the clinical study presented in Chapter 5, failure to detect any effect of pulse consumption on thiamine and folate status and glycemia could be due to several reasons such as length of the study, the relatively low dose of pulses, variable background diets, recruitment of normoglycemic subjects and overall small power to detect differences due to preliminary analysis nature of the study. When designing future trials, several important factors need to be taken into consideration. First, the study population should ideally be recruited based on criteria specific to impaired glucose tolerance and/or T2D. Second, a more controlled background diet would help with reducing the impact of differential dietary patterns in different individuals. Third, using a higher dose of pulses, at levels still tolerable, might help accentuate the plausible beneficial effects of pulse-containing diets. Finally, an intervention with a longer duration would increase the probability of detecting more long-term effects of pulse consumption on glucose control and B vitamin status. Furthermore, given the promising evidence on the impact of PSC feeding on microbial composition from our animal trial and the fact that clinical studies of this scale are mostly limited to assessment of circulating metabolites, one reasonable and feasible approach to examine mechanistic pathways in humans would be examining the effect of pulse feeding on the microbial composition in feces.

6.5 Implications of Thesis Findings

The studies presented in this thesis contribute significantly to existing literature in a number of ways. Besides reproducing the previously reported results

regarding glucose-lowering properties of pea-derived fractions, and more importantly, we were able to demonstrate that cooking enhances the benefits of PSC on glucose tolerance. This is an important finding since firstly, it contradicts previous reports of unfavourable impact of processing on the effectiveness of pulses in improving glycemia (Jenkins et al., 1982); secondly, it shows that the promising effects observed in animal studies can be translatable to humans considering that cooking would be the preparation method of choice. Moreover, the current findings provide novel health claims for PSC fractions based on their ability to reduce glucagon secretion and positively modify microbial composition of the gut and elements of intestinal barrier function. To my knowledge, these properties of PSC fractions have not been reported prior to this work or have been addressed at significantly different scales. In the food industry, the use of health claims is recognized as a valuable approach to increase the marketability of the novel functional foods (Marinangeli & Jones, 2013). Considering the potential of PSC fractions for use as a nutraceutical agent in the development of functional foods, the importance of additional evidence for health claims of these components can be appreciated. In addition, information on the components responsible for the actions of PSC in glycemia can help food scientists and pulse growers with optimizing the cultivar structure. For instance, evidence on increased abundance of xylan-utilizing bacteria upon PSC feeding, which is indicative of fermentation of xylose-containing insoluble fibre, could be used to effectively target the cultivars with the highest amount of these types of fibre.

Finally, while animal feeding trials are shown to be a more unbiased option with fewer complexities compared to the human feeding trials, the translatability of the results obtained in animals can sometimes be challenging. Another major implication of the present study findings is that it proves incorporating peas and their fractions into diets of humans is feasible from a practical standpoint. This is particularly important since we used doses that provide significant amounts of dietary fibre similar to those recommended to diabetic patients by CDA. This was true in the clinical trial of pulse consumption as well, backed up by a very high rate of adherence reported by participants. A clinical trial to specifically examine the benefits of pea seed coats prepared according to our protocol is a logical next step from this research.

6.6 Conclusion

In summary, this research demonstrates that supplementation with cooked PSC fractions improves glucose tolerance in glucose intolerant rats. This was associated with reduced insulin secretion and decreased fasting glucagon levels likely as a result of smaller alpha-cell area. CP preparations also resulted in improved plasma incretin concentrations, which was possibly due to an increase in the production of butyrate by microbial fermentation. The same fractions also conferred beneficial properties that could reverse the adverse effects of HFD on the expression of mucin proteins and TLR2, hence it may contribute to protective functions of the mucus layer and reduced activation of TLR2 signaling pathway. Taken together, our findings contribute to the growing body of literature that

supports a beneficial role for dried peas and/or their components in glucose control mainly in the presence of diet-induced insulin resistance and obesity. Due to the multifaceted nature of glucose homeostasis, it was not surprising to discover that PSC exerts an array of effects on multiple organs and pathways, which interact with each other in a complex manner.

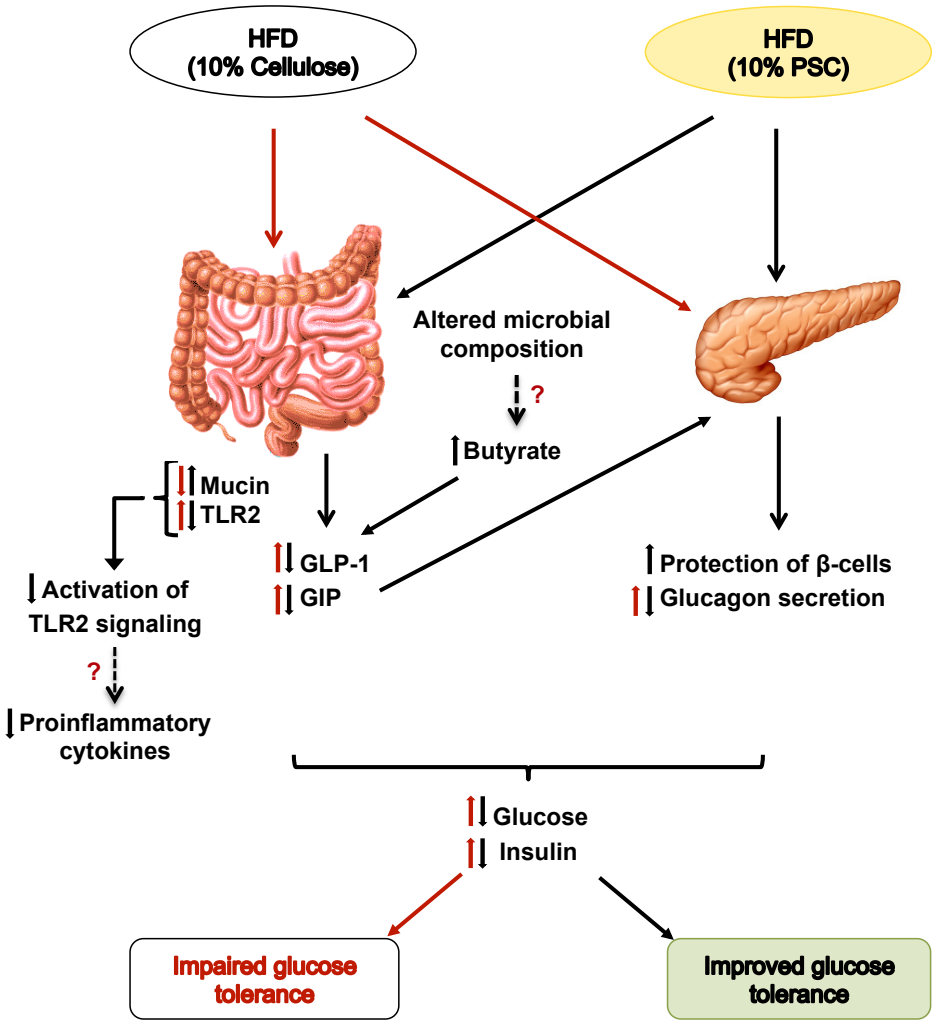

Figure 6-3. Schematic representation of plausible mechanisms of actions through which PSC influences pathways involved in glucose homeostasis in gastrointestinal tract and pancreas.

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Appendix A: Applied Physiology, Nutrition and Metabolism manuscript

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ARTICLE

Cooking enhances beneficial effects of pea seed coat consumption on glucose tolerance, incretin, and pancreatic hormones in high-fat-diet–fed rats

Zohre Hashemi, Kaiyuan Yang, Han Yang, Alena Jin, Jocelyn Ozga, and Catherine B. Chan

Abstract: Pulses, including dried peas, are nutrient- and fibre-rich foods that improve glucose control in diabetic subjects compared with other fibre sources. We hypothesized feeding cooked pea seed coats to insulin-resistant rats would improve glucose tolerance by modifying gut responses to glucose and reducing stress on pancreatic islets. Glucose intolerance induced in male Sprague–Dawley rats with high-fat diet (HFD; 10% cellulose as fibre) was followed by 3 weeks of HFD with fibre (10%) provided by cellulose, raw-pea seed coat (RP), or cooked-pea seed coat (CP). A fourth group consumed low-fat diet with 10% cellulose. Oral and intraperitoneal glucose tolerance tests (oGTT, ipGTT) were done. CP rats had 30% and 50% lower glucose and insulin responses in oGTT, respectively, compared with the HFD group ($P < 0.05$) but ipGTT was not different. Plasma islet and incretin hormone concentrations were measured. α- and β-cell areas in the pancreas and density of K- and L-cells in jejunum and ileum were quantified. Jejunal expression of hexose transporters was measured. CP feeding increased fasting glucagon-like peptide 1 and glucose-stimulated gastric inhibitory polypeptide responses ($P < 0.05$), but K- and L-cells densities were comparable to HFD, as was abundance of *SGLT1* and *GLUT2* mRNA. No significant difference in β-cell area between diet groups was observed. α-cell area was significantly smaller in CP compared with RP rats ($P < 0.05$). Overall, our results demonstrate that CP feeding can reverse adverse effects of HFD on glucose homeostasis and is associated with enhanced incretin secretion and reduced α-cell abundance.

Key words: peas, insulin resistance, GLP-1, GIP, glucagon, pancreatic islets.

Résumé : Comparativement à d'autres sources de fibres alimentaires, les légumineuses, incluant les pois secs, sont des aliments riches en nutriments et en fibres qui améliorent le contrôle de la glycémie chez les sujets diabétiques. Nous posons l'hypothèse selon laquelle l'alimentation de rats insulinorésistants au moyen de l'enveloppe du grain de pois cuit améliore la tolérance au glucose en modifiant les réponses de l'intestin au glucose et en diminuant le stress appliqué sur les ilots pancréatiques. On induit l'intolérance au glucose à des rats mâles Sprague–Dawley par un régime riche en gras (« HFD », 10 % de fibres de cellulose) puis on les soumet durant 3 semaines à l'un des trois régimes HFD comprenant des fibres (10 %) provenant de la cellulose, de l'enveloppe du grain de pois cru (« RP ») et de l'enveloppe du grain de pois cuit (« CP »). Un quatrième groupe est soumis à un régime faible en gras comprenant 10 % de cellulose. On passe des tests de tolérance au glucose par voie orale (« oGTT ») et intrapéritonéale (« ipGTT »). Les rats alimentés au CP présentent respectivement 30 % et 50 % moins de réponses au glucose et à l'insuline dans la condition oGTT comparativement au groupe HFD ($P < 0,05$); on n'observe pas de différence dans la condition ipGTT. On mesure la concentration plasmatique d'incrétine et des hormones des ilots. On quantifie la surface des cellules alpha et bêta dans le pancréas ainsi que la densité des cellules K et L dans le jéjunum et l'iléon. On mesure aussi l'expression jéjunale des transporteurs des hexoses. CP suscite une augmentation de GLP-1 (*glucagon-like peptide 1*) et du polypeptide inhibiteur gastrique médié par le glucose à jeun ($P < 0,05$), mais la densité des cellules K et L est semblable à la condition HFD tout comme l'abondance de l'ARNm de *SGLT1* et *GLUT2*. On n'observe pas de différence significative de surface des cellules bêta entre les groupes suivant un régime. La surface des cellules alpha est significativement plus petite dans le groupe CP comparativement au groupe RP ($P < 0,05$). Globalement, nos résultats démontrent que CP peut inverser les effets néfastes d'un HFD sur l'homéostasie du glucose et est associé à une augmentation de la sécrétion d'incrétine et à une diminution de l'abondance des cellules alpha. [Traduit par la Rédaction]

Mots-clés : pois, insulinorésistance, GLP-1, GIP, glucagon, ilots pancréatiques.

Introduction

Pulse grains, including dried peas, are rich sources of fibre with low glycemic indices. Their unique nutritional profile has led to many investigations of the health benefits of different varieties of pulses. In a meta-analysis of randomized control trials, Sievenpiper et al. (2009) found that consumption of nonoil-seed pulses was associated with enhanced long-term glycemic control. Consumption of dried peas has specifically been linked with enhanced glycemic control in several human intervention studies. Patients with type 2 diabetes (T2D) consuming a mixed meal containing whole dried peas had a delayed increase in postprandial plasma glucose and insulin concentration compared with controls eating

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potato-based meals (Schafer et al. 2003) and whole yellow pea flour-containing foods, when compared with the same foods made from whole-wheat flour, improved postprandial glucose response in healthy subjects (Marinangeli et al. 2009). Most studies identifying beneficial effects of pulses on glycemia have used the whole grain (Sievenpiper et al. 2009) but some studies of pulse fractions are emerging. In hamsters, feeding a hypercholesterolemic diet with partial substitution of cornstarch with pea hull flour resulted in significant decreases in circulating glucose and insulin levels (Marinangeli et al. 2011). Lunde and colleagues (2011) also showed that consumption of pea fibre-enriched breads resulted in improved postprandial glucose tolerance and increased satiety in human subjects with a high risk of developing T2D. In humans, whole or fractionated pea flour (pea hulls) muffins ameliorated insulin resistance in overweight subjects compared with wheat flour muffins (Marinangeli and Jones 2011). Another study reported yellow pea fibre as the most beneficial fraction, compared with pea flour or starch, in terms of lowering blood glucose (Eslinger et al. 2014). One study, however, suggests that pea protein is the fraction accountable for improved acute glucose control following consumption of pea-containing meals in healthy adults (Smith et al. 2012).

The pea seed (*Pisum sativum* L.) consists of an embryo (cotyledons and an embryo axis), which is enclosed in a seed coat (hull). The nutrient components of the embryo are mostly starch and protein, while the seed coats are largely soluble and insoluble fibre (Dueñas et al. 2004; Guillon and Champ 2002; Whitlock et al. 2012). Many studies have shown that dietary fibre, particularly soluble fibre, has positive effects on postprandial glucose control (reviewed by Babio et al. 2010). However, deeper insights into the mechanisms by which different sources of fibre affect glucose metabolism are yet to be elucidated. It is known that dietary fibre is fermented by colon microflora, producing short-chain fatty acids (SCFA) like acetate, propionate, and butyrate (Jenkins et al. 2000). SCFAs have been linked with enhanced secretion of incretins and increased abundance of beneficial microbes in the gut (Freeland and Wolever 2010; Lecerf et al. 2012). Soluble fibre also dissolves in water to form a viscous slow-moving solution that results in slowed gastric emptying; however, the effect of this increased transit time on digestion and absorption is controversial (Lattimer and Haub 2010).

We previously showed that insulin-resistant rats fed a raw pea seed coat-supplemented diet had better glucose homeostasis compared with embryo-supplemented diet fed rats, suggesting that the beneficial effects are associated with the seed coat fraction (Whitlock et al. 2012). One limitation of that study was that raw pea seed coats incorporated into the diet were not suitable for human consumption. Some studies have suggested that processing reduces the effectiveness of pulses in improving glycemia (Jenkins et al. 1982). Therefore, this study was undertaken to examine the effects of grinding and cooking followed by freeze-drying on the ability of pea seed coat fractions to improve glucose control and to identify potential physiological mechanisms. We hypothesized that supplementing diets with pea seed coat fractions would ameliorate glucose tolerance by modulating glucose handling by the gut and reducing high-fat diet (HFD)-induced stress on pancreatic islets. We further hypothesized that the beneficial effects of pea seed coat fibre consumption would not be lost following cooking.

Methods and materials

Animals and diets

Our animal care protocols were all in accordance with the guidelines of the Canadian Council on Animal Care. They were reviewed and approved by the Health Sciences Animal Care and Use Committee at the University of Alberta. Eight-week-old male Sprague–Dawley rats were purchased from the Department of Biology, University of Alberta, or Charles River Canada (St. Constant, Que., Canada). They were housed 2 per cage with ad libitum access to normal chow and water for 1 week. After acclimatization, rats received 6 weeks of HFD (20% *w*/*w*) to induce insulin resistance, except for the low-fat diet (LFD) control group, which remained on chow. The HFD-fed rats were then randomly assigned to the following 3 diets: HFD, raw-pea seed coat (RP; HFD supplemented with raw seed coats), cooked-pea seed coat (CP; HFD supplemented with cooked seed coats). All these diet groups were isocaloric and maintained a macronutrient ratio of 42:33:25 for fat, carbohydrate, and protein. The chow-fed rats were put on LFD (6% *w*/*w*), in which carbohydrate replaced the fat resulting in a macronutrient ratio of 15:55:30 for fat, carbohydrate, and protein. As noted in Table 1, the fibre source in the treatment groups, which was 10% *w*/*w* cellulose in HFD and LFD, was replaced by prepared pea seed coat fractions so that the total fibre weight per gram of chow was identical. The protein was adjusted as necessary to ensure the diets were isonitrogenous. The animals were on the pea seed coat diets for 4 weeks with ad libitum access to food and water.

Table 1. Diet composition (g/kg).

	HFD	HFD+PSC	LFD
Ingredients			
Canola sterine	99.5	99.5	29.85
Flaxseed oil	6	6	1.8
Sunflower oil	94.5	94.5	28.35
Casein	270	263	270
Dextrose	189	189	255
Corn starch	169	169	245
Cellulose	100	0	100
Pea seed coat (dwt)*	0	107	0
L-methionine†	2.5	2.5	2.5
Mineral mix‡	51	51	51
Vitamin mix‡	10	10	7.6
Inositol†	6.3	6.3	6.3
Choline chloride†	2.8	2.8	2.8
Total weight (g)	1000.6	1000.6	1000.2
Macronutrient composition (% dwt)			
Carbohydrate	36	36	51
Fat	20	20	6
Protein	27	27	27
Fibre	10	10	10

Note: dwt, dry weight; HFD, high fat diet; HFD+PSC, high fat diet supplemented with pea seed coats; LFD, low fat diet.

*The composition of 'Canstar' pea seed coats has been reported previously (Whitlock et al. 2012); per 107 g dwt it includes 100 g fibre and 7 g protein. Therefore, the amount of PSC was adjusted to provide 100 g fibre per kg diet, thereby matching the amount of cellulose added to the HFD and LFD. The amount of casein was decreased in the PSC diets by the amount of protein in the PSC.

†MP Biomedicals.

‡Mineral mix (Bernhart & Tomarelli) and vitamin mix (AIN-93-VX) from Harlan.

Pea seed coat preparation and analysis

Seed coat preparation

The pea seed coat fractions used in this study were produced from the seeds of the pea (*Pisum sativum* L.) cultivar 'Canstar' that were grown in Alberta, Canada. 'Canstar' is a yellow-seeded field pea cultivar with little to no proanthocyanidins present in its seed coats. Whole peas were dehulled and the hulls (seed coats) screened and ground as described in Whitlock et al. (2012). A portion of the ground samples were used unprocessed (raw seed coat material) and a portion was subjected to a cooking treatment (cooked seed coat material), which consisted of boiling the samples at 100 °C in deionized water (approximately 10 volumes of water to 1 volume of seed sample) for 30 min. After 30 min of

cooking, the samples were cooled down to room temperature and stored at –20 °C until lyophilization of samples (using a freeze dryer; Virtis Ultra 35L Freeze Dryer, SP Scientific, Stone Ridge, N.Y., USA) for 7 days. For starch, protein, and fibre analyses, both raw and cooked seed coat material were lyophilized for 7 days and further ground using a Retsch ZM 200 (Retsch; Newtown, Pa., USA) mill to produce finely ground powder that passed through a 0.5-mm screen.

Starch and protein analysis

The ground lyophilized samples were assayed for total starch content using the Total Starch Assay Procedure AA/AMG 11/01 (Megazyme International Ireland Ltd., Bray, Ireland; AOAC Method 996.11). A nitrogen analyzer (LECO TruSpec CN Carbon/Nitrogen Determinator; Leco Corp., St. Joseph, Mich., USA) was used to estimate the total protein content, which was calculated by multiplying the nitrogen content with a conversion factor of 6.25 (AOAC Method 968.06). Caffeine (150 mg) and ethylenediaminetetraacetic acid (100 mg) were used as standards for instrument calibration.

Nonstarch polysaccharides (NSP) analysis (fibre)

The total, water insoluble, and water soluble NSP (fibre) components of seed coats were determined using the methods described in Englyst and Hudson (1987) and Englyst (1989) using 45 to 50 mg of ground seed coat per sample. The resulting starch-free sample residues were processed for total and insoluble NSP determination in independent samples (2 replicates per sample). After processing, myo-inositol (20 mg/mL; 0.1 mL) was added as an external standard to the total NSP and insoluble NSP residue samples. For conversion of the hydrolyzed sugars to their alditol acetates, the hydrolysate was vortexed and centrifuged at 2000*g* for 5 min. NH_4OH (12 mol/L, 0.2 mL) was added to a 1-mL aliquot of the hydrolysate and the mixture was vortexed, then freshly prepared $NaBH_4$ solution (0.1 mL; 100 mg $NaBH_4$ per mL of 3 mol/L aqueous NH_4OH solution) was added and the solution was incubated for 1 h in a 40 °C water bath. Subsequently, glacial acetic acid (0.1 mL) was added to the solution, followed by vortexing. A 0.2-mL aliquot of the acidified solution was added to 0.3 mL 1-methylimidazole. Acetic anhydride (2 mL) was then added to this solution and vortexed continuously for 10 min. Distilled water (5 mL) was subsequently added to the solution to decompose excess acetic anhydride and aid in phase separation. After the solution was cooled to room temperature, dichloromethane (4 mL) was added and mixed for 15 s. After centrifugation at 700*g* for 5 min, the top layer was aspirated off and distilled water (5 mL) was added. The solution was again centrifuged at 700*g* for 5 min, the top layer was aspirated off, and the bottom layer was dried in a 50 °C evaporator. Dichloromethane (1 mL) was added to the residue and a 0.5-μL aliquot of the derivatized sample was injected onto a DB-17 fused silica capillary column (0.25 mm inside diameter × 30 m; J&W Scientific, Folsom, Calif., USA) connected to a Varian 3400 gas chromatograph equipped with a cool-on-column injector. Helium was used as the carrier gas with a flow rate of 1.5 mL/min. The injector temperature was increased from 60 °C to 270 °C at the rate of 150 °C/min and maintained for 20 min. Oven temperature was raised from 50 °C to 190 °C at a rate of 30 °C/min, and maintained for 3 min, then increased to 270 °C at the rate of 5 °C/min, and maintained for 5 min. The flame ionization detector temperature was set at 270 °C. Peak area integration for carbohydrate analyses was according to a Shimadzu Ezchrom Data System (Shimadzu Scientific Instruments Inc., Columbia, Md., USA). The soluble NSP values were estimated by subtracting the insoluble NSP value from the total NSP value for a given sample.

Glucose and insulin tolerance tests

After 3 weeks of experimental diets (9 weeks in total on HFD), rats were appointed to either an oral glucose tolerance test (oGTT) or an intraperitoneal glucose tolerance test (ipGTT). The tests were performed as described previously (Sutherland et al. 2008; Whitlock et al. 2012). Dipeptidyl peptidase IV inhibitor (Millipore, Billerica, Mass., USA) was added to aliquots obtained at baseline and 30 min to assay gastric inhibitory polypeptide (GIP). Insulin tolerance test was conducted as published (Saleh et al. 2008). Area under the curve and incremental area under the curve (IAUC) were calculated in accordance with established methods (Wolever 2004).

Body weight, food intake, and measurement of body composition

Body weights were measured on a weekly basis. After introduction of the supplemented diets, food intake was measured for 24 h twice during the 4-week period. In addition, 1 day prior to tissue collection, magnetic resonance imaging (MRI) technique was applied to specify lean and fat mass body composition using an EchoMRI Whole Body Composition Analyzer (Echo Medical Systems LLC, Houston, Tex., USA).

Tissue collection

At the end of the 10th week, animals were fasted overnight and then euthanized by an overdose of xylazine/ketamine via intraperitoneal injection. A 3- to 5-mL blood sample was obtained by cardiac puncture and serum obtained following centrifugation, which was then stored at –80 °C. Intestinal segments and pancreatic tissue were collected and fixed in buffered formalin, dehydrated in graded ethanol, and embedded in paraffin. They were then cut to generate 5-μm cross sections using a microtome and adhered to glass slides.

Assays of serum

Samples from the ipGTT were assayed for insulin using an ELISA kit (Alpco Diagnostics, Salem, N.H., USA). GIP was assayed by Meso Scale Discovery human total GIP kit (validated for use with rat samples). Serum obtained at euthanasia was assayed for triglyceride (Sigma–Aldrich, St. Louis, Mo., USA) and free fatty acids (Waco Diagnostics, Richmond, Va., USA) by colourimetric assays, glucagon (Sceti K.K., Tokyo, Japan), and active glucagon-like peptide-1 (GLP-1) (Millipore, Billerica) by ELISA according to manufacturers' instructions.

Immunohistochemistry and morphometric tissue analysis

Tissue slides were rehydrated, endogenous peroxidases quenched, and nonspecific binding reduced using techniques described previously (Whitlock et al. 2012). For pancreas, rabbit anti-glucagon (Linco) and guinea pig anti-insulin primary antibodies (Dako) were diluted 1:100 in phosphate buffered saline (PBS), applied to the tissue sections, and incubated overnight at 4 °C. For jejunum and ileum, mouse anti-GIP (generously provided by Dr. Timothy Kieffer, University of British Columbia) and rabbit anti–GLP-1 (Epitomics, Burlingame, Calif., USA) were respectively diluted 1:1000 and 1:250 in PBS, then applied and incubated under the same conditions. Following washes, appropriate peroxidase-coupled secondary antibodies (1:200) were applied to the sections and the slides were incubated for 1 h at room temperature. Positive reactions were identified by peroxidation of diaminobenzidene in the presence of H_2O_2. Imaging was performed using an Axiovert microscope connected to an AxioCam MRm digital camera (Carl Zeiss, Toronto, Ont., Canada), and controlled with AxioVision version 4.6 software.

For pancreas, total pancreatic tissue area as well as α- and β-cell areas were quantified as published (Whitlock et al. 2012). The ratios of the α-cell and β-cell to total pancreatic area were calculated for each rat. For jejunum and ileum, random sections of each tissue were selected and photographed under 20 times magnification, and total number of GIP-positive and GLP-1–positive cells were calculated. The number of positive cells was then normalized to the number of villi.

Table 2. Sugar components of raw and cooked seed coats of 'Canstar' by gas chromatography analysis.

Milligrams/100 mg dry weight (%)									
Fibre class	Rhamnose	Ribose	Fucose	Arabinose	Xylose	Mannose	Glucose	Galactose	Total
Raw seed coat fraction									
Total	0.73±0.01	0.05±0.01	0.27±0.02	3.73±0.33	10.59±0.87	0.19±0.01	51.81±1.11	0.77±0.03	68.13±2.04
Insoluble	0.43±0.05	0.02±0.01	0.14±0.00	2.02±0.12	9.99±0.56	0.17±0.01	52.07±0.65	0.43±0.02	65.28±0.17
Soluble	0.30±0.07	0.03±0.01	0.12±0.02	1.71±0.21	0.60±0.31	0.02±0.00	1.00±1.00	0.34±0.02	4.11±1.50
Cooked seed coat fraction									
Total	0.62±0.01	0.06±0.01	0.25±0.01	3.71±0.36	10.64±0.91	0.20±0.01	51.50±0.56	0.86±0.05	67.84±1.84
Insoluble	0.34±0.01	0.01±0.00	0.14±0.01	1.76±0.16	9.50±0.76	0.17±0.00	52.64±1.15	0.40±0.03	64.96±1.97
Soluble	0.28±0.02	0.04±0.01	0.11±0.01	1.95±0.21	1.14±0.15	0.03±0.00	0.30±0.30	0.46±0.07	4.33±0.31

Note: Data are means ± SE, $n = 3$. The total % (sum of sugar components) of the raw seed coat fraction was compared with the cooked seed coat fraction within each fibre class (total, insoluble, and soluble fibre) using a Student's t test analysis, and no significant treatment effects were detected ($P > 0.05$).

Quantification of glucose transporters gene expression (*Glut2, Glut5, SGLT1*)

Total RNA was isolated from ileal tissue using Trizol reagent and purified with an RNeasy Mini Kit (Qiagen, Valencia, Calif., USA) per manufacturer's instructions. The cDNA (cDNA) was generated from RNA samples using a cloned AMV first-strand cDNA synthesis kit (Invitrogen). The cDNA samples were amplified using primers synthesized by the IBD core at the University of Alberta and analyzed by quantitative reverse transcription polymerase chain reaction. Primer sequences used for amplifications were as follows: *Glut2* (Accession Number NM_012879) forward primer, 5′-GACACCCCACTCATAGTCACAC-3′; *Glut2* reverse primer, 5′-CAGCAATGATGAGAGCATGTG-3′; *Glut5* (Accession Number NM_031741) forward primer, 5′-AACTTTCCTAGCTGCCTTTGGCTC-3′; Glut5 reverse primer, 5′-TAGCAGGTGGGAGGTCATTAAGCT-3′; *SGLT-1* (Accession Number NM_013033) forward primer, 5′-ATGGTGTGGTGGCCGATTGG-3′; *SGLT-1* reverse primer, 5′-GTGTAGATGTCCATGGTGAAGAG-3′. The housekeeping gene 18S ribosomal RNA was used for normalization (forward primer 5′-AGCGATTTGTCTGGTTAATTCCGATA-3′; reverse primer 5′-CTAAGGGCATCACAGACCTGTTATTG-3′). All sample reactions were prepared using Evolution Eva Green qPCR mastermix (Montreal Biotech, Montreal, Que., Canada) and run in duplicate on a Corbett Rotor-Gene 6000 cycler.

Statistical analysis

Two-way repeated measures ANOVA was performed on the oGTT, ipGTT, and insulin ELISA data. One-way ANOVA and Student's t test were used to compare the other data, as appropriate. All data are expressed as means ± SE; Bonferroni and Dunn's post hoc comparison tests were performed to assess differences between diet groups and a P value <0.05 was considered to be significant.

Results

Fibre analysis

Analysis of the fibre constituents from raw and cooked pea seed coats is reported in Table 2. The total fibre (NSP) content of the raw seed coat fraction was 68% *w*/*w*, with 64%–65% composed of insoluble fibre and 3%–4% soluble fibre. The total fibre fraction was composed mainly of glucose moieties (52%), while the total and insoluble NSP fibre fraction was also rich in xylose. Arabinose (4%) and mannose (0.2%) were also present in the total fibre of pea seed coats, but at low levels. Consistently, the amount of rhamnose was enriched in the soluble fraction compared with the total and insoluble fibre fractions. Galactose, xylose, and a small amount of fucose were also detected in the soluble fibre fraction. The cooking treatment did not affect the fibre classes of the pea seed coats ($P > 0.05$).

The total protein content of the raw pea seed coat fraction was 6%–7% by weight, and the total starch content was less than 1% (Table 3). Cooking treatment significantly increased the total protein and starch content of the pea seed coat fractions (Table 3, $P < 0.05$).

Table 3. Protein and total starch components of raw and cooked pea seed coats of 'Canstar'.

Pea seed coat fraction	Protein (%)[a]	Total starch (%)[a]
Raw	6.65±0.05	0.16±0.01
Cooked[b]	6.91±0.03*	0.59±0.02*

Note: Data are means ± SE, $n = 3$. The raw seed coat fraction was compared with the cooked seed coat fraction for percent protein and percent total starch using a Student's t test analysis.

*Significantly different ($P < 0.05$) from the raw seed coats fractions within each nutrient component.

[a]% = mg/100 mg dry weight of sample.

[b]Placed in boiling water for 30 min.

Body weight and body composition analysis

Rats in all groups gained the same amount of weight, calculated as % of baseline, at the end of the study (Table 4, $P > 0.05$). Food intake data were also comparable between groups, indicating that the palatability of the diets did not affect the results. MRI data revealed higher fat mass in RP compared with LFD when normalized to total body weight ($P < 0.05$, Table 4).

Circulating metabolites and hormones

These data are reported in Table 4. Fasting blood glucose was significantly higher in RP than LFD ($P < 0.05$). Fasting serum insulin and glucagon were significantly lower in CP than HFD ($P < 0.05$). Serum triglyceride concentrations were significantly higher ($P < 0.05$) in HFD than CP or LFD, but no differences in serum nonesterified fatty acids were detected (Table 4). Serum GLP-1 was significantly higher only in CP compared with HFD (Table 4, $P < 0.05$). As shown in Fig. 1, in fasted rats, fasting serum GIP concentrations were 50% higher in RP and CP than HFD ($P < 0.05$ for both). A similar trend was observed with GIP measured 30 min after glucose administration in the oGTT, in which RP was 2-fold higher than HFD (Fig. 1A and 1B, $P < 0.05$).

Glucose tolerance tests and insulin tolerance test

oGTT and ipGTT results are shown as responses over 120 min (Fig. 2A and 2C), and as IAUC (Fig. 2B and 2D). As expected, LFD had lower glucose response compared with HFD at $t = 10$ min ($P < 0.05$), and $t = 20$, 30, and 60 min ($P < 0.001$). CP but not RP rats had lower glucose response compared with the HFD group at $t = 10$ ($P < 0.05$), $t = 20$ ($P < 0.001$), and $t = 30$ ($P < 0.01$) min. IAUC during oGTT showed that both CP and LFD groups had glucose values that were significantly lower than HFD (Fig. 2B, $P < 0.05$ and $P < 0.001$, respectively). Although neither CP nor RP had different ipGTT from the HFD group (Fig. 2C), LFD had improved response at $t = 20$ ($P < 0.001$) and $t = 30$ ($P < 0.01$) min. Trends for LFD and CP to lower

Table 4. Metabolic profile of rats fed diets containing pea fractions.

Diet group	HFD	RP	CP	LFD
BW (g); baseline	401.1±11	402.2±10	394.2±14.1	397±13.3
BW (g); final	627±12.3	649.3±13.9	660.3±16.1	608±14.07
Change (% of baseline BW)	65.84±3.79	67.61±3.12	74.52±3.33	62.54±3.91
Fat mass (% of final BW)	18.9±1.21	19.1±0.97	18.1±0.92	16.2±1.08
Food intake (kcal/day)	134.4±4.9	138.1±8.4	143±14.5	153±10.2
Fasting blood glucose (mmol/L)[a]	5.3±0.18	5.5±0.14†	4.9±0.28	4.4±0.08
Fasting serum insulin (ng/mL)[a]	1.04±0.13	1.24±0.19	0.49±0.08*	0.72±0.15
Serum TG (mg/dL)[b]	52.2±2.3	45.3±8.7	30.4±4.4*	32.6±4.0*
Serum NEFA (mmol/L)[b]	0.5±0.1	0.49±0.07	0.37±0.09	0.37±0.08
Fasting serum glucagon (pg/mL)[b]	308±32.31	286.6±12.28	167.5±26.9*	246.7±17.61
Fasting serum GLP-1 (pg/mL)[b]	18.3±0.7	23.1±2.5	27.9±1.6*	23.4±1.5

Note: Data are means ± SE analyzed by 1-way ANOVA followed by Bonferroni's post hoc comparison test, $n = 4–25$. BW, body weight; HFD, high-fat diet; RP, raw-pea seed coat (HFD supplemented with raw seed coats); CP, cooked-pea seed coat (HFD supplemented with cooked seed coats); LFD, low-fat diet.

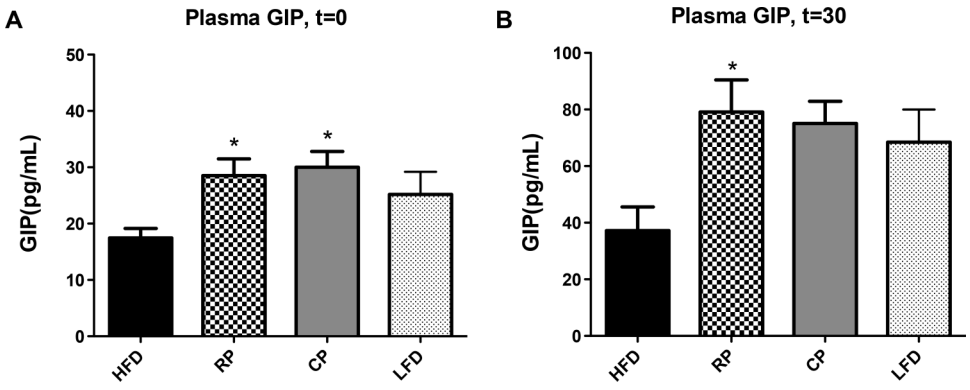
*Significant difference compared with HFD ($P < 0.05$).

†Significant difference compared with LFD ($P < 0.05$).

[a]Blood sampling was done at the end of the feeding trial during oral glucose tolerance test.

[b]Serum for TG, NEFA, glucagon and GLP-1 assessment was obtained from blood samples collected from fasted rats by cardiac puncture at the time of tissue collection.

Fig. 1. Plasma gastric inhibitory polypeptide (GIP) concentrations measured during oral glucose tolerance test at (A) fasting ($t = 0$ min) and (B) following administration of 1 g/kg glucose ($t = 30$ min). The bars are the mean ± SE, $n = 4–7$. Asterisks indicate significant difference compared with high-fat diet (HFD) by 1-way ANOVA followed by Bonferroni's post hoc comparison test (*$P < 0.05$). CP, cooked pea seed coat; LFD, low-fat diet; RP, raw-pea seed coat.



IAUC during ipGTT were attenuated and not statistically significant (Fig. 2D).

During oGTT, RP rats had lower insulin concentrations than HFD rats at $t = 10$ and 20 min (Fig. 2E, $P < 0.001$); CP group had decreased insulin concentrations compared with the HFD group at $t = 10$ ($P < 0.001$), $t = 20$ ($P < 0.001$), $t = 30$ ($P < 0.01$), $t = 60$ ($P < 0.01$), and $t = 120$ ($P < 0.05$) min. The LFD group had lower serum insulin than the HFD group at $t = 10$ ($P < 0.01$) min. Both RP and CP groups had smaller IAUC values when compared with HFD (Fig. 2F, $P < 0.05$ and $P < 0.01$, respectively); LFD rats also had significantly lower IAUC ($P < 0.01$). Insulin concentrations of CP and RP groups during ipGTT were not different than those of HFD (Fig. 2G). However, LFD had decreased concentrations at $t = 20$ and 30 min ($P < 0.05$). IAUC data also only revealed a difference between HFD and LFD (Fig. 2H, $P < 0.05$). Blood glucose levels during the glucose disappearance phase (0–30 min) of the insulin tolerence test were comparable among the groups (Fig. 3A and 3B). During the recovery phase (60–120 min), HFD rats rebounded most quickly and this was significantly faster than for RP rats (Fig. 3C, $P < 0.05$).

Pancreatic β- and α-cell mass analysis

Pancreatic β- and α-cell area at the end of the study are shown in Fig. 4A and 4B. After 4 weeks of pea seed coat intervention, no significant difference in β-cell area between diet groups was observed (Fig. 4A, $P > 0.05$). As shown in Fig. 4B, α-cell area in the 4 diet groups followed a similar pattern with β-cell area; however, significant differences were found between diet groups ($P < 0.05$), with CP fed rats having a significantly smaller α-cell area compared with the RP fed rats ($P < 0.05$). Total islet area was estimated by adding α- and β-cell areas and are presented in Fig. 4C ($P = 0.16$; denoting trend to increased islet area in the RP group). Representative micrographs depicted in Fig. 4D suggest that the increase in islet area of the RP group is due to an increase in the number of islets, rather than the size of individual islets.

K- and L-cell quantification

There was no significant difference in the number of K-cells expressing GIP in the jejunum shown in Fig. 5A ($P > 0.05$). Similarly, the number of GLP-1 positive L-cells in the ileum was comparable between all the groups (Fig. 5B, $P > 0.05$).

mRNA expression of glucose transporters

Jejunal mRNA expression of *Glut2* and *SGLT1* were similar between the groups (Fig. 6A and 6B); however, *Glut5* expression was significantly different between diet groups ($P = 0.005$), with a lower expression in RP when compared with LFD (Fig. 6C, $P < 0.01$).

Discussion

The present study demonstrated that supplementing a HFD with cooked pea seed coats improved glucose tolerance, whereas raw seed coat supplementation was not as beneficial. We also observed that the effect of the pea seed coat fibre on postprandial

Fig. 2. Effect of feeding pea seed coat on oral (oGTT) and intraperitoneal (ipGTT) glucose tolerance in rats. (A, C) Effect of 4 weeks of feeding a high-fat diet (HFD, 20% *w*/*w*) supplemented with raw (RP) or cooked (CP) pea seed coats on blood glucose levels measured basally and following oral or intraperitoneal administration of 1 g/kg glucose. (B, D) Incremental area under the curve (IAUC) was calculated for glucose during oGTT and ipGTT. (E, G) Plasma insulin levels measured using the blood samples collected during oGTT and ipGTT (baseline and in response to administration of 1 g/kg glucose). (F, H) IAUC for insulin during oGTT and ipGTT. The data are means ± SE, n = 4–14. Significant differences seen at different time points are explained in the text, while differences between IAUCs are depicted here. Asterisks show significant difference compared with HFD by 1-way ANOVA followed by Bonferroni's post hoc comparison test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). LFD, low-fat diet.

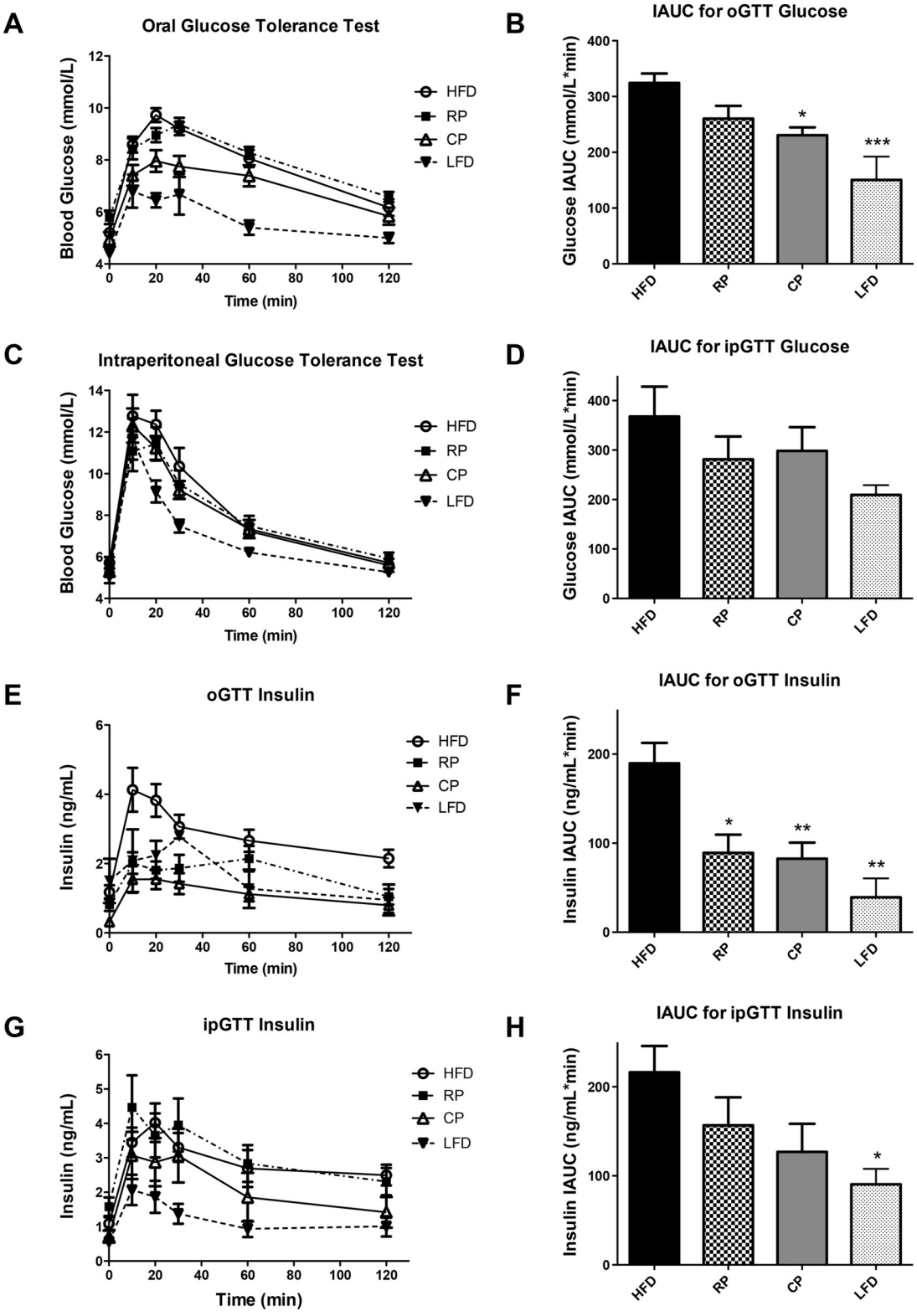
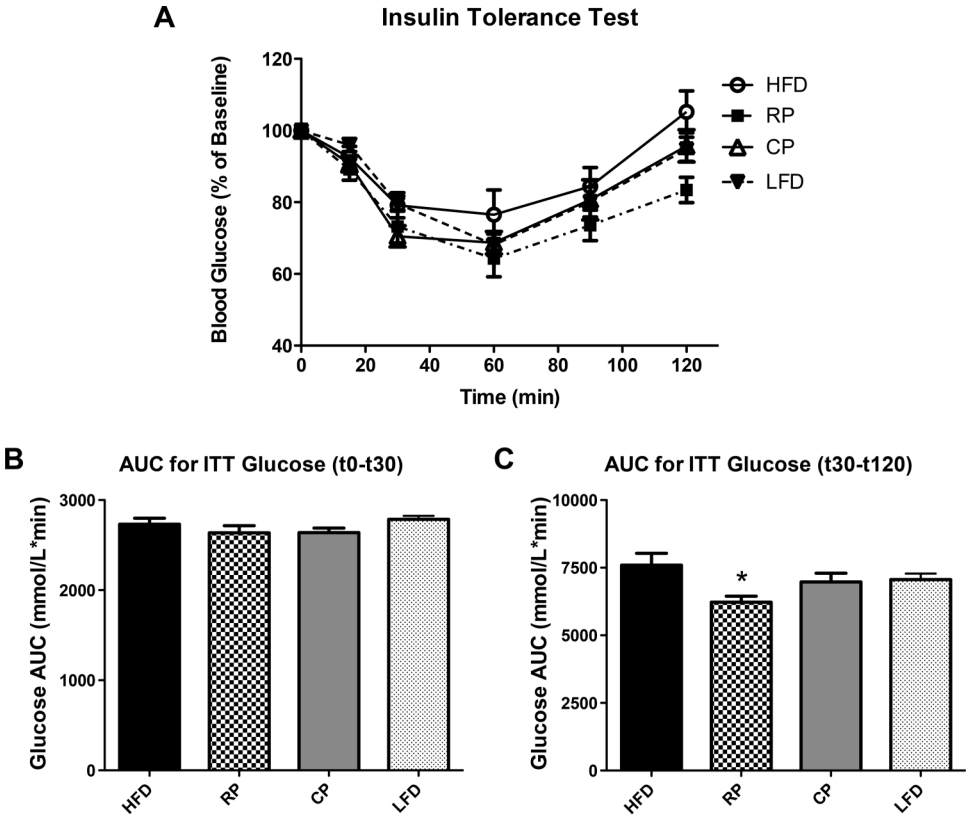


Fig. 3. Insulin tolerance test was performed on rats after a 4-h fast. Blood glucose levels are shown as (A) percentage of basal glucose, and area under the curve (AUC) from (B) $t = 0$ to $t = 30$ min and (C) $t = 30$ to $t = 120$ min for blood glucose. A significant decrease in glucose for raw-pea seed coat (RP) compared with high-fat diet (HFD) was observed at $t = 30$–120 min (*, $P < 0.05$). Data are means ± SE analyzed by 1-way ANOVA followed by Bonferroni's post hoc comparison test, $n = 8$. CP, cooked-pea seed coat; ITT, insulin tolerance test; LFD, low-fat diet.



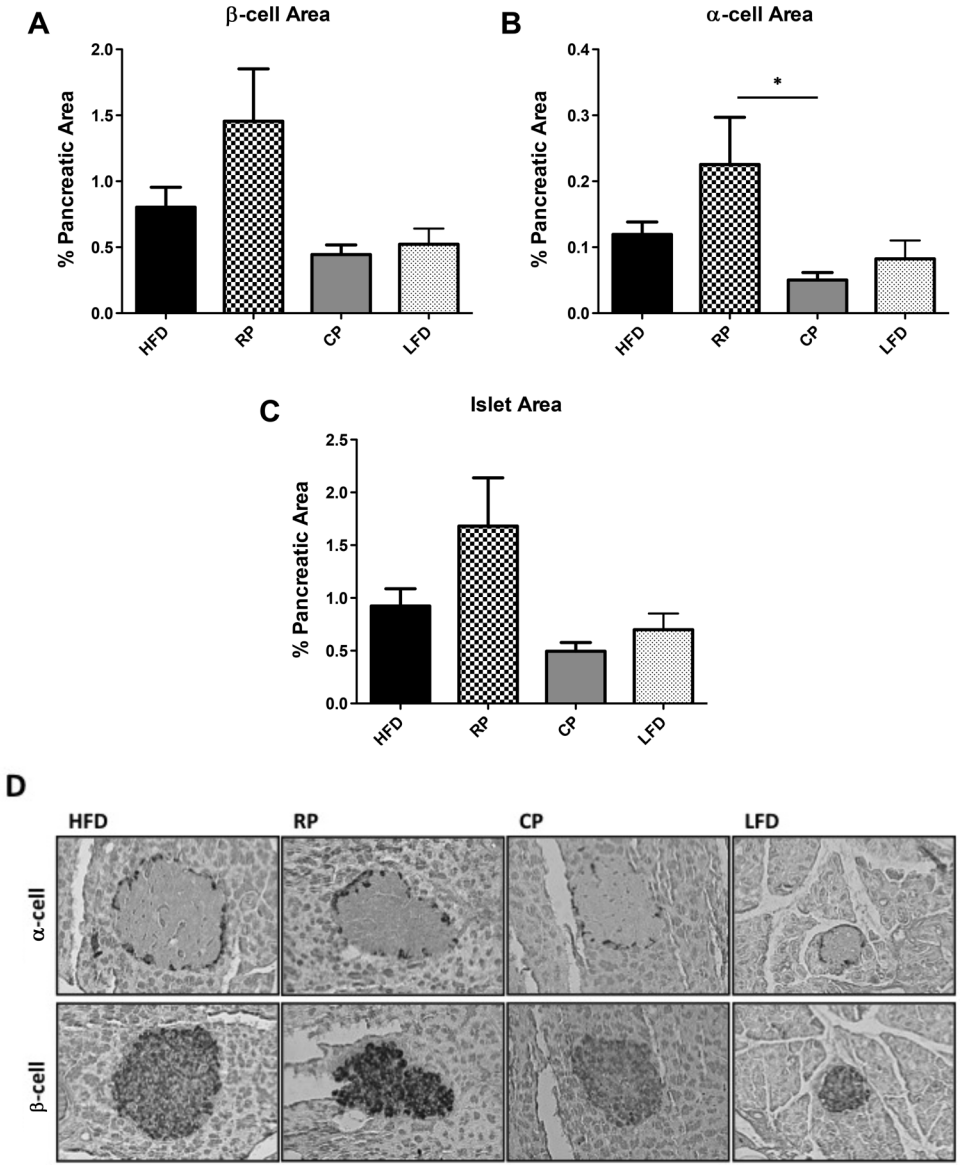
glucose excursions was only detectable when glucose was administered orally and not intraperitoneally. In other words, bypassing the gastrointestinal tract during ipGTT diminished the improved glycemic excursions in the pea fibre groups to a high degree. These divergent outcomes on oral versus intraperitoneal glucose tolerance led us to consider mechanisms of action involving the gastrointestinal tract.

The total fibre fraction of pea seed coats was mainly composed of monosaccharide glucose (Table 2), indicating that the most abundant polysaccharide present was cellulose (made up of linear chains of glucose). Because cellulose is a water-insoluble polysaccharide, the insoluble fibre component was also mainly made up of cellulose. The glucose content determined in the total NSP fibre of pea seed coats in this study is consistent with that of 58% reported by Weightman et al. (1994). The higher percentage of xylose (also consistent with Weightman et al. 1994), along with the occurrence of fucose, galactose and glucose in the total and insoluble NSP fibre fraction indicates the presence of the cell wall cellulose microfibril cross-linking polysaccharide, fucogalactoxyloglucan, commonly found in legume family members (Carpita and McCann 2000). The presence of arabinose in the total fibre of pea seed coats (Weightman et al. 1994 reported 3.9% arabinose in this fraction) suggests the presence of glucuronoarabinoxylans and/or pectins (Carpita and McCann 2000). Very low levels of mannose indicate minimal presence of glucomannans, galactoglucomannans, or galactomannans in interlocking microfibrils of the cell wall (Carpita and McCann 2000). The major noncellulosic neutral sugars, arabinose and xylose, detected in the soluble fibre pea seed coat fraction indicate the presence of pectin (Carpita and McCann 2000; Weightman et al. 1994). Rhamnose, which is another constituent of pectins, was also enriched in the soluble fraction of pea seed coats. Galactose, xylose, and the small amount of fucose also indicate the presence of fucogalacto-xyloglucans in the soluble fibre fraction.

Cooking treatment improved glucose homeostasis but did not alter the fibre classes of the pea seed coat fraction; however, the boiling process may have caused separation and/or hydration of the fibre components that were stabilized by the subsequent lyophilization. Other studies showed that cooking procedures did not affect the total dietary fibre (Goodlad and Mathers 1992; Marconi et al. 2000), although some inconsistencies are noted (De Almeida Costa et al. 2006; Kutoš et al. 2003). Other thermal procedures such as autoclaving also changed the composition of fibre that led to less fat accumulation upon consumption (Harding et al. 2014). In addition, boiling partially solubilizes and depolymerizes hemicelluloses and insoluble pectic substances (Marconi et al. 2000), which may change the properties of the fibre with respect to gut fermentation. Altered microstructures of pea flour, as a result of thermal treatments in general, can promote its nutritional and functional characteristics, among which are increased fat and water absorption capacity, and emulsifying and gelling activity (Ma et al. 2011). In addition to higher nutritional value, the thermal processing-derived characteristics of pea flour improve its practicality for food application (De Almeida Costa et al. 2006; Ma et al. 2011).

In the current study, we reported enhanced glucose control in the CP group during oGTT versus no improvement during ipGTT. This effect could be explained by several different mechanisms. Soluble dietary fibre (SDF) can be fermented to SCFA in the colon, which are absorbed into the blood and suppress glucose production in the liver (Higgins 2004) and stimulate skeletal muscle uptake of glucose (Lu et al. 2004). A previous study by our group

Fig. 4. (A) β-cell and (B) α-cell areas of rats fed a high- fat diet (HFD), raw-pea seed coat (RP), cooked-pea seed coat (CP), and low-fat diet (LFD) for 4 weeks, presented as percentage of pancreatic area. (C) Estimated total islet area. α-cell area was significantly different between the groups where CP rats revealed a smaller α-cell area (*P < 0.05) when compared with RP. Data are means ± SE analyzed by 1-way ANOVA followed by Dunn's post hoc comparison test, n = 6–8. (D) Representative insulin- and glucagon-stained islets of all the groups.



showed increased circulating 3-hydroxybutyrate believed to be derived from butyrate of dietary origin because butyrate dehydrogenase expression in the liver was suppressed (Chan et al. 2014). On the other hand, insoluble dietary fibre also regulates glucose homeostasis but not via effects on viscosity (Schenk et al. 2003; Weickert et al. 2006). We speculated that CP might improve oGTT by downregulating the expression of intestinal glucose transporters. However, HFD appeared to be the main negative driver of glucose transporter expression, and there was no subsequent modulation upon addition of RP or CP. Contrary to our results, a study performed on dogs showed that a diet containing high fermentable dietary fiber resulted in increased jejunal *SGLT1* and *Glut2* mRNA abundance (Massimino et al. 1998), but that would not explain enhanced glucose tolerance.

Pea seed coat-supplemented diets significantly enhanced plasma incretin concentrations. GLP-1 has insulinotropic effects and acts directly on pancreatic islets to stimulate insulin secretion from β-cells, promote β-cell proliferation, and suppress apoptosis (Seino et al. 2010) as well as noninsulinotropic effects such as inhibiting gastric emptying. GLP-1 also inhibits glucagon secretion, and decelerates endogenous production of glucose (Seino et al. 2010). In several animal studies, consumption of fermentable dietary fibres has been linked with elevated plasma GLP-1 (Grover et al. 2011; Massimino et al. 1998; Wang et al. 2007). In addition, in both healthy (Johansson et al. 2013; Tarini and Wolever 2010) and hyperinsulinemic (Freeland et al. 2010) human subjects, diets high in SDF increased GLP-1 in plasma. In our study, we observed 50% higher fasting GLP-1 in CP group relative to HFD. This could be positive given that it has previously been reported that diabetic patients had significantly lower fasting serum GLP-1 when compared with nondiabetic overweight subjects (Legakis et al. 2003); however, the physiological significance of fasting GLP-1 levels remains elusive. Pannacciulli et al. (2006) reported a positive association between fasting plasma GLP-1 concentration and energy expenditure and fat oxidation. In another study, both in vitro and in vivo results showed that GLP-1 increased basal uptake of

Fig. 5. (A) Number of K-cells and (B) L-cells as detected using gastric inhibitory polypeptide and glucagon-like peptide 1 immunoreactivity in jejunum and ileum, presented as number of positive cells per villus. Data are means ± SE analyzed by 1-way ANOVA followed by Bonferroni's post hoc comparison test, $n = 4$. No significant differences were observed. CP, cooked pea seed coat; HFD, high-fat diet; LFD, low-fat diet; RP, raw-pea seed coat.

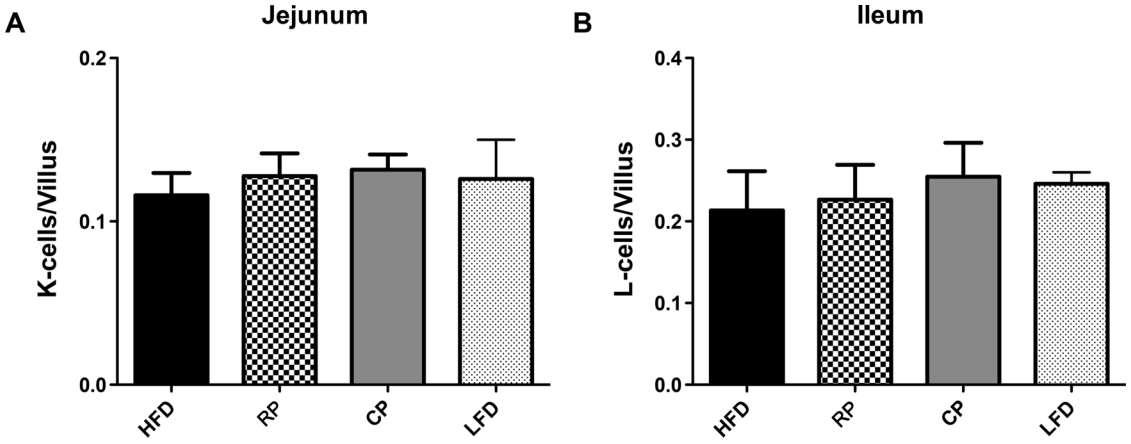
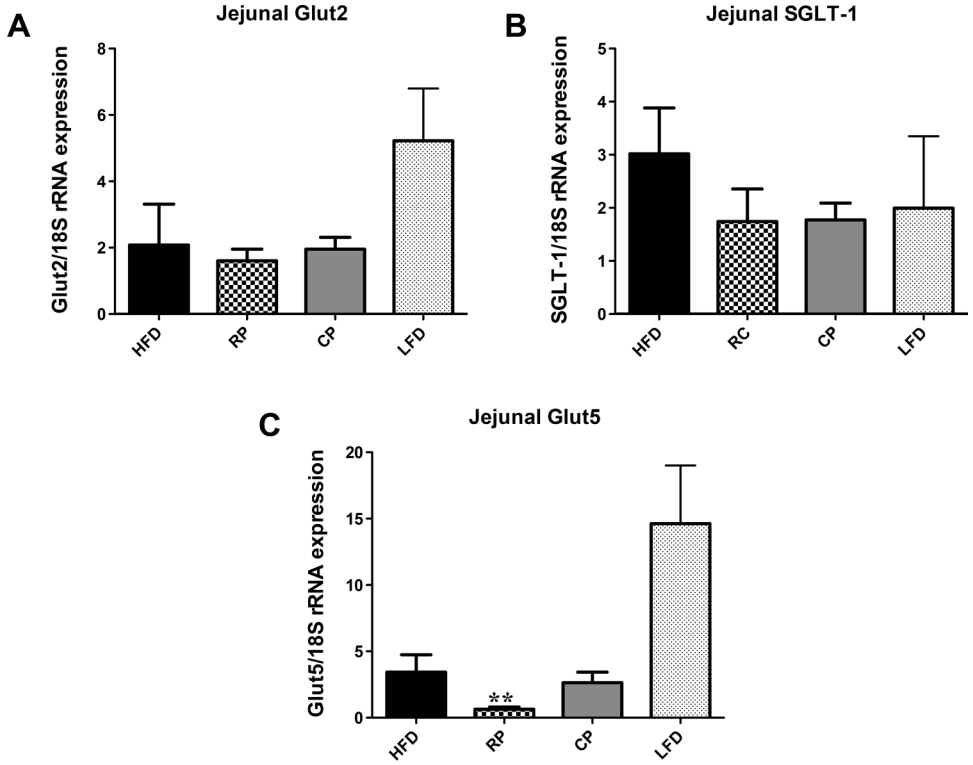


Fig. 6. (A) mRNA expression of *Glut2*, (B) *SGLT1*, and (C) *Glut5* in jejunum of the rats normalized to 18S rRNA expression. Data are means ± SE analyzed by 1-way ANOVA followed by Dunn's post hoc comparison test, $n = 5–12$. Asterisk indicates significant difference compared with LFD (**, $P < 0.01$).



glucose in the muscle through a nitric oxide-dependent pathway, although the concentration of the GLP-1 used was higher than the fasting levels seen in our rats (Chai et al. 2012).

GIP is another incretin that is secreted in response to nutrient ingestion resulting in many similar actions as GLP-1 in the pancreas. GIP secretion is normal or increased in impaired glucose tolerance and T2D, whereas its insulinotropic effect is diminished in T2D (Kim and Egan 2008). Studies of the effects of dietary fibre intake on circulating GIP have produced diverse results (Johansson et al. 2013; Juntunen et al. 2002; Weickert and Pfeiffer 2008). In our study, we observed higher GIP responses in the pea fibre-fed rats before and postglucose ingestion, independent of changes in K-cell number, suggesting increased sensitivity to stimulation. However, because GIP secretion changes were similar in RP and CP, this could not account for the differential effects on oGTT between the groups. Furthermore, improved oGTT could not be accounted for by differences in body weight gain or body fat amongst the groups. In contrast, male Wistar rats, following a high fibre diet (21% *w*/*w*) comprising inulin and oligofructose, had a lower percentage of body fat (Reimer et al. 2012). In our study, we failed to detect a significant change in body composition, which may be related to the short length of our study (10 weeks) versus that of Reimer et al. (2012) (21 weeks).

A novel finding upon dietary intervention with pea seed coat was the significant decrease in α-cell mass after supplementation

with CP, comparable to that seen in the LFD group. While HFD-induced insulin resistance results in expansion in β-cell area (Ahren et al. 2010; De Almeida Costa et al. 2006; Goodlad and Mathers 1992; Hull et al. 2005; Marconi et al. 2000; Pick et al. 1998), the impact on α-cells is unclear. Dysregulated glucagon secretion has been proposed as an early hallmark of T2D (D'Alessio 2011; Liu et al. 2011; Weiss et al. 2011). In diabetic mice increased number of α-cells and α-cell mass was reported as diabetes developed over time (Liu et al. 2011). In our study, increased α-cell area in HFD rats may be due to elevated insulin and glucagon levels in the plasma. At the same time, rats in the CP group had significantly lower plasma insulin concentrations compared with those in the RP and HFD groups, which could explain the smaller α-cell area and lower fasting glucagon in that group. In addition, lower glucagon secretion could result in downregulation of hepatic gluconeogenesis and hence reduced fasting plasma glucose concentration.

These data show that daily intake of cooked pea seed coats improves glucose tolerance and decreases postprandial insulin in glucose intolerant rats. Moreover, feeding cooked pea seed coats decreases fasting glucagon levels likely as a result of smaller α-cell area. The same diet also enhances plasma incretin concentrations. The most likely component of the pea seed coats exerting these effects was the fibre, because the amount of protein contributed to the overall diet was only 0.7% of the diet (*w*/*w*) and this variety of pea did not contain appreciable amounts of tannins (Whitlock et al. 2012). Current Canadian guidelines (Canadian Diabetes Association Clinical Practice Guidelines Expert Committee 2013) recommend that the diabetic population increase its dietary fibre intake to 15–25 g per 1000 kcal. In the present study, rats received 3.3 g of pea fibre for every 140 calories of diet consumed daily, which corresponds to a daily intake of 23 g fibre per 1000 kcal. Given that this amount is still within the range of the current recommendations of the Canadian Diabetes Association for dietary fibre intake in diabetic adults (Canadian Diabetes Association Clinical Practice Guidelines Expert Committee 2013), incorporating a similar amount of pea fibre into diets seems feasible, from a practical perspective, when translating our results to human interventions.

Conflict of interest statement

The authors report no conflicts of interests with the funding agencies.

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Appendix B: Study information sheet and consent

Title: Substantiating a Health Claim for Pulses (Beans and Peas) and Cholesterol - Lowering

Investigators:

Investigator	Position, Dept	Phone Number	
Dr. Rhonda Bell	Professor, AFNS	780-492-7742	Rhonda.bell@ualberta.ca
Dr. Linda McCargar	Professor, AFNS	780-492-4987	Linda.mccargar@ales.ualberta.ca
Dr. Cathy Chan	Professor, AFNS	780-492-9939	cchan@ualberta.ca
Dr. Spencer Proctor	Professor, AFNS	780-492-4672	Spencer.proctor@ales.ualberta.ca
Dr. Jocelyn Ozga	Professor, AFNS	780-492-2653	Jocelyn.ozga@ales.ualberta.ca
Dr. David Wishart	Professor, AFNS	780-492-0383	David.wishart@ualberta.ca
Dr. Peter Senior	Professor, MD	780-407-3636	petersenior@ualberta.ca
Ms. Janis Baarda	Research Assistant, AFNS	780-492-4182	pulseRCT@ualberta.ca

Background

Beans, peas, lentils and chickpeas (also known as "pulses") are healthy foods that contain twice the amount of protein as grains, are very high in fibre, key vitamins and minerals like folate and iron and are low in fat. This study will compare how regularly eating beans or peas may be good for heart health, especially blood lipids such as cholesterol and triglyceride levels.

Also, Canada is among the world's largest producers of pulses and is interested in promoting these items as part of a healthy diet. Currently people in Canada do not eat a lot of pulses, in part, because not many convenient food products contain pulses. Experts agree that we need specific studies, like this one, to compare the effects of different types of pulses such as navy beans, red beans yellow and green peas, on important indicators of health. We hope the results from this study will be used to show parts of the Canadian government the healthy effects of beans and peas. This information could stimulate the industries that grow and process these foods.

What is the study for?

The aim of this study is to look at how regularly eating pulses may improve risk factors for good heart health, especially blood lipids like cholesterol and triglyceride levels. People in this study will be asked to eat beans or peas or rice in amounts recommended in Canada's Food Guide.

What do participants do in this study?

- Eligible participants will be randomly assigned (similar to drawing a name from a hat) to a group and will be asked to consume either:
    - a) foods containing ¾ cup of cooked beans per serving for 5 days per week for six weeks, or
    - b) foods containing ¾ cup of cooked peas per serving for 5 days per week for six weeks, or;
    - c) foods containing ¾ cup of cooked rice per serving for 5 days per week for six weeks.
- The foods provided such as soups, side dishes, and entrees, should be eaten with the foods you normally eat. The study food will replace something else in your meal. You will receive a 3 week supply of the study food items at the baseline visit and again at the week three visit.
- You will be asked to not consume additional foods containing beans, lentils, chickpeas or dried peas, or make diet changes or changes to your physical activity during the study period.
- You will need to come to the University of Alberta Human Nutrition Research unit four times, and have one short telephone conversation with the study coordinator.

    - <u>Screening Visit (approximately 1.5 hours)</u>:
        - Ask you to fill out questionnaires about yourself (e.g age, education, occupation), and about your medical history, any medications you take, and your smoking habits
        - We will give you some questionnaires to take home. You will only be asked to fill them out if you are eligible for the study. We will call you in the next 10 days, after we get the results of your blood work, to confirm whether you should fill these out or not. One questionnaire asks about what you normally eat; it will take you ~1 hour to fill out.
        - One questionnaire is a 3-day food diary. We ask that you fill out everything you eat and drink for 2 weekdays and 1 weekend day on these forms. We will give you some training on how to do this before you leave this screening visit.
        - One asks about how your stomach/intestines feels after eating.
        - Measure your height, weight, waist and hip circumference.
        - If you are in a fasting state (no food or drink, besides water, for 12 hours before appointment) a blood sample will be collected (approximately 2 teaspoons). If you are not in a fasting state, you will be asked to come in for an additional appointment to collect a blood sample. This sample will determine if you are able to participate in the study.

When we know whether or not you are eligible for the study, we will contact you to confirm that you still want to participate, and schedule your next visit to the

study center. You will be reminded to bring your questionnaires you were asked to fill out at home and to fast before your next appointment.

- Study Visit 1, Baseline (~1.5 hours):
  - We will review the questionnaires you completed.
  - We will measure your weight, blood pressure, waist and hip circumference, and collect a urine and fasting blood sample. The urine and blood sample will be used to measure different chemicals in your body such as cholesterol, different hormones, and vitamins.
  - You will be asked to complete questionnaires about any changes in your medical history since you were last here.
  - You will be given enough food items so that you can eat one item every day for 5 days/week for about 3 weeks (you will pick up more meals at the 3 week visit). We will teach you how to prepare the food; usually this will mean defrosting the food item and heating it.
- Throughout the first 3 weeks, you will fill out questionnaires about the meals, your food intake, how your stomach/intestines feel after eating, and your feelings of hunger and fullness.
- Short Telephone Call, Week 2 (~15 min): We will talk to you on the phone about how easy or hard it has been for you to incorporate the study food items into your diet, and to confirm that you will be continuing in the study. This will make sure we can have your food items ready for you when you come to your next study visit. We can answer any questions you have about the study as well, you will be reminded to fast (no food or drink besides water for 12 hours) before the next appointment.
- Study Visit 2, Week 3 (~1 hour): We will measure your weight, blood pressure, waist and hip circumference, and collect urine and fasting blood sample. We will review the questionnaires you completed during the first 3 weeks of the project and will discuss your experiences so far with the meals, how you feel, and any changes you've made to your medications or lifestyle. You will be given the rest of the food items for the study.
- Study Visit 3, Final visit (~1 hour): We will measure your weight, blood pressure, waist and hip circumference and collect a urine and fasting blood sample. We will review the questionnaires you completed during the last 3 weeks of the project and will discuss your experiences with the meals, how you feel, and any changes you've made to your medications or lifestyle. We will ask you to fill out a few final questionnaires and give us any other feedback you wish, on the study.

What Do We Want to See?
We hope to see that intake of beans or peas will improve heart health and lower cholesterol and other blood lipids like triglycerides.

How Do I Benefit?
You may or may not benefit from participating in this study. You may see a lowering of cholesterol and improved heart health through the duration of the study. There is no cost associated with participating.

Are There Any Risks if I Participate?
There is a blood draw at the appointments. This is a routine procedure that will be used for obtaining samples. A needle will be inserted into a vein and blood will be withdrawn into tubes for laboratory tests. It is possible you may experience mild pain, fainting, bleeding, discoloration or bruising, and/or an infection at the place where the needle enters the vein to draw blood. Bruising is very common and usually goes away after a few days. Infection is rare, as is dizziness or fainting during the procedure.

Some participants may experience gastrointestinal changes due to the dietary changes, such as bloating, flatulence (gas), or softer stools. The study doctor, Dr. Peter Senior, will monitor the results of your blood tests while you are participating in the study. If the results of your blood tests show anything out of the ordinary, the blood tests will be repeated (within ~ 5 working days). If the results remain the same, you will be withdrawn from the study and advised to consult your regular doctor.

If you experience any adverse reactions or notice any unusual signs or symptoms, you must contact the study doctor, Co-Investigator Dr. Peter Senior at 780-407-3636, immediately.

Do I have to participate?
No, taking part in this study is your choice. You may end your involvement with the study at any time without affecting your health care. You can withdraw from the study by contacting the Study Coordinator, Ms. Janis Baarda at 780-492-4182.

Will I be paid if I Participate?
You will be reimbursed for your parking or for the cost of your transportation for each study visit, up to a maximum of $10/visit. You will be provided with a maximum of $75 upon completion of the study (or partial payment if you withdraw from the study) to reimburse and thank you for your time. Reimbursement will be according to the number of completed visits as follows: $30 for Baseline Visit, $15 for Week 3 Visit, and $30 for Week 6 Visit.

Will My Records be Kept Private?
The consent forms and any questionnaire information you provide will be kept in locked filing cabinets or scanned and shredded in a locked confidential bin. Your

privacy and your identity will be kept confidential. The study database will be stored on a computer drive protected by a password. All blood and urine samples will be stored in locked freezers in a secure research facility. These samples will be labelled with a study identification number and will not identify you by name or initials. Each participant will be issued a specific study identification code. All information contained in our summaries will be anonymous, and based on group data. Any report published as a result of this study will not identify you by name, address or any other personal information.

During research studies it is important that the data we get is accurate. For this reason your study information, including your name, may be looked at by people from the Health Research Ethics Board or University of Alberta. By signing the consent form you give permission for the collection and use of information for research purposes.

After the study is completed, your study information will be stored at the University of Alberta for 5 years. If you end your involvement during the study, the information that is obtained from you for study purposes up to that point, will not be destroyed unless you specifically request it. **If at any point during the study you decide you do not want researchers to keep your samples and data, you can ask the researchers to destroy it.**

What if I suffer a research-related injury?
If you become ill or injured as a result of being in this study, you will receive necessary medical treatment, at no additional cost to you. By signing this consent form you are not releasing the investigator(s), institution(s) and/or sponsor(s) from their legal and professional responsibilities.

Contact Information
If you have further questions related to this research, please contact: Dr. Rhonda Bell **780-492-7742**

If you have any concerns about any part of the study, please contact Health Research Ethics Board office, University of Alberta 780-492-9724.

UNIVERSITY OF ALBERTA

Consent

Title: Substantiating a Health Claim for Pulses (Beans and Peas) and Cholesterol - Lowering

Investigators:

Investigator	Position, Dept	Phone Number
Dr. Rhonda Bell	Professor, AFNS	780-492-7742
Dr. Linda McCargar	Professor, AFNS	780-492-4987
Dr. Cathy Chan	Professor, AFNS	780-492-9939
Dr. Spencer Proctor	Professor, AFNS	780-492-4672
Dr. Jocelyn Ozga	Professor, AFNS	780-492-2653
Dr. David Wishart	Professor, AFNS	780-492-0383
Dr. Peter Senior	Professor, MD	780-407-3636
Ms. Janis Baarda	Research Assistant, AFNS	780-492-4182

Please circle your answers:

Do you understand that you have been asked to be in a research study?

YES NO

Have you read and received a copy of the attached Information Sheet?

YES NO

Do you understand the benefits and risks involved in taking part in this research study? YES NO

Have you had an opportunity to ask questions discuss this study?

YES NO

Do you understand that you can quit taking part in this study if you notify the researchers?

YES NO

Has confidentiality been explained to you?

YES NO

Do you understand who will be able to see your study information?

YES NO

Do you agree to maintain your medication regime and physical activity, and not eat any additional bean or pea products?

YES

NO

Do you give us permission to use your data for the purpose specified?

YES

NO

I agree to take part in this study.

Name (please print)_____

Signature
Date_____

I believe that the person signing this form understands what is involved in the study and voluntarily agrees to participate.

Signature
Date_____

Appendix C: Inclusion/exclusion criteria (case report form)

⇨ INCLUSION / EXCLUSION CRITERIA - SCREENING VISIT ⇦

CASE REPORT FORM

Substantiating a Health Claim for Pulses (Beans and Peas) and Cholesterol Lowering.

Participant Screening #:	Date of Visit (dd-Mmm-yyyy):

	INCLUSION CRITERIA (all responses must be marked "yes" in order to proceed)	YES	NO
1.	Male or non-pregnant, non-lactating female, 20 to 75 years of age;	❑	❑
2.	LDL Cholesterol ≥3.00 mmol/L and ≤5.00 mmol/L;	❑	❑
3.	Fasting triglycerides <4.00 mmol/;	❑	❑
4.	Stable body weight (± 5 kg) for past 3 months and BMI 20 – 40;	❑	❑
5.	Must be on a stable regime for the past 3 months if taking medications or if taking vitamin and mineral/dietary/herbal supplements;	❑	❑
6.	Able to read, write and communicate orally in English;	❑	❑
7.	Willing to maintain a stable level of physical activity during the study;	❑	❑
8.	Willing not to consume other pulse-containing foods consumed during the participation in the study;	❑	❑
9.	Willing to comply with protocol requirements and procedures;	❑	❑
10.	Willing to provide informed consent.	❑	❑

	EXCLUSION CRITERIA (all responses must be marked "no" in order to proceed)	YES	NO
1.	High pulse consumption (≥2 servings per week);	❑	❑
2.	Individuals who are vegan (consume only plant-based sources of protein);	❑	❑
3.	Use of medications to lower blood lipids or lower blood glucose;	❑	❑
4.	Medical history of liver disease, renal insufficiency, inflammatory bowel disease or other gastrointestinal disorders influencing gastrointestinal mobility or nutrient absorption;	❑	❑
5.	Any acute medical condition or surgical intervention within the past 3 months precluding study participation;	❑	❑
6.	Conditions or medications which are likely to increase the risk to the participants or study personnel, or to reduce the ability of the participant to comply with the protocol, or affect the results;	❑	❑
7.	History of gastrointestinal reactions or allergies to lentils or potato-based foods, or to one or more ingredients in the study foods which significantly limits the number of study foods that can be consumed;	❑	❑
8.	Currently participating in or having participated in a food intervention study within the last 3 months.	❑	❑

⇨ INCLUSION / EXCLUSION CRITERIA - SCREENING VISIT ⇦

CASE REPORT FORM

Substantiating a Health Claim for Pulses (Beans and Peas) and Cholesterol Lowering.

Participant Screening #:	Date of Visit (dd-Mmm-yyyy):

Is the Participant Eligible to Participate in the Study? ❑ **YES** ❑ **NO** (Please add comment)

Comments:

I hereby verify that the above information is accurate:

Rhonda Bell _____ _____

Name of Principal Investigator Signature

Appendix D: Study foods Information

a. Preparation of study beans, peas, and rice

Peas and Beans

Soaking

1. Weigh dried beans and peas according to table below.
2. Wash under cold running water (1 min)
3. Place beans in bowl in cold water equal to 3 times the weight of the beans.
4. Refrigerate for at least 12 hours.

Cooking

1. Drain soaked beans in strainer.
2. Weigh.
3. Wash under cold running water (1 min)
4. Place beans in saucepan in cold water equal to 3 times the weight of the beans.
5. Place saucepan on high heat and bring to boil. Reduce heat to maintain cook water temperature at ~97°C.
6. Cook for time as noted below stirring every 9 min. Drain.

Rice

Cooking

1. Uncle Bens Converted
2. Place 330 g raw rice in cooker.
3. Add 900 g water.
4. Cook until cook light goes out. Check at 25 min and monitor thereafter.
5. Turn off when water just gone.

b. Background ingredients and recipes

Zucchini Casserole

Ingredients	1 portion (g)	4 portions (g)
Canola oil	1	4
Onion, diced	30	120
Garlic, minced in jar	4	16
Mushrooms	25	100
Zucchini	40	160
Tomatoes, canned, diced	60	240
Pasta, small shells	15	60
Thyme	0.12	0.49
Oregano	0.16	0.64
Salt	0.06	0.24
Cumin	0.03	0.12
Water	60	240
Total	**235.37**	**941.49**
Total Weight of ingredients for nutrient values	**175.00**	**700.00**

- Heat oil with onion and garlic on low in saucepan for about 4 minutes or until onion is translucent. Stir occasionally.
- Cut mushrooms into quarters. Cut zucchini lengthwise in quarters then into ½ inch thick slices.
- Add mushrooms and zucchini; cook for 2 minutes or until softened.
- Stir in tomatoes, salt, pasta, thyme, oregano and water.
- Bring to a boil; reduce heat and simmer covered for 10 minutes or until pasta is tender but still firm.
- Ensure that an internal temperature of 65°C is reached.

Vegetable Soup

Ingredients	**1 portion (g)**	**4 portions (g)**
Canola oil	1	4
Onion, diced	50	200
Garlic, minced in jar	3	12
Carrot, sliced	25	100
Corn, frozen	45	180
Chicken OXO powder low sodium	4.5	18
Tomatoes, canned, diced	70	280
Tomato Sauce, low sodium	20	80
Thyme, dried	0.1	0.4
Summer Savory, dried	0.1	0.4
Parsley, dried	0.1	0.4
Black pepper	0.1	0.4
Cumin	0.03	0.12
Water	80	320
Total	**298.93**	**1195.72**
Total Weight of ingredients for nutrient values	**218.50**	**874.00**

- Heat oil with onion and garlic on low in saucepan for about 4 minutes or until onion is translucent. Stir occasionally.
- Stir in carrot and corn and cook another 2-3 minutes.
- Combine chicken powder and water and add to saucepan. Add tomatoes, thyme, savory, parsley, pepper and cumin and bring to a boil.
- Reduce heat to a simmer, uncovered and cook approximately 30 min.
- Ensure that an internal temperature of 65°C is reached.

Chicken Casserole

Ingredients	**1 portion (g)**	**4 portions (g)**
Canola oil	1	4
Onion, diced	30	120
Garlic, minced in jar	3	12
Chicken, thigh, diced	40	160
Green pepper, chopped	30	120
Red pepper, chopped	30	120
Chicken OXO powder low sodium	4.5	18
Water	13	52
Yogurt, plain, low fat	6	24
Mozzarella cheese, part skim, shredded	10	40
Oregano	0.1	0.4
Black pepper	0.03	0.12
Cumin	0.03	0.12
Total	**167.66**	**670.64**
Total Weight of ingredients for nutrient values	**144.50**	**578.00**

- Heat oil with onion and garlic on low in saucepan for about 4 minutes or until onion is translucent. Stir occasionally.
- Add chicken, increase heat to medium and cook until chicken reaches an internal temperature of 75 °C.
- Add green and red pepper and cook until softened. Add chicken powder to water and dry seasonings and mix well.
- Reduce heat and simmer covered for 10-15 min. Add yogurt. Add cheese slowly while stirring.
- Ensure that an internal temperature of 65°C is reached.

Hamburger Soup

Ingredients	**1 portion (g)**	**4 portions (g)**
Onion, diced	20	80
Garlic, minced in jar	3	12
Ground Beef, lean	40	160
Tomatoes, canned, Italian style	40	160
Tomato Sauce, low sodium	10	40
Mixed vegetables, frozen	40	160
Onion Soup, dried	4.5	18
Cumin	0.03	0.12
Water	100	400
Total	**257.53**	**1030.12**
Total Weight of ingredients for nutrient values	**157.50**	**630.00**

- Cook onion and garlic on low in saucepan for about 4 minutes or until onion is translucent. Stir occasionally.
- Add beef, increase heat to medium and cook until beef reaches an internal temperature of 75 °C. Drain juices.
- Add tomatoes, tomato sauce, vegetables, dried onion soup, cumin and water; bring to a boil.
- Reduce heat and simmer covered for 30 min.
- Ensure that an internal temperature of 65°C is reached.

Tortellini Soup

Ingredients	**1 portion (g)**	**4 portions (g)**
Canola oil	1	4
Onion, diced	25	100
Garlic, minced in jar	3	12
Mushroom, diced	20	80
Red Pepper, diced	20	80
Green Pepper, diced	20	80
Zucchini, diced	20	80
Vegetable Powder	4.5	18
Italian seasoning	0.2	0.8
Cumin	0.03	0.13
Water	80	320
Tortellini, cheese	20	80
Total	**213.73**	**854.93**
Total Wt of ingredients for nutrient values	**133.50**	**534.00**

- Heat oil with onion and garlic on low in saucepan for about 4 minutes or until onion is translucent. Stir occasionally.
- Stir in mushrooms, peppers and zucchini and cook another 2-3 minutes until vegetables softened.
- Combine vegetable powder and water and add to saucepan. Add seasoning and bring to a boil.
- Add tortellini. Reduce heat to a simmer, cover, and cook approximately 5 to 7 min.
- Ensure that an internal temperature of 65°C is reached.

c. List of study food ingredients and brand names

- Bay leaves, dried, no name
- Black Pepper
- Canola Oil, No Name
- Carrot, fresh, sliced
- Chicken powder, OXO Knorr, 25% less salt than regular OXO
- Chicken thigh, boneless, skinless, Superstore
- Chili powder, club house
- Corn, niblets, frozen, no name
- Cumin, dried, no name
- Garlic, minced in jar, Derlea Brand Foods Inc
- Green pepper, fresh, chopped
- Ground beef, lean, Superstore
- Italian seasoning, no name
- Mixed vegetables, Green Giant
- Mozzarella, part-skim (18% fat) Best Buy, (Safeway), shredded
- Mushrooms, white fresh
- Onion Soup, dried, lipton
- Onion, fresh large yellow
- Oregano, leaves, dried, Safeway
- Parsley, dried, No Name
- Pasta, Unico, baby shells
- Red pepper, crushed, club house
- Red pepper, sweet, fresh, chopped
- Salt
- Summer Savory, dried, McCormick
- Thyme, dried leaves, no name
- Tomato Sauce, low sodium, hunts
- Tomatoes, canned diced Italian style, PC
- Tomatoes, canned diced, aylmer
- Tortellini, rainbow three cheese, gourmet Safeway select (ricotta, parmesan, romano)
- Vegetable powder, Knorr
- Yogurt, low fat (1.6% fat) plain, Lucerne
- Zucchini, fresh
- Rice, White, Uncle Ben's converted

Appendix E: Three-day food record sample

THREE-DAY
DIETARY INTAKE RECORD

Week to be completed:_____

Study ID:_____

Phone Number: _____

Record Dates: _____ _____ _____ _____ _____ _____
(Day) (Month) (Day) (Month) (Day) (Month)

University of Alberta
Department of Agricultural, Food and Nutritional Science

INSTRUCTIONS FOR RECORDING DAILY FOOD INTAKE

Please record your food intake for **2 week days and one weekend day.**
It is important to record ALL foods and beverages – whether it is a full course meal at home or a quick can of pop at school/work.

The Three-Day Dietary Intake Record has a separate section for every day Each day is broken up into 6 eating times:

1. Morning meal	2. Midmorning snack	3. Midday meal
4. Afternoon snack	5. Evening meal	6. Evening snack

It is a good idea to carry your Dietary Intake Record book with you and record your entries as soon after eating as possible. Please include the following information on your food record:

1. **FOOD AND BEVERAGE ITEMS** Column: Enter all foods and beverages consumed at the meal or snack time. Please record the specific type of food (for example: *WHOLE WHEAT* bread, *FROSTED FLAKES* cereal). In the same column, record all toppings or items added at the time of eating (for example: sugar, syrup, jam, butter, mayonnaise, gravy, milk, salt, etc.). For combination foods, please include detailed information on each item. For example: If you had a tuna sandwich, you would list the following foods and include detailed information for each of them: white bread, mayonnaise, celery, solid white tuna, salt.

2. **DESCRIPTION OF ITEM** Column: For every food or beverage item listed, include the following (if applicable):
   - **Brand**: *MIRACLE WHIP* mayonnaise, *PIZZA HUT DEEP DISH* pizza, *OREO* cookie, *ACTIVITA* yogurt.
   - **Type of flavour**: *BLUEBERRY* muffins, *STRAWBERRY* yogurt
   - **Method of cooking**: *FRIED, BAKED, BBQ'D, HOMEMADE*

   All other relevant information included on food label: *LOW FAT,* ranch fat free salad dressing, *28% M.F. (MILK FAT*) cheddar cheese, *LEAN* Ground Beef. For fruits and vegetables specify the varieties if possible e.g. "Granny smith" apple and other information's such as frozen, canned sweetened/unsweetened, sliced etc.

3. **AMOUNT** Column**:** Specify number and units of measure of food or beverage item and the amount of any topping or items added.. E.g. 2 cups, 1 Teaspoon. Use appropriate units of measures necessary e.g. “cup”, “grams”, “piece”, “ounce”, “number”, size of fruit (small, medium, large), “teaspoon”, or “tablespoon. Use measuring cups and spoons whenever possible.

Homemade foods - attach the recipe to the additional information sheet and mention the portion you had eaten E.g. $1/5^{th}$ of a batch of stew, and how many servings the recipe yields.
Restaurants: Include as much information as possible. Make sure you include the name of the restaurant, name of the dish and the options that you have chosen.
Please attach the food labels of processed foods if possible and do ensure that you have entered “label attached”

Fill in the blanks on the bottom of each record. Please list any vitamin or mineral supplements and/or herbal products taken, including quantities and detailed label information along with the Drug Identification number (DIN). Indicate the time of your meal or snack and where it was eaten (for example: at home, at a restaurant). If you did not eat a meal or snack, please place a check mark (✓) in the space provided on the bottom of the page, so that we do not think you forgot to record it.

In the evening do a check over your day to ensure you haven’t missed anything.
Dietary record should reflect the way you usually eat. Please do not change your normal eating habits for the 3 days you are recording your food intake. Your honesty is crucial to the success of this research study.

Sample Meal

FOOD AND BEVERAGE ITEMS	DESCRIPTION OF ITEM	AMOUNT
Enter all foods and beverages consumed. For combination foods, please include detailed information on each item.	Include a detailed description of each food and drink item consumed including: - Brand name - Flavour - Method of cooking - All other relevant information on food/drink label	Enter number of units and units of measure: for example: cup, grams, ounce, piece, teaspoon, tablespoon
Spaghetti with tomato/meat sauce:		
Pasta	Whole wheat Spaghetti, cooked	2 Cup
Tomato sauce	Hunt's canned sauce, roasted garlic flavour	1 Cup
Meat balls	Made with extra lean ground beef	5 Number (1 oz/ball)
Parmesan cheese, grated	Kraft, 30% Milk Fat (M.F.)	1 Tablespoon
Garlic Bread:		
Italian Bread	Toasted	3 Piece (large slice)
Garlic Butter		3 Teaspoon
Caesar salad:		
Lettuce	Romaine	1 Cup
Croutons	Safeway brand, garlic flavor	2 Tablespoon
Bacon bits	Simulated flavour, No Name Brand	2 Tablespoon
Caesar salad dressing	Kraft, Fat free	2 Tablespoon
Milk	1%	1 Cup
Tiramisu	Sarah Lee	1 Slice
Coffee	Brewed, Black	1 Cup

Vitamin/Mineral Supplements or Herbal Products taken:_____

Fill in blanks: Time of meal/snack: 6:00 pm **Location meal/snack was consumed:** at home

Please CHECK (✓) if you did not eat or drink at this meal or snack time:_____

Sample Meal

FOOD AND BEVERAGE ITEMS	DESCRIPTION OF ITEM	AMOUNT
Enter all foods and beverages consumed. For combination foods, please include detailed information on each item.	Include a detailed description of each food and drink item consumed including: - Brand name - Flavour - Method of cooking - All other relevant information on food/drink label	Enter number of units and units of measure: for example: cup, grams, ounce, piece, teaspoon, tablespoon
Strawberry - Kiwi Juice	Sunrype 100% fruit juice	½ Cup (125 ml)
Bacon	Maple leaf regular	1 piece
Whole wheat bread:	Toasted, homemade (recipe attached)	2 Piece (small slice)
Margarine	Becel, polyunsaturated salt reduced spread	3 teaspoon
Peanut butter	Compliments, 100% natural crunchy	2 teaspoon (10 ml)
Jam	Blueberry haven, blueberry, no sugar	3 teaspoon
Granola bar	Nature valley, sweet and salty, gluten free, Almond (Label attached)	1 Bar
Apple	Granny smith	1 medium size

Vitamin/Mineral Supplements or Herbal Products taken:_____

_____

Fill in blanks: Time of meal/snack: 6:00 pm **Location meal/snack was consumed:** at home

Please CHECK (✓) if you did not eat or drink at this meal or snack time:_____

Day 1-Morning Meal

FOOD AND BEVERAGE ITEMS	DESCRIPTION OF ITEM	AMOUNT
Enter all foods and beverages consumed. For combination foods, please include detailed information on each item.	Include a detailed description of each food and drink item consumed including: - Brand name - Flavour - Method of cooking - All other relevant information on food/drink label	Enter number of units and units of measure: for example: cup, grams, ounce, piece, teaspoon, tablespoon

Vitamin/Mineral Supplements or Herbal Products taken:_____

_____

Fill in blanks: Time of meal/snack:_____ **Location meal/snack was consumed:**_____

Please CHECK (✓) if you did not eat or drink at this meal or snack time:_____

Day 1 - Mid-Morning Snack

FOOD AND BEVERAGE ITEMS	DESCRIPTION OF ITEM	AMOUNT
Enter all foods and beverages consumed. For combination foods, please include detailed information on each item.	Include a detailed description of each food and drink item consumed including: - Brand name - Flavour - Method of cooking - All other relevant information on food/drink label	Enter number of units and units of measure: for example: cup, grams, ounce, piece, teaspoon, tablespoon

Vitamin/Mineral Supplements or Herbal Products taken:_____

_____

Fill in blanks: Time of meal/snack:_____ **Location meal/snack was consumed:**_____

Please CHECK (✓) if you did not eat or drink at this meal or snack time:_____

Day 1 - Midday Meal

FOOD AND BEVERAGE ITEMS	DESCRIPTION OF ITEM	AMOUNT
Enter all foods and beverages consumed. For combination foods, please include detailed information on each item.	Include a detailed description of each food and drink item consumed including: - Brand name - Flavour - Method of cooking - All other relevant information on food/drink label	Enter number of units and units of measure: for example: cup, grams, ounce, piece, teaspoon, tablespoon

Vitamin/Mineral Supplements or Herbal Products taken:_____

_____

Fill in blanks: Time of meal/snack:_____ **Location meal/snack was consumed:**_____

Please CHECK (✓) if you did not eat or drink at this meal or snack time:_____

Day 1 - Afternoon Snack

FOOD AND BEVERAGE ITEMS	DESCRIPTION OF ITEM	AMOUNT
Enter all foods and beverages consumed. For combination foods, please include detailed information on each item.	Include a detailed description of each food and drink item consumed including: - Brand name - Flavour - Method of cooking - All other relevant information on food/drink label	Enter number of units of units and measure: for example: cup, grams, ounce, piece, teaspoon, tablespoon

Vitamin/Mineral Supplements or Herbal Products taken:_____

_____

Fill in blanks: Time of meal/snack:_____ **Location meal/snack was consumed:**_____

Please CHECK (✓) if you did not eat or drink at this meal or snack time:_____

Day 1 - Evening Meal

FOOD AND BEVERAGE ITEMS	DESCRIPTION OF ITEM	AMOUNT
Enter all foods and beverages consumed. For combination foods, please include detailed information on each item.	Include a detailed description of each food and drink item consumed including: - Brand name - Flavour - Method of cooking - All other relevant information on food/drink label	Enter number of units of units and measure: for example: cup, grams, ounce, piece, teaspoon, tablespoon

Vitamin/Mineral Supplements or Herbal Products taken:_____

_____

Fill in blanks: Time of meal/snack:_____ **Location meal/snack was consumed:**_____

Please CHECK (✓) if you did not eat or drink at this meal or snack time:_____

Day 1 - Evening Snack

FOOD AND BEVERAGE ITEMS	DESCRIPTION OF ITEM	AMOUNT
Enter all foods and beverages consumed. For combination foods, please include detailed information on each item.	Include a detailed description of each food and drink item consumed including: - Brand name - Flavour - Method of cooking - All other relevant information on food/drink label	Enter number of units and units of measure: for example: cup, grams, ounce, piece, teaspoon, tablespoon

Vitamin/Mineral Supplements or Herbal Products taken:_____

_____

Fill in blanks: Time of meal/snack:_____ **Location meal/snack was consumed:**_____

Please CHECK (✓) if you did not eat or drink at this meal or snack time:_____

Appendix F: Tracking document

Beans and Peas – Tracking Document Study ID: _____
Weeks 1-3 are shown as an example. Study Week : _____

Week 1:

Instructions – For each study food item you eat, please add a short comment about the food, such as how it tasted, whether you enjoyed it, or any other comments you think are important for us to know. On the days where a study food was not consumed, mark an X over that day of the week. You must consume **5** study foods per week. *Study Coordinator to fill in dates.*

Day 1 DATE	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**
Study Food Name	Study Food Name	Study Food Name	Study Food Name	Study Food Name	Study Food Name	Study Food Name
How much did you eat? (*circle one*) **None** ¼ ½ ¾ **All**	How much did you eat? (*circle one*) **None** ¼ ½ ¾ **All**	How much did you eat? (*circle one*) **None** ¼ ½ ¾ **All**	How much did you eat? (*circle one*) **None** ¼ ½ ¾ **All**	How much did you eat? (*circle one*) **None** ¼ ½ ¾ **All**	How much did you eat? (*circle one*) **None** ¼ ½ ¾ **All**	How much did you eat? (*circle one*) **None** ¼ ½ ¾ **All**
Comments:	Comments:	Comments:	Comments:	Comments:	Comments:	Comments:

Remember! Please eat one serving of each study food item per day for 5 days of the week. The study foods should be eaten as a part of your regular diet (i.e. Replace something in your meal with the study food item). Do not eat more than one study food item per day. Drink fluids when you eat the study foods and consume fluids each day.

This week remember to fill out each of the following questionnaires: □ GI Questionnaire □ Satiety Questionnaire

Week 2:

Instructions – For each study food item you eat, please add a short comment about the food, such as how it tasted, whether you enjoyed it, or any other comments you think are important for us to know. On the days where a study food was not consumed, mark an X over that day of the week. You must consume **5** study foods per week. *Study Coordinator to fill in dates.*

Day 1 DATE	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**
Study Food Name	Study Food Name	Study Food Name	Study Food Name	Study Food Name	Study Food Name	Study Food Name
How much did you eat? (*circle one*) **None** ¼ ½ ¾ **All**	How much did you eat? (*circle one*) **None** ¼ ½ ¾ **All**	How much did you eat? (*circle one*) **None** ¼ ½ ¾ **All**	How much did you eat? (*circle one*) **None** ¼ ½ ¾ **All**	How much did you eat? (*circle one*) **None** ¼ ½ ¾ **All**	How much did you eat? (*circle one*) **None** ¼ ½ ¾ **All**	How much did you eat? (*circle one*) **None** ¼ ½ ¾ **All**
Comments:	Comments:	Comments:	Comments:	Comments:	Comments:	Comments:

Remember! Please eat one serving of each study food item per day for 5 days of the week. The study foods should be eaten as a part of your regular diet (i.e. Replace something in your meal with the study food item). Do not eat more than one study food item per day. Drink fluids when you eat the study foods and consume fluids each day. This week remember to fill out each of the following questionnaires:

□ 3-day food record (remember to track your food for at least one weekend day) □ This week you can expect a call from the study coordinator

Week 3:

Instructions – For each study food item you eat, please add a short comment about the food, such as how it tasted, whether you enjoyed it, or any other comments you think are important for us to know. On the days where a study food was not consumed, mark an X over that day of the week. You must consume **5** study foods per week. *Study Coordinator to fill in dates.*

Day 1 DATE	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**
Study Food Name	Study Food Name	Study Food Name	Study Food Name	Study Food Name	Study Food Name	Study Food Name
How much did you eat? (*circle one*) **None** ¼ ½ ¾ **All**	How much did you eat? (*circle one*) **None** ¼ ½ ¾ **All**	How much did you eat? (*circle one*) **None** ¼ ½ ¾ **All**	How much did you eat? (*circle one*) **None** ¼ ½ ¾ **All**	How much did you eat? (*circle one*) **None** ¼ ½ ¾ **All**	How much did you eat? (*circle one*) **None** ¼ ½ ¾ **All**	How much did you eat? (*circle one*) **None** ¼ ½ ¾ **All**
Comments:	Comments:	Comments:	Comments:	Comments:	Comments:	Comments:

Remember! Please eat one serving of each study food item per day for 5 days of the week. The study foods should be eaten as a part of your regular diet (i.e. Replace something in your meal with the study food item). Do not eat more than one study food item per day. Drink fluids when you eat the study foods and consume fluids each day.

This week remember to fill out each of the following questionnaires: □ GI Questionnaire □ Satiety Questionnaire

For your next study visit on _____ at _____, please remember to fast (i.e. no food or beverage intake for 12 hours), and bring your study binder with completed questionnaires. Remember, it is okay to drink water during a fast and may actually make the blood draw easier if you are hydrated!