THE CONSUMPTION OF SORGHUM PRODUCTS AND PHLORETIN; IMPLICATIONS FOR HEALTH AND METABOLIC SYNDROME

A Thesis submitted by

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Abstract

Metabolic syndrome is a precursor to non-communicable diseases including type 2 diabetes and cardiovascular disease. Diet and lifestyle choices are major contributors to the development and progression of metabolic syndrome. Adding functional foods and functional compounds into the diet of those at risk of developing metabolic syndrome will improve metabolic status and, in the long term, minimise the incidence of the disease. This research aims to identify and quantify the impacts that sorghum and phloretin have on diet-induced metabolic syndrome.

Methods: A 16 week rat model of diet-induced metabolic syndrome was used to determine responses to 5% whole red sorghum, 20% red sorghum flour, 20% black sorghum flour, 20% wet cake sorghum, or phloretin at 50mg/kg/day or 200mg/kg/day. Cardiovascular, physiological, and metabolic variables were measured. Studies were conducted to determine whether there were changes in intestinal permeability caused by alterations in colonic tight junction proteins which would lead to reduced serum LPS concentrations and TLR4-mediated inflammation which is known to contribute to metabolic syndrome.

Results: Phloretin, a glucose transporter 2 inhibitor, normalised post-prandial blood glucose responses when consumed at 200mg/kg/day showing potential for the pre-diabetes aspect of metabolic syndrome. On the contrary this dose increased total plasma cholesterol which is considered to be a risk factor for increased cardiovascular events in metabolic syndrome.

Whole sorghum reduced total cholesterol concentrations in a rat model of diet-induced metabolic syndrome, showing potential for reducing risk of cardiovascular events. Black and red sorghum flour showed remarkably few changes in a high fat-high carbohydrate diet, and the implications for metabolic syndrome are low. Improvements were observed in the liver and gastrointestinal tract, with reduced steatosis and a modest reduction in small intestinal permeability.

Wet cake sorghum, the waste product of ethanol fermentation, showed the greatest changes of all the sorghum products with normalisation of post-prandial glucose, improved liver morphology, reduction in left ventricular collagen, and a modest

reduction in small intestinal permeability. A number of changes were seen when adding sorghum into the control diet which did not develop metabolic syndrome. The outstanding change was a distinct increase in lean mass.

There was little evidence to suggest that changes in the gastrointestinal tract were major contributors to improving metabolic syndrome through reducing metabolic endotoxaemia.

Conclusions: Overall both phloretin and sorghum products show benefits to their consumption for attenuation of metabolic syndrome, which can be used as leverage for further clinical studies and for promotion of sorghum as a health food.

Certification of Thesis

This Thesis is entirely the work of Anna Balzer except where otherwise acknowledged. The work is original and has not previously been submitted for any other award, except where acknowledged.

Principal Supervisor: Lindsay Brown

Associate Supervisor: Sunil Panchal

Associate Supervisor: Mark Lynch

Student and supervisors signatures of endorsement are held at the University.

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I would like to make special mention of the animals that sacrificed their lives for the completion of this degree. My stance and opinions on animal research have changed drastically during this time and while animal research has its place, I believe nutrition research with whole foods is better restricted to human trials.

I'd like to dedicate this thesis to my Mum and Dad. Thankyou sincerely for your support throughout my entire education from day one of my life. For the emotional support, tech support, financial support, any support imaginable. I hope one day I can repay you for even a small part of what you have done for me. I owe every success in my life to you both. And the same goes for my siblings Jess, Tim, Nick and Jake for putting up with my stressed state and having a beer, coffee or laugh with me every time I needed it, and not just throughout this PhD.

Eat sorghum. Eat well. Exercise. Your future is in your hands, make it a long and healthy one. The greatest wealth is health, invest in sorghum.

Associated Publications

Carnahan S, Balzer A, Panchal S, & Brown L (2014) Prebiotics in obesity. Panminerva Med 56(2):165-175.

Table of Contents

Abstract ii

Certification of Thesis iv

Acknowledgements....................v

Associated Publications vii

Table of Contents.................... viii

List of Figures xiii

List of Tables.................... xviii

Chapter 1 - Introduction....................1

Chapter 1.1 – Sorghum Literature Review- An ancient grain with modern potential..1

1.1.1 Sorghum in traditional diets....................1

1.1.2 Sorghum significance....................2

1.1.3 Sorghum varieties and types....................4

1.1.4 Sorghum proteins....................5

1.1.4.1 Protein digestibility 7

1.1.4.2 Prolamins - kafirin....................8

1.1.5 Sorghum carbohydrates and fibres....................10

1.1.5.1 Starches – amylose, amylopectin, resistant and digestible starches, and glycaemic properties 10

1.1.5.2 Fibre - Non-starch polysaccharides, soluble fibre, insoluble fibre 15

1.1.6 Lipids & lipid soluble sterols & alcohols....................17

1.1.6.1 Phytosterols....................18

1.1.6.2 Policosanols 18

1.1.6.3 Tocochromanols....................19

1.1.6.4 Fatty acids....................20

1.1.7 Other bioactive compounds....................20

1.1.7.1 Phenolics....................20

1.1.7.1.2.2 Flavanones....25
1.1.7.2 Other compounds – stilbenoids, anthraquinones, terpenoids, alkaloids.30
1.1.8 Potential for metabolic syndrome and health....30
1.1.8.1 Metabolic syndrome....30
1.1.8.2 Anti-dyslipidaemic effects....31
1.1.8.3 Anti-diabetic effects....33
1.1.8.4 Effect on central adiposity and obesity....35
1.1.8.5 Effects on cardiovascular disease and hypertension....35
1.1.8.6 Gastrointestinal health....37
1.1.9 Sorghum integration into diets....39
1.1.10 Cautions....39
1.1.11 Processing, growth, and breeding....40
Chapter 1.2 – Prebiotics in Obesity....42
1.3 Phloretin Literature Review....53
Chapter 2 – Materials and Methods....61
2.1 Ethics....61
2.2 Rat Diet and Experimental Structure....61
2.3 Oral Glucose Tolerance....62
2.4 Body Composition (Dual Energy X-Ray Absorptiometry)....63
2.5 Systolic Blood Pressure....63
2.6 Termination Process....63
2.6.1 Euthanasia....63
2.6.2 Serum collection....64
2.6.3 Plasma Collection....64
2.6.4 Left ventricular diastolic stiffness....64
2.6.5 Vascular reactivity....66
2.6.6 Ileum and colon reactivity....67
2.6.7 Organ weights (normalised to tibial length) and tissue collection....68
2.7 Plasma Biochemistry....68
2.7.1 Plasma ALT and AST activity....68
2.8 Plasma Lipid Profile....68

2.9 Histology analyses 69
2.9.1 Tissue Collection....................69
2.9.2 Manual Tissue Processing 69
2.9.3 Automated Tissue Processing....................69
2.9.4 Tissue Sectioning 70
2.9.5 Staining....................70
2.9.6 Microscopy and histological analysis....................71
2.10 Endotoxin analysis 72
2.11 Intestinal permeability....................72
2.11.1 Urine collection....................72
2.11.2 Urine sample preparation and analysis....................73
2.11.3 Urine chromatography and analysis....................73
2.12 Semi-quantitative ELISA (enzyme linked immunosorbent assay)....................75
2.12.1 Protein Extraction....................75
2.12.2 Protein Quantification....................75
2.12.3 GLUT2 and TLR4 determination....................75
2.12.4 Occludin and claudin-1 determination 77
2.13 Sorghum extract analysis....................78
2.13.1 Sorghum phenolic extraction 78
2.13.2 Sorghum phenolic analysis 78
Chapter 3 – 5% Wholegrain sorghum in a rat model of diet-induced metabolic syndrome....................79
3.1 Introduction 79
3.1.1 Hypothesis 81
3.2 Materials and Methods....................81
3.2.1 Ethics....................81
3.2.2 Rat Diet and Experimental Structure....................82
3.2.3 Statistics....................83
3.3 Results....................84
3.3.1 Sorghum extraction 84
3.3.2 Physiological and metabolic parameters 85
3.3.3 Blood lipids, and glucose control....................87

3.3.4 Cardiovascular and hepatic parameters 90
3.3.5 Gastrointestinal health 91
3.4 Discussion 96
3.5 Conclusion 106
Chapter 4 - 20% Red and black sorghum flour in a rat model of metabolic syndrome 107
4.1 Introduction 107
4.2 Materials & Methods 110
4.3 Results 112
4.3.1 Black and red sorghum flour composition 112
4.3.2 Physiology and metabolism indicators 115
4.3.3 Blood glucose regulation, dyslipidaemia and lipopolysaccharides 118
4.3.4 Liver and cardiovascular structure and function 123
4.3.5 Gastrointestinal function 131
4.4 Discussion 137
4.5 Conclusion 147
Chapter 5 – 20% wet cake sorghum in a dietary model of metabolic syndrome 148
5.1 Introduction 148
5.2 Materials and Methods 149
5.3 Results 152
5.3.1 Phytochemical composition 152
5.3.2 Feed intake, energy intake and body composition 154
5.3.3 Glucose control and blood lipid profile 158
5.3.4 Cardiovascular and liver structure and function 160
5.3.5 Gastrointestinal function 165
5.4 Discussion 169
5.5 Other considerations and limitations 177
5.6 Conclusions 177
Chapter 6 – Phloretin in a rat model of metabolic syndrome 179
6.1 Introduction 179
6.2 Materials & Methods 180

6.2.1 Phloretin analysis 180
6.2.2 Rats and diets 181
6.3 Results 182
6.3.1 Physiological parameters 182
6.3.2 Plasma lipids and glucose control 186
6.3.3 Cardiovascular and hepatic structure and function 188
6.3.4 Gastrointestinal parameters 194
6.4 Discussion 195
6.5 Conclusions 201
Chapter 7 – Conclusions 202
7.1 Conclusions and future directions of sorghum in diets 202
7.1.1 Summary of key findings and their implications 202
7.1.2 Limitations and considerations for future research 203
7.1.3 Key recommendations for future studies 206
7.2 Conclusions and future directions for phloretin in diets 207
7.2.1 Summary of key findings and their implications 207
7.3 Post script 208
References 210

List of Figures

Figure 1.2.1:Wild type sorghum originating in Africa and modern domesticated sorghum grown on the Darling Downs. 1

Figure 1.2.2: Long-term sorghum predicted average yields over a 115 year period in Queensland and northern New South Wales........3

Figure 1.2.3: A selection of the sorghum grain colours that can be produced........5

Figure 1.2.4 : Stylised indication of the structure of sorghum protein bodies........9

Figure 1.2.5: Predicted structure of α-prolamin structures indicating many α-helices 9

Figure 1.2.6: Structure of amylose and amylopectin........11

Figure 1.2.7 : Scanning electron micrograph of sorghum starch granules throughout the digestion process........14

Figure 1.2.8: An example of the anticipated structure of sorghum non-starch polysaccharides........17

Figure 1.2.9: Structures of sorghum sterols. 18

Figure 1.2.10: Structure of tocopherols & tocotrienols........19

Figure 1.2.11: Basic structures of phenolic acids and flavonoids 20

Figure 1.2.12: Structure of flavonoids........23

Figure 1.2.13: Catechin and epicatechin 24

Figure 1.2.14: Structures of naringenin and eriodictyol. 25

Figure 1.2.15: Structures of apigenin and luteolin. 26

Figure 1.2.16: Structure of 3-deoxyanthocyanidins. 28

Figure 1.2.17: Development process of hypertension in metabolic syndrome........36

Figure 1.3.18: Structure of phloretin and phlorizin........53

Figure 1.3.19: Function of SGLT1 in glucose transport........54

Figure 1.3.20: The role of GLUT2 is gastrointestinal glucose uptake as a mobile element........54

Figure 2.21: Experimental diet groups. 62

Figure 2.22: Isolated heart perfusion schematic. 65

Figure 2.23: Schematic of organ baths used for assessment of aortic vascular reactivity...67

Figure 2.24: Urine sample example chromatogram. 74

Figure 3.25:210nm UV detection of HPLC of acidified methanol extracts of whole red sorghum. 84

Figure 3.26: 330nm UV detection of HPLC separated acidified methanol extracts of whole red sorghum. 85

Figure 3.27: 510nm UV detection of HPLC extracts of whole red sorghum 85

Figure 3.28:Pancreatic islet alpha and beta cell density, cell number per islet, and islet area. 88

Figure 3.29: Example of haematoxylin and eosin-positive inflammatory cells and fat vacuoles...91

Figure 3.30: TLR4 expression in the liver determined by semi-quantitative ELISA.91

Figure 3.31: GLUT2 expression in the liver determined by semi-quantitative ELISA. 92

Figure 3.32: Diastolic stiffness constant (κ, unitless), and picrosirius red area %, ventricular sections of CS, HCHF, CSb and HSb. 92

Figure 3.33: Left ventricular sections of CS, HCHF, CSb and HSb stained with picrosirius red. 93

Figure 3.34: Thoracic aortic contractions in response to noradrenaline, sodium nitroprusside and acetylcholine 91

Figure 3.35: ex-vivo proximal colon contractility in response to acetylcholine stimulation and ex-vivo ileal contractility in response to acetylcholine stimulation .92

Figure 3.36: Percent recovery of sugars in urine over time. 93

Figure 3.37: Ratio of lactulose and mannitol recovery..93

Figure 3.38: Occludin expression in the colon determined by semi-quantitative ELISA 94

Figure 3.39: Claudin-1 expression in the colon determined by semi-quantitative ELISA 94

Figure 3.40: TLR4 expression in the colon determined by semi-quantitative ELISA. 94

Figure 4.41: Sorghum's potential for improving metabolic syndrome. 110

Figure 4.42: 210nm UV detection of HPLC of acidified methanol extracts of red sorghum flour and black sorghum flour..113

Figure 4.43: 510nm UV detection of HPLC extracts of red sorghum flour and black sorghum flour 113

Figure 4.44: 330nm UV detection of HPLC separated acidified methanol extracts of red sorghum flour and black sorghum flour..114

Figure 4.45: Body weight changes over 16 weeks of the dietary protocol..............115

Figure 4.46: Lean:fat mass ratio at 16 weeks and skeletal muscle myofibril width 118

Figure 4.47: Changes in glucose TAUC (area under curve), and fasting glucose blood concentrations between the 8-week test, and 16 week tests...................................119

Figure 4.48: Pancreatic islet cell area, cells per islet and cell density.....................120

Figure 4.49: Serum LPS concentrations 122

Figure 4.50: Hematoxylin and eosin staining of liver sections (10µm) under 20x magnification...124

Table 4.19: Cardiovascular and liver structure and function parameters of rats fed mCS, mCRed, mCBlack, mHCHF, mHRed, and mHBlack diets...........................126

Figure 4.51: GLUT2 expression in liver determined by semi-quantitative ELISA .127

Figure 4.52: Liver TLR4 expression determined by semi-quantitative ELISA.......127

Figure 4.53: Picrosirius red staining indicating the % type I and type III collagen deposition in the left ventricle 128

Figure 4. 54: Picrosirius red stained left ventricle sections, 20x magnification. 129

Figure 4.55: Thoracic aortic reactivity of isolated vessel in response to noradrenaline, sodium nitroprusside or acetylcholine 130

Figure 4.56: Force of contraction of isolated ileum sections in response to acetylcholine exposure (a) total area under curve (b) and minimum concentration required for contraction (c). 131

Figure 4.57: Dose-response curves of force of contraction of isolated proximal colon sections in response to acetylcholine exposure 132

Figure 4.58: Colon TLR4 expression as determined by semi-quantitative ELISA133

Figure 4.59: Claudin expression as determined by semi-quantitative ELISA 133

Figure 4.60: Colon occludin expression 134

Figure 4.61: Lactulose:mannitol ratio of percent recovery 135

Figure 4.62: Cellular location of tight-junction proteins 145

Table 5.21: Macronutrient profiles of diets, per 100g. 150

Figure 5.63: 210nm UV detection of HPLC of acidified methanol extract of WS and local whole red sorghum 152

Figure 5.64: 510nm UV detection of acidified methanol extracts separated by HPLC of WS. 153

Figure 5.65: 330nm UV detection of HPLC separated acidified methanol extracts of WS 153

Figure 5.66: Weekly bodyweight gain. 154

Figure 5.67: Change in feed intake and energy intake. 156

Figure 5.68: Feed efficiency 0-8 weeks (hollow bars) and 8-16 weeks (solid colour). 157

Figure 5.69: Protein intake weeks 8-16 157

Table 5.23: Blood lipids and glucose tolerance. 159

Figure 5.70: Pancreatic islet density. 160

Figure 5.71: Serum lipopolysaccharide concentrations determined by horseradish peroxidase activity. 160

Figure 5.72: Hematoxylin and eosin stain of liver section at magnification 20x 161

Figure 5.73: Liver TLR4 and GLUT expression determined by semi-quantitative ELISA. 163

Figure 5.74: Ex vivo thoracic aortic reactivity in response to acetylcholine, sodium nitroprusside and noradrenaline. 164

Figure 5.75: Percent type I and III collagen stained by picrosirius red...................165

Figure 5.76: Reactivity of isolated ileum and proximal colon sections to acetylcholine...166

Figure 5.77: Colon occludin, claudin-1 and TLR4 expression 166

Figure 5.78: Total mannitol recovery and lactulose/mannitol recovery ratio over a 20 hour period. 169

Figure 6.79: Mass spectrum of phloretin. 181

Figure 6.80: Weekly body weight. 183

Figure 6.81: Hematoxylin & eosin stain of pancreatic islets at 10x magnification. 188

Figure 6.82: Picrosirius red staining indicating deposition of collagen type I and III fibres in the left ventricle. 191

Figure 6.83: Thoracic aortic responses to acetylcholine, sodium nitroprusside and noradrenaline of control and phloretin-treated rats. 192

Figure 6.84; Hematoxylin and eosin stains of liver indicating ballooning and fat deposition, at 20x magnification. 193

Figure 6.85: Liver GLUT2 expression (left) and liver TLR4 expression (right). 193

Figure 6.86: Colon (top) and ileum (bottom) reactivity to acetylcholine................194

Figure 6.87: Liver TLR4 expression determined by semi-quantitative ELISA. 195

Figure 6.88: Claudin-1 expression in the colon (left) and occludin (right) expression in the colon determined by semi-quantitative ELISA. 195

List of Tables

Table 1.2.1: Amino acid composition of white sorghum (Liberty) and red sorghum (Buster), and predicted amino acid requirements for adults 6

Table 1.2.2: Comparison of sorghum, brown rice, maize and wheat amino acid composition in proteins. 7

Table 1.2.3: Total phenolic content of sorghum types, and FRAP (Ferric reducing ability of plasma) value. 21

Table 1.2.8: Phenolic acids content of various sorghum products. 22

Table 1.2.4: Indicative flavanol/proanthocyanidin concentrations in sorghum and sorghum fractions. 25

Table 1.2.5: Concentration ranges of naringenin and eriodictyol in sorghum...........26

Table 1.2.6: Indicative content of the flavones luteolin and apigenin in sorghum. Values show combined concentrations of apigenin and luteolin. 27

Table 1.2.7: Concentrations of 3-deoxyanthocyanidins in different sorghum grain types. 29

Table 2.9: Retention times of lactulose, sucrose, D-mannitol, and sucralose............74

Table 2.10: Linearity and % relative standard deviation of a serial dilution of protein, assessed for GLUT2 and TLR4 protein expression..76

Table 2.11: Linearity and % relative standard deviation (precision) of a serial dilution of protein, assessed for occludin and claudin-1 protein expression. 78

Table 3.12: Physiological and metabolic variables of CS, CSb, HCHF and HSb fed rats...86

Table 3.13: Blood glucose and lipid profiles with sorghum supplementation in a HCHF and CS diet. 89

Table 3.14: Hepatic and cardiovascular parameters of CS, CSb, HCHF and HSb fed rats at 16 weeks of protocol...94

Table 3.15: Pearson's correlation P-values indicating no significant correlation between urinary sugar recovery and expression of proteins in the colon. 95

Table 4.16: Macronutrient profiles of red and black sorghum flour used in the dietary protocols. 114

Table 4.17: Physiological parameters of rats fed mCS, mCRed, mCBlack, mHCHF, mHRed, and mHBlack diets. 116

Table 4.18: Blood glucose and plasma lipids of rats fed mCS, mCRed, mCBlack, mHCHF, mHRed, and mHBlack diets. 121

Table 4.20: Percent recovery of orally gavaged sugars from urine over a 20-hour period. 136

Table 5.22: Body composition of rats fed on mCS, mCWS, mHCHF, and mHWS protocols. 155

Table 5.24: Cardiovascular and liver structure and function parameters of rats fed on mCS, mCWS, mHCHF, and mHWS protocols. 162

Table 5.25: Total percent recovery of sugars. 168

Table 6.26: Phloretin daily intake of rats and equivalent human doses 183

Table 6.27: Physiological and metabolic parameters of CS, CPH50, CPh200, HCHF, HPh50, and HPh200 fed rats. 185

Table 6.28: Blood glucose control and plasma lipids of CS, CPH50, CPh200, HCHF, HPh50, and HPh200 fed rats. 187

Table 6.29: Cardiovascular and hepatic structure and function of CS, CPH50, CPh200, HCHF, HPh50, and HPh200 fed rats. 190

Chapter 1 - Introduction

Chapter 1.1 – Sorghum Literature Review - An ancient grain with modern potential

1.1.1 Sorghum in traditional diets

Sorghum is a grass of the *Poaceae* genus, producing round, edible grains with between 12000 and 16000 seeds per pound (1). Modern cultivated sorghum compared with its traditional, undomesticated counterpart is shown in Figure 1.2.1.

Figure 1.2.1:Wild type sorghum originating in Africa (left) (2), and modern domesticated sorghum grown on the Darling Downs (Photo courtesy of Patricia Balzer).

Sorghum is not considered one of the major cereal grains for human consumption, outcompeted by wheat, maize, oats and rice. Only approximately 50% of the 60MMT (million metric tonnes) of the sorghum produced worldwide is used for human consumption, while around 70% of the 650MMT of wheat is consumed by humans (3). Despite this, sorghum remains a staple food for 500 million people in 30 countries (4), partly due to its tolerance to environmental conditions and the diverse ways it can be prepared as food.

Prior to agricultural domestication, sorghum is believed to have been consumed by humans as early as 8000BC in its wild form, with domestication following somewhere between 4000BC and 3000BC (5). The original domestication of sorghum occurred in Africa, which then spread throughout the world, and became part of the diets of India

and Asia (6). Traditional dishes made from sorghum include fermented beverages such as Mahewu (7), fermented porridges in South Africa (8), ground sorghum boiled porridges in Sudan (9), baking of Dduk cake in Korea (10) and boiled sorghum ‘on the cob’ in Botswana (11).

Sorghum, on the scale of breeding, was described as having more underdeveloped genetic potential than any other major food crop in the world (4). This suggests that the current genetic status of sorghum will allow breeding for specific human consumption. Sorghum’s potential as a nutraceutical or health food is still in its infancy.

1.1.2 Sorghum significance

Almost all sorghum grown in Australia is produced in the Northern Grains Region of Queensland and New South Wales, with less than 1% estimated to be produced in other states (12). Sorghum is one of the major crops of Queensland, with value of agricultural production being an average of $340 million per year over a 5-year period to 2017. This was the second highest value cereal crop following wheat ($401 million) for the same period (13). Sorghum represents a high value commodity crop in the region and is important to the local economy, with 2200 producers in Queensland and 3200 Australia-wide as of 2010-2011, as reported in the GRDC (Grain Research & Development Corporation) Australian Grains Focus 2010-2012 (14). Sorghum and wheat are both volatile in price due to overseas demand, production and local weather patterns. Production in the Queensland and northern New South Wales regions is shown in Figure 1.2.2 (15).

Sorghum is primarily used in Australia for livestock feed, rather than human consumption. According to calculations based on FAO (Food and Agriculture Organization) statistics, domestic sorghum usage in Australia (including both imports and exports) has stabilised since 1994 with over 96% used as animal feed (16). Grain sorghum markets also exist for biofuel production. Any grains containing starches can be used for ethanol production, and sorghum is ideal for this purpose as it has one of the highest starch contents of grains (17). Ethanol from grain sorghum is produced locally by Dalby Bio-Refinery Limited (18), although current capacity is low (19).

The difficulties for sorghum to break into the human food consumption market in Australia come from traditional or cultural attitudes, in addition to market competition against stock feed and biofuel dominance. The conventional use of sorghum exclusively as stock feed means that promoting it for human consumption is likely to be difficult, especially when competing against more traditional cereals such as wheat. Traditionally, sorghum has been used by some communities as a staple cereal for human consumption, primarily Africa and India (20). The predominant regions for human consumption of sorghum are still Asia and Africa at around 97%, however Central and South America account for a small proportion (21).

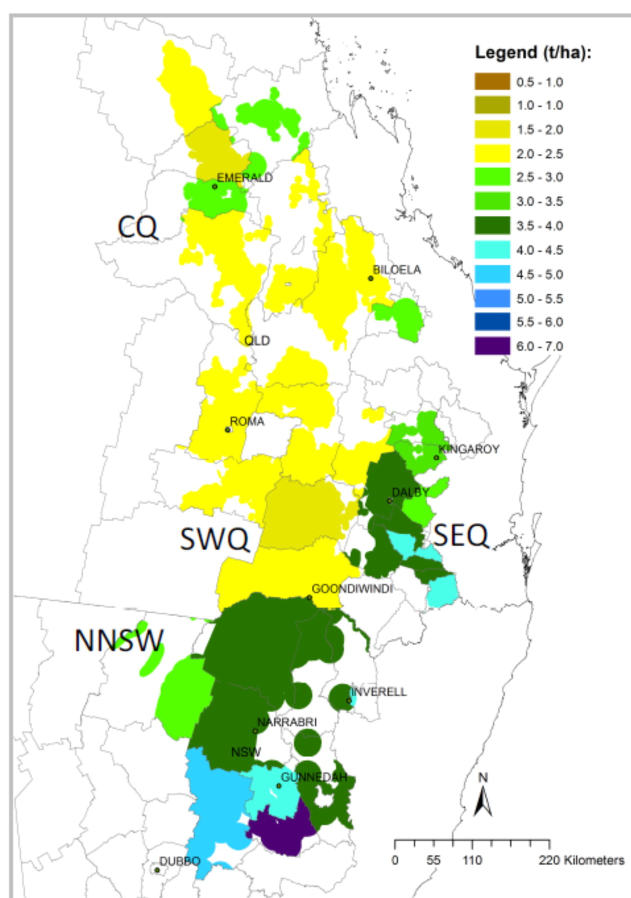


Figure 1.2.2: Long-term sorghum predicted average yields over a 115 year period in Queensland and northern New South Wales (15)

1.1.3 Sorghum varieties and types

Sorghum is considered to have an untapped genetic potential by many plant breeders and researchers (4). There is still significant variation in the types of sorghum that fall under the *Sorghum biocolor* species that are of commercial interest, all of which have their origins in Africa. Sorghum varieties which are commercially grown are typically classed by the colour of grain that they produce, although nomenclature is not standardised. The typical classes of grain colour are red, bronze or tan, and white. Although not commercially grown in Australia, black sorghum is also gaining traction as a niche crop overseas (22). Despite the "colour classes", there are at least 10 genes which dictate the colour of the pericarp and glume (23). Further genes alter the appearance including pericarp thickness (8-160μm) and the presence or absence of testa (24). Due to the large number of possible combinations, there is an almost continuous range of colours (25).

Other type-classes of sorghum are waxy and non-waxy sorghum which are defined by the presence or absence of amylopectin in the endosperm, respectively (26). The waxy trait in sorghum is controlled by a single gene where two recessive alleles will result in a "waxy" phenotype (26). High tannin sorghum is also controlled by one known gene, *Tan1* (27). High levels of tannins are considered to be detrimental in most breeding cases, as high tannin content reduces protein digestibility, a detriment for stockfeed production (27). Reduced starch digestion due to tannins also occurs through interaction with amylase (28). However, tannins have been implicated as beneficial for human health (29). There are a number of *Sorghum* species that are native to Australia (30) and will act as a source of breeding material, or as a potential health-food source.

For the purposes of this research, I have focussed on red and black sorghum as they have been used in the researched literature. White sorghum is also mentioned in this review as it is the type commonly used in the production of sorghum-based gluten-free flours, although not studied in the ensuing research.

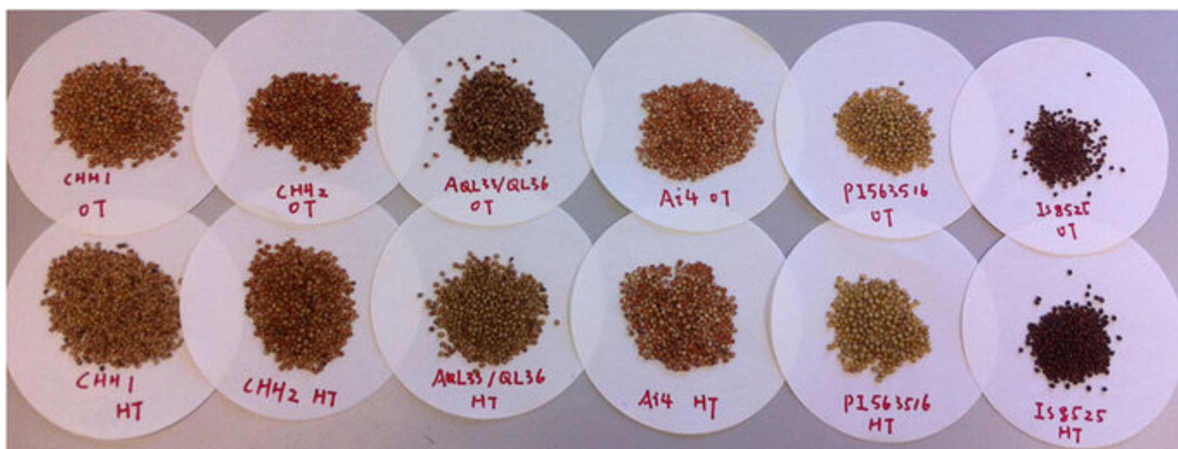


Figure 1.2.3: A selection of the sorghum grain colours that can be produced (31).

1.1.4 Sorghum proteins

Sorghum is comparable in protein content to most other common cereal crops. White sorghum flour has a protein content of approximately 12.5% (32), with the sorghum wholegrain of around 12.1% protein (33). For comparison, corn, wheat and rice contain approximately 9-12%, 8-15% and 7-9% protein, respectively (34). Variation in sorghum proteins are significant being from 10% up to 15% (35), and 8.6% up to above 18% (36) depending on the variety and growing conditions. The storage protein fraction of grains are prolamins, called kafirins in sorghum. These account for up to 50% of the total protein (37). Characteristically, prolamins are high in proline and glutamine, with low solubility (38).

Other proteins include albumins, globulins and glutelins (36, 37). The non-kafirin proteins participate in formation of the endosperm matrix which contains starch, protein bodies, and functional proteins (39). Little research has been done on the potential health effects of kafirins, especially outside feedstock models however recent research is emerging (40-43). Sorghum is gluten-free, in that it does not contain the proteins glutenin or gliadin (44), which are unique glutelins, meaning that sorghum is suitable for patients with gluten intolerance or coeliac disease (45).

The amino acid profile of sorghum varies depending on the cultivar and growth conditions, particularly those which change the protein content. As shown in Table 1.2.1, sorghum is generally high in both leucine and glutamate (36, 46, 47). This

content is higher than barley, wheat and rice which contain below 9g/kg leucine (48). Sorghum is considered as deficient in lysine, hence other dietary sources are required (49).

Table 1.2.1: Amino acid composition of white sorghum (Liberty) and red sorghum (Buster)(47), and predicted amino acid requirements for adults (50),(51). Table adapted from Liu et al (47) and FAO (52).

Amino Acid	White g/kg	Red g/kg	White g/kg protein	Red g/kg protein	mg/kg requirement (Adult, per day)
Arginine	2.5	3.2	28.7	27.1	-
Histidine*	1.7	2.5	19.5	21.2	8-12
Isoleucine*	3	4.1	34.5	34.7	10
Leucine*	10	14.6	114.9	123.7	12
Lysine*	1.7	2	19.5	16.9	12
Methionine*	0.9	1.1	10.3	9.3	13 (combined with cysteine)
Phenylalanine*	4	5.7	46	48.3	14 (combined with tyrosine)
Thrreonine*	2.4	3.2	27.6	27.1	7
Valine*	3.8	5.3	43.7	44.9	10
Alanine	6.3	9.2	72.4	78	-
Aspartate	4.8	6.7	55.2	56.8	-
Glutamine	15.7	23.4	180.5	198.3	-
Glycine	2.1	2.7	24.1	22.9	-
Proline	6.2	8.6	71.3	72.9	-
Serine	3.4	4.7	39.1	39.8	-
Tyrosine	1	1.4	11.5	11.9	
Tryptophan*	-	-	-	-	3.5

When compared with brown rice, wheat and maize, sorghum shows the highest content of leucine, phenylalanine and glutamic acid (Table 1.2.2). Both leucine and phenylalanine are essential amino acids.

Table 1.2.2: Comparison of sorghum, brown rice, maize and wheat amino acid composition in proteins. Essential amino acids are indicated by as asterisk (53).

	Brown Rice	Wheat	Maize	Sorghum
	% in protein	% in protein	% in protein	% in protein
Tryptophan*	1.08	1.24	0.61	1.12
Threonine*	3.92	2.88	3.98	3.58
Isoleucine*	4.69	4.34	4.62	5.44
Leucine*	8.61	6.71	12.96	16.06
Lysine*	3.95	2.82	2.88	2.72
Methionine*	1.8	1.29	1.86	1.73
Cysteine	1.36	2.19	1.3	1.66
Phenylalanine*	5.03	4.94	4.54	4.97
Tyrosine	4.57	3.74	6.11	2.75
Valine*	6.99	4.63	5.1	5.71
Arginine	5.76	4.79	3.52	3.79
Histidine*	1.68	2.04	2.06	1.92
Alanine	3.56	3.5	9.95	
Aspartic Acid	4.72	5.46	12.42	
Glutamic Acid	13.69	31.25	17.65	21.92
Glycine	6.84	6.11	3.39	
Proline	4.84	10.44	8.35	
Serine	5.08	4.61	5.65	5.05

1.1.4.1 Protein digestibility

Protein digestibility of sorghum has been extensively studied using both *in vivo* and *ex vivo* models. Typically, the digestibility of proteins indicates how quickly and to what extent a protein will undergo proteolysis in the gastrointestinal system, and therefore bioavailability of amino acids (54). Sorghum has a low digestibility compared to other cereal proteins (55) (54). Estimated digestibility ranges from 75% (56) to as low as 59% (55); however this changes with variety and preparation (56). In comparison, wheat, maize and rice have digestibility of over 85%, 85% and 83%, respectively (55). One of the causes of poor digestibility is the high degree of disulphide linkages in kafirin proteins (57). Some non-kafirin proteins of sorghum are indigestible, or have low digestibility by pepsin (35, 57). Research in animals models can only be considered indicative of human digestibility, as rats are more efficient at digesting proteins than humans (58).

Methods to improve protein digestibility have been widely studied for sorghum use in stock-feeds. The process of fermentation improved the digestibility (32) and reduced the concentrations of phytates and tannins which may reduce digestibility (56). The protein digestibility was reduced with cooking (59-62) making sorghum unique when compared to other cereals (54) which do not have large decreases in digestibility with cooking such as maize (48, 62), rice (62, 63), legumes (64), barley and wheat (62). This is an important consideration when determining whether humans are likely to consume sorghum, and how processing or the preparation will affect protein digestibility and availability of other nutrients.

The low digestibility may be benefical as there will be gastrointestinal effects of undigested proteins, and interactions with gut microflora. Although literature is currently scarce, some animal models have been studied. Pre-digesting samples with α-amylases increased digestibility of protein by improving enzymatic access which is more akin to an *in vivo* system (61). The interactions with starch granules can affect protein digestion; however, these interactions have not been fully elucidated (35). Decorticating to remove the bran layer (pericarp and germ layer) leaving only endosperm increases the percentage of digestible protein (61).

1.1.4.2 Prolamins - kafirin

Prolamin size is one reason sorghum protein has a such a low digestibility when compared to other cereal crops (61). Kafirin is the major prolamin in sorghum, and has 4 subgroups defined as α- γ- β- and δ-kafirin (59). Kafirin accounts for a high proportion of the sorghum protein, so it is important to note the features of each group and how this affects their biological activity. The structures of kafirins are tightly connected to their role in the sorghum grain. The highly cross-linked β- and γ- kafirins encase the α-kafirin inside the protein body (65). α-Kafirin is the predominant kafirin and typically makes up the majority of kafirin protein bodies (59). Prolamin structure is indicated in Figure 1.2.4.

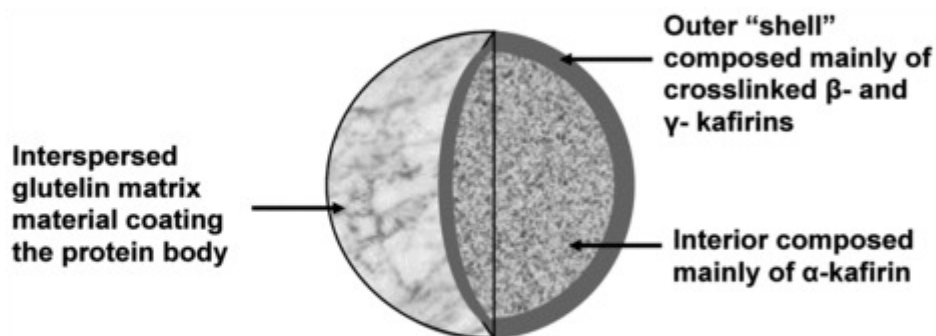


Figure 1.2.4 : Stylised indication of the structure of sorghum protein bodies (66)

Cross-linking is important in kafirins, as it relates to their digestibility (59). The degree of cross-linking varies for each kafirin; with the relative proportions of uncross-linked:cross-linked protein following the general trend α- > β- > γ-kafirin (35, 59).

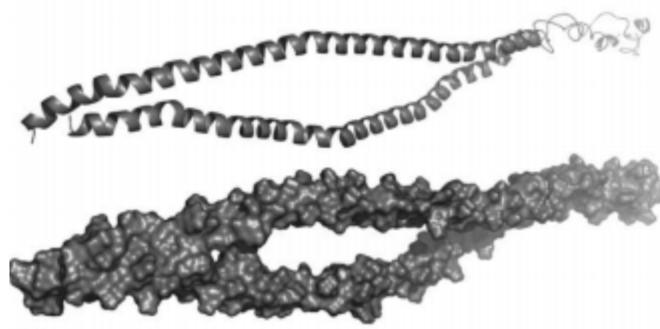


Figure 1.2.5: Predicted structure of α-prolamin structures indicating many α-helices (67, 68).

The highly cross-linked β- and γ-kafirins encase the less cross-linked α-kafirins. α-Kafirin is comprised primarily of alpha sheets, shown in Figure 1.2.5, and is typically the most digestible isolated kafirin (59). Due to its positioning in the protein body, α-kafirin digestion is impaired by the β- and γ-kafirin coating. The surface proteins are the physically restricting factor for access to the chemically least resistant protein (59). β-Kafirin is high in methionine and cysteine, being 9.3 and 5.8% molar percentages of the molecule (69). This allows for moderate disulphide crosslinking and low solubility which impair digestion. The structure of the protein is a combination of α-helices and β-sheets. γ-Kafirin is primarily a mix of random coil structures and many β-sheets, and is high in cysteine which contributes to its high level of disulphide crosslinking (70).

As with other kafirins, solubility is low which impairs digestion. The sizes of the kafirins also varies, with α-, β-, γ-, and δ- having molecular weights of 26000-27000Da (Daltons), 18745Da, 20278Da and 12961Da respectively (68).

Kafirin digestion is further complicated by the presence of phenolics in the sorghum. Condensed tannins and tannic acid, which are present in certain non-white varieties, are able to bind to kafirins *in vitro* (71). This decreased digestibility, primarily through binding to granule surface γ-kafirins (72), and subsequently decreased digestion of the encased α-kafirin.

As sorghum has a unique protein profile so peptidomics and proteomics may become of increasing interest, although little literature is currently available. In addition to kafirin proteins, sorghum also contains globulins, albumins and glutelins; these concentrations can vary but are consistently lower than the kafirins (73, 74). Little research exists on these proteins in their isolated forms.

The interest in gluten-free flours has been spurred by the rise in diagnosis of coeliac disease, the somewhat questionable evidence for "gluten sensitivity" in the non-coeliac population, and the scapegoating of gluten as a driver in a number of metabolic conditions (75). Sorghum is considered a gluten-free grain. Gluten is comprised of two major prolamin proteins, glutenin and gliadin, neither of which exist in sorghum (76).

1.1.5 Sorghum carbohydrates and fibres

1.1.5.1 Starches – amylose, amylopectin, resistant and digestible starches, and glycaemic properties

Starches in cereal grains are comprised of amylose and amylopectin, in differing ratios. Both are comprised of glucose units. Amylose is glucose units with α 1-4 glycosidic linkages in a helical structure. Amylopectin is glucose units which contain α 1-4 glycosidic linkages, however has branching units from α 1-6 glycosidic linkages. Resistant starches have some chemical or physical property that results in resistance to this digestion. They are partially digested by the human gastrointestinal system, however final digestion occurs in the colon by microbial populations.

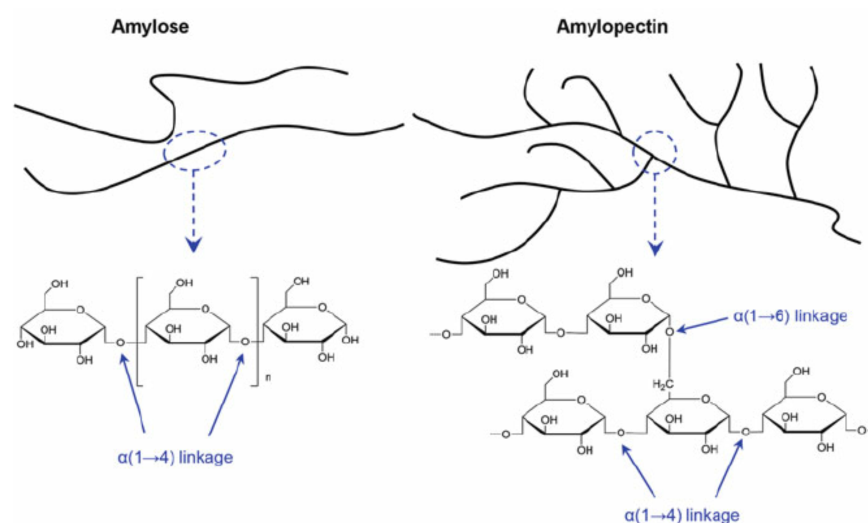


Figure 1.2.6: Structure of amylose and amylopectin (77).

The waxy and non-waxy sorghums are two distinct classes of sorghum that differ considerably in starch composition. Waxy sorghum endosperm contains only amylopectin, whereas non-waxy sorghums contain a mixture of amylose and amylopectin (78). High amylose starches typically have a lower hydrolysis rate, and higher resistance to small intestinal digestion (79); this is important in human gastrointestinal function. Amylose content of non-waxy sorghum is usually around 20% depending on the variety (80), with ranges between 18-30% (81). Amylose content typically shows an inverse relationship with digestibility (82).

Amylose and amylopectin are chemically digestible by the human body by both salivary and pancreatic amylases. However, the physical structure of amylose can lead to a lack of digestion in the upper gastrointestinal tract which results in undigested amylose entering the colon where bacterial fermentation can occur (83). Sorghum is quite high in slowly digestible starch, at around 60-70% for waxy, heterowaxy and non-waxy sorghum. The percentage of resistant starch, however, differs greatly. Heterowaxy sorghum has 23.7% resistant starch on a dry weight basis, normal sorghum has 17.9%, and waxy sorghum less than 10% (78). Estimates in raw sorghum have been up to 43.7% of total starch in some studies (84). Among cereals, sorghum has a relatively high resistant starch as a proportion of its total starch, with estimates

of above 40%, although this can be markedly decreased during preparation of foodstuffs (84).

Digestibility is also affected by both branching and chain length of amylopectin (85). In sorghum the degree of polymerisation is variable with genotype and conditions. Polymerisation degree is heavily in favour of the regions 6-15, and 16-36 glucose units per branch, comprising approximately 90% of the amylopectin chains (78). Digestion by α-amylase increases with degree of polymerisation of up to a certain point, approximately 10 units, however decreases once this is surpassed (85).

Resistant starches are important in colonic health and might influence metabolic syndrome and obesity. Resistant starch (RS) is divided into four classes RS1, RS2, RS3 and RS4. RS1 is physically inaccessible starch, which may be caused by inhibition to digestion by a non-digestible matrix (86). RS2 is found in raw starch granules and resistance is caused by the tightly packed nature of the amylopectin and amylose chains in the granules (86). RS3 is typically formed after cooking and dehydration to produce a crystalline amylose structure (87) and RS4 is chemically modified. In the case of sorghum, RS1 and RS2 are the most important for unprocessed grains. Typically, wholegrains will have higher concentrations of RS1 than flours, due to the intact nature of the starch granules (88). Sorghum also has a large starch granule size compared to other ceral grains (89). Both the amylose content and the starch granule size contribute to the resistance of starch polysaccharides to digestion (90, 91). A large portion of sorghum resistant starch is likely be RS1 as the starch granules are typically encased by a matrix of proteins with low digestibility. Milling and reduction of starch particle size in sorghum will improve digestion (92), such as is done in flour production which is important for the digestibility of RS2. Resistant starch type 3 may be relevant for cooking and preparation purposes. It has been shown that cooking of sorghum increases the amount of resistant starch (93). Resistant starch increased with soaking while resistant starch decreased with autoclaving (94) which is akin to cooking. Raw non-waxy sorghum contained around 6g resistant starch per 100g wholegrain on a dry weight basis (94). Soaking allows permeation of water into starch granules to induce swelling, then subsequent re-association of starch into a crystalline form after dehydration, which is then resistant to enzymatic digestion (95). Cooking

disrupts native, crystalline starch structure which increases susceptibility to digestion (95).

As with all cereals, methods of preparation affect the resistance of amylose and hence the level of resistant starch. Sorghum is unique in its starch granule structure which makes it of particular interest to resistant starch. In sorghum, proteins including albumins, globulins and glutelins attach a matrix of kafirins to the outside of starch granules (96). This protein matrix results in reduced digestibility of the granules (96). Processing will inevitably lead to alterations in the structure of these granules and must be considered when using sorghum as a functional food, with focus on starch and digestion. The effect of digestion on starch granules is shown in Figure 1.2.7. Even within the sorghum grain itself, there is differing structure and digestibility of different endosperm fragments. The whole food matrix is an important consideration when determining its functional properties.

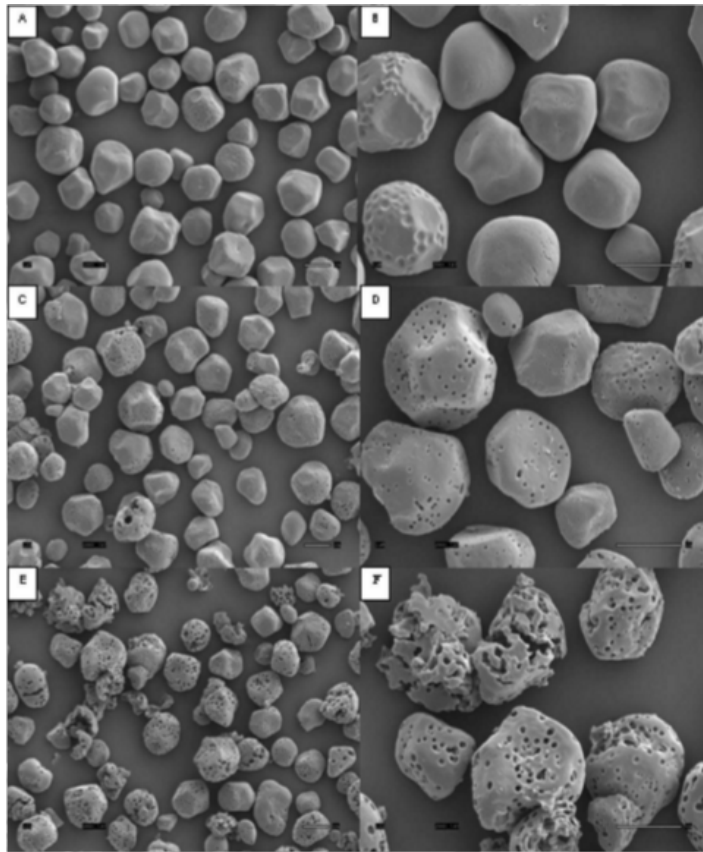


Figure 1.2.7 : Scanning electron micrograph of sorghum starch granules throughout the digestion process, A) undigested x 1000, B) undigested x 2500, C) 30 minute digestion x 1000, D) 30 minute digestion x 2500, E) 60 minute digestion x 1000, and F) 60 minute digestion x 2500. Bar equals 1000μm (97).

The carbohydrate and fibre composition of cereals is important in relation to the glycaemic index (GI) and glycaemic load (GL), in addition to their rate of hydrolysis by digestive enzymes. The glycaemic index is proportional to the concentration of rapidly available glucose (98). Glycaemic load is a representation of the effect of consumption determined by multiplying the glycaemic index by the quantity of carbohydrate in a meal (99). Non-waxy varieties of sorghum, having high concentrations of slowly digestible starch, are likely to fall into low glycaemic category. Interactions with tannins and phenolics of sorghum inhibit starch digestibility and can reduce the glycaemic index of starches (100). Additionally, the presence of phytic acids correlated negatively with starch digestibility and glycaemic index (101), with sorghum having concentrations of phytic acid above 300mg/kg (56). Similarly, sorghum has α-amylase inhibitory properties (56). This has been shown in other trials to be a plausible mechanism for the reduction of glycaemic index and

slowing digestion (102). Addition of brown sorghum bran to a food matrix reduced rapidly digestible starch content and increased the proportion of slowly digestible starch (103).

Preparation affects the glycaemic values; for example, sorghum bread has a glycaemic load of approximately 10 (104). When compared with wheat counterparts, sorghum showed a lower glycaemic load when prepared as coarse or fine semolina, flakes or pasta (105). The glycaemic load of sorghum was also lower in a biscuit preparation, however higher when prepared as roti (105). The low glycaemic load and index of sorghum can be attributed to its slow digestibility.

Colonic digestion has not been fully characterised and may add another level of complexity to the process of digestion of sorghum, and therefore its role in metabolic processes in the body. Interactions of sorghum components with starches from other ingested foods will be altered in the presence of tannins and proteins (103). These concentrations are important, as the sorghum grain is predominantly carbohydrates. The majority of metabolisable energy will be derived from carbohydrates when integrated into the diet. Some have noted that it is the interaction of the other components such as proteins, fats and phenols which may lead to the health benefits of sorghum, rather than the carbohydrate components (106).

1.1.5.2 Fibre - Non-starch polysaccharides, soluble fibre, insoluble fibre

Polysaccharides that contain sugars other than glucose are considered as non-starch polysaccharides. They can be comprised of all other sugar monosaccharides, in differing sequences, with differing glycosidic linkages, with or without branching and are typically not digested fully by human enzymes (107). Soluble and insoluble fibre are both non-starch polysaccharides. Insoluble fibre includes cellulose, hemicellulose, chitin, lignin and xanthan. Soluble fibre includes arabinoxylans, fructans, pectins, polyuronides, alginates, raffinose, xylose and polydextrose.

In sorghums, there can be important variations in the concentrations of carbohydrates, both digestible and indigestible. This can be due to varietal differences or environmental conditions. Different gentoypes of sorghum cultivated under the same

conditions show arabinoxylan content from 0.95-1.35%, with environmental conditions responsible for between 5-14% of this variation, and genotype 69-74% (108). Similarly, genetics and environment affect total starch content (108), amylose content (109) and fibre content (110). Most of the fibre is concentrated in the bran fraction of the grain, so removal of this layer in processing will remove a major proportion of these compounds. In sorghum brans, the apparent range is 20-40% hemicellulose, 10-20% cellulose and <10% lignin on a dry basis (111). In sorghum, the predominant form of fibre is insoluble as opposed to soluble at a ratio of around 90:10 (112).

Non-starch polysaccharides are not able to be cleaved by human enzymes. The enzymes required to break down the sugar linkages are lacking in the human digestive tract although these polysaccharides are comprised of monosaccharides that are a normal part of the human diet including glucose, fructose, arabinose, xylose, mannose, rhamnose and galactose (113). The most typical way for these non-starch polysaccharides to be assessed is by their differing sugar content, with the ability to characterise sequential linkages and structural characteristics having taken major steps forward in recent years. The primary sugars in these fractions are xylose, arabinose and glucose with much lower percentages of the other sugars (113, 114). In sorghum, the composition can vary depending on the method of extraction and processing. However, in all cases with sorghum arabinoxylans, the ratio of arabinose to xylose is generally high (113, 115). These can also contain glucose (116). Mannose and galactose are uncommon (117, 118). In total, sorghum contains 2.6-6.5% pentosans or non-starch polysaccharides on a dry weight basis. Arabinoxylans from sorghum have a xylan backbone with β 1-4 linkages, as shown in Figure 1.2.8, with substitutions to the xylan backbone at the O-3 position (113, 114).

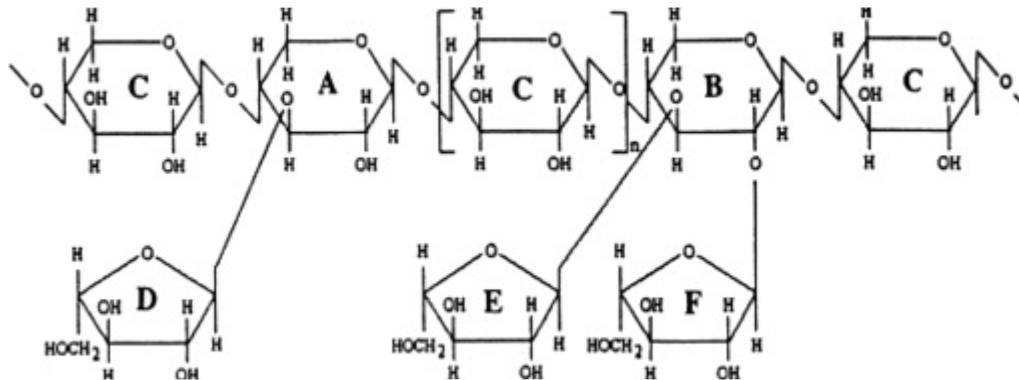


Figure 1.2.8: An example of the anticipated structure of sorghum non-starch polysaccharides (113).

Sorghum contains β-glucans but the concentration of 0.12g/100g dry weight (94) is much lower compared with 4.0% in wholegrain oat flour on a dry weight basis (14).

Structural characteristics are important with relation to fermentation. Ethanol extractable arabinoxylans from sorghum have rapid faecal fermentation when compared with endoxylanase-hydrolyzates of corn, wheat arabinoxylan, and corn arabinoxylans (119). As with most arabinoxylans, there is preferential production of acetate, over propionate, and even more so butyrate (119). There is a suggestion that slow-fermenting arabinoxylans have a higher degree of terminal xylose units (119), such as is found in sorghum at around 70% compared with wheat at around 56% (117).

Glucuronoarabinoxylans from sorghum varieties are not necessarily fermented by the same species as arabinoxylans from other cereals (120), an indication that structural differences may produce different biological effects.

1.1.6 Lipids & lipid soluble sterols & alcohols

Although grain sorghum is low in fat, the composition of the lipid-soluble fraction may still have relevance to health. The fat content of wholegrain sorghum was around 3% (121), and approximately 10% (122, 123) to 14% in sorghum dry distillers grain (124). Sorghum lipids are comprised of triacylglycerides, diglycerides, policosanols, sterols, free fatty acids, tocopherols and fatty aldehydes (125, 126).

1.1.6.1 Phytosterols

Phytosterols are present in sorghum wholegrains, where they can account for around 50mg/100g grain weight, however these values can increase approximately four-fold after distillation (123). This is similar to the median value associated with cereal flours, grains and germs (127). Total sterols as a percent of dry distiller grain lipid extract have been calculated at 9.9mg/g (126). These may prove to be an economical source of these compounds. In sorghum, the specific sterols found are campesterol, stigmasterol and sitosterol (123, 126), with structures indicated in Figure 1.2.9. Genotype and growing conditions alter the composition of the phytosterols in sorghum (128). Sigmasterol and sitosterol are found in quantities typically around twice that of campesterol (126). Although sorghum contains these compounds, it contains 72-93% less than maize (129).

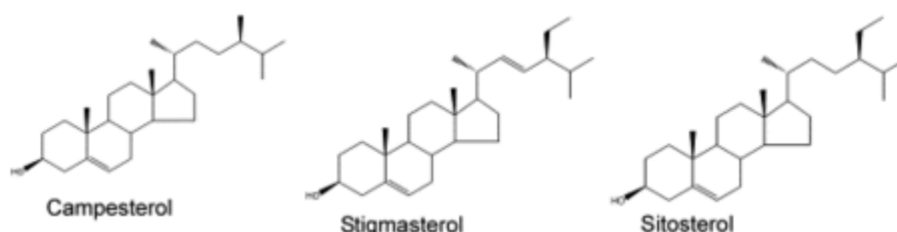


Figure 1.2.9: Structures of sorghum sterols (130).

1.1.6.2 Policosanols

Policosanols are long-chain alcohols found in many plants. Policosanols differ based on their chain length which may affect their biological activity. Policosanols are particularly high in sorghum dry distillers grain when compared with maize dry distillers grain and wholegrains, with up to 100mg/g of dry material (123). The ratio of chain lengths shifts during the fermentation process to produce DDGS (dry distillers grain with solubles) (123, 131). In dry distillers grain, 3.9% of the lipid fraction may be policosanols (126). In sorghum, the chain lengths of policosanols are predominantly 28:0 and 30:0, which are present at over 40% each (131).

1.1.6.3 Tocochromanols

Tocochromanols encompass tocopherol and tocotrienol compounds, or "Vitamin E" (132). Tocopherols are a group of compounds found in many plants with a basic structure as shown in Figure 1.2.10. The α-, δ-, β-, and γ- tocopherols differ in their methylation patterns at positions R1 and R2.

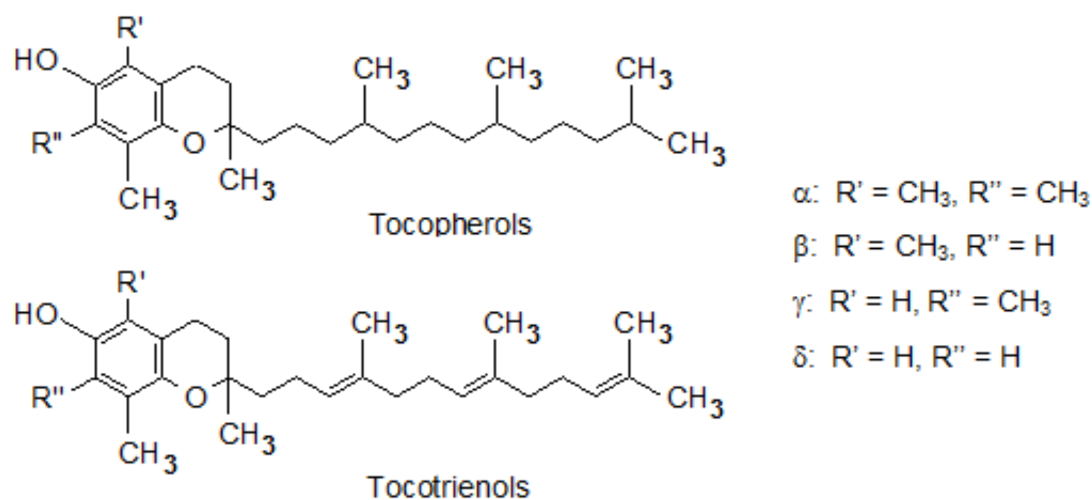


Figure 1.2.10: Structure of tocopherols & tocotrienols (133)

Some tocopherols are in quite high abundance in the lipid fraction of red and white sorghums. γ-Tocopherol, δ-tocopherol, α-tocopherol and β-tocopherol are present in sorghum but concentrations vary (122, 128). These tocopherols can be present at concentrations up to several hundred μg/g dry distillers grain weight (122, 128) and 476.5-2515.5 μg/100g of grain (134). Growing conditions alter the composition, based on geography, however distinct effects of climate and location have not been defined (128).

Tocotrienols resemble the structure of tocopherol but the hydrophobic tail is unsaturated at three positions (Figure 1.2.10). Raw, red sorghum had an α-tocotrienol content of 241.7 μg/100g and δ-tocotrienol content of 37.6μg/100g (135). The concentration of tocotrienol is affected by processing (135), and genotype (132). Ranges for different genotypes were 37.2 μg/100g to 160.9 μg/100g α-tocotrienol, undetectable to 135.3 μg/100g β-tocotrienol, 4.2 to 32.8 μg/100g γ-tocotrienol and undetectable to 43.2 μg/100g δ-tocotrienol (132).

1.1.6.4 Fatty acids

Fatty acid composition of the lipid fraction from sorghum differs between white and red varieties. In both varieties, the percentage of unsaturated fatty acids is around 80-90% (122). The predominant fatty acids are C18:1 and C18:2 unsaturated fatty acids, and C16:0 saturated fatty acid (122, 126, 136). Total saturated fatty acids vary between 12.82-22.46%, polyunsaturated fatty acids between 27.9-53.7% and monounsaturated fatty acids between 31.63-49.52% of total fatty acids (137). A high proportion of linoleic acid (C18:2) is present, with one study suggesting up to 42-56% of the oil fraction (138).

1.1.7 Other bioactive compounds

1.1.7.1 Phenolics

Phenolic compounds are any compounds which contain a phenol ring, either single or multiple. These have varying constitutive moieties at different positions on the phenolic rings, as indicated in Figure 1.2.11. Many metabolites fall into this class of compound. These are mostly secondary plant metabolites and many also elicit a biological response (139). Plant polyphenols are chemically classed into subgroups of flavonoids, phenolic acids, esters, stilbenes and tannins. Interest in these compounds has been due to the large variety and noted antioxidant, antimicrobial and anti-inflammatory properties (140-142).

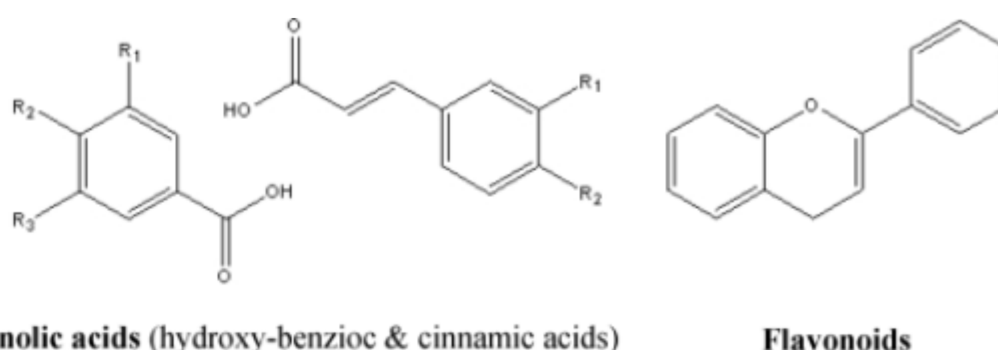


Figure 1.2.11: Basic structures of phenolic acids and flavonoids (143)

Sorghum is high in total phenolics compared to many other cereal grains. Reported values vary markedly depending on the extraction and analysis methods, as shown in

Table 1.2.3: Total phenolic content of sorghum types, and FRAP (ferric reducing ability of plasma) value, an indicator of "antioxidant" capacity (144).

Extract	Phenolic value (mg/g)	FRAP value (mmol/100 g)
Sorghum brans		
Sumac Sorghum bran	62.4 ± 0.9	28.4 ± 0.1
Shanqui Sorghum bran	47.7 ± 0.7	15.3 ± 0.1
Black Sorghum bran	22.7 ± 0.5	7.9 ± 0.2
Mycogen Sorghum bran	5.6 ± 0.1	2.2 ± 0.1
Fontanelle Sorghum bran	2.5 ± 0.1	1.0 ± 0.1
White Sorghum bran	3.9 ± 0.1	1.2 ± 0.1
Non-Sorghum brans		
Wheat bran	2.0 ± 0.1	0.7 ± 0.1
Oat bran	0.6 ± 0.1	0.4 ± 0.1
Rice bran	6.0 ± 0.3	5.3 ± 0.6

Data represent mean ± SEM of triplicate determinations.

1.1.7.1.1 Phenolic acids and phenolic esters

Phenolic acids present in red sorghum are ferulic (145, 146), vanillic (145), *p*-coumaric (145, 146), caffeic (146, 147), protocatechuic (146) and 4-hydroxybenzoic (146) acids (Table 1.2.4). Phenolic acids are descrbed as being present either as their "free" or "bound" forms (145). Bound phenolic acids may be bound to cell walls and non-starch polysaccharides. Although bound to these large compounds, they may still elicit a biological effect, particularly if released on ingestion by hydrolysis. Their quantification and detection is improving with better methods of release and assessment. In sorghum, the concentration of free and bound acids, and the phenolic acid composition can also vary with genotype, environment and processing (145, 148). They also vary depending on the specific phenolic acid, and how they respond to processing. For example, vanillic acid exists almost exclusively in the unbound form, whereas caffeic acid exists almost exclusively in the bound form (145). Phenolic acids tend to be in lower concentrations than many of the flavonoids.

Table 1.2.4: Phenolic acids content of various sorghum products.

Reference	Phenolic acids detected	Free (μg/g)	Bound (μg/g)	Total (μg/g)
Hahn, et al. [115]	Gallic, protocatechuic, ρ-hydroxybenzoate, vanillic, caffeic, p-coumaric, ferulic, cinnamic	54.1-230.4	276.7-598.3	
Chiremba, et al. [116]	caffeic, ρ-coumaric, ferulic, sinapic			2711-4395
Chiremba, et al. [112]	ρ-coumaric, ferulic			107-383 (flour)
Salazar-Lopez, et al. (149)	Caffeic, ρ-coumaric, ferulic, sinapic	784.3	2107.9	

The major phenolic ester associated with sorghum is coumaroyl-glycerol. The newly identified phenolic esters of caffeoyl-glycerol (147, 150), dicaffeoyl-glycerol (147, 150), coumaryl-caffeoyl glycerol (147, 150), ferruloyl-caffeoyl (147, 150) and coumaroyl-feruloylglycerol (150) are present in some red and white sorghum varieties. These phenolic esters are much lower in black sorghum (147) varieties however present at varying concentrations in red and white sorghums. As more powerful chemical analysis techniques become available, the identification of the more complex and lower concentration compounds is improving the knowledge of the cereal's chemistry.

The phenolic acid aldehyde, p-hydroxybenzoic acid aldehyde, present in sorghum is now joined by a list of others including protocatechuic acid aldehyde (150). Whether these exist in high enough concentrations to elicit a biological effect is still yet to be studied. However, in extracts, there is an indication that they may have involvement in apoptosis (147).

1.1.7.1.2 Flavonoid-based polyphenols

Sorghum is very rich in bioactive phytochemicals, particularly the coloured bran layer that gives the characteristic colours of red, brown and black sorghums (151). White sorghum typically has lower concentrations of polyphenols (152) but still contains many other potentially bioactive compounds. The profiles vary significantly depending on the type (22, 153), variety (154), growing conditions (22, 151, 153, 155), processing and age of samples (156). There are several major recurring components that may have health effects. Processing impacts the concentration and bioavailability of these compounds. Typically, soaking decreases the concentrations of polyphenols and their *in vitro* antioxidant capacity (148), hence these processes will need to be taken into account when discussing the biological activities of sorghum and how human altering and processing can enhance or reduce potential health effects.

Flavonoid-based polyphenols have structures as indicated in Figure 1.2.12.

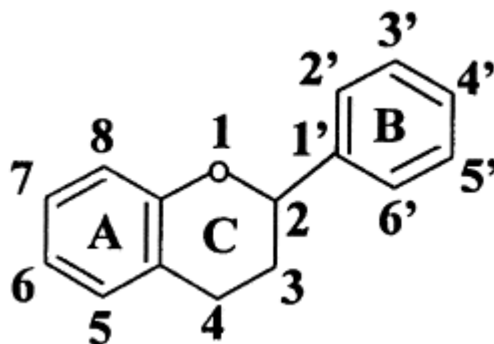


Figure 1.2.12: Structure of flavonoids. Different classes contain substituents at differing positions of the molecule (139). Hydroxyl groups are present at one or more positions 1 through 6'.

Flavonoids consist of several different subclasses. In sorghum, the predominant classes are flavonols, catechins, proanthocyanidins, 3-deoxyanthocyanidins and flavones.

1.1.7.1.2.1 Flavanols/catechins/proanthocyanins

Flavanols, or catechins, are built from the same backbone as noted above but with a hydroxyl moiety located at carbon 3 of benzene ring C (Figure 1.2.13). Substituent

groups at other positions lead to a wide array of molecules in this class. Stereochemistry can affect their biological activity (157).

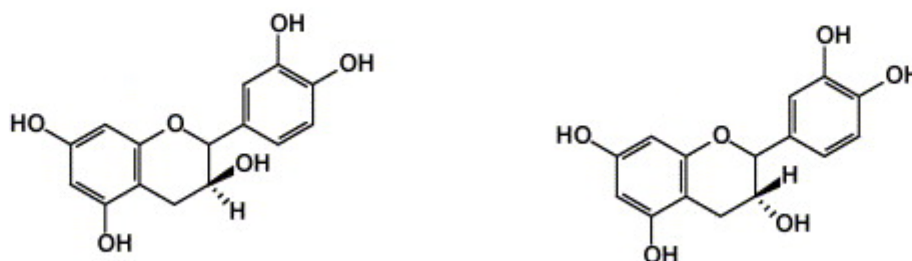


Figure 1.2.13: Catechin (left) and epicatechin (right) (158), indicating hydroxyl substituents on carbon 3, changing their stereochemistry. Methylated derivatives have methoxy moieties at positions 3' and 4'.

In sorghum, there are many flavanols, which will vary depending on the sorghum type. Catechin (146, 159) and epicatechin (159) were present in addition to their methylated derivatives. 3'-O-methylcatechin, 4'-O-methylcatechin, 3'-O-methylepicatechin and 4'-O-methylepicatechin have been identified in many varieties of coloured sorghums (159). Proathocyanidins are oligomers of flavanols. Typically, in sorghum, the degree of polymerisation ranged from 2 to >10 (160), with many having degree of polymerisation over 10. Catechins and epicatechins were close to absent in white sorghum, when compared with red varieties (152, 159). Extrusion of sumac and bran combinations reduced proanthocyanidin content but increased their bioavailability, as indicated by plasma concentration after ingestion (159). Table 1.2.5 indicates the expected ranges of flavanols/procyanidins in sorghum varieties. Oligomeric procyanidins are considered to be tannins, and are generally referred to as condensed tannins.

Table 1.2.5: Indicative flavanol/proanthocyanidin concentrations in sorghum and sorghum fractions.

Sorghum Product	Reference	Concentration
Red sorghum	Gu, *et al.* (161)	447.3-1919.5 mg/100g wholegrain
Hi Tannin Bran	Gu, *et al.* (162)	23.3mg/g
Hi-tannin sorghum grain	Awika, *et al.* (160)	20.5 mg/g
Sumac sorghum	Awika, *et al.* (160)	21.97 mg/kg
White sorghum	Gu, *et al.* (159)	Not detected
Sumac sorghum	Gu, *et al.* (159)	36.2mg/kg

1.1.7.1.2.2 Flavanones

Flavanones are classed based on their ketone substituent on position 4. They are distinct from flavones as they lack planarity and do not contain a double bond between carbons 2 and 3, shown in Figure 1.2.14.

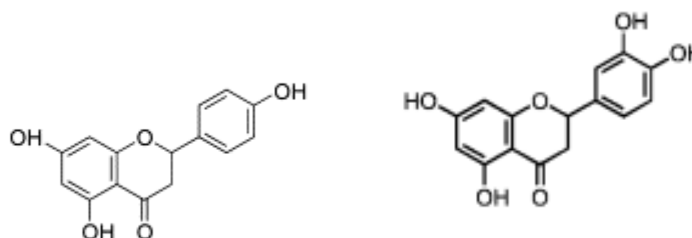


Figure 1.2.14: Structures of naringenin (left) (163) and eriodictyol (right) (164).

In sorghum, the predominant flavanones include naringenin (146), eriodictyol (146, 153) and eriodictyol glycosides (146). Concentrations can range from undetectable, to combined total flavanone concentration of over 48µg/g (153). At present, there does not appear a correlation with colour and concentration so this may be a factor of growing conditions, as most polyphenols are secondary plant metabolites.

Table 1.2.6: Concentration ranges of naringenin and eriodictyol in sorghum

Reference	Concentration Range
Dykes, *et al.* (153)	0-48.4 µg/g
Dykes, *et al.* (154)	0-1779.6 µg/g
Taleon, *et al.* (22)	96-164 µg/g
Dykes, *et al.* (165)	88.6-119.1 µg/g
Cardoso, *et al.* (134)	51.2-267.3 µg/100g dry basis

1.1.7.1.2.3 Flavones – apigenin and luteolin

Flavones have structures which include a ketone substituent at position 4, and varying hydroxyl additions at other positions. In addition, they are planar molecules, resulting from the double bond between carbons 2 and 3 on the 2-phenylchromen-4-one backbone, Figure 1.2.15.

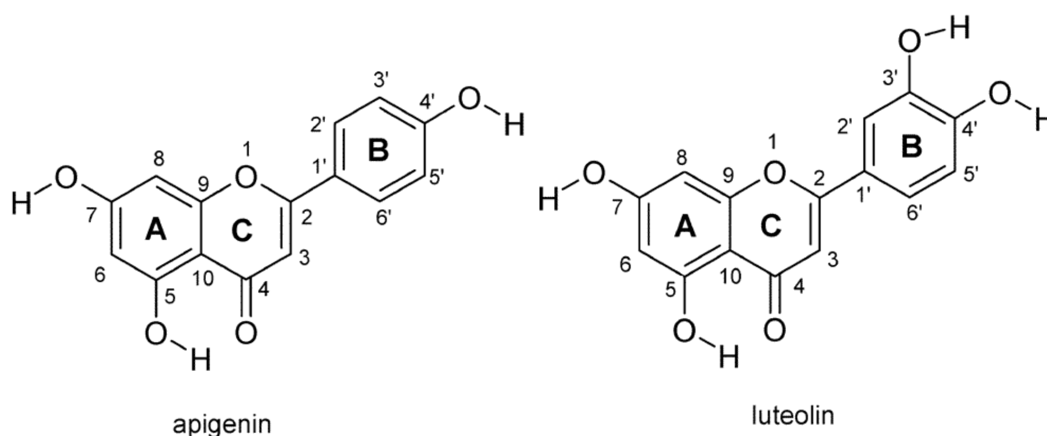


Figure 1.2.15: Structures of apigenin and luteolin (166), indicating keto-substituents at position 4, and hydroxyl-substituents at the 3' and 4' carbons.

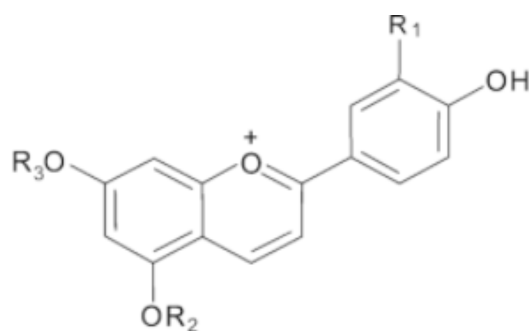
In sorghum, apigenin (146) and luteolin (146) are two common flavones. These can vary from undetectable to up to 385µg/g in certain red-appearing varieties (153), while they appear to be completely absent in some other red varieties (147). Red sorghums are typically free of isoflavones, which are structurally distinct from flavones based on the positioning of the phenyl group being at C4 as opposed to C2 (146). Indicative contents of flavones in sorghum types are shown in Table 1.2.7.

Table 1.2.7: Indicative content of the flavones luteolin and apigenin in sorghum. Values show combined concentrations of apigenin and luteolin.

Reference	Concentration	Colours/varieties
Dykes, *et al.* (153)	0-385.9 μg/g	Red, tan, black
Dykes, *et al.* (154)	0-362.2 μg/g	Lemon yellow varieties
Taleon, *et al.* (22)	14-48 μg/g	Black varieties
Dykes, *et al.* (165)	18.2-56.1 μg/g	Black varieties
Cardoso, *et al.* (134)	13.9-44.9μg/100g	Red varieties

1.1.7.1. 3-deoxyanthocyanidins

3-deoxyanthocyanidins are unique due to their stability in the environment. They can exist in their non-glycosylated form unlike their oxygenated counterparts, the anthocyanins which will exist as anthocyanidins. Luteolinidin and apigeniniden are the major 3-deoxyanthocyanidins present in red sorghum (147, 153) and black sorghum (22, 147, 165) varieties. Tan or white varieties contained very low concentrations of this class of compound (147, 153), while total concentrations can reach over 600μg/g in varieties with a black kernel appearance (153). In contrast, the concentrations of flavones luteolin and apigenin were lower in black than white/tan and red varieties (153). Typically 3-deoxyanthocyanidins are at the highest concentrations in the black varieties of sorghum (147, 153). Some varieties of black sorghum contained over 1mg of total 3-deoxyanthocyanidins per gram of grain weight (165). Their relationship to their flavone counterparts lutelin and apigenin is the loss of the ketone at position 4, and they typically exist as a salt with a positive charge on the oxygen at position 1 (Figure 1.2.16).



1. R_1 = OH, R_2 = Glc, R_3 = H: luteolinidin 5-glucoside (MW = 433)
2. R_1 = OH, R_2 = CH_3, R_3 = Glc: 5-methoxyluteolinidin 5-glucoside (MW = 447)
3. R_1 = H, R_2 = Glc, R_3 = H: apigeninidin 5-glucoside (MW = 417)
4. R_1 = H, R_2 = Glc, R_3 = CH_3: 7-methoxyapigeninidin 5-glucoside (MW = 431)
6. R_1 = OH, R_2 = H, R_3 = H: luteolinidin (MW = 271)
7. R_1 = H, R_2 = H, R_3 = H: apigeninidin (MW = 255)
8. R_1 = OH, R_2 = CH_3, R_3 = H: 5-methoxyluteolinidin 5-glucoside (MW = 285)
10. R_1 = H, R_2 = H, R_3 =CH_3: 5-methoxyapigeninidin (MW = 269)

Figure 1.2.16: Structure of 3-deoxyanthocyanidins (167).

As discussed further in this review, the biological activities of 3-deoxyanthocyanidins have huge potential. Black sorghum represented a more economically accessible source of these compounds compared to other highly promoted sources such as blueberries (168). Two of the major deoxyanthocyanins, luteolinidin (orange pigment) and apigeniniden (yellow pigment), exist in non-glycosylated forms and represent a major component of the phytochemical profile of black sorghum (151),

Table 1.2.8.

Table 1.2.8: Concentrations of 3-deoxyanthocyanidins in different sorghum grain types.

Reference	Predominant Compounds	Concentration
Dykes*, et al.* (153)	Luteolinidin, apigeninidin, 5-methoxyluteolinidin, 7-methoxyapigenidin	0-679.7 ug/g
Dykes*, et al.* (154)	Luteolinidin, apigeninidin, 5-methoxyluteolinidin, 7-methoxyapigenidin	0-186.9 ug/g
Taleon*, et al.* (22)	Luteolinidin, apigeninidin, 5-methoxyluteolinidin, 7-methoxyapigenidin	251-804 ug/g
Dykes*, et al.* (165)	Luteolinidin, apigeninidin, 5-methoxyluteolinidin, 7-methoxyapigenidin	329.1-1053.9 ug/g

Cardoso, *et al.* (134)	Luteolinidin, apigeninidin, 5-methoxyluteolinidin, 7-methoxyapigenidin	50.6-342.4 ug/100g (dry basis)

Anthocyanins can exist in either polymerised or un-polymerised forms (151), which may alter their bioavailability and activity, and their *in vivo* effects. Similarly, de-glycosylated procyanidins in sorghum have varying degrees of polymerisation (169). However, it has been difficult to characterise the individual combinations of anthocyanins in these compounds.

1.1.7.2 Other compounds – stilbenoids, anthraquinones, terpenoids, alkaloids

Stilbenoids have also been identified in sorghums at very low concentrations, for example 1 mg/kg concentrations of *trans*-piceid and up to 0.2 mg/kg *trans*-resveratrol (170).

Sorghum varieties also contain anthraquinones, terpenoids, carotenoids and alkaloids (171). Carotenoids are higher in red varieties when compared to white, and anthraquinones are absent in white sorghums from the few lines tested (171). The major carotenoids found in sorghum are lutein, zeaxanthin and β-carotene. These occur in quantities of approximately 0.112-0.309 mg/kg in coloured varieties, with yellow endosperm types being the most concentrated, and white endosperm types being the lowest containing 0.010-0.016 mg/kg (172). Anthraquinones and terpenoids in sorghum have not been well-studied.

1.1.8 Potential for metabolic syndrome and health

1.1.8.1 Metabolic syndrome

Metabolic syndrome is caused by a cluster of pathologies that lead to increased risk of type 2 diabetes mellitus and cardiovascular disease. The diagnosis is based on the presence of central adiposity and dyslipidaemia, hypertension and raised fasting glucose concentrations (173, 174). Additional measurements which are of importance include dysregulated adipose tissue biomarkers or hormones (such as adiponectin and leptin), fatty liver, insulin resistance, elevated free fatty acids in plasma, vascular dysregulation, endothelial dysfunction, pro-inflammatory state indicated by high

concentrations of inflammatory cytokines, pro-thrombotic state, fibrinolytic factors and clotting factor dysregulation, and hormonal characteristics (173). It has been suggested that there are links between gastrointestinal health and the development of metabolic syndrome (175-179) which are also discussed.

1.1.8.2 Normalisation of blood lipids

1.1.8.2.1 Clinical and animal trials

Dyslipidaemia is a key pathology of metabolic syndrome and has been linked to high cardiovascular risk (180). Sorghum, its fractions and extracts of sorghum can improve the blood lipid profile. Ethyl acetate extracts of *Sorghum bicolor* reduced serum triglycerides and total cholesterol in rats with diet-induced obesity (181). Similar results have been found in hamster models of atherosclerosis using hexane-extractable lipids, where plasma non-HDL (high-density lipoprotein) cholesterol was reduced, in addition to reduced esterified cholesterol in the liver. This was found to be due to reduction in cholesterol reabsorption (182). These results appear consistent with the reductions caused by sorghum distillers dried grain lipids (183) and non-wax lipids (184). Methanol extracts have similar effects on serum triglycerides in mouse models (185). Isolated kafirin fractions of white sorghum improved serum cholesterol profiles in rat models, however equivalent sorghum flour-based studies increased total cholesterol, primarily HDL cholesterol (186). Unrefined sorghum consumed at a rate of 100g per day in human studies improved serum lipid profiles (187). Extruded *Sorghum bicolor* reduced degree of steatosis and liver cellular fat by 30% in a diet induced obesity model of rat (188).

1.1.8.2.2 Mechanism and potential

The most likely mechanism by which sorghum reduced serum triglycerides and cholesterol was by alteration of the reabsorption of bile and bound fats. The major effect is likely to be the prevention of reabsorption of cholesterol. Sorghum contains sterols which have been shown to prevent the reabsorption of cholesterols. Policosanols, also found in sorghum (131), reduced plasma cholesterol concentrations by inhibition of the absorption of bile acids (189). In addition, the presence of policosanols reduced endogenous cholesterol synthesis, although this has been proven

only in *in vitro* studies (190). Although it is not completely clear, it is believed that the mechanism lies in changes to either the synthesis or degradation of HMG CoA (3-hydroxy-3-methyl-glutaryl-coenzyme A) reductase (190), the rate-limiting step in cholesterol biosynthesis. It has been further suggested that policosanols increase the phosphorylation of AMP (adenosine monophosphate) (191), by the activation of AMP kinase (192). The effects of policosanols are restricted to serum, rather than to particular tissues (189). The favourable policosanol profile of sorghum containing a high ratio of C24-C38 chains (193) may have beneficial effects for cardiovascular health. Policosanols improved serum cholesterol profiles in human studies (194, 195) although this has been disputed by some studies (196, 197). These results indicate that the lipid fraction of sorghum could be responsible for the reduction in triglycerides and normalisation of blood lipids.

Further, catechins and tannins have been implicated in the regulation of blood lipids. Although direct studies of sorghum tannins on cholesterol regulation have not been carried out, several other tannins have been studied, with promising results. Structural similarities indicate that there is potential for the sorghum tannins to have similar effects. High molecular weight persimmon tannin improved lipid profiles by increasing the activity of key enzymes required for the synthesis and transport of cholesterol and its esters (198). These promising results *in vivo* suggest that high tannin foods such as sorghum may help improve serum lipid profiles, one of the key diagnostic criteria for metabolic syndrome.

Oxidation of LDL (low-density lipoprotein) cholesterols has been implicated in the development of atherosclerosis. This is initiated by a lipoprotein, followed by adherence and penetration of monocytes which mature into macrophages. Oxidised cholesterols then lead to development of plaques (199). Macrophage-mediated oxidation of LDL cholesterols were reduced both *in vitro* (200-202) and *in vivo* (202, 203) by flavonoids. Although these studies were not conducted with sorghum flavonoids, this may be one mechanism by which sorghum reduces cardiovascular risk.

1.1.8.3 Anti-diabetic effects

1.1.8.3.1 Clinical and animal trials/current evidence

Sorghum has similarly been linked to hypoglycaemic effects (204) and is a low glycaemic index food with slow digestion. The development of pathologies of metabolic syndrome is increased with high glycaemic index and load foods in the overweight population (205). Ingestion of whole sorghum flour muffins lowered insulin fluxes more than a wheat-based counterpart and marginally reduced fluctuations in glucose (206) as a mechanism for the management and prevention of development of type 2 diabetes mellitus, associated with metabolic syndrome.

Whole sorghum food consumption caused a lower rise in plasma glucose than the dehulled version and similar rice and wheat foodstuffs (204). Methanol extracts of sorghum in models of insulin resistance in mice reduced fasting glucose and insulin concentrations when administered in a high lipid diet designed to induce dysfunction of glucose regulation (185). This was correlated with increased PPAR-γ (peroxisome proliferator-activated receptor gamma) expression in adipose tissue (185). Ethanol extracts showed insulin-independent normalisation of glucose responses in diabetic rats (207). In addition, there were minor improvements in plasma glucose concentrations, which were similar to that of glibenclamide, when sorghum extract was administered (208). Fermented and dried whole-grain red sorghum flour reduced severity of alloxan-induced diabetes, with blood glucose as low as half that of diabetic controls (209). Human trials showed that consumption of extruded sorghum beverage containing proanthocyanidins and 3-deoxyanthocyanidins reduced peak postprandial glucose and total insulin release of a subsequent meal by up to a half (210).

Diabetic complications such as reduced endothelial reactivity can arise from glycation of proteins (211). Sorghums high in phenolics, such as red or black varieties, reduced the glycation of these proteins *in vitro* (144).

1.1.8.3.2 Mechanisms and potential

The reversal of type 2 diabetes is possible with the primary requirement being re-establishment of pancreatic β-cell function. In the progression of type 2 diabetes, there

are distinct changes in the release and activity of insulin (212). These are caused by the dysfunction of many factors with β-cell dysfunction being one of the earliest changes (213). Apoptosis of pancreatic β-cells has been implicated in the impaired insulin release associated with type 2 diabetes mellitus. The presence of proteins associated with apoptosis and histological assessment of apoptotic pancreatic cells was markedly reduced by treatment with anthocyanins extracted from black soybeans (214). This may be one mechanism by which flavonoids, especially the deoxyanthocyanidins found in sorghum, result in reduced progression or potential reversal of the disease with long-term consumption.

Inflammation of the pancreatic cells, mediated by several pathways, can lead to cell death. Inflammatory responses have been implicated in the development of type 2 diabetes mellitus. In instances where dietary or genetic susceptibility has led to development of type 2 diabetes mellitus, inflammation has been implicated as one of the mechanisms at play. *In vivo*, flavonoids such as apigenin and luteolin reduced NFκB (nuclear factor kappa-B) activation and subsequently reduced β-cell damage, albeit in an *in vitro* setting (141). These results are promising, especially given that apigenin is absorbed from the gastrointestinal tract (215). Hydrolysed kafirin proteins similarly show *in vitro* oxygen radical scavenging properties, suggested to be due to the release of bound compounds within the protein matrix (42).

Ethyl acetate extracts of rice bran containing over 50% phenolic acids, dosed at 0.2g/kg per day, reduced fasting blood glucose concentrations in diabetic rats, linked to the presence of ferulic acid (216). Rice bran extract contains similar phenolic acids to sorghum such as *m*-hydroxybenzoic acid, benzoic acid, *p*-coumaric acid, ferulic acid and sinapic acid.

Further to this, inflammation may be regulated, at least to some degree, by the colonic microflora, the colonic environment and the interaction with the host (217, 218). Faecal lipopolysaccharide concentrations were increased with high fat feeding, in addition to the concentrations of pro-inflammatory cytokines in faecal lysates (219). The consumption of wheat-derived arabinoxylans has the potential to reduce circulating endotoxins (220). Sorghum, containing galacturoarabinoxylans, may result in similar effects. These studies, however, were unable to determine whether these

changes were due to reduced production, increased degradation, or reduced permeation.

1.1.8.4 Effect on central adiposity and obesity

Whole sorghum has not been correlated with weight reduction or improvements in body composition (186). In a rat model of diet induced obesity, extruded sorghum flour consumption reduced the weight of epididymal adipose tissue and adipocyte diameter (221). Long-term consumption has been linked to improvements in hormonal regulation of satiety and food consumption (222).

Sorghum resistant starch, however, decreased body weight and abdominal fat pads (223). With this change, there was an altered gastrointestinal microflora with a decrease in *Bifidobacterium* and *Lactobacillus* and an increase in *Enterobaceriaceae* (223). Although food intake did not change, there were decreased leptin and increased adiponectin responses with sorghum resistant starch administration (223), which may help to regulate food intake.

Due to the high proportion of leucine, interest in sorghum may grow due to the increasing evidence of the “leucine threshold” required for muscle growth that has been implicated as a potential way of managing obesity (224). The provision of 2.5g of leucine is sufficient to initiate this response when ingested in a single meal (224).

1.1.8.5 Effects on cardiovascular disease and hypertension

1.1.8.5.1 Clinical trials

The effects of sorghum and its extracts on hypertension have not been extensively studied. A review of literature has found no evidence to date of sorghum being specifically linked to improvements in blood pressure. There have, however, been studies which link several of the chemical compounds in sorghum to improved blood pressure. Sorghum vinegar extracts which contain phenolic acids protected from pulmonary thrombosis in animal models through anticoagulation and platelet activities (225). Thrombosis has a higher risk factor in those with metabolic syndrome (226). The process behind this is thought to be the induction of hypercoagulability,

potentially through increased activity of PAI-1 (plasminogen activator inhibitor-1) which is associated with higher visceral fat, and insulin release (227).

1.1.8.5.2 Mechanisms and Potential

Angiotensin converting enzyme (ACE) inhibition is a well-known mechanism by which compounds may improve hypertension. Apigenin and luteolin have shown 50% inhibition of ACE activity at 280 and 290µM concentrations *in vitro*, respectively (228). Further, endothelial dysfunction, which precedes the vascular dysfunction leading to hypertension (229), may be changed. Flavonoids reduced blood pressure in rat models and *ex vivo* vascular reactivity to acetylcholine (230).

The development of hypertension in obesity is thought to be caused by the interaction of several pathways, as shown in Figure 1.2.17 (231).

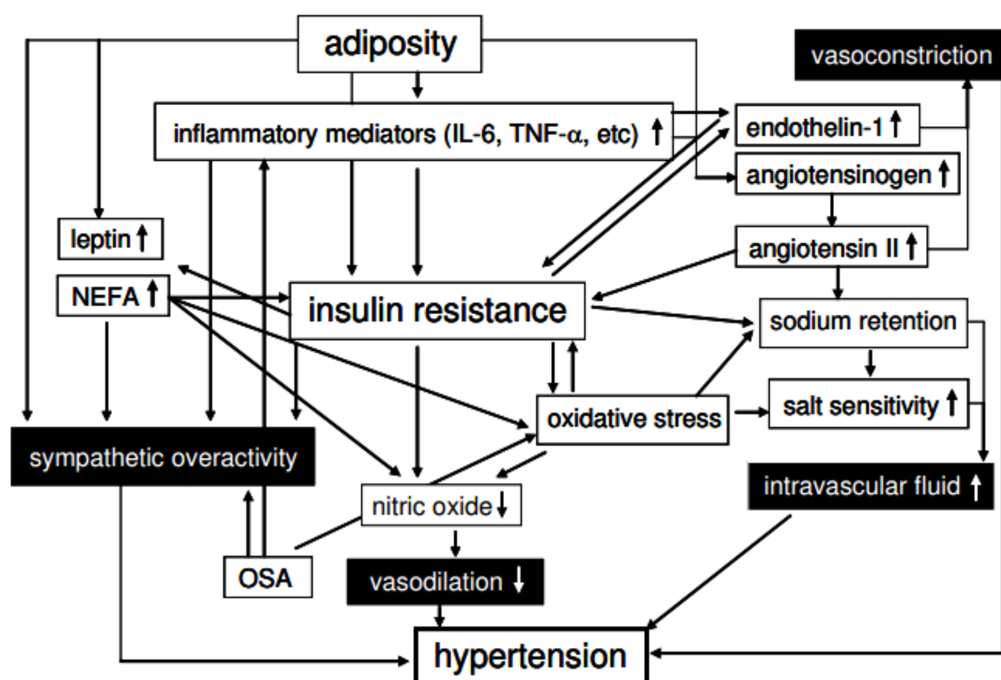


Figure 1.2.17: Development process of hypertension in metabolic syndrome (231)

Reduction in the concentrations of inflammatory markers may be mediated by sorghum supplementation. Certain sorghum genotypes when integrated into diets of

rats reduced the transcription of TNF-α (tumour necrosis factor-alpha), IL-10 (interleukin-10) and IL-8 (interleukin-8) (232). TNF-α protein expression was reduced with sorghum extract administration in high fat diets (185). Sorghum kafirin fractions extracted in ethanol reduced lipopolysaccharide-induced macrophage inflammation of TNF-α by up to 81.4% (41).

1.1.8.6 Gastrointestinal health

1.1.8.6.1 Clinical trials

Very little information exists so far that sorghum, as a whole food supplement, moderates the endotoxaemic state. However, many studies suggest that sorghum has the potential, based on its composition, to improve metabolic syndrome via modulation of endotoxaemia (217, 233, 234).

Sorghum lipid extracts are capable of increasing the *Bifidobacteria* genera in the colon (233). These micro-organisms are gram-positive, non-LPS (lipopolysaccharide) producing bacteria that increase short chain fatty acid production. They also ameliorate LPS-induced inflammation (234) which is associated with metabolic syndrome factors.

Providing sufficient substrates for these beneficial bacteria is one way to enrich their population. Resistant starches and non-starch polysaccharides, such as those in sorghum and other wholegrain cereals, will improve gastrointestinal health (107, 235, 236). Although not a controlled study, investigations into populations which consume large amounts of sorghum, such as the Mossi people of Africa, have shown a favourable gut microbial profile with large proportions of *Bacteroidetes* and higher concentrations of faecal short chain fatty acids (217).

1.1.8.6.2 Potential mechanisms

Recently, it has been suggested that intestinal microflora have an important role to play in the development of insulin resistance (237) by the initiation of inflammatory pathways caused by an endotoxaemic state (238). The term "low-grade endotoxaemia" is associated with metabolic syndrome (238), and management of this state markedly

improved insulin resistance (239). Modulation of gastrointestinal dysfunction is one way by which prevention or reversal of aspects of metabolic syndrome is plausible. Inflammation of the gastrointestinal tract itself is mediated by flora in a high fat diet, and precedes many of the pathologies of metabolic syndrome (240).

There are multiple pathways this state can be altered. One approach is the modulation of the gut bacteria to reduce the production of LPS. The "optimal" gut flora balance has not been characterised, and indeed may be different on an individual basis. It is generally accepted that the balance of Bacteriodetes:Firmicutes may be of importance, where a greater proportion of Bacteroidetes is desirable (217). Although this is an initial starting point, there are approximately 823 known species of bacteria that inhabit the human gastrointestinal tract (241). The composition varies greatly with geographical location, age, lifestyle and diet (242, 243). It will take a significant amount of time to determine what the "ideal" microbiome is and how to manipulate it for full health. Certain species and smaller groups of bacteria have been linked to improvements in pathologies of metabolic syndrome, including *Akkermansia muciniphila,* a mucin-degrading bacteria that is present at around 3-5% of the total community (244).

Several studies have defined the alterations in the microbiome composition with high fat feeding. This typically leads to an unfavourable decrease in Bifidobacteria (239) and an increase in Firmicutes and Proteobacteria (245). This finding has been questioned, with some suggesting that obese individuals have a higher ratio of *Bacteriodetes* (246). Additionally, there has been interest in the role of *Mollicutes*, which show a significant increase in high fat, Western-style diets (247).

Increasing the breakdown of LPS by intestinal alkaline phosphatase has also been suggested as a mechanism as intestinal alkaline phosphatase is responsible for detoxification of LPS (248). Concentrations of intestinal alkaline phosphatase are negatively correlated with obesity and high fat diets (175). Restricting the movement of LPS through the gastrointestinal barrier, by reduction of intestinal permeability, and improvement in tight junctions reduced the circulating LPS concentrations (237). The development of inflammation cascades initiated by compounds such as LPS can then

lead to atherosclerosis, insulin resistance and impaired glucose tolerance (249). A high fat diet increased intestinal permeability and reduced tight junction proteins (175).

Several purified non-starch polysaccharides improved the endotoxaemic state (220, 250, 251), some of which may be derived from sorghum. Increased consumption of resistant starches is linked to increased short chain fatty acid production, and increased Bifidobacteria (252). The increased short chain fatty acids lead to increased hepatic adipogenesis and subsequently control of satiety and feeding hormones. Sorghum does not appear to damage intestinal morphology, and assists in maintaining changes associated with a high fat diet (232, 253).

1.1.9 Sorghum integration into diets

Sorghum is being investigated as a potential replacement for wheat in many food products. The popularity of "gluten-free" diets has been increasing in recent years (254, 255). This movement has helped sorghum gain traction as a grain for human consumption (256). The use of sorghum in products such as Gluten Free WeetBix™ is potentially the beginning of the evolution of sorghum from "feed" to "food". Studies have indicated that it is an appropriate substitute, or partial substitute, in wheat-based products including breads, cakes, biscuits and cereal flakes (257). Bobs Red Mill™ sorghum flour, commercially available in Australia, contains 33.5% resistant starch and can be used in the preparation of muffins and other products (206).

1.1.10 Cautions

Sorghum has the potential to act as a chelating agent for trace minerals and contains "anti-nutritive compounds", but this is likely only when consumed in excessive amounts (258, 259). Sorghum is prone to ergot infections, but, with appropriate management and processing, products will be free of toxic compounds, of particular concern are fumonisins and other mycotoxins (260). Processing of sorghum will alter its composition and potentially change the concentration and total contents of beneficial compounds. This is also especially true for glycaemic responses after ingestion. The preparation method can change the insulin and glucose responses (261). Consumers should be aware that any increase in processing of flours will likely reduce the benefits, similar to the case of white wheat flour, compared to whole wheat grains

(262). Concentration of phytochemicals and sorghum extracts are likely to enhance certain aspects of sorghums functionality in the context of metabolic syndrome parameters.

1.1.11 Processing, growth, and breeding

Sorghum is a wide-scale, commercially produced crop, with multiple varieties available for use, so this variability needs to be addressed when promoting sorghum for health. Sorghum grown in different locations, and under different environmental conditions, can vary drastically in the concentrations of flavonoids. Environmental variability can account for almost 60% of the variance component of total flavanones, 21% for flavones and approximately 16% of 3-deoxyanthocyanidins (22). These differences are not trivial, with one study showing that environmental conditions can consistently result in less than half the comparable flavone, flavanone or 3-deoxyanthocyanidin concentrations when the same genotype is grown under less favourable conditions (22). Since the concentrations in the bran fraction of sorghum are highest for flavonoids, phenolics and tannins (263), any process which removes the bran layer will reduce the potential health effects associated with these compounds. The research into processing methods has become increasingly of interest over recent years, as knowledge of how these methods change the nutritional and nutraceutical profile have been recognised (91, 264-267). With the increased consumption of pre-prepared and packaged foods, this knowledge is required for promotional purposes and education of consumers.

Sorghum is often fermented for the purpose of ethanol production (268), gluten-free beer production (170), for spirit production (269), and for gluten-free sourdough production (150). Improved bioavailability of phenolics has been found after fermentation in oat and millet (270), and fermentation with strains of *Lactobacilli* can both increase and decrease availability of catechin, quercetin and gallic acid depending on the strain (271). The fermentation process will also alter the existing profile of phenolics (160, 272, 273).

Sorghum tea is also noted to be a traditional way for sorghum to be prepared, while the preparation can also alter the chemical profile of sorghums (145). Cooking typically reduces flavonoid concentrations as shown during the preparation of

“porridges” (146) and alters the profile by increasing certain flavonoids and decreasing others (146). The cooking process also decreased phenolic acid concentrations (146).

Conscious efforts have been made to breed for higher flavonoid sorghums with the potential use as a functional food or for nutraceutical supplements. This is in stark contrast to previous efforts to reduce these compounds for use in the stockfeed industry, where they are often considered “anti-nutritive”, and are associated with impaired weight gain in cattle, pigs and poultry (274-276).

Chapter 1.2 – Prebiotics in Obesity

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Prebiotics in obesity

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Obesity was probably rare in ancient times, with the current increase starting in the Industrial Revolution of the eighteenth century, and becoming much more widespread from about 1950, so concurrent with the increased consumption of carbohydrates from cereals in the Green Revolution. However, dietary components such as oligosaccharides from plants including cereals may improve health following fermentation to short-chain carboxylic acids in the intestine by bacteria which constitute of the microbiome. Such non-digestible and fermentable components of diet, called prebiotics, have been part of the human diet since at least Palaeolithic times, and include components of the cereals domesticated in the Neolithic Revolution. If consumption of these cereals has now increased, why is obesity increasing? One reason could be lowered prebiotic intake combined with increased intake of simple sugars, thus changing the bacteria in the microbiome. Processing of food has played an important role in this change of diet composition. Since obesity is a low-grade inflammation, changing the microbiome by increased consumption of simple carbohydrates and saturated fats may lead to obesity via increased systemic inflammation. Conversely, there is now reasonable evidence that increased dietary prebiotic intake decreases inflammation, improves glucose metabolism and decreases obesity. Would widespread increases in prebiotics in the modern diet, so mimicking Palaeolithic or Neolithic nutrition, decrease the incidence and morbidity of obesity in our communities?

KEY WORDS: Obesity - Prebiotics - Microbiota - Inflammation.

Food is essential to life, with famine as a result of too little food, and obesity as a symptom of excessive food intake. Both famine and obesity are an integral part of human history. They have been connected through the thrifty gene hypothesis, referring to metabolic traits that allowed humans to store energy during times of excess, to be used in times of famine. This attractive hypothesis has generated intense discussion and interesting arguments including that genetic variability in metabolic genes within populations is a means of distributing risk across offspring in the absence of strong selective pressures.[1] However, the thrifty gene hypothesis has been deemed untenable for many reasons, including that hunter-gatherers do not become obese between famines, with body mass index values remaining between 17 and 21.[2] Further, the presence of both slim and obese humans in modern societies suggests that gene mutations are essentially neutral, suggesting genetic drift rather than selection.[3]

The prevalence of obesity has increased markedly in the last two centuries, but there is sufficient evidence for obesity dating back tens of thousands of years.[4] From prehistoric times until the eighteenth century, overweight and obesity were probably rarely seen, and this remains a characteristic of modern hunter-gatherer societies. Prehistoric statuettes such as the so-called Venus figurines found across southern and central Europe show exaggerated female abdomen, breasts, hips and thighs. The oldest of these Palaeolithic statuettes, found in 2008 at Hohle Fels in southern Germany, is between 35,000 and 40,000 years old [5] while the well-known Venus of Willendorf in Austria is about 25,000 years old.[6] Since the first statuettes were found in the 1860's, arguments have focused on whether they are depictions of actual obesity or rather symbols of fertility

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and abundance. The historical evidence for obesity continues with figurines from the Neolithic period,[7] defined as the beginning of farming between 10,200 and 8,800 BC and ending between 4500 and 2000 BC. In ancient civilisations, only the wealthy could afford to be obese; one documented example is the Ptolemy rulers of Egypt who succeeded Alexander the Great, reigning until 30 BC.[8] The concept that obesity is evidence of conspicuous consumption is an ancient one, but it became clearly developed in the Age of Enlightenment (eighteenth century) as a negative social perception of obesity together with an association with suspect morals and excess, and as an outward representation of the soul.[9]

Prevention of obesity also has a long history, beginning with the writings of Hippocrates (c. 460 BC-c. 370 BC) [5] and continued with the writings of Soranus of Ephesus (2nd century AD) and Caelius Aurelianus (5th century AD).[10-12] Treatments included purging and vomiting, fasting and exercise.[4, 10-12] Many physicians of antiquity recognised that diet was an important factor in both the cause and the attenuation of the disease. Dietary treatments included foods such as cheese, vegetables, mustard seeds and herbs. Alexander Trallianus wrote in the 6th century AD of a diet including indigestible foods, vegetables, barley, rice and legumes as well as fish, seafood and fruit to treat obesity, so an early version of functional foods.[12] The ideas of the ancients appear to be a foundation for modern conservative management of obesity.

This review will discuss the relationships between prebiotics and obesity, without attempting to cover the enormous literature on either prebiotics or obesity. A prebiotic is defined as a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health.[13] The WHO defines overweight and obesity as abnormal or excessive fat accumulation that presents a risk to health for a number of chronic diseases including diabetes, cardiovascular diseases and cancer.[14]

The Neolithic Revolution – successful in providing healthy diets?

The domestication of plants including cereals in the Fertile Crescent during the Neolithic period,[15, 16] termed the Neolithic Revolution, allowed a sedentary life-style with the formation of permanently settled farming towns, later developing into cities. Domestication of cereals is part of a continuum from gathering to cultivation of wild plants, their domestication and then agriculture that probably occurred over thousands of years in widely spread areas.[17] There have been fascinating discoveries and vigorous discussions on the domestication of cereals; some of the more recent reviews discuss the domestication of rice, wheat and maize.[16, 18-22]

Maize was domesticated in modern Mexico, while another ancient crop from the Andean countries of modern Bolivia and Peru has gained recent attention. Quinoa (*Chenopodium quinoa* willd.), technically known as a pseudocereal, has been cultivated for 5000-7000 years with transmission throughout South America via livestock migrations and trade from Venezuela in the north to the southern extremities of Argentina and Chile.[23] Quinoa was considered by the Incas as the mother of grains, with the Emperor using a golden spade to dig the soil to plant the first seed of the new crop each spring. After the Spanish conquest in the 1500's, quinoa cultivation retreated to isolated pockets above 3500m where introduced European crops such as wheat, rye and oats could not be grown. Quinoa has all the properties of an ancient cereal with high protein content (12-17%), dietary fibre content (2-10%), lipids (5-9%) and carbohydrates (60-70%), with 11% as amylose.[23] Quinoa contains essential amino acids, with similar values to casein, the protein found in milk. Quinoa flour is high in xylose and maltose, and low in glucose and fructose, along with ribose and galactose, giving it a low glycaemic index value. The total lipid content of quinoa is 14% with linoleic (39%) and oleic (28%) acids being the most prevalent.[23] The rediscovery of quinoa and other ancient grains by consumers in developed countries may be no more than a consumer fad, but these functional foods may change the way we think about the components of our foods, especially prebiotics.

The human diet has changed dramatically since the introduction of agriculture in the Neolithic Revolution, unlike the human genome. Many factors including geography, agricultural advances and technology, the advent of food processing and food technologies, wars, climatic extremes and modern marketing have changed the food we eat. For most of human history, people only ate what was avail-

able in their locality. There were exceptions, but only for the very wealthy, such as the importation of spices from India to Europe. From the 15th century, when Western Europeans started exploring the world, exposure to new foods occurred with new species being introduced to new cultures. Agricultural advances saw the rise of certain crops and reduced production and consumption of others. In our more recent history, food processing has changed the way many humans hunt and gather their food, and with this has come a dramatic change in the intake of both micro- and macro-nutrients. Further, advances in agricultural productivity have changed the source and quality of the food.

The modern Green Revolution - unsuccessful in providing healthy diets?

Since the Industrial Revolution, there have been several periods of major changes in agricultural production and productivity, including famines such as the potato famine in Ireland from 1845-1852. In the following generation, Northern Europe went through a Green Revolution from 1870 to 1914 with marked increases in productivity.[24] The much more publicised Green Revolution from about 1960 to 2000 was hailed as the world's answer to imminent famine due to a rapidly increasing population. Production increased dramatically, with increases of 208% in wheat production, 109% for rice production and 157% for maize in developing countries between 1960 and 2000.[25] Further, these increased yields came with shorter growing periods. Without this Green Revolution, it has been estimated that food prices would have been 35-65% higher, with the risk of political instability, and caloric availability would have decreased by 11-13%; these changes have benefited all consumers, especially the poor.[25] Gains have been uneven, with China and India showing large improvements with much smaller improvements in sub-Saharan Africa. Even in countries with successful Green Revolution changes in productivity, poverty and food insecurity still exist as does micronutrient malnutrition. Further, environmental impacts have been mixed, with increased production allowing marginal land to return to forest cover, but with increased soil degradation and chemical run-off from productive land. Another critical issue has been the reduced research expenditure as agricultural production increased.[25] Although the Green Revolution is hailed as one of the most positive changes in modern agriculture, did the increased yield and profit come at the expense of nutritional quality and variety in the modern-day diet?

During the Green Revolution, emphasis was placed on increased production, with high-yielding strains with low economic input becoming predominant, specifically of rice, maize and wheat. The use of powerful breeding tools, increased nutrient inputs and shared knowledge resulted in provision of large quantities of food. The doubling in cereal production came largely from increased carbohydrate content, with grain weights typically being inversely proportional to the protein percent.[15, 16, 26] Increases in carbohydrate content require much less nitrogen input than improved protein content. Further, the selective increases in the major cereals may have led to decreased availability and hence consumption of other staple foods such as legumes, grains and cereal-based crops. Production of legumes in the Punjab region of India, a major food production area, has halved during the Green Revolution, with the difference being filled by rice and wheat cultivation.[27] This change decreased the nutritional diversity, purely due to availability.

The microbiome

The processes of absorption of nutrients from food are complex, with important roles of enzymes and hormones released into the gastrointestinal tract. Further, the essential role of the bacteria, especially in the colon, is now being realised. These bacteria, termed the microbiome or microbiota, are diverse with around 500 to 1000 bacterial species present accounting for 2 to 4 million genes (about 100-fold more genes than the human genome) and about 100 trillion bacteria (about 10-fold more cells than the human cells).[28] These bacteria obtain their nutrients from the food we eat, and they also change the components of the food before absorption by the human host. The different bacteria of the microbiome have very specific environmental needs, which are mostly met by the conditions in the distal colon. They are often unable to survive outside this environment, so culture of these species *ex vivo* is relatively unsuccessful with estimates suggesting only 30-40% can

be cultured.[29] The advent of molecular techniques has allowed the potential extent of the relationship between the microbiome and the human host to be investigated.

There is debate about the inheritability of the microbiome with some suggesting it is high,[30, 31] whilst others suggest it is low,[32] leading to ambiguity as to whether there may be directed co-evolution. Among different primate species, there are typical microbiomes, with the host phylogeny being the driving determinant, showing significant evidence for co-evolution.[30] There appears to be geographical differences among human populations,[33] likely to be the result of local traditions and exposure to external elements.[32] The local diet is one of the elements that undoubtedly influence both the long-term changes and the immediate composition of the microbiome. The proportion of fermentable components in the diet influences the species of bacteria present, and the species of bacteria influences the host, allowing complex interactions.[34] The microbiome is very susceptible to short-term changes in the diet,[35, 36] suggesting that the relationship between the diet and the microbiome could be both the cause and the treatment of obesity. The microbiomes of ancient and modern humans are distinct, and geographical variation has probably decreased in the post-Columbian period.[37] These changes continue and so may stunt the evolution of the microbiome, due to reduced diversity and possible gene transfers between species.[34]

Prebiotics

Fermentation of dietary carbohydrates provides the energy for the growth and activity of the microbiome; this is the first part of the definition of these carbohydrates as prebiotics.[13] All known and suspected prebiotics include resistant starches, and non-starch polysaccharides with different sugar monomers and chemical linkages.[38] Prebiotics must be able to resist gastric acidity, resist human enzymatic hydrolysis, resist absorption in the upper gastrointestinal tract, be fermentable by one or more intestinal microflora, and selectively stimulate growth or activity of intestinal bacteria.[39] Prebiotics can be found naturally in many foods including leeks, asparagus, chicory, Jerusalem artichokes, onions, garlic, wheat, rye, barley, oats and soybean.[40] Typically, any food containing dietary fibre is likely to contain some form of prebiotics, although in varying amounts. The definition of a prebiotic concludes by stating that these bacteria must improve the health of the host. There is a long history of prebiotic consumption by humans, but scientific evaluation of the health benefits is relatively recent.

Many ancient foods contain prebiotics as well as nutrients, so the daily prebiotic intake was significant. One of many examples is the yacon, a traditional root used by many Andean tribes in Ecuador, Peru, Bolivia and Argentina. The dietary fibre content of the root, usually eaten raw, is approximately 10.4% of dry matter, with an insoluble fraction of approximately 8.7% and soluble fraction of 1.7% of dry matter.[41] The fibre consists of cellulose, galactose, arabinose and galactose, with lower amounts of xylose, mannose and rhamnose.[41] The fructose oligosaccharide content of yacon varies between 24 and 35% with some studies indicating higher content of 54-62%.[41, 42]

Prebiotics were present in the components of ancient diets and were consumed routinely. In the ancient Indian diet, unlike the modern European diet, prebiotics were a major component through consumption of grains, fruits and vegetables.[43] Also, the Indian diet contained spices and herbs in home-made food which were quite different from the processed food in European diet.[43] Palaeodietary studies, for example in the Chihuahuan Desert of Mexico, have shown that prehistoric people consumed a wide variety of plants, including inulin-rich sotol, agave and onion.[44] Conservative estimates indicate that the average male hunter-forager consumed 135g prebiotics per day, while females consumed 108g/day.[44] When the whole diet was considered as the energy source, it was estimated that a larger proportion of the energy was derived from fermentation of prebiotics in the large intestine on a daily basis. However, the total dietary intake of prebiotics has reduced in the modern era, for example, to less than 20g/day in the USA.[44] Although there are current social trends to target refined carbohydrates as the cause of the obesity epidemic, it is likely more caused by what these originally plant-based, yet highly processed products lack, as opposed to what they contain. The increase in simpler starches with a high digestibility has certainly come at the cost of non-digestible starches and other dietary fibres, which are not useful economically or in a processing context.

The advent of processed foods

Changes in lifestyle due to modernisation created the need for processed and fast foods. The delivery of large volumes of food to a widespread population resulted in industries realising the potential economic gains. Industries aimed to produce low cost, highly-consistent foods with palatable tastes and textures. Raw grains and non-refined materials are mostly unsuitable for these processed foods, hence the industries aimed to include non-fibre carbohydrates, mostly including simple sugars. Highly-refined white bread, wheat, rice and maize products became a norm of the modern westernised diet. Furthermore, the addition of these derivatives to non-wheat-based products increased the amounts of rapidly digestible carbohydrates with no addition of fibrous components. The estimated intake of refined grain was approximately 89% of the cereal intake in the USA in 2005.[45]

The increased consumption of processed foods has been a product of both convenience and cost. Although some credit to this may be derived from the Green Revolution, it would appear that the major driver is the consumer markets pushing toward convenient, cheap and consistent foods. Globalisation may be responsible for the nutritional changes resulting in increased processed food consumption and increased obesity.[46] The increased consumption of processed foods has come together with increased foreign investment in food processing in developing countries.[47] The advances in industrialisation and commercialisation of processed foods since the late 1800s has outpaced increases in dietary knowledge which may explain the current situation.[48] Consumers have then perpetuated the decline in whole food consumption with their increased desire for processed foods.

The marked differences between the whole-food and processed food diets are likely to have contributed heavily to the rising health epidemics of obesity and metabolic syndrome. Epidemiological studies have correlated decreased whole grain intake and increased refined grain intake with weight gain in short-term studies.[49] This evidence suggests that long-term dietary changes contribute to increased body weights and related diseases. The most obvious differences are the increased addition of sweeteners, the decreased amounts of dietary fibres, the addition of preservatives, and the increases in hydrogenated fats.[50]

Processing and prebiotics

The shift toward increased processing has decreased the fibre intake in many countries. It can be clearly seen that the increased processed food consumption mimics the trends in obesity and diet-related diseases. Many link the decreased fibre consumption with a decreased intake of grain and cereal crops. Japan decreased its average fibre consumption per capita by almost 10 grams over the period of 1911-1980.[51, 52] This change can be directly attributed to a decrease in whole grain intake.[52] Alterations in the American diet were marked with fibre intake declining by 28% from 1909 to 1975. The cause is a reduced intake in whole plant-based foods, with the greatest decline coming from the grain intake.[53] In Brazil, the consumption of ultra-processed foods increased over the period of 1987-2003, which led to much lower dietary fibre intake over this period.[54]

Grain crops contain both fermentable and non-fermentable fibres that may confer health benefits. These fermentable fibres are of particular interest as they typically act like prebiotics to change the composition of the gut microbiome. Of particular interest are resistant starches and non-starch polysaccharides. Grains, cereals and legumes have different amounts of non-starch polysaccharides and resistant starches depending on their species, variety and growing conditions. Of particular significance to the prebiotic concept are soluble dietary fibres, many of which are fermentable, and non-starch polysaccharides, all of which are fermentable. The primary grains that increased in production throughout the Green Revolution were rice, maize and wheat with total dietary fibre of 19.6%, 5.7% and 17%, respectively and total soluble dietary fibres of approximately 3.6%, 1.4% and 2.3%, respectively. Other cereals such as millet contain 5.4% total dietary fibre and 3.8% soluble fibres and oats contain 37.7% total dietary fibre and 3.8% soluble fibres.[55] Barley, depending on variety, can contain from 4.5-26.9% soluble non-starch polysaccharides.[56] By replacing 100g of white rice with 100g quinoa, carbohydrate content decreases by 17.5 g and protein content increases by 7.3 g.[57] Gram for gram, ancient grains such as quinoa, amaranth, barley and millet offer fewer carbohydrates and more protein as well as containing beneficial fibres.

Processing reduces the content of soluble dietary fibres.[55] Common food additives such as corn starch and potato starch do not include fibre, processed

white rice contains 0.3% soluble dietary fibre, and processed oats contain less than a third of the total dietary fibres of their unprocessed equivalent.[55] The soluble non-starch polysaccharide content of commonly consumed processed foods while obesity was rapidly increasing were much lower than the raw whole grains. Kellogg's Cornflakes™ contain 0.48% non-starch polysaccharides, puffed rice 0.41%, brown rice 0.89%, white rice 0.92%, Kellogg Krispies™ 0.32%, and white spaghetti 1.82%.[58] With processing comes a reduced intake of the dietary fibre which have potential prebiotic affects and as a result, our overall health is likely to have been impacted.

Obesity-related disease

The changes in nutrient composition and intake due to processing of food are partly to blame for the current prevalence of obesity. Many countries now report more than 50% of their population as overweight or obese with marked increases since the 1980s.[59] The prevalence of obesity today varies from 3-4% in Japan and Korea, to 30% or more in the USA and Mexico.[59] The incidence has appeared to plateau in the USA over the past decade.[60] In Australia, 60% adults and 25% children, or more than 12 million people, are classified as being overweight or obese.[61] Due to these increases, obesity-associated illnesses have also increased, along with an increased financial burden.[62, 63]

Obesity has a major impact on the energy metabolism and regulatory mechanisms that lead to metabolic diseases including type 2 diabetes, cardiovascular disease, dyslipidaemia, hormone-linked cancers and gastrointestinal diseases including inflammatory bowel disease and colon cancer.[64] Type 2 diabetes is closely associated with obesity and insulin resistance, and is affected by the inflammatory status of adipose and other tissues. The associations are clear with increasing incidence of diabetes, hypertension, asthma and arthritis as body mass index increases.[65]

Obesity is a very slowly developing metabolic disorder. While there is evidence showing the relationship between obesity and the above-mentioned diseases, the clear development of obesity is not identified until the person looks obese. During the asymptomatic development of obesity, metabolic dysfunction develops. The process is unclear but it is speculated that the gut microbiome changes with the presence of components in the diet such as saturated fatty acids. Hence, this metabolic dysfunction can be described as a dysfunctional gut microbiome. In contrast, the incidence of obesity and obesity-related diseases in communities that follow a more traditional whole-food diet is lower.[66] Pimo Indians eating traditional diets showed lower body mass index and cholesterol concentrations than people of similar heritage eating a westernised diet. The traditional Pimo diet contains over 50 g/day of potentially prebiotic dietary fibre, primarily from corn and beans.[67] Traditional diets in urban Brazil have a protective effect against obesity, with possible reasons including the high-fibre intake in traditional diets.[68] A similar trend is evident in tribes of the Solomon Islands. In communities where a westernised diet has begun to infiltrate, the plasma cholesterol concentrations are higher and the percentage of males with high blood pressure is increased.[69] Female teachers in Tehran consuming westernised diets high in refined carbohydrates, processed meats and low in dietary fibre have higher body mass index and increased risk of developing metabolic syndrome.[70] These are a few examples, with most studies indicating similar trends following consumption of highly-refined, acellular diets.[66]

The ever-increasing obesity epidemic also increases the financial costs of health care.[50] It has been estimated that, in the year 2000, the burden of obesity from both direct health care and indirect costs was as high as 4.06% of gross national product of China. In the USA and the United Kingdom, estimated costs are likely to increase by $48-66 billion and £1.9-2 billion per year over the next 15 years, respectively.[71] Other countries with rising obesity will have similar increases in health costs.

Chronic inflammation in obesity

Metabolic endotoxaemia, a chronic low-grade inflammatory condition, has been identified as an early predictor of obesity and diabetes.[72] Multiple studies associate obesity with this chronic low-grade inflammatory status. The inflammatory state is characterised by the presence of elevated plasma concentrations of cytokines and by the presence of macrophages and monocytes within the adipose tissue. As the adipose tissue is an endocrine tissue, imbalances and disruptions in adipose tissue status can have marked effects

on metabolic processes. The increased plasma cytokines in obesity are in response to increased plasma microbial components and lipotoxicity. The presence of lipotoxic substances, including the saturated fatty acids, palmitic, stearic and lauric acid,[73-76] and microbial components [77, 78] have the ability to activate toll-like receptors (TLR) which then initiate immune responses.

Obesity has been associated with distinct gut microbiomes and increased gut permeability leading to a low-grade endotoxaemic state.[79] This state leads to the initiation of several inflammatory pathways in adipose tissue. TLR4 responds to increased plasma lipopolysaccharides and lipids, and the induction of the NF-κB gene transcription.[80, 81] The result is an increased expression and secretion of cytokines leading to recruitment and infiltration of macrophages to adipose tissue. The macrophages then further the inflammatory response by increased cytokine expression mediated by NF-κB.[82, 83] TLR2 induces a similar response to the presence of lipopolysaccharide, with the induction of the NF-κB mediated transcription of cytokines;[81] however, it interacts with a wider variety of ligands than TLR4.

Prebiotic interventions

There is increasing evidence that prebiotics have the potential to attenuate obesity, obesity-related inflammation and obesity-related diseases.[84-87] The mechanism of action of prebiotics involves their fermentation in the colon, which changes the gut microbiome.[88] The prebiotics appear to improve gut barrier function, potentially reduce pathogenic bacterial populations and produce compounds which can improve host metabolism and endocrine function.[79] Bacteria produce by-products when fermenting prebiotics including hydrogen, lactate, methane, carbon dioxide and short-chain carboxylic acids including acetate, propionate and butyrate. Much research into the mechanism of action of prebiotics on inflammation and obesity has revolved around the benefits to gut health of these short-chain carboxylic acids.[38]

Prebiotic effects on inflammation

Supplementation with prebiotics increases the plasma and colonic concentrations of short-chain carboxylic acids. Plasma short-chain carboxylic acids initiated activation of specific receptors classified as GPR43 leading to reduced lipolysis and reduced plasma free fatty acids.[89] Reduction of plasma free fatty acids by this mechanism should reduce the inflammatory response by reducing the exposure of membrane TLR to plasma free fatty acids. GPR43-knockout mice showed exacerbated inflammation mediated by immune cells and increased release of reactive oxygen species.[90] Thus, GPR43 plays a role in metabolic inflammation.[90]

Several studies have investigated the effects of short-chain carboxylic acids produced by colonic fermentation on the inflammatory state. The presence of short-chain carboxylic acids reduced the presence of TLR4 mRNA leading to reduced active protein levels and subsequent inflammatory responses.[91] Treatment of neutrophils with butyrate, propionate and acetate at concentrations of 3 mM can counteract the release of TNF-a induced by lipopolysaccharide.[92] Similar effects have been noted in cultured colon epithelial cell lines where short-chain carboxylic acids inhibit NF-κB activity.[92] Similar anti-inflammatory responses have been noted in adipose tissue [93] and cultured macrophages where pro-inflammatory cytokine release can be decreased.[93, 94] These results have not been consistent with evidence suggesting that the presence of short-chain carboxylic acids can enhance the TLR2-induced expression of TNF-a and IL-8.[95]

Whole grains with their combination of fibre, protein, vitamins and minerals appear to have protective effects in the body as they are digested more slowly than refined grains and have the potential to stimulate the growth of appropriate gut bacteria. They also generally maintain lower blood glucose concentrations and improved insulin responses in the body. Subjects with high dietary fibre intake have lower blood pressure and serum cholesterol concentrations, reduced glycaemia, improved insulin sensitivity with lower risk of developing stroke, hypertension, type 2 diabetes, obesity and gastrointestinal diseases.[52]

Prebiotic effects on obesity

Obesity is a complex metabolic disorder with impairments in lipid and carbohydrate metabolism. Different prebiotics have been tested for their effects in obesity as the microbiome fermentation products

of prebiotics can affect host metabolism.[96] Many prebiotics alter blood lipid profiles by binding cholesterol and bile acids in the upper gut, increasing sterol excretion and causing a bulking effect in the intestine improving satiety.[38, 97] Animal studies have shown that dietary interventions by prebiotics, especially fructans, inulin and oligofructose, decrease serum triglycerides.[97] A Japanese study found that when Japanese millet was fed to diabetic mice, plasma concentrations of adiponectin and high density lipoprotein cholesterol increased while glucose and triglyceride concentrations decreased.[98] In human studies, decreases in serum triglycerides by inulin and oligofructose are independent of gender, type of prebiotic, background diet, overweight, dyslipidaemia or diabetes.[99] Despite lowering triglyceride and cholesterol concentrations, prebiotic dietary supplementation in both animal and human trials showed inconsistent modulation of lipid concentrations.[99] Of the studies reviewed, 8 out of 21 showed no change in blood lipid concentrations while the rest indicated lower lipid biomarkers (triglyceride, total cholesterol) for cardiovascular disease risk.[99] More studies of prebiotics targeting selected symptoms or disease such as metabolic syndrome and obesity are required.

Several studies have linked diets supplemented with indigestible oligosaccharides (fructo-oligosaccharides, galacto-oligosaccharides and arabinoxylan) to reduced dietary intake in mice [86, 87] and rats[84, 85]. Most studies in mouse and rat models resulted in decreased body weight gain where high-fat diets were administered with supplementation against a high-fat diet alone; however, the decrease in caloric intake was not sufficient to account for the total weight loss.[84, 86, 87] Human studies of fructo-oligosaccharides supplementation have shown promise in obesity attenuation with long-term supplementation leading to reduction in body weight, body mass index and waist circumference in obese women.[100, 101] A study with fructo-oligosaccharies has indicated gender differences in responses with reduced calorie intake in women and increased intake in men.[102] It may be possible to have responsive and non-responsive hosts to galacto-oligosaccharides despite having similar gut microbiomes before the treatment.[103] This trend is likely to be similar for other prebiotics; however the mechanisms and interactions require further investigation. Supplementation with xylo-oligosaccharides and fructo-oligosaccharides in streptozotocin-induced diabetic rats showed that the improved body weight, reduced mortality and increased population of *Bifidobacteria* and *Lactobacilli* in the caecum may be due to a decreased pH with increased concentrations of short-chain carboxylic acids.[104]

Butyrate is rapidly absorbed from the lumen of the cecum to influence glucose and cholesterol concentrations and lipid metabolism. Supplementation with 5% butyrate to high-fat diet-fed mice inhibited the development of obesity and insulin resistance due to reduced adipose tissue,[104] which suggests it may be an effective compound in the treatment of obesity and insulin resistance. Butyrate also has an important function in maintaining metabolism and the proliferation and differentiation of epithelial cells types.[104] Similarly, propionate reduced cholesterol in rats possibly through inhibition of HMG CoA reductase, redistribution of cholesterol from plasma to liver and enhanced synthesis and secretion of bile acids.[105]

The interrelationships between satiety hormones and the gut microbiota are complex; however, these hormones reduce energy consumption and metabolic processes and may be important in the development and attenuation of obesity. Satiety hormones change with administration of prebiotics. Prebiotics appear to have a stimulatory effect on peptide YY secretion[85, 101, 106] and tend to increase glucagon-like peptide-1.[107, 108] This change is associated with better regulation of post-prandial glucose concentrations, improved insulin tolerance and improved satiety.

Conclusions

There is a widespread concept that our genome and our modern diet are mis-matched, with only the genome remaining unchanged since the Neolithic Revolution. Discussion of the optimal human diet to decrease modern diseases such as obesity leads to passionate discussions, with many of the proposals being incompatible. As one example, should we consume high protein, high saturated fat and almost no carbohydrates as in the Atkins diet? Or is the Ornish diet more appropriate, with 80% carbohydrates and minimal animal protein and fat? Would widespread substitution of the modern diet with Palaeolithic or pre-Palaeolithic nutrition decrease the incidence and morbidity of obesity in our communities? If so, this

would argue that our diet should be optimised to the time when the modern human genome stabilised. As we have had the Green Revolution to increase the production of cereals, do we need a Nutrition Revolution to increase wellness? This could include incorporation of the Palaeolithic diets into current nutritional recommendations but this requires a thorough examination of these diets.[108, 109] Is it possible to become a 21st century hunter-gatherer[110] and still have the same quality of life as currently enjoyed in developed countries? To answer these questions, we need a better understanding of the complex relationships between components of the diet, especially prebiotics, and the bacteria of the gastrointestinal tract, the microbiome. Ultimately, this increased understanding will use past nutritional experience to provide the foundation for a healthy future, including decreasing chronic disorders such as obesity.

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S. Carnahan (40%) & A. Balzer (40%) contributed equally to this paper.

1.3 Phloretin Literature Review

Dietary-induced glucose dysregulation is one of the key parameters of metabolic syndrome, an affliction which results in a significant burden to the health care industry. Sugar transport inhibitors have been tested as a potential treatment target for pre-diabetes and type 2 diabetes mellitus (277, 278). Phloretin, a flavonoid isolated from apples, is an inhibitor of solute carrier family 2 (facilitated glucose transporter), member 2 (SCLA2) also called GLUT2 (glucose transporter 2). It is a weaker inhibitor of the sodium/glucose co-transporter 1 (SGLT1). Its glycoside, phlorizin, inhibited sodium/glucose co-transporter 1 and 2 (SGLT1 and SGLT2).

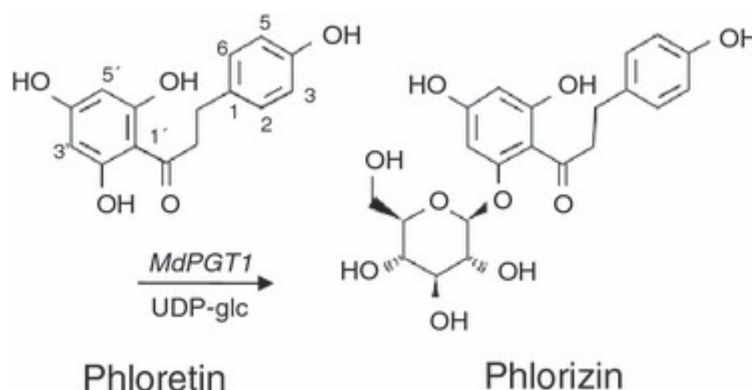


Figure 1.3.18: Structure of phloretin and phlorizin (279)

As functional glucose transporters are present in almost all living cells in the body, the administration of phloretin, and other sugar transport inhibitors, has the potential to inhibit several pathways to diabetes and metabolic syndrome. *In vivo* effects have not been fully studied and the beneficial and detrimental effects on the symptoms of metabolic syndrome have not been elucidated.

Sugar transporters have substrate selectivity. SGLT1 is an active transporter capable of transporting glucose and galactose, present in the intestine tract, kidney, heart, brain, testis and prostate. SGLT2 is also responsible for glucose and galactose transport, however in the kidney, brain, liver, thyroid, muscle and heart (280). SGLT1 is non-mobile and is sodium-dependent (Figure 1.3.19).

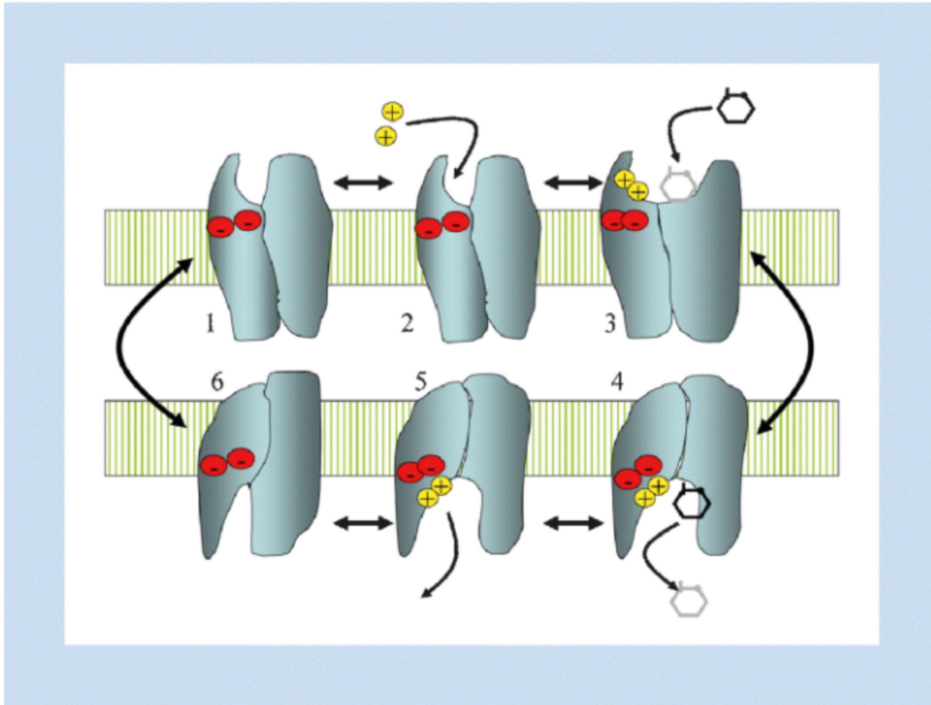


Figure 1.3.19: Function of SGLT1 in glucose transport (280)

GLUT2 is a mobile sugar transport element, which is non-sodium dependent (Figure 1.3.20). It is present in the gastrointestinal tract, pancreas, liver, brain and other parts of the nervous system (281). It is not only responsible for sugar transport of glucose, galactose and fructose (282), but hepatoportal glucose sensing, pancreatic sensing, taste preference and thermoregulation (281).

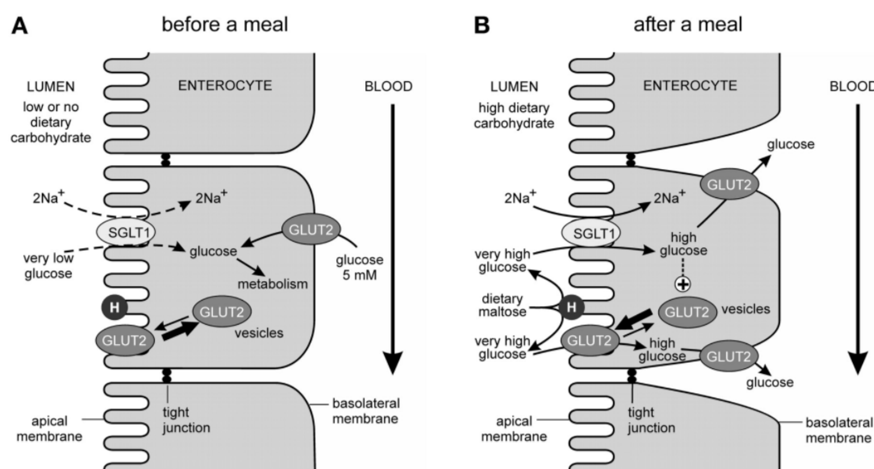


Figure 1.3.20: The role of GLUT2 is gastrointestinal glucose uptake as a mobile element (283)

Flavonoids including quercetin, isoquercetin and myricetin have been implicated in the inhibition of sugar transporters (284). Catechins (flavonols/proanthocyanidins) competitively inhibit SGLT1-mediated transport (285) and are found in sorghum, barley, blueberries, cranberry, chokeberry, apples, peaches, nectarines, pinto beans, chocolate, red wine, and walnuts among others (161). Flavanones also show sugar uptake inhibiting properties. Naringenin inhibits gastrointestinal uptake of glucose (286-288), however eriodictyol can improve glucose uptake in hepatocytes (289) which show benefits for both glucose uptake regulation and peripheral clearance. Flavanones are found in food such as sorghum (153), lemon juice (290), orange juice and grapefruit (291). The flavone apigenin inhibits GLUT1-mediated sugar uptake (292, 293), while luteolin inhibits GLUT2-mediated sugar transport (284). Apigenin and luteolin can be found in sorghum (153), parsley, celery and capsicum (290).

1.3.2 Sugar transporter expression in metabolic dysfunction

1.3.2.1 Gastrointestinal lumen

Phloretin is an inhibitor of GLUT2-mediated glucose uptake, while phloridzin is an inhibitor of SGLT1-mediated uptake in the gastrointestinal lumen (294). SGLT1 and GLUT2 work in conjunction to absorb glucose from the gastrointestinal lumen. SGLT1, unlike GLUT2, is a non-mobile membrane element responsible in part for the apical uptake of glucose and other hexoses from the lumen (295). GLUT2 is primarily responsible for the basolateral flux of glucose and fructose (296). GLUT2 is a mobile element of the sugar transport system, which is present in the apex of brush border cells in response to food ingestion (283). Expression of GLUT2 in the gastrointestinal lumen is typical, however location of the protein is altered under certain metabolic conditions. Translocation of GLUT2 is stimulated by the ingestion of meals and the concentration of glucose in the lumen (282).

Obesity and insulin resistance result in apical presence of GLUT2 in gastrointestinal enterocytes, whereas in lean phenotypes, the primary location is the basolateral membrane, with transient localisation in the apex upon ingestion of foods (297). In diabetic rat models, SGLT2 expression was increased with presence in the brush border membrane of jejunal epithelial cells (298).

1.3.2.2 Pancreas

GLUT2 is the primary glucose sensor of pancreatic β-cells in rats (299). Preceding pancreatic β-cell death, there is an indication of dysregulation of sugar transporter function. Transcription and expression of GLUT2 was decreased in animal models of streptozotocin-induced diabetes (300). GLUT2 had a reduced expression in pancreatic β-cells of diabetic mice, however was reversible at least to some extent (301, 302). Serum non-esterified fatty acids also reduced GLUT2 expression in the pancreas (303). Similarly a high fat diet resulted in reduced pancreatic GLUT2 expression in rats and suggested that a high fat diet, rather than a high-carbohydrate diet, resulted in impaired insulin secretion (304, 305). The loss of GLUT2 appeared to be reversible (306) (301, 302). This reduction was prevented by the administration of a known GLUT2 inhibitor, 5-thio-D-glucose (300). 5-thio-d-glucose acts via competitive inhibition (307) while phloretin acted to inhibit glucose transport by preventing translocation of the GLUT2 from vesicle to the apical and basolateral cell membranes (308). Although the mechanisms differ, the potential for improvement must be investigated.

Rat models indicated that GLUT2 is the primary sugar glucose transporter in pancreatic β-cells, however conflicting evidence exists in human models where GLUT2 does not appear altered with diabetes (309, 310). GLUT1 and 3 are present in higher concentrations and may be the primary glucose transporters or sensing molecules in human islet cells (311). This may have implications for transfer to human models.

1.3.2.3 Liver

GLUT2 is expressed in the liver and, in a normal physiological model, its role is to control blood glucose concentrations (312). The role of GLUT2 in peripheral glucose clearance was primarily in liver tissue (313, 314). Expression of GLUT2 was variable, where expression was dose-dependent with respect to glucose, which suggests a role in glucose metabolism (315). On the contrary, insulin presence had an inhibitory short-term effect on GLUT2 expression in the liver (316). In the liver, it appears that GLUT2 has a role in regulating blood sugar, by releasing glucose into the bloodstream (317) but also for uptake (312).

The expression of GLUT2 in the liver with diabetes and pre-diabetes has not been unequivocally defined. Many researchers suggest that GLUT2 is not affected by the pre-diabetic state (301, 318). However, the expression may fluctuate depending on the insulin and glucose status of the rat (319). Research has suggested that increased PPAR-γ, responsible for adipogenesis, was able to bind the GLUT2 promoter region and upregulate expression, linking the adipogenic state and glucose uptake (320). GLUT2 expression in the liver of streptozotocin-induced diabetic rats was almost doubled, yet was reduced by the presence of phlorizin (313). Similar increases have been seen in Wistar diabetic rats and Zucker fatty rats (321), models of oleic-induced fatty liver (322). While normal fluctuations occur with glucose and insulin in a "healthy" liver, the increases seen in obese and diabetic rats occur independently of these controls, contributing to the dysregulation of glucose control (321). A number of treatments for diabetes correct or improve the elevated liver GLUT2. These have included ginseng (323) and vanadate (324), while other treatments failed to correct the upregulation including pioglitazone (325).

1.3.2.4 Kidney

SGLT1 is present at low levels in the late part of the proximal renal tubule, and SGLT2 is highly expressed in the early proximal renal tubules (326). The primary roles of SGLT1 and SGLT2 in the renal tubules are the re-uptake of glucose with approximately 90% reabsorbed by SGLT2 (327). GLUT2 was significantly upregulated in proximal tubular epithelial cells from patients with type 2 diabetes, when hyperglycaemia was present (328), and in Zucker diabetic rats (329). Similar studies have suggested that this facilitates glucose reuptake under high glucose load in the kidney brush border, which is transient in the diabetic state (330).

Renal uptake inhibitors such as dapaglifozin, a synthetic dihydrochalcone, are effective as inhibitors of renal SGLT2 uptake and in the treatment of diabetes (331).

1.3.3 Phloretin biological activity

Early studies indicated that phloretin was an extremely effective glucose and fructose transport inhibitor in isolated hepatocytes (332). Phloretin inhibited glucose uptake in

erythrocytes with concentrations as low as 1μM inhibiting approximately 20% of glucose uptake, where 72 and 100 percent inhibition were achieved at 10μM and 100 μM (333). Inhibition of glucose transport by 50% at 20μM phloretin has been recorded in adipocytes, however transport can be increased with exposure to insulin (334). Comparative studies indicated that phloretin is a better hexose transport inhibitor than phlorizin in models of hepatocyte transport, where 1mM concentration of phloretin reduced uptake to a greater extent than phlorizin (332). This may be due to the expression or efficiency of the respective transporters the two compounds interact with (283). GLUT2 is the major glucose transporter in the liver, and GLUT2 is inhibited by phloretin (308).

Phloretin ingested in meals typically resulted in circulating conjugated forms of phloretin. Urinary excretion after an acute dose in the form of a meal is estimated to be approximately 10.4% (294). *In vivo* studies indicated that absorption of phloretin occurs primarily in the small intestine, with approximately 25% being absorbed (335). Phloretin is generally considered as a safe food flavouring ingredient by the Flavour Extract Manufacturers Panel (Flavouring Substances 23), number 4390, therefore its ingestion should be of limited concern.

The systemic effects of phloretin with relation to metabolic syndrome have not been fully characterised. Oral ingestion with food is one mechanism by which phloretin may ameliorate the progression of type 2 diabetes mellitus, or alter the manifestation of some of the associated pathologies of metabolic syndrome.

1.3.3.1 Anti-diabetic potential for phloretin

The vast majority of research has focused on the anti-diabetic effects of phloretin due to its sugar transport inhibiting properties.

In vivo, SGLT inhibitors lowered post-prandial blood glucose concentrations in diabetic rats (331, 333). In streptozotozin-induced diabetic rats, phloretin at 25mg/kg/day corrected blood glucose and insulin concentrations to the same extent of glibenclamide (336). The effects on diabetes in streptozotozin-induced rats appeared to be dose dependent, even at low concentrations between 5 and 40mg/kg/day (337).

Some ingested phloretin was converted to its glycoside within the lumen, and prior to absorption into the blood stream (335). Dietary ingestion of phlorizin at 0.05% of the diet reduced fasting glucose concentrations by 23%, and showed that approximately 88% of the dihydrochalcone ingested circulated as the aglycone, phloretin (338). Other studies where phloretin was ingested at 75mg/kg/day resulted in normalised fasting glucose, and greatly reduced glycation of proteins in the kidney and heart, which is a major cause of oxidative stress and diabetic complications (339).

It has been shown that inhibition of GLUT2 can assist in ameliorating loss of its expression in pancreatic β-cells in rat models of streptozotocin-induced diabetes, exhibiting potential for phloretin (300).

1.3.3.2 Anti-lipidaemic potential for phloretin

Dyslipidaemia is one of the pathologies of metabolic syndrome. In a mouse model of dietary induced metabolic syndrome, there was no improvement on blood lipid profiles despite improvements in fat infiltration in the liver (340). In diets of choline-induced hepatic damage and vascular changes that mimic the changes in metabolic syndrome, phloretin at a dose of 200mg/kg reduced triglyceride concentrations and alanine transferase activity. At 400mg/kg/day, phloretin reduced total cholesterol concentrations and liver non-esterified fatty acids, and at 100mg/kg/day, it reduced plasma aspartate transferase activity (341).

1.3.3.3. Cardio-protective potential of phloretin

In vitro, phloretin corrected impaired arterial vaso-relaxation that is caused by human immune-virus protein (342). The cause may differ, however this shows potential in a metabolic syndrome context. Phloretin ingestion has been shown to maintain vascular tone and nitric oxide release (341). Phloretin reduced expression of pro-inflammatory cytokines, in addition to adhesion molecules intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and endothelial leukocyte adhesion molecule-1 (E-selectin) involved in the progression of atherosclerotic lesions (343). Although the study was done in tissue culture, it may be transferrable to *in vivo* applications.

1.3.3.4 Anti-inflammatory potential of phloretin

Inflammation is one of the causes of the progressive decline in pancreatic β-cell mass that leads to type 2 diabetes. Further to this, chronic low-grade inflammation was been linked to multiple pathologies of metabolic syndrome. Inflammation caused progression of the diabetes and apoptosis/necrosis of insulin producing cells (344). Inflammation also caused vascular dysfunction (345), development of liver steatosis markers (346), and progressed atherosclerosis (347). Intestinal barrier function has been linked to the development of metabolic syndrome and systemic inflammation. Studies of colonic epithelial cell lines have shown that exposure to apple polyphenol phloretin results in increased expression of occludin mRNA (348).

There is significant evidence that phloretin has anti-inflammatory activities in *in vitro* studies of LPS-stimulated mouse macrophages as indicated by reduced production of the pro-inflammatory cytokines, IL-6 (interleukin-6), TNFα and prostaglandin E2 (349). Lu*, et al.* (350) indicated suppression of nitric oxide production in T-lymphocytes stimulated by LPS and interferon. Production of pro-inflammatory cytokines was suppressed in cell lines exposed to phloretin (351).

Chapter 2 – Materials and Methods

2.1 Ethics

All animal handling and experimentation was approved by the Animal Ethics Committee (AEC) of the University of Southern Queensland. Experiments were carried out under the AEC approval numbers 13REA005 (10/9/2013-10/9/2015), 15REA001 (11/3/2015-11/3/2017) and 15REA005 (20/7/2015-20/7/2018). All rats were treated and housed as per the NHMRC (National Health and Medical Research Council) 2014 guidelines for the ethical treatment of animals (352). All rats were individually housed in temperature-controlled, 12hr light-dark conditions and provided *ad libitum* access to food and water, except for periods of specific experimentation as indicated in procedural details. Food and water consumption, and body weight were assessed daily.

2.2 Rat Diet and Experimental Structure

Male Wistar rats weighing between 330 and 340g were sourced from the Animal Research Centre (Perth, Australia) and were divided randomly into experimental groups. CS and HCHF diets were as described by Panchal et al (353). The CS diet was composed of 570 g of corn starch (Agri Food Ingredients, Kew East, Victoria Australia), 155 g of powdered rat food (Specialty Feeds, Glen Forest, Western Australia, Australia), 25 g of Hubble, Mendel, and Wakeman salt mixture (MP Biomedicals LLC, Illkirch, France), and 250 g of water per kilogram of diet. HCHF diet consisted of 175 g of fructose (Tate & Lyle ANZ Pty Ltd, Wacol, Qld, Australia), 395 g of sweetened condensed milk (Coles, Australia), 200 g of beef tallow (Carey Brothers Butchers, Warwick), 155 g of powdered rat food (Specialty Feeds, Glen Forest, Western Australia, Australia), 25 g of Hubble, Mendel and Wakeman salt mixture (MP Biomedicals LLC, Illkirch, France) and 50 g of water per kilogram of diet. The energy of the diets were 11.2 kJ/g food for the CS diet and 17.8 kJ/g for the HCHF diet (353). An additional 3.85kJ/mL energy was added to energy calculations for the HCHF diet to account for fructose water intake.

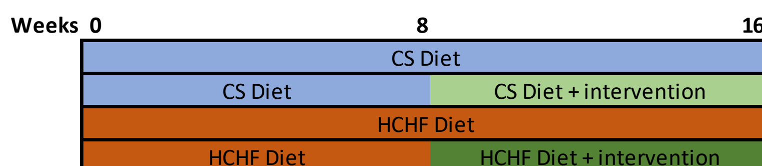
The total metabolisable energy of the mCS diet was determined to be 9.94 kJ/g, and the mHCHF diet was 14.65 kJ/g. An additional 3.85 kJ/mL energy for water intake was added to any energy intake for rats consuming the mHCHF diet or mHCHF with

added interventions. The mCS diet was composed of 570g corn starch (Agri Food Ingredients, Kew East, Victoria Australia), 25g Hubbel, Mendel & Wakemen Salt Mixture (MP Biomedicals LLC, Illkirch, France), 45g skim milk powder (Coles, Australia), 5g Vitamin Diet Fortification Mixture (MP Biomedicals LLC, Illkirch, France), 5g canola oil (Coles, Australia) and 350mL of water per kilogram. mHCHF diets were composed of 395g sweetened condensed milk (Coles, Australia), 175g fructose (Tate & Lyle ANZ Pty Ltd, Wacol, Qld, Australia), 25g Hubbel, Mendel & Wakemen Salt Mixture (MP Biomedicals LLC, Illkirch, France), 45g skim milk powder (Coles, Australia), 5g Vitamin Diet Fortification Mixture (MP Biomedicals LLC, Illkirch, France), 5g canola oil (Coles, Australia) and 200mL of water per kilogram.

Individual dietary inclusions and interventions are described in Chapters 3 through 6. Interventions replaced the water portion of the diet, while all other components remained the same.

Rats were in the protocol for 16 weeks in total, with control rats ingesting the same diet for the duration, and intervention rats ingesting the intervention or supplement in food for weeks 8-16, Figure 2.21.

Figure 2.21: Experimental diet groups.



2.3 Oral Glucose Tolerance

Rats were fasted for 12 hours and all groups were given *ad libitum* access to normal water with no fructose. Blood glucose concentrations were assessed prior to glucose administration and at 30 minute intervals after administration for a period of two hours. Glucose was administered by oral gavage (USQ AEC SWP (University of Southern Queensland Animal Ethic Committee Standard Work Procedure) HP006) at a dose of 2.0g/kg body weight as a 40% D-glucose (Sigma Aldrich, St Louis, USA) solution. Blood glucose concentrations were determined in blood drawn by tail prick (USQ AEC SWP HP001) and analysed using a Medisense Precision Q.I.D glucose meter (Abbott

Laboratories, Bedford, MA). Fasting glucose concentrations and total area under the curve were analysed by one-way and two-way ANOVA (GraphPad Prism 6.0, GraphPad Software, La Jolla, California USA).

2.4 Body Composition (Dual Energy X-Ray Absorptiometry)

Body composition was determined by Dual Energy X-ray Absorptiometry using a Norland XR36 Densitometer (Norland Corporation, Ft. Atkinson, Wisconsin, USA) and Illuminatus 4.2.4a software. Rats were anaesthetised by intra-peritoneal injection of tiletamine HCl 15mg/kg and zolazepam HCl 15mg/kg (Zoletil®; Virbac, Milperra, New South Wales), and xylazine 10mg/kg (Ileum Xylazil-100®, Troy Laboratories) prior to scanning. Rats were scanned at a resolution of 1.5 x 1.5mm at a rate of 30mm/s to determine bone mineral density, bone mineral content, body area, lean mass and fat mass. Outcomes were analysed by one-way and two-way ANOVA (GraphPad Prism 6.0, GraphPad Software, La Jolla, California USA).

2.5 Systolic Blood Pressure

Sedation was achieved by intra-peritoneal injection of 0.1-0.13mL Zoletil® (Virbac, Carros, France) equivalent to tiletamine HCl 10mg/kg and zolazepam HCl 10mg/kg (USQ AEC SWP HP003). Systolic blood pressure was recorded using a MLT1010 Piezoelectric Pulse Transducer connected to an MLT844 Physiological Pressure Transducer and PowerLab data acquisition unit (ADInstruments, Sydney, New South Wales, Australia). An inflatable tail cuff was placed at the base of the tail, adjacent and proximal to the pulse transducer until a steady pulse rate was identified. The tail cuff was inflated to 200mmHg and slowly deflated, to identify the pressure (mmHg) of steady systolic flow return (USQ AEC SWP DP003). Outcomes were analysed by one-way and two-way ANOVA (GraphPad Prism 6.0, GraphPad Software, La Jolla, California USA).

2.6 Termination Process

2.6.1 Euthanasia

Rats were euthanised by intra-peritoneal injection of pentobarbital sodium 325mg/mL (Virbac, Carros, France) at a dose of 100mg/kg body weight. Rats were placed in a soft, dark box to minimise stress of euthanasia. Adequate anaesthesia was determined

by assessment of pedal reflex, tail reflex and palpebral reflex. Immediately following determination of anaesthesia, 200IU ammonium heparin of porcine origin (1.87mg/mL) (Sigma-Aldrich St Louis, USA) was injected through the femoral vein to prevent clotting.

2.6.2 Serum collection

The abdominal cavity was opened and upon exposure of the abdominal aorta, approximately 2mL of blood was drawn using a 5mL endotoxin free syringe (Terumo, Somerset, New Jersey USA) and a 30 gauge x ½ inch endotoxin-free needle (Terumo, Somerset, New Jersey USA). Blood was placed in a 10mL endotoxin-free tube and (Sarstedt Numbrecht, Germany) and held on ice for 20 minutes until clotting had occurred, followed by centrifugation at 5000g for 10 minutes. Endotoxin-free tips (Eppendorff, Hamburg, Germany) were used to aliquot 50µL samples of serum in to endotoxin-free tubes (Astral Scientific, New South Wales, Australia). Samples were stored at -20°C.

2.6.3 Plasma Collection

Upon exposure of the abdominal aorta, approximately 4mL of blood was drawn as noted in method 2.6.2 Serum collection and placed into heparinised tubes (16 I.U/mL lithium heparin) (Sarstedt, Numbrecht, Germany). Plasma was collected following centrifugation at 5000g for 10 minutes and stored at -20ºC.

2.6.4 Left ventricular diastolic stiffness

Left ventricular function was determined at termination by isolated heart preparation (USQ AEC SWP DP006). Prior to termination, the system was equilibrated such that the balloon catheter under normal atmospheric pressure with 500µL of fluid was equal to zero (0) mmHg (millimetres of Mercury).

Hearts were removed with approximately 5-10mm of aorta attached and intact. Hearts were perfused (Figure 2.21) in modified Krebs-Henseleit bicarbonate buffer containing 119.1mM NaCl, 4.75mM KCl, 1.19mM $MgSO_4$, 1.19mM KH_2PO_4, 25.0mM $NaHCO_3$, 11.0mM glucose and 2.16mM $CaCl_2$. The buffer was oxygenated

by steady infusion with 95% O_2 and 5% CO_2 carbogen gas (BOC Australia, North Ryde, New South Wales) maintained at a temperature of 37ºC.

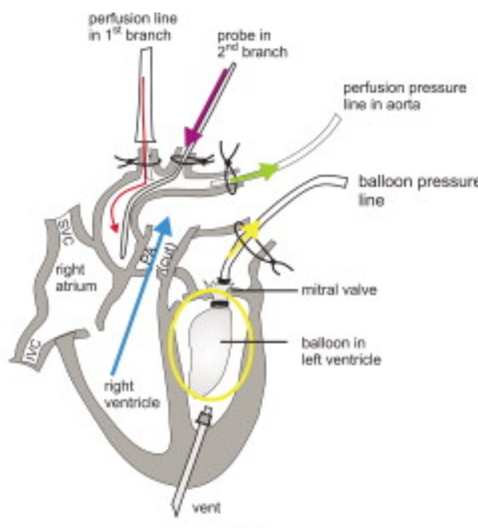


Figure 2.22: Isolated heart perfusion schematic (354).

Iso-volumetric ventricular function was determined by a Capto SP844 MLT844 physiological pressure transducer and LabChart software on a MacLab system (AD instruments, Castle Hill Australia), connected to a latex balloon catheter inserted into the left ventricle of the isolated heart (Figure 2.22). All measurements were performed at an induced heart rate of 250 beats per minute. Pressure-volume relationships were determined at increments from 0 to 30mmHg by injection of water into the latex balloon catheter and determination of the resultant pressure. The end-diastolic stiffness function (κ, unitless) was calculated as described by Brown, Duce, Miric and Sernia (355).

Pressure-volume data were used to generate stress and strain values. Stress was determined assuming sphericity and the hydrostatic pressure exerted on the left ventricle at each volume interval. Outcomes were generated in dynes/cm^2. The differential of the curve generated at each pressure-volume point indicated the stress exerted by the fluid.

Strain was calculated as described by Mirsky and Parmley (356). A line of the formula $y=ae^{bx}$ was generated by the stress-strain relationship, such that the values of *a* and *b* were used to determine the differential equation and plot the tangent of the line at each point. This tangential line is equal to the elastic modulus of the heart at any given

volume. The linear equation of the stress-strain relationship was determined of the formula y= mx + c, where m is equal to the diastolic stiffness constant. Diastolic stiffness values were analysed by one-way and two-way ANOVA (GraphPad Prism 6.0, GraphPad Software, La Jolla, California USA).

2.6.5 Vascular reactivity

Prior to experimentation, all pressure transducers were calibrated to a 15mN weight. A section of descending aorta was collected immediately after removal of the heart and placed in cold Tyrode's buffer (136.9mM NaCl, 5.4mM KCl, 1.05mM $MgCl_2$, 0.42mM NaH_2PO_4, 22.6mM $NaHCO_3$, 1.8mM $CaCl_2$, 5.5mM D-glucose, 0.28mM ascorbic acid and 0.1mM Na_2EDTA) with a steady flow of carbogen gas (BOC, 5% CO_2, 95% O_2). 3-4 mm sections of aorta were suspended in organ baths (schematic, Figure 2.23) equilibrated to 37°C, and rinsed twice in equilibrated Tyrode's buffer. The force generated by contraction or relaxation was measured by a Chart MacLab System (ADInstruments, Castle Hill Australia). Aortic contraction or relaxation responses to sodium nitroprusside and acetylcholine were pre-contracted with a single addition of noradrenaline resulting in a 2 micromolar noradrenaline concentration in the organ bath; and allowed to stabilise to >95% maximum contraction. Cumulative concentration-response curves were generated for noradrenaline, sodium nitroprusside or acetylcholine at half-log units from $1x10^{-9}$mol/L to $3x10^{-5}$mol/L or until no further contractions were observed. Outcomes were analysed by one-way and two-way ANOVA (GraphPad Prism 6.0, GraphPad Software, La Jolla, California USA).

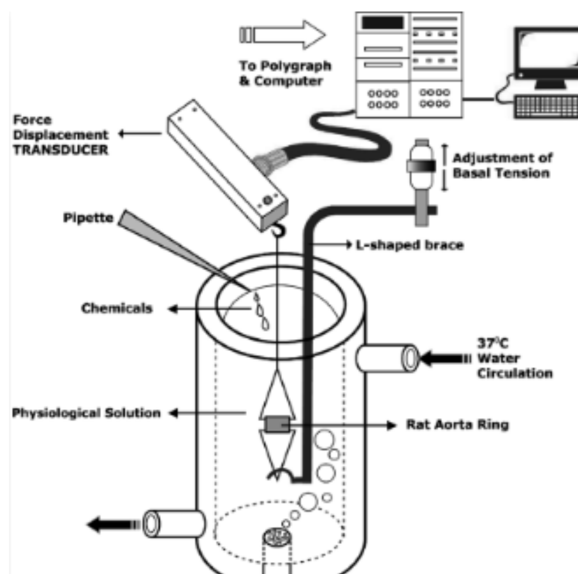


Figure 2.23: Schematic of organ baths used for assessment of aortic vascular reactivity (357).

2.6.6 Ileum and colon reactivity

Approximately 10mm sections of ileum and proximal colon were collected from euthanised rats and washed gently in cold Tyrode's buffer. Ileal sections were taken leaving approximately 10mm proximal to the ileocecal valve, and colon sections were collected approximately 10mm distal to the cecocolic valve. Remaining chyme and faeces were removed using cold buffer and syringe with gentle washing. Sections were strung to a tension of ~10mN in Tyrode's buffer in 25mL organ baths, equilibrated to 37°C, oxygenated by carbogen gas (BOC, 5% CO_2, 95% O_2). Force of contraction was measured by a Chart MacLab System (ADInstruments, Castle Hill Australia). Maximal force of contraction (mN tension) was measured at half-log increases in acetylcholine from $1x10^{-9}$mol/L to $3x10^{-5}$mol/L in a concentration-response manner or until no further increases in contraction force occurred. Tissues were washed twice in equilibrated Tyrode's buffer between each addition and allowed to equilibrate at approximately 10mN tension. Outcomes were analysed by one-way and two-way ANOVA (GraphPad Prism 6.0, GraphPad Software, La Jolla, California USA).

2.6.7 Organ weights (normalised to tibial length) and tissue collection

The wet weights of organs (kidneys, liver, spleen, epididymal fat, omental fat, retroperitoneal fat, left ventricle plus septum and right ventricle) were determined and normalised against the tibial length of the individual rat. Tibial length was measured to the nearest 10th of a millimetre using vernier callipers. Outcomes were analysed by one-way and two-way ANOVA (GraphPad Prism 6.0, GraphPad Software, La Jolla, California USA).

All samples were washed in cold (4°C) Tyrode's buffer and placed in a sterile 1.5mL screw cap tube and placed on ice immediately. Samples were stored at -80°C long term. Sections of colon were taken from the distal colon containing the 3 terminal faecal pellets, rinsed in Tyrode's buffer and stored similarly.

2.7 Plasma Biochemistry

2.7.1 Plasma ALT and AST activity

Plasma was analysed for aspartate transaminase (AST) and alanine transaminase (ALT) activity using an Olympus AU400/AU480 analyser (Olympus, Tokyo, Japan) at The University of Queensland Veterinary School, Gatton with commercial kits and controls (Olympus OSR6107 kinetic UV test & Olympus OSR6109 kinetic UV test). Creatine kinase and lactate dehydrogenase were assessed using kinetic UV tests OSR6179 and OSR6128. All tests were carried out as per manufacturer's instructions. Outcomes were analysed by one-way and two-way ANOVA (GraphPad Prism 6.0, GraphPad Software, La Jolla, California USA).

2.8 Plasma Lipid Profile

Plasma was collected as described in method 2.6.3. Lipid concentrations were determined using an Olympus AU400 analyser. Plasma concentrations of total cholesterol were determined using an Olympus OSR6216 enzymatic colour test and triglycerides were determined using an Olympus OSR6133 enzymatic colour test. Plasma NEFA (non-esterified fatty acids) were determined by a Wako Diagnostics HR Series NEFA-HR enzymatic kit (Wako, Osaka, Japan). Outcomes were analysed by one-way and two-way ANOVA (GraphPad Prism 6.0, GraphPad Software, La Jolla, California USA).

2.9 Histology analyses

2.9.1 Tissue Collection

Colon sections were taken immediately proximal to the cecocolic junction and were approximately 10mm in length. Ileum samples were collected immediately anterior to the ileocecal junction and were approximately 10mm in length. Samples of kidney, liver, spleen, pancreas, retroperitoneal fat, ileum and colon were washed in cold Tyrode's buffer and placed immediately in 10% neutral buffered formalin, pH 7.0. Samples were stored in formalin for 72-96 hours prior to processing.

Prior to processing, either automated or manual, sections were removed from the formalin solution and sectioned to no greater than 1.0 x 0.5cm sections. Samples were placed in labelled tissue cassettes and stored in 100% ethanol until processing.

2.9.2 Manual Tissue Processing

Manual tissue processing was carried out by removing residual formalin in running tap water for 20 minutes. The following sequence was carried out to fix sections: 45 minutes in 70% ethanol; 45 minutes in 90% ethanol; 45 minutes in 100% ethanol, 45 minutes in 100% ethanol, 45 minutes 100% in xylene and 45 minutes in 100% xylene. After removal from xylene, sections were placed in wax at 60°C overnight. Sections were embedded in paraffin wax at 60°C and allowed to cool completely prior to sectioning.

2.9.3 Automated Tissue Processing

Automated processing was carried out using a Thermo Scientific™ Shandon™ Pathcentre™ Tissue Processor (ThermoFisher Waltham, MA, USA). The following sequence was carried out: 1 hour each 70%, 90%, 95%, 100% x 3 washes of ethanol, followed by re-hydration with xylene 3 times. Samples were then soaked in four sets of wax (Tissue Embedding Medium (Paraplast® Regular, melting point 56°C) under vacuum for 1 hour each.

Sections were allowed to remain in water at 60°C under a vacuum of 1000 millibar overnight before embedding into wax blocks (Paraplast® Regular, melting point 56°C).

2.9.4 Tissue Sectioning

Paraffin sections were sectioned to 5µm using a Leica Microtome (Wetzlar, Germany). 3-5 concurrent sections were placed in a 60°C water bath and were collected on to a Superfrost™Plus (ThermoFischer Waltham, MA, USA) slide and allowed to dry at room temperature prior to storage.

2.9.5 Staining

Prior to staining, sections were dewaxed and rehydrated by pre-heating sectioned slides to 60°C for 15 minutes. Dewaxing was carried by 3 subsequent washes in 100% xylene. Rehydration was carried out by 2 consecutive washes in 100% ethanol, single washes in 90% ethanol, 70% ethanol and running tap water. All washes were carried out for 2 minutes.

2.9.5.1 Hematoxylin and Eosin Staining

Liver, left ventricle, ileum, colon, kidney, skeletal muscle, and pancreas were stained. Hematoxylin (Sigma Aldrich, Darmstadt, Germany) was prepared at a concentration of 6.4g/L in a solution of 20% ethanol, 16% glycerol and 0.6% aluminium persulfate, and allowed to stand for 6 weeks in the dark prior to use. Eosin Y (Sigma Aldrich, Darmstadt, Germany) was prepared at a concentration of 10g/L in 90% ethanol. Prior to use, the solution was diluted 1:1 in 90% ethanol.

After dehydration, samples underwent the following sequence: hematoxylin (6 minutes), running water (2 minutes), 70% ethanol (2 minutes), eosin Y (7 minutes), 95% ethanol (2 minutes), 100% ethanol (2 minutes, 3 times), 100% xylene (2 minutes, 3 times). After removal of excess xylene, slides were fixed with a cover-slip using DPX Mountant for histology (Sigma Aldrich, St Louis, USA).

2.9.5.2 Picrosirius red staining

Only left ventricular sections were stained for collagen. Picrosirius red stain (specific for type I and III collagen) was prepared at a concentration of 1.0g/L Sirius Red (Sigma Aldrich, St Louis, USA) in saturated picric acid.

After dehydration, samples underwent the following sequence: 0.5% phosphomolybdic acid (5 minutes), running water (2 minutes), 0.1% picrosirius red

(90 minutes), 0.1M hydrochloric acid (2 minutes), 95% ethanol (2 minutes), 100% ethanol (2 minutes, 3 times) and 100% xylene (2 minutes, 3 times). After removal of excess xylene, slides were fixed with a cover-slip using DPX Mountant for histology (Sigma Aldrich, St Louis, USA).

2.9.6 Microscopy and histological analysis

2.9.6.1 Liver Histology Imaging

Liver sections were imaged at 20x magnification using a standard white light for inflammatory cell presence and steatohepatitis indicators of ballooning, and fat deposition (steatosis).

2.9.6.2 Picrosirius left ventricular histology imaging

Picrosirius red slides were imaged using a UV light source and narrow band emission filter, to allow green light of the wavelengths 510-560nm to pass. Two non-overlapping fields per section were imaged. ImageJ (National Institute for Health, United States of America) software was used to determine the percent collagen deposition. Outcomes were analysed by one-way and two-way ANOVA (GraphPad Prism 6.0, GraphPad Software, La Jolla, California USA).

2.9.6.3 Pancreatic islet imaging

Pancreatic α and β cell density was determined by counting the number of nuclei in an islet, stained with hematoxylin and eosin. Counts were carried out using ImageJ (National Institute for Health, United States of America) by selecting the periphery of the islet and counting nuclei in an 8 bit image. Outcomes were analysed by one-way and two-way ANOVA (GraphPad Prism 6.0, GraphPad Software, La Jolla, California USA).

2.9.6.4 Gastrointestinal histology imaging

All gastrointestinal images were taken at a 10x magnification. A representative section was chosen to visualise any differences. Due to the highly variable nature of sectional morphology and microscale differences, no statistical analyses were performed on these sections.

2.10 Endotoxin analysis

Plasma endotoxin concentrations were assessed using the Lonza QCL1000 Limulus Amebocyte Lysate (LAL) kit (Lonza Basel, Switzerland) as per manufacturer's instructions. All reagents were allowed to equilibrate to room temperature prior to preparation. Reference endotoxin was prepared by diluting endotoxin stock solution with supplied LAL endotoxin-free reagent water to produce a concentration of 1.0EU/mL. A serial dilution was carried out to produce standard EU (endotoxin unit) concentrations of 1.0, 0.5, 0.25 and 0.1 EU/mL. 96 well endotoxin-free (Techno Plastic Products, Trasadingen, Switzerland) plates were pre-equilibrated to 37ºC. In each well, 50µL of limulus amoebal lysate and 50µL of sample (plasma 1:10 dilution), blank (LAL reagent water) or standard (1.0, 0.5, 0.25, 0.1 EU/mL) was added. Each well was mixed by pipetting. Samples were prepared in duplicate. Plates were allowed to incubate at 37ºC for 10minutes. 100µL of chromogenic substrate solution (*p*-nitroalanine, 2mM) was added to each well and allowed to incubate at 37ºC for 6 minutes. The reaction was then stopped by the addition of 100µL of concentrated acetic acid in pyrogen-free water. Absorbance was read at 405nm using a FLUOStar Omega (BMG Labtech Offenburg, Germany).

Absorbance was assessed against reactions of a standard curve of *E. coli* between 0.1 and 1.0EU/mL endotoxin concentration. Outcomes were analysed by one-way and two-way ANOVA (GraphPad Prism 6.0, GraphPad Software, La Jolla, California USA).

2.11 Intestinal permeability

2.11.1 Urine collection

For consistency, all starvation periods were initiated at approximately 6am each day. During intestinal permeability, rats were housed in individual metabolic cages and urine collected with separation from faeces. Initially rats were food-deprived for 4 hours, during which time all rats had *ad libitum* access to normal tap water. At 4 hours, rats were dosed with 2.0 mL of probe containing 0.5 g/mL sucrose, 0.04 g/mL mannitol, 0.06 g/mL lactulose and 0.03 g/mL sucralose by oral gavage (USQ AEC SWP HP006). Following probe administration, water was withdrawn for 3 hours. Collection tubes were replaced at 3 hour, 8 hour and 20 hour intervals post-probe

administration. Collection was done in to 15mL tubes (Sarstedt Numbrecht, Germany) containing 100µL of 50mg/mL thymol in 1-propanol to retard bacterial growth. Food and water were returned *ad libitum* at 3 hours post-gavage, however all rats received normal water regardless of treatment group. Samples were stored at -20ºC until further analysis.

2.11.2 Urine sample preparation and analysis

Samples were thawed on ice. Volumes of urine were recorded to the nearest 100µL. A 2.0mL sample was removed for further analysis and centrifuged at 12000rpm for 20 minutes at 4°C. Samples were the filtered through a 0.45µm polytetrafluoroethylene (PTFE) (Grace Davidson Discovery Sciences, Maryland USA) into a 2.0mL glass screw-cap vial (ThermoScientific Massachusetts, USA), closed with a 0.25mm PTFE septum. Samples were analysed by HPLC-RID (high-performance liquid chromatography-refractive index detection).

The system comprised a Shimadzu (Kyoto, Japan) DGU-20ASR degassing unit, LC-20AT solvent delivery unit, SIL-20A Autosampler, CBM-20A communication bus module, CTO-20A column oven, RID-10A refractive index detector. The chromatography conditions were as follows: isocratic elution using 100% Milli-Q water, 10µL injection volume, 0.300mL/minute flow rate, 50°C column oven. Detection was by RID (refractive index detection). Percent recovery of each sugar for each rat was determined and used in analysis. Concentration of urine was assessed against an external curve generated for each compound. Concentration was multiplied by the total urine volume collected for each time period and determined as the percent recovery of the total amount of administered sugar. Outcomes were analysed by one-way and two-way ANOVA (GraphPad Prism 6.0, GraphPad Software, La Jolla, California USA).

2.11.3 Urine chromatography and analysis

Each component was run individually to determine retention times. Retention times are shown in Table 2.9.

Table 2.9: Retention times of lactulose, sucrose, D-mannitol, and sucralose.

Compound	Retention time (minutes)
Lactulose	18.341
Sucrose	20.452
D-Mannitol	22.41
Sucralose	27.349

Linearity was determined by preparation of solutions at 5 concentrations between 0.02mg/mL and 2mg/mL. This curve was also used as the external curve for determination of sugar concentration. A contaminant of urine origin, determined by running blank urine samples, was found at elution time of 12 minutes however did not interfere with peaks identification of quantitation (Figure 2.24).

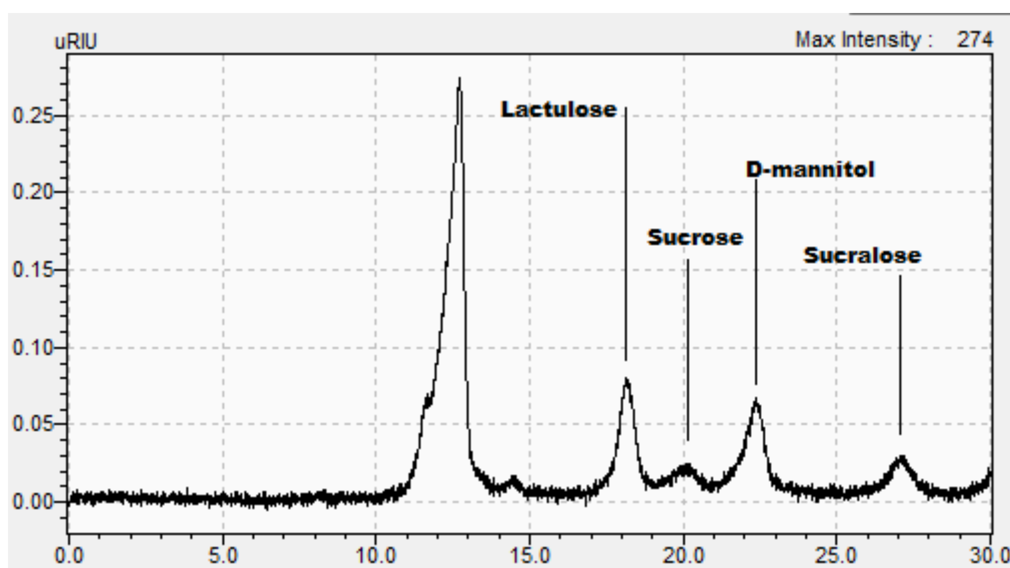


Figure 2.24: Urine sample example chromatogram. Initially, a mixture of the gavage compounds was diluted to 1:100 in distilled water. This was used for optimisation of separation.

2.12 Semi-quantitative ELISA (enzyme-linked immunosorbent assay)

2.12.1 Protein Extraction

100mg of tissue samples were homogenised in RIPA buffer (150 mM sodium chloride, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris, pH 8.0) in 1% Halt^{TM} EDTA-Free Protease Inhibitor (ThermoScientific Massachusetts, USA). Samples were homogenised for 2 minutes with a GentleMacs dissociator (Miltenyi Biotech, Bergisch Gladbach, Germany), and mixed for 2 hours on ice. Supernatants were retained after centrifugation at 12000g for 20 minutes (4°C), then stored at -80°C. Protein concentration was determined using a BCA (bicinchoninic acid) assay kit (ThermoScientific Massachusetts, USA).

2.12.2 Protein Quantification

Protein quantification was carried out using a ThermoScientific Pierce BCA protein Assay. Colon samples were diluted 1/10, and liver samples were diluted 1/20 for analysis. A BSA (bovine serum albumin) standard were prepared to manufacturer's instruction at 2000, 1500, 1000, 750, 500, 250, 125, 25 and 0 µg/mL. Working reagents A (sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1M sodium hydroxide) and B (containing 4% cupric sulfate) were combined at a ratio of 50:1.

25µL of diluted sample or standard were added to each well of a 96 well TPP (Techno Plastic Products, Trasadingen, Switzerland) plate in duplicate. 200µL of working reagent was added to each well and incubated for 20 minutes at 37°C. Cooled plates were read using a FLUOStar Omega (BMG Labtech Offenburg, Germany) plate reader at 562nm. The sample's protein concentration was determined from the standard curve.

2.12.3 GLUT2 and TLR4 determination

To determine relative GLUT2 and TLR4 (toll-like receptor-4) expression in liver, 0.2µg/mL rabbit polyclonal antibody (Santa Cruz Biotechnology, Dallas Texas, USA) was prepared in coating buffer (15mM Na_2CO_3, 35mM $NaHCO_3$, pH 9.6) and 100µL added to each well of a TPP 96 well cell culture plate (Techno Plastic Products, Trasadingen, Switzerland). Plates were coated overnight at 25°C in a ShelLab

incubator (Sheldon Manufacturing Inc. Cornelius, USA). Each well was washed 3 times with 200µL of Tris-buffered saline (TBS) (25mM Tris-HCl; 0.13M NaCl; 2.7mM KCl; pH 7.0). Liver protein extracts were diluted to a concentration of 100µg/mL in sterile distilled water based on the BCA assay. 10µg in 100µL was loaded per well. Positive control was a combined liver sample and the negative control was 10µg/100µL BSA in sterile water. Plates were coated for 2 hours at 25°C for two hours, then washed 3 times per well with TBS. 0.2µg/mL primary antibody in sterile distilled water was loaded per well (100µL) and allowed to bind for 2 hours at 25°C. Each well was then washed 3 times with TBS. Goat anti-rabbit IgG-HRP (immunoglobulin-G horseradish peroxidase) conjugated secondary antibody was diluted to 0.1µg/mL and 100uL added to each well and allowed to incubate at 25°C for two hours. Each well was washed 3 times with TBS. Substrate solution was prepared by diluting 10mg/mL OPD (o-phenylenediamine dihydrochloride) (Sigma Aldrich, Darmstadt, Germany), in 10x Stable Peroxide Buffer (ThermoScientific Massachusetts, USA) diluted 10x in distilled water. 100uL per well was added and allowed to mix at room temperature for 15 minutes on an orbital shaker. Plates were then read using a FLUOStar Omega (BMG Labtech, Offenburg, Germany) plate reader to measure absorbance at 450nm. Analysis was based on the AU reading after deduction of the negative control value for each plate. Outcomes were analysed by one-way and two-way ANOVA (GraphPad Prism 6.0, GraphPad Software, La Jolla, California USA).

The detection of the protein was determined to be linear across the range of 0-1000ug/mL as indicated in Table 2.10. Percent relative standard deviation (%RSD) was determined using three replicates at each of the concentrations across the linear curve range.

Table 2.10: Linearity and % relative standard deviation of a serial dilution of protein, assessed for GLUT2 and TLR4 protein expression.

	Linear range 0-1000µg/mL	Regression value	% RSD range
GLUT2	y = 5.8975x + 0.181	0.9588	5.92-11.90%
TLR4	y = 8.9176x + 0.1604	0.9537	4.63-11.7%

2.12.4 Occludin and claudin-1 determination

To determine relative occludin expression in colon, 0.2µg/mL rabbit polyclonal antibody (Santa Cruz Biotechnology, Dallas Texas, USA) was prepared in coating buffer (15mM Na_2CO_3, 35mM $NaHCO_3$, pH 9.6) and 100µL added to each well of a clear, Greiner Bio-one (Kremstunster, Austria) 96 well polystyrene plate. Claudin-1 was treated similarly using a mouse monoclonal antibody (Santa Cruz Biotechnology, Dallas Texas, USA).

Plates were coated overnight at 25°C in a ShelLab incubator (Sheldon Manufacturing Inc. Cornelius, USA). Each well was washed 3 times with 200µL of Tris-buffered saline (TBS) (25mM Tris-HCl; 0.13M NaCl; 2.7mM KCl; pH 7.0). Colon protein extracts were diluted to a concentration of 100µg/mL in sterile distilled water based on the BCA assay. 10µg in 100µL was loaded per well. Positive control was a combined liver sample and the negative control was 10µg/100µL BSA in sterile water. Plates were coated for 2 hours at 25°C for two hours, then washed 3 times per well with TBS. 0.2µg/mL primary antibody in sterile distilled water was loaded per well (100µL) and allowed to bind for 2 hours at 25°C. Each well was then washed 3 times with TBS. Goat anti-rabbit IgG-HRP conjugated secondary antibody was used for detection of occludin, diluted to 0.1µg/mL and 100uL added to each well and allowed to incubate at 25°C for two hours. Mouse anti-rabbit IgG-HRP was similarly used for claudin-1 detection. Each well was washed 3 times with TBS. Substrate solution was prepared by diluting 10mg/mL OPD (o-phenylenediamine dihydrochloride) (Sigma Aldrich, Darmstadt, Germany) in 10x Stable Peroxide Buffer (ThermoScientific Massachusetts, USA) diluted 10x in distilled water. 100uL per well was added and allowed to mix at room temperature for 15 minutes on an orbital shaker. Plates were then read using a FLUOStar Omega (BMG Labtech Offenburg, Germany) plate reader to measure absorbance at 450nm. Analysis was based on the AU (atomic unit) reading after deduction of the negative control value for each plate. Outcomes were analysed by one-way and two-way ANOVA (GraphPad Prism 6.0, GraphPad Software, La Jolla, California USA).

The detection of the protein was determined to be linear across the range of 0-1000ug/mL as indicated in Table 2.11. Percent relative standard deviation was

determined using three replicates at each of the concentrations across the linear curve range.

Table 2.11: Linearity and % relative standard deviation (precision) of a serial dilution of protein, assessed for occludin and claudin-1 protein expression.

	Linear range 0-1000µg/mL	Regression value	% RSD range
Occludin	y = 40.097x + 0.6529	0.9900	10.40-21.71%
Claudin-1	y = 48.598x + 0.286	0.9797	4.22-20.27%

2.13 Sorghum extract analysis

2.13.1 Sorghum phenolic extraction

5.0g of whole sorghum, red sorghum flour, black sorghum flour or wet cake sorghum was weighed into 50mL Falcon tubes (Sarstedt Numbrecht, Germany). Samples were prepared in triplicate. Sorghum was extracted by addition of 10.0mL of 1% acetic acid in 100% methanol. Sorghum was mixed on a platform shaker for 2 hours and allowed to stand with intermittent mixing for 4 hours. Samples were centrifuged for 10 minutes at 3000*g* and supernatants retained. Extraction was repeated three times and supernatants pooled. Samples were evaporated to dryness and stored at room temperature until analysis.

2.13.2 Sorghum phenolic analysis

Samples were analysed by HPLC-DAD (high performance liquid chromatography – diode array detector) at 210nm, 330nm and 510nm. Approximately 1mg of sample was re-suspended in 1% acidified methanol. Samples were analysed by Southern Cross University Plant Science Analytical Research Laboratory (Lismore, New South Wales).

Chapter 3 – 5% Wholegrain sorghum in a rat model of diet-induced metabolic syndrome

3.1 Introduction

Poor diet and lack of physical activity are the major contributors to the development of the non-communicable disease of metabolic syndrome (358). Prevention and reversal of the disease rely heavily on changing dietary habits (359). Changes in metabolic function have been noted with dietary changes sych as switching to a Mediterranean diet (360, 361) or Nordic diet (362). A wholegrain-based diet improved metabolic disease with reduced insulin fluctuations, improved glucose regulation and lowered triglyceride concentrations (363-365). Changing diet is effective in reversing metabolic syndrome, and understanding the benefits of certain foods will allow better education of the public.

The improved changes noted with wholegrain consumption have led to a wider variety of cereal products becoming popular for human consumption. Sorghum products have entered the Australian marketplace for human consumption in products like GlutenFree WeetBix (Sanitarium), Gluten Free Flour (Bobs Red Mill), Pearled Sorghum (SorgPlus) and Red Sorghum Puffs (Friendship Foods), and its inclusion into rice mixes, breakfast cereals and muesli bars.

Consumption of sorghum in Australia has previously been limited to livestock animals (16). Overseas, the use of sorghum differs. According to FAO (16), in 2011, only 41,000 tonnes of the domestic supply of sorghum in Australia of 1,959,000 tonnes was used in food manufacturing, representing 2.11%, and none as a direct food source for human consumption. Comparatively, in countries such as Nigeria, approximately 75% of sorghum is used as a direct food source with an additional 2.36% used in food manufacturing (16). The prevalence of metabolic syndrome between these countries vastly differs, as do their overall diets. The rural Nigerian community which consumes a more traditional diet has an estimated metabolic syndrome rate of 5% in females and 8% in males by the IDF (International Diabetes Federation) criteria in 2015 (366), while in Australia the rates were 29.1% by these IDF criteria in 2015 (359).

The physiological effects of sorghum consumption have been investigated predominantly in chickens and beef cattle (243-245). In livestock, the aim is to maximise weight gain and feed efficiency for profitability. However, in mono-gastric animals, such as pigs and broilers, sorghum is detrimental for maximising weight gain (243-245). The factors inhibiting the conversion of ingested sorghum to body weight include the large starch granules, inhibitory proteins around starch granules, and the presence of phytochemicals such as tannins and phenolic acids. Studies on mono-gastric nutrition of livestock discuss the anti-nutritive properties of sorghum (103, 274, 367). Although the term "anti-nutritive" may be a deterrent toward consumption in humans, these components may improve the physiological status of individuals following sorghum consumption.

These components, although detrimental in commercial livestock production, may be useful in humans to circumvent the increasing obesity and metabolic syndrome epidemic in Western societies. The coloured compounds in sorghum have anti-inflammatory actions (168, 253). In obesity and metabolic syndrome, alleviation of the inflammation can minimise long-term pathologies associated with being overweight including impaired glucose tolerance, dyslipidaemia, liver steatosis, high blood pressure and poor cardiovascular function (219, 240, 368-371).

Sorghum contains compounds which may reduce the digestion of proteins and carbohydrates *in vitro* including flavonoids, phenolic acids, tannins and kafirin proteins (43, 54, 55, 59). These compounds have differing bio-accessibilities and bio-availabilities, however many of the bioactive compounds appear in the plasma after dietary supplementation. Phenolic acids are bioavailable, however larger procyanidins (flavonoids, condensed tannins) and catechins undergo bacterial degradation in the colon prior to absorption (162). 3-deoxyanthocyanidins, one of the most effective antioxidant components of sorghum, and related anthocyanins are bioavailable and appear in plasma after ingestion (372).

A diet high in fat increases metabolic endotoxaemia, leading to a cascade of inflammatory responses which progress to metabolic syndrome (373, 374). These diets have been linked to both dysbiosis in the gut and increased permeability of the colon to lipopolysaccharides (373). Consumption of resistant starches and non-starch

polysaccharides reduced diet-induced endotoxaemia (375). Consumption of wheat-derived arabinoxylans improved metabolic endotoxaemia and improved the reductions in colonic tight junction mRNA in animal models (220). The effects on endotoxaemia with consumption of wholegrain cereals containing these carbohydrates have not been widely studied. Reviews speculate that protection from type 2 diabetes with wholegrain consumption is mediated by the large intestine (218, 376, 377). LPS is a known cause of metabolic syndrome (238), and activator of TLR4, one of the innate immune receptors (219). Some evidence suggests that components of sorghum reduce LPS mediated inflammation (168, 378), yet little information is available on whether sorghum reduces the total plasma concentration of LPS.

Components of wholegrain sorghum have theoretical potential to alleviate the pathologies of metabolic syndrome. This chapter investigates whether a supplement of 5% sorghum into an obesogenic diet alters the pathology of metabolic syndrome in a rat model of diet-induced metabolic syndrome.

3.1.1 Hypothesis

My hypothesis is that consumption of 5% sorghum in the diet will correct dyslipidaemia, improve liver structure and function, improve cardiovascular health, and improve glucose tolerance.

One of the mechanisms for this improvement is the reduction of circulating lipopolysaccharides concentration. This will result in changes to TLR4 expression in the colon and liver. There will be a reduction in gastrointestinal permeability with sorghum consumption and that will be linked to an increased expression of colonic tight junction proteins.

3.2 Materials and Methods

3.2.1 Ethics

Approval for experimentation with 5% sorghum was granted under AEC approval number 13REA005 (10/9/2013-10/9/2015) by the Animal Ethics Committee of the University of Southern Queensland. Please refer to chapter 2.1 for further information.

3.2.2 Rat Diet and Experimental Structure

Forty-eight male Wistar rats were divided into 4 experimental groups of n=12. Control diets were comprised as outlined in method 2.2. Experimental groups were CS (corn starch diet), HCHF (high carbohydrate, high fat diet), CSb (corn starch diet + 5% w/w *Sorghum bicolor*), HSb (high carbohydrate, high fat diet + 5% w/w *Sorghum bicolor*).

For the first 8 weeks of the protocol, all CS and CSb rats received the CS diet and all HCHF and HSb rats received the HCHF diet as described by Panchal et al (353), detailed in Chapter 2. Energy contents of the diets were 11.2 kJ/g food for the CS diet, 17.8 kJ/g for the HCHF diet (353), 11.98 kJ/g for CSb diet and 18.58 kJ/g for HSb diet, based on an energy density for sorghum of 361 kJ/100g. An additional 3.85 kJ/mL was added to calculations for the HCHF and HSb diets for the fructose in the drinking water.

At eight weeks, CSb rats were transferred to the treatment diet which included supplementation with 5% raw red sorghum, and similarly HSb treatment rats were placed on the HCHF diet supplemented with 5% raw red sorghum. Red sorghum was sourced from Crest Seeds (Toowoomba, Queensland) and is a commercially available stock feed supplement.

After eight weeks, systolic blood pressure and oral glucose tolerance were determined prior to dietary intervention, and again after 8 weeks (at 16 weeks after start of protocol) on continuous supplementation.

At 16 weeks, rats were assessed for the following parameters, with corresponding methods indicated in parentheses; oral glucose tolerance (method 2.3), dual x-ray absorptiometry (method 2.4), systolic blood pressure (method 2.4) and intestinal permeability urine collection (method 2.10.1). All rats were food-deprived for 2 hours prior to termination. Rats were euthanised as per method 2.6.1. At termination, rats underwent serum collection (method 2.6.2) plasma collection (method 2.6.3), vascular reactivity (method 2.6.5), ileum and colon contractility (method 2.6.6), measurement of organ weights (method 2.6.7) and histological tissue collection (method 2.8.1). A minimum of 8 rats per group underwent isolated heart perfusion (method 2.6.4), with the remaining two hearts preserved in formalin for histological analysis as per method

2.8.1. Plasma biochemistry was assessed on 8 rats per group for liver enzyme activity (method 2.7.1) and plasma lipid profile (method 2.7.2). Collected serum was analysed for presence of endotoxins as per method 2.9. Liver sections were assessed for expression of GLUT2 and TLR4 (method 2.11.3). Distal colon sections were also assessed for occludin and claudin expression using the method in method 2.11.4, and TLR4. Urine samples collected prior to termination were prepared (method 2.10.2) and analysed as per method outlined in method 2.10.3.

Sorghum phenolics were extracted from 5.0g of whole sorghum (method 2.12.1) and analysed as in method 2.12.2.

Histological tissue samples were processed manually (method 2.8.2) and sectioned (method 2.8.3). Liver, ileum, colon, pancreas and left ventricle were stained with haematoxylin and eosin (method 2.8.5.1). Separate sections of left ventricle were stained with picrosirius red (method 2.8.5.2). Microscopy and imaging were performed as per methods 2.8.6.1, 2.8.6.2, 2.8.6.3 and 2.8.6.4.

3.2.3 Statistics

Statistics were performed using GraphPad Prism (GraphPad Software, La Jolla, California USA). Individual groups were compared using a one-way ANOVA. Where data were normally distributed, a normal one-way ANOVA was used. Where data were not normally distributed, a non-parametric one-way ANOVA was used. Tests for normality were done using the D'agostino-Pearson normality test. Two-way ANOVA was carried out, where indicated, to determine the influence of the two categorical variables of "diet" (CS or HCHF), or "intervention" (5% sorghum, no sorghum).

Multiple comparisons were carried out comparing all groups to test the following null hypotheses:

There is no difference in the outcome of metabolic syndrome parameters between the CS, CSb, HCHF and HSb-fed groups at a given time point for a given parameter.

For two-way ANOVA, we tested the following null hypotheses:

There is no effect of diet on metabolic syndrome parameters,

There is no effect of sorghum on the metabolic syndrome parameters, and

There is no interaction between diet and sorghum, in affecting metabolic syndrome parameters.

3.3 Results

3.3.1 Sorghum extraction

Whole sorghum extracts showed a range of compounds extracted by 1% acidified methanol.

Detection of flavonoids can be carried out using a 210nm diode array absorbance (379). Furthermore, it will also detect compounds containing carboxylic acid, propylamide or ester fucntional groups (380). Acidified methanol extracts of whole red sorghum flour detected at 210nm (Figure 3.25) showed that there were 3 major compounds present at 2 minutes, 20.3 minutes and 20.8 minutes. The peak present at 1.1 minute represents acetic acid in all 3 chromatograms of Figure 3.25, 3.26, 3.27.

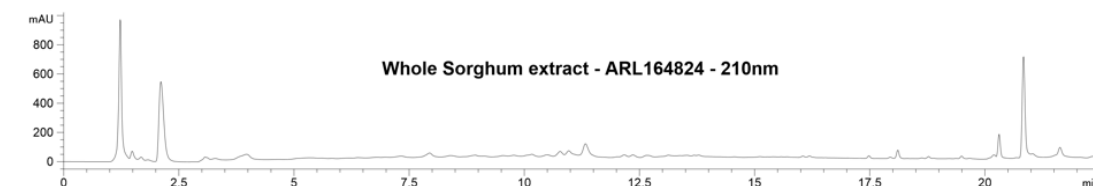


Figure 3.25:210nm UV detection of HPLC of acidified methanol extracts of whole red sorghum. 210nm detection detects primarily hydrophilic compounds.

The 330nm profile (Figure 3.26) primarily indicates phenolic acids which are present (381). This wavelength is also suitable for detection of flavones (153) and flavonols (379).

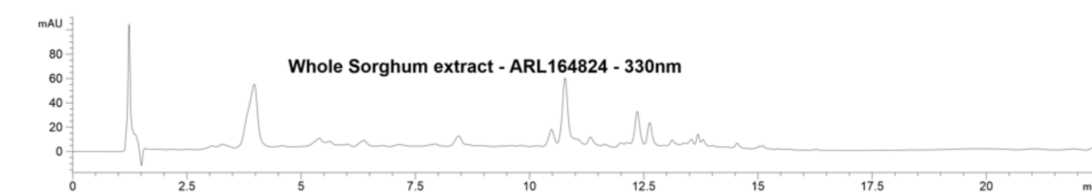


Figure 3.26: 330nm UV detection of HPLC separated acidified methanol extracts of whole red sorghum. 330nm detection primarily indicates presence of phenolic acids.

Detection of anthocyanins can be carried out by diode array absorbance at 510nm (379). Very few anthocyanin compounds appeared to be present in the extracts, with a minor peak at 7.5 minutes.

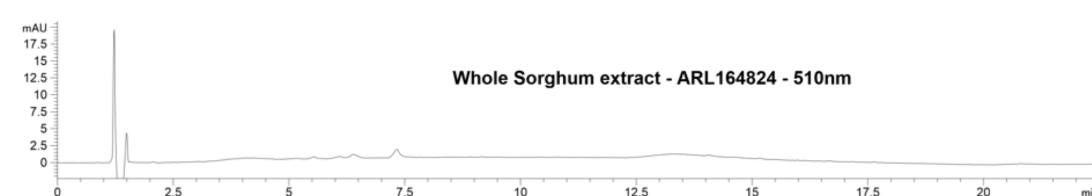


Figure 3.27: 510nm UV detection of HPLC extracts of whole red sorghum. 510nm detection primarily indicates anthocyanin presence.

3.3.2 Physiological and metabolic parameters

After 8 weeks of the protocol, there were no differences in tested parameters between the HCHF and HSb groups, nor the CS and CSb groups, Table 3.12. The HCHF diet groups had higher body weight, increased basal blood glucose, impaired glucose tolerance, higher energy intake, lower food weight intake, and higher feed efficiency.

Table 3.12: Physiological and metabolic variables of CS, CSb, HCHF and HSb fed rats.

Parameter	CS	Csb	HCHF	HSb	Diet	Sorghum	Interaction
Average weight gain week 8-16(g)	30.8 ± 3.0 ^{a}	42.08 ± 5.0 ^{a}	80.42 ± 7.2 ^{a}	62.55 ± 9.5 ^{ab}	<0.0001	0.6183	0.0306
Body weight week 8 (g)	349.9 ± 5.45 ^{a}	355.8 ± 4.58 ^{a}	449.5 ± 8.46 ^{b}	450.7 ± 7.6 ^{b}	<0.0001	0.6044	0.7296
Final body weight week 16 (g)	380.8 ± 6.8 ^{a}	397.8 ± 6.6 ^{a}	529.9 ± 12.1 ^{b}	508 ± 11.4 ^{b}	<0.0001	0.8017	0.0475
Food intake week 1-8 (g/day)	36.6 ± 1.1 ^{a}	37.1 ± 0.8 ^{a}	25.4 ± 0.5 ^{b}	24.5 ± 0.7 ^{b}	<0.0001	0.8507	0.377
Food intake week 8-16 (g/day)	36.3 ± 1.0 ^{a}	35.3 ± 1.0 ^{a}	24.3 ± 0.6 ^{b}	21.5 ± 0.6 ^{b}	<0.0001	0.0266	0.2681
Energy intake week 1-8 (kJ/day)	410.4 ± 12.4 ^{a}	416.9 ± 9.1 ^{a}	540.6 ± 12.3 ^{b}	509.3 ± 13.2 ^{b}	<0.0001	0.2992	0.1184
Energy intake week 8-16 (kJ/day	407.4 ± 10.7 ^{a}	423.2 ± 11.9 ^{a}	524.7 ± 13.3 ^{b}	483.6 ± 11.7 ^{b}	<0.0001	0.2969	0.0218
BMI (g/cm^2)	0.59 ± 0.01 ^{a}	0.62 ± 0.02 ^{ab}	0.75 ± 0.03 ^{c}	0.67 ± 0.02 ^{bc}	<0.0001	0.2649	0.0155
Feed efficiency week 8-16 (g gained/kJ)	1.4 ± 0.1 ^{a}	1.8 ± 0.2 ^{ab}	2.7 ± 0.2 ^{c}	2.3 ± 0.3 ^{bc}	<0.0001	0.9495	0.0777
Epididymal fat (mg/mm tibial length)	172.0 ± 11.8 ^{a}	183.0 ± 16.5 ^{a}	483.0 ± 53.6 ^{b}	435.0 ± 35.6 ^{b}	<0.0001	0.5954	0.3855
Retroperitoneal fat (mg/mm tibial length)	96.4 ± 8.8 ^{a}	105.0 ± 7.5 ^{a}	231.0 ± 24.7 ^{b}	219.0 ± 14.8 ^{b}	<0.0001	0.9364	0.5183
Omental fat (mg/mm tibial length)	123.0 ± 6.2 ^{a}	145.0 ± 11.7 ^{a}	251.0 ± 23.2 ^{b}	248.0 ± 15.6 ^{b}	<0.0001	0.528	0.4147
Abdominal fat pad mass (mg/mm tibial length)	390.5 ± 22.8 ^{a}	433.6 ± 34.0 ^{a}	964.2 ± 98.9 ^{b}	902.4 ± 60.7 ^{b}	<0.0001	0.8802	0.3997
Bone mineral density (g/cm^2)	0.179 ± 3.E-03 ^{ab}	0.17 ± 2.E-03 ^{a}	0.187 ± 1.E-03 ^{b}	0.184 ± 2.E-03 ^{b}	0.0001	0.027	0.2928
Bone mineral content (g)	11.75 ± 0.28 ^{a}	11.71 ± 0.35 ^{a}	16.53 ± 0.51 ^{b}	15.69 ± 0.61 ^{b}	<0.0001	0.3423	0.3873
Lean Mass (g)	276.8 ± 6.71 ^{a}	276.9 ± 8.28 ^{a}	269.3 ± 14.29 ^{a}	278.1 ± 13.7 ^{a}	0.7814	0.6952	0.7017
Fat Mass (g)	111.2 ± 6.4 ^{a}	114.0 ± 12.6 ^{a}	252.2 ± 29.8 ^{b}	223.9 ± 25.0 ^{b}	<0.0001	0.5427	0.4586

Results are expressed as mean±SEM. Values with the same superscript letter do not differ statistically at $p<0.05$ determined by one-way ANOVA. Effect of diet, intervention or interaction at $p<0.05$, determined by two-way ANOVA.

Sorghum supplementation did not alter final body weight or body weight gain in either the CS or HCHF model determined by one-way ANOVA. There was a significant interaction of diet and 5% sorghum supplementation, whereby the direction that body weight in the two diet models were opposing. These results appear consistent with the significant interaction effect noted in body mass index (BMI) associated with sorghum supplementation. The lean body mass was maintained in all groups. Food intake by weight was not altered with sorghum supplementation, nor was energy intake by one-way ANOVA. Two-way ANOVA showed a significant effect of sorghum and diet on week 8-16 feed intake, and interaction between diet and sorghum for energy intake, Table 3.12.

3.3.3 Blood lipids, and glucose control

At 8 weeks, the HCHF and HSb groups showed increased basal blood glucose concentrations and increased glucose TAUC. At 16 weeks, basal blood glucose concentrations were increased in the CSb group, above the value of the CS control. Sorghum normalised TAUC to the CS control in the HSb group (Table 3.13). No changes in fasting blood glucose concentrations were found with consumption of whole red sorghum.

Pancreatic islet density, islet size and β-cell count did not differ between groups, Figure 3.28.

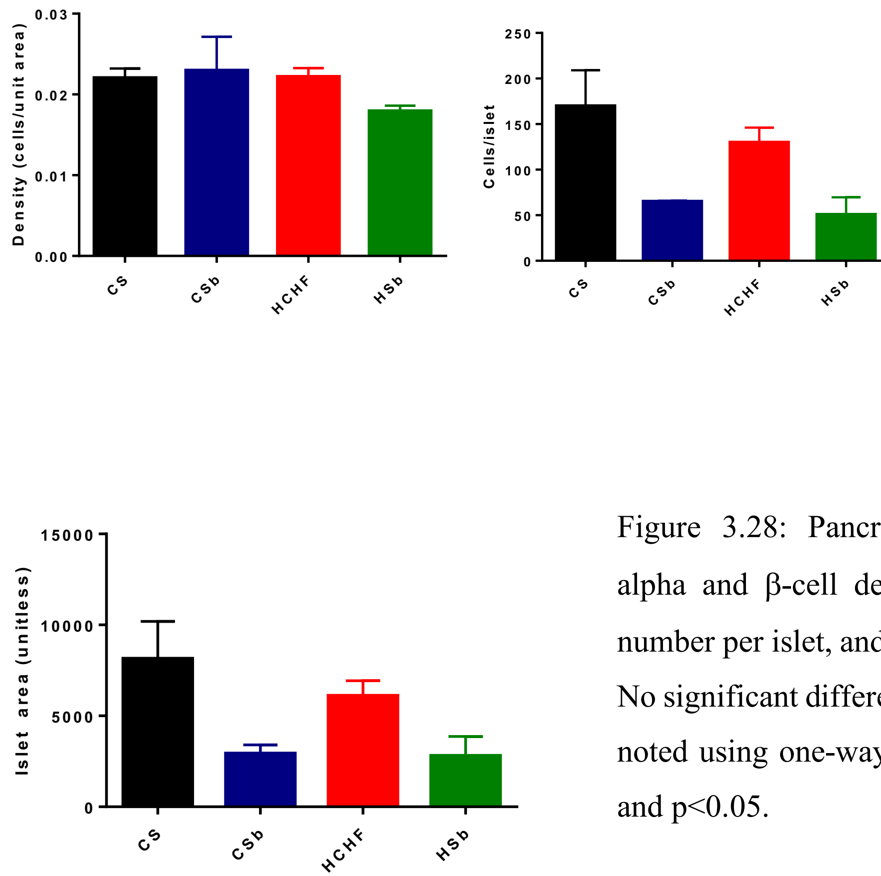


Figure 3.28: Pancreatic islet alpha and β-cell density, cell number per islet, and islet area. No significant differences were noted using one-way ANOVA and $p<0.05$.

Table 3.13: Blood glucose and lipid profiles with sorghum supplementation in a HCHF and CS diet.

	CS	**CSb**	**HCHF**	**HSb**	**Diet**	**Sorghum**	**Interaction**
Basal blood glucose 8 week (mM)	3.6 ± 0.3 ^{a}	3.5 ± 0.10 ^{a}	5.4 ± 0.08 ^{b}	4.6 ± 0.19 ^{ab}	<0.0001	0.0197	0.0651
TAUC 8 week	615.2 ± 17.05 ^{a}	688.4 ± 34.97 ^{a}	805.2 ± 23.70 ^{b}	775.6 ± 14.17 ^{b}	<0.0001	0.4618	0.0888
Basal blood glucose 16 week (mM)	3.6 ± 0.18 ^{a}	4.3 ± 0.21 ^{ab}	4.8 ± 0.21 ^{b}	4.6 ± 0.19 ^{b}	0.0004	0.2575	0.0304
TAUC 16 week	660.8 ± 11.76 ^{a}	681.5 ± 17.23 ^{a}	847.8 ± 35.1 ^{b}	736.3 ± 14.12 ^{a}	<0.0001	0.2544	0.0504
Plasma total cholesterol (mM)	1.46 ± 0.04 ^{a}	1.54 ± 0.05 ^{a}	1.79 ± 0.08 ^{b}	1.51 ± 0.06 ^{a}	0.0191	0.1085	0.0702
Plasma triglycerides (mM)	0.45 ± 0.03 ^{a}	0.44 ± 0.04 ^{a}	1.6 ± 0.33 ^{b}	1.96 ± 0.16 ^{b}	<0.0001	0.3551	0.3223
Plasma NEFA (mM)	1.84 ± 0.20 ^{a}	2.16 ± 0.17 ^{a}	5.02 ± 0.61 ^{b}	5.49 ± 0.36 ^{b}	<0.0001	0.3041	0.8408
Serum LPS (EU/mL)	1.63 ± 0.84 ^{a}	2.26 ± 0.58 ^{a}	0.93 ± 0.23 ^{a}	1.61 ± 0.47 ^{a}	0.2069	0.1970	0.9651

Letters on each row with differing superscript lower-case letters were statistically different at $p<0.05$ by one-way ANOVA. Significant effects of diet, intervention and interaction determined by two-way ANOVA. TAUC – total area under curve, LPS – lipopolysaccharide, NEFA – non-esterified fatty acids.

Plasma triglycerides and NEFA (non-esterified fatty acid) concentrations were increased in the HCHF diet from the CS control. The addition of 5% raw sorghum to the HCHF diet normalised plasma total cholesterol concentrations to the CS control, as shown in Table 3.13, however did not affect NEFA or triglycerides.

3.3.4 Cardiovascular and liver parameters

Liver weight was 50-60% higher in the HCHF and HSb groups than the CS control (Table 3.14). Liver weight was unchanged by sorghum consumption. There was a decrease in the indicators of liver steatohepatitis with the administration of 5% raw sorghum into the HCHF diet, determined by histology. This primarily came from a reduction in the steatosis of liver cells (Figure 3.29), reduced cell size, and apparent lack of fatty deposits. In HCHF rats, steatosis was typically macro-vesicular and very pronounced. There was no reduction in inflammatory cells with 5% sorghum supplementation, shown in Figure 3.29. Plasma ALT and AST activities were not different between any groups (Table 3.13).

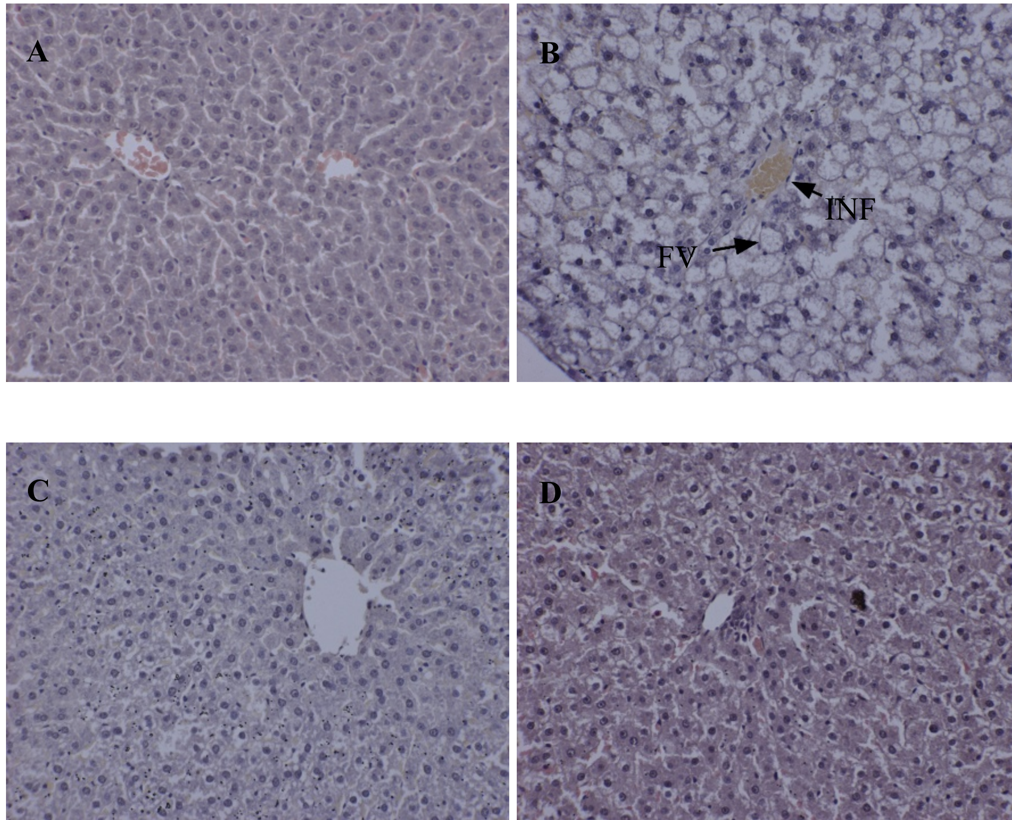


Figure 3.29: CS (A), HCHF (B), CSb (C), HSb (D). Examples of haematoxylin and eosin-positive inflammatory cells are indicated by "INF", and examples of fat vacuoles are indicated by "FV".

Log-transformed TLR4 expression in the liver was lower in the CS and CSb groups. Expression of TLR4 was increased in the HSb groups compared to HCHF, Figure 3.30. two-way ANOVA indicated a significant effect of diet (<0.0001), 5% whole sorghum (0.0167), and no interaction effect (0.3947).

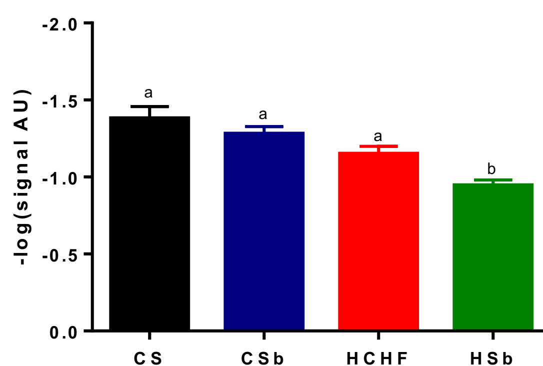


Figure 3.30: TLR4 Expression in the liver determined by semi-quantitative ELISA. Differing lower-case superscript letters indicate significant difference at $p<0.05$, determined by one-way ANOVA.

GLUT2 liver values were tested for outliers. One value was removed from the CS group, while two each were removed from the HCHF, CSb and HSb groups. Log-transformed data showed that only the HSb group had increased GLUT2 expression, higher than the two control groups (Figure 3.31). Two-way ANOVA indicated that only intervention was a significant main effect (p=0.008).

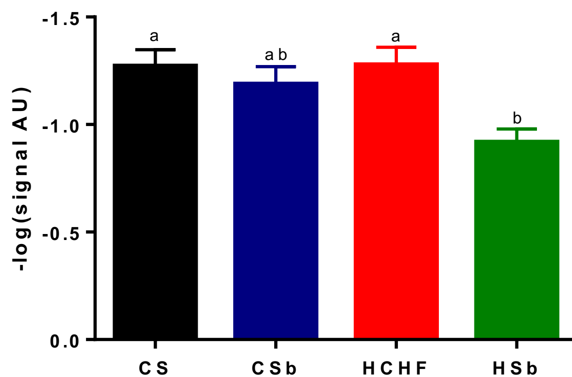


Figure 3.31: GLUT2 expression in the liver determined by semi-quantitative ELISA. Differing lower-case superscript letters indicate significant difference at p<0.05, by one-way ANOVA.

Left ventricle and septum, and right ventricle wet weights were increased in the high fat feeding model, but were not corrected by the 5% sorghum supplement (Table 3.14). Systolic blood pressure was increased by HCHF feeding, and not changed by sorghum. Collagen deposition in the heart did not differ between any groups. These changes are consistent with there being no difference in diastolic stiffness with 5% raw sorghum consumption, although correlation between the diastolic stiffness and collagen deposition was not significant (r^2=0.3293, p=0.4261), as shown in Figure 3.32 and Figure 3.33.

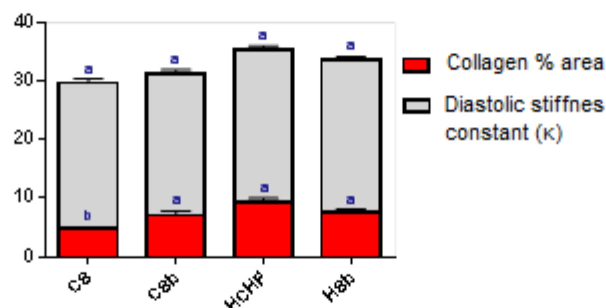


Figure 3.32: Diastolic stiffness constant (κ, unitless), and picrosirius red area %, ventricular sections of CS, HCHF, CSb and HSb. Different lower-case letters indicate a significant difference at p<0.05 by one-way ANOVA.

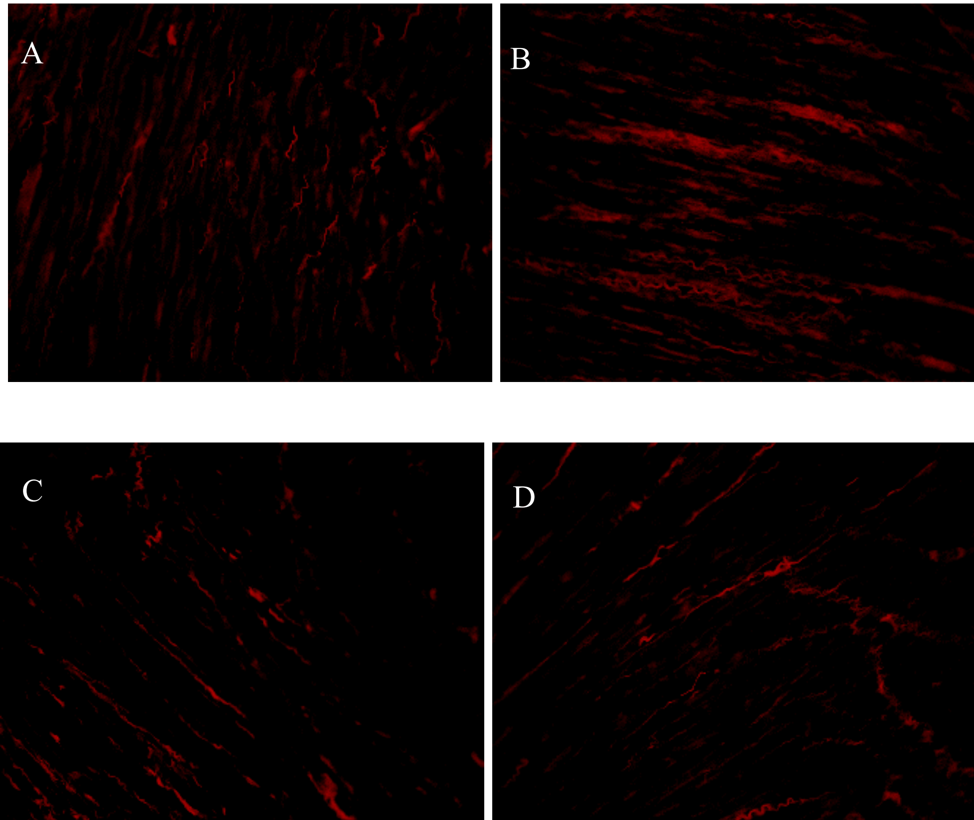


Figure 3.33: Left ventricular sections of CS (A), HCHF (B), CSb (C) and HSb (D) stained with picrosirius red and illuminated under polarised light at 510-560nm at 20x magnification.

Table 3.14: Hepatic and cardiovascular parameters of CS, CSb, HCHF and HSb-fed rats at 16 weeks of protocol.

	CS	CSb	HCHF	HSb	Diet	Sorghum	Interaction
Liver wet weight (mg/mm tibial length)	215 ± 5 ^{a}	222 ± 7 ^{a}	339 ± 23 ^{b}	298 ± 8 ^{b}	<0.0001	0.1965	0.068
Plasma ALT (U/L)	27.0 ± 2.0 ^{a}	25.4 ± 2.2 ^{a}	26.4 ± 1.4 ^{a}	23.1 ± 1.3 ^{a}	0.4173	0.1734	0.6458
Plasma AST (U/L)	70.4 ± 5.0 ^{a}	61.9 ± 2.1 ^{a}	67.8 ± 7.1 ^{a}	60.1 ± 4.0 ^{a}	0.6593	0.1117	0.9296
Systolic blood pressure (mmHg)	136 ± 2.0 ^{ab}	131 ± 1.0 ^{a}	140 ± 1.8 ^{b}	142 ± 1.5 ^{b}	0.0006	0.2648	0.0642
Left ventricle + septum (mg/mm tibial length)	20.1 ± 0.9 ^{a}	20.9 ± 1.0 ^{ab}	23.8 ± 0.5 ^{b}	22.4 ± 0.6 ^{ab}	0.0018	0.7269	0.1483
Right ventricle (mg/mm tibial length)	4.2 ± 0.3 ^{a}	4.6 ± 0.3 ^{ab}	5.32 ± 0.2 ^{b}	5.42 ± 0.2 ^{b}	0.0005	0.339	0.5469

Differing superscript, lower-case letters indicate a significant difference at $p<0.05$ determined by one-way ANOVA. Significant sources of variation are indicated in the far left three columns determined by two-way ANOVA.

Aortic relaxation in response to acetylcholine was impaired in the HCHF model compared to the CS. This was improved with the addition of 5% raw sorghum in the HCHF model, with no detrimental effects when sorghum was added to the CS diet (Figure 3.34). Diet and sorghum were major sources of variation in the noradrenaline curve based on a two-way ANOVA, however statistical significance was not reached between the HCHF and CS model responses. Maximal force of contraction in response to noradrenaline was increased in the HSb from HCHF. No difference was found for the potency or maximal responses between any groups for responses to noradrenaline, sodium nitroprusside or acetylcholine.

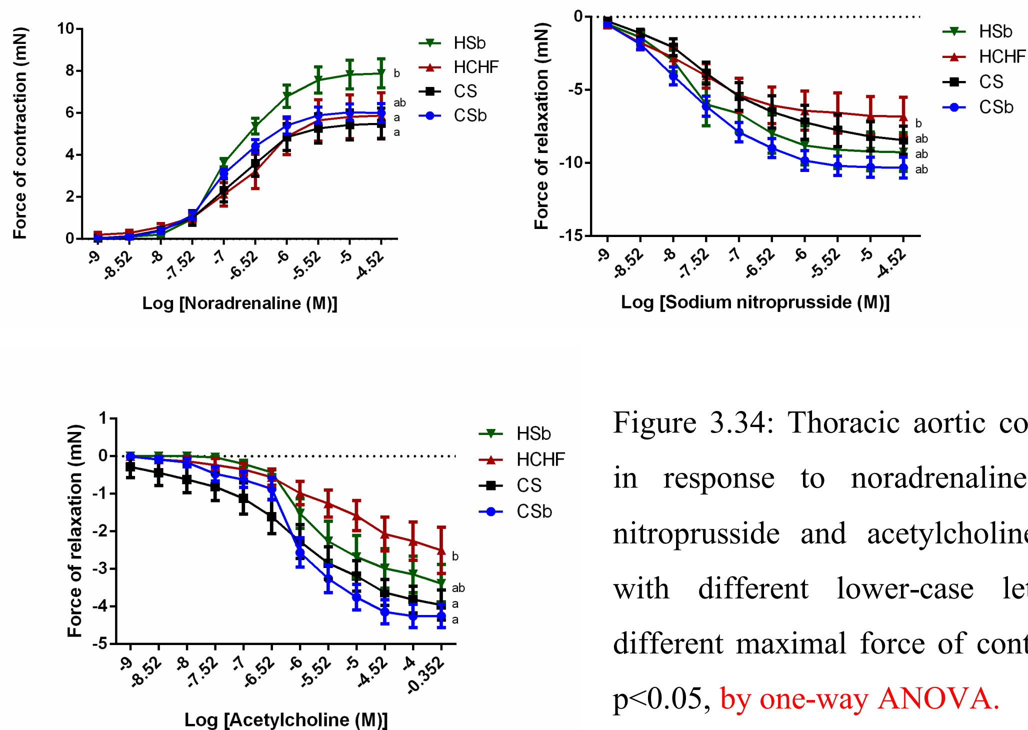


Figure 3.34: Thoracic aortic contractions in response to noradrenaline, sodium nitroprusside and acetylcholine. Values with different lower-case letters had different maximal force of contraction at $p<0.05$, by one-way ANOVA.

3.3.5 Gastrointestinal structure and function

No effects of diet were evident in ileal reactivity to acetylcholine (Figure 3.35). A HCHF diet increased maximum contraction force in the colon, and the presence of sorghum in the CS diet reduced the colonic reactivity to acetylcholine (Figure 3.35). Potency did not differ between any diet groups for either gastrointestinal section.

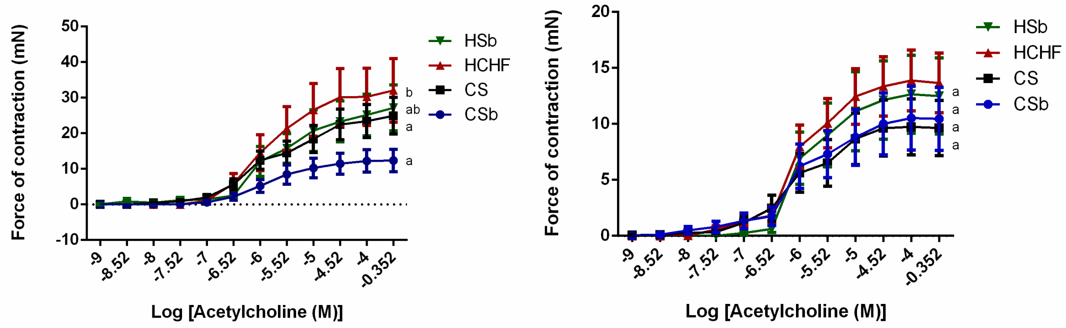


Figure 3.35: Left; *ex vivo* proximal colon contractility in response to acetylcholine stimulation. Right; *ex vivo* ileal contractility in response to acetylcholine stimulation.

Results shown are mean ±SEM, lines showing differing lower-case letters had a significantly different maximal force of contraction, by one-way ANOVA $p<0.05$.

Sucrose acted as a representative test for permeability of the stomach, with lactulose and mannitol as tests for the permeability of the stomach and small intestine, and sucralose as a measure for the entire gastrointestinal tract. Recovery of sugars in the urine was higher in the HCHF model for lactulose, sucralose, mannitol and sucrose for the 8-20 hour time period, Figure 3.36. Only lactulose recovery differed between the HCHF and HSb groups, where recovery in the 8-20 hour bracket was higher in the HSb group. Intervention with 5% sorghum in the CS diet did not affect the urinary recovery of any sugar (Figure 3.36 and Figure 3.37).

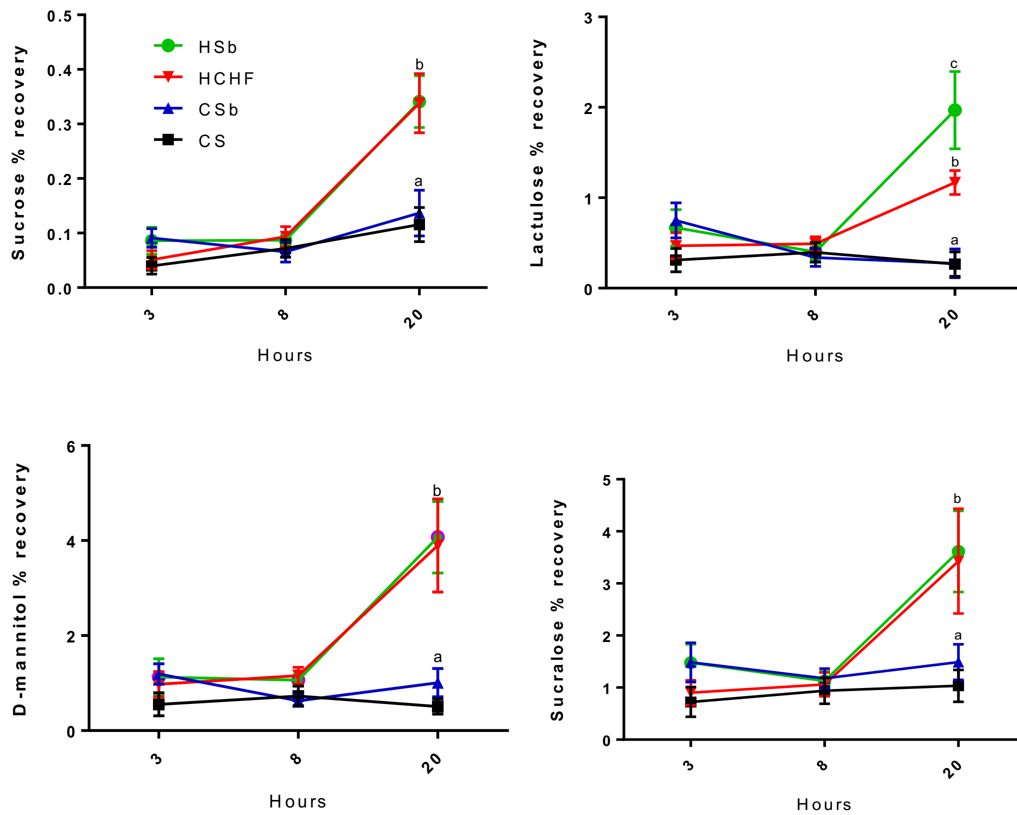


Figure 3.36: Percent recovery of sugars in urine over time. Differing lower-case letters indicate significant difference at p<0.05, determined by two-way ANOVA.

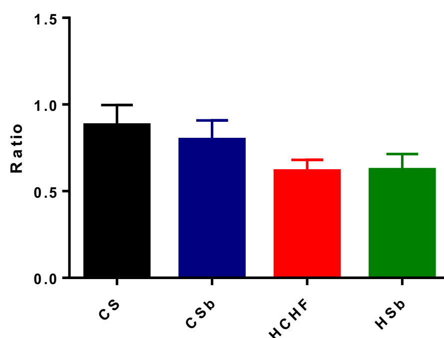


Figure 3.37: Ratio of lactulose and mannitol recovery. No differences were found between any groups.

Identification of outliers for occludin expression resulted in removal of two values for the CS, CSb, and HSb groups, and one from the HCHF group. Expression of occludin was increased in CSb from CS control. CS and HCHF did not differ and no effect of sorghum on occludin expression occurred in the high fat diet.

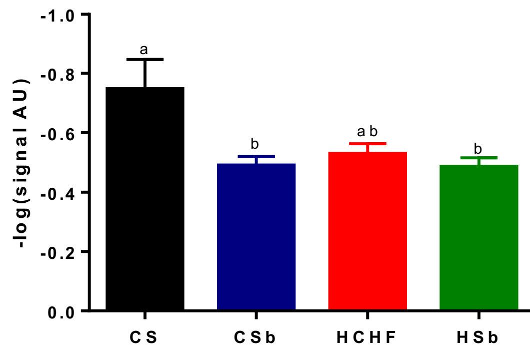


Figure 3.38: Occludin expression in the colon determined by semi-quantitative ELISA. Differing lower-case superscript letters indicate significant difference at $p<0.05$, by one-way ANOVA.

Two outlier values were removed from the CSb group data of claudin-1 expression. Claudin-1 expression was unchanged by diet or intervention with sorghum.

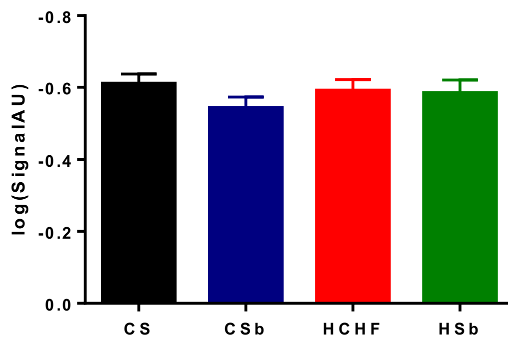


Figure 3.39: Claudin-1 expression in the colon determined by semi-quantitative ELISA. Differing lower-case superscript letters indicate significant difference at $p<0.05$.

One outlier was removed from the HCHF data, and two from the HSb TLR4 data. TLR4 expression did not differ between the CS and HCHF diets. Addition of sorghum showed no significant change in either diet.

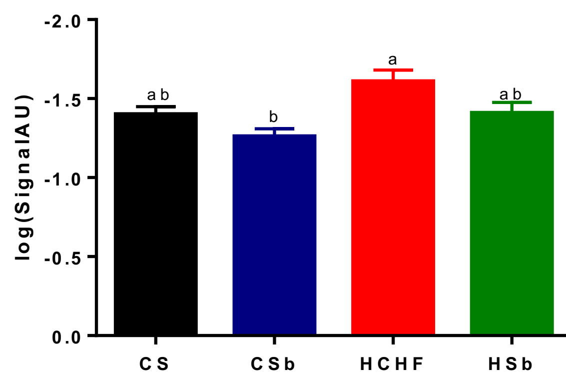


Figure 3.40: TLR4 expression in the colon determined by semi-quantitative ELISA. Differing lower-case superscript letters indicate significant difference at $p<0.05$ by one-way ANOVA.

Occludin and colon expression changes did not correlate with the changes in sugar recovery from the intestinal permeability analysis, as shown in Table 3.15.

Table 3.15: Pearson's correlation P-values indicating no significant correlation between urinary sugar recovery and expression of proteins in the colon.

		Expression		
		TLR4	Claudin-1	Occludin
% urinary recovery	Lactulose	0.634	0.930	0.341
	Mannitol	0.481	0.982	0.369
	Sucralose	0.726	0.748	0.200
	Sucrose	0.557	0.925	0.320

3.4 Discussion

Supplementation of sorghum into an obesogenic diet showed modest changes in several parameters of metabolic syndrome. Cholesterol reduction, improved liver physiology and improved glucose tolerance were the major findings, while average weight gain, final body weight, energy intake and body mass index were the physiological changes noted. Biochemical changes included increased liver TLR4 liver expression and increased GLUT2 liver expression. Sorghum had no impact on gastrointestinal permeability or serum lipopolysaccharide concentrations.

Scaling equations from rat to human models based on body surface area (382) or body weight (383) show that 5% sorghum in food equates to a daily dose of approximately 28g or 34g sorghum in humans. These calculations are based on an estimated rat dietary intake of ~25g of food per day containing 5% sorghum, for a 500g rat, scaled to a 70kg human.

Dyslipidaemia

The most significant changes in metabolic parameters was normalised total plasma cholesterol with 5% sorghum in the HCHF model. This has significant ramifications for promotion of sorghum as a health food, placing it in similar realms to oats for marketing purposes. Other literature has shown that the lipid fraction of sorghum can contribute by inhibiting cholesterol absorption from the gastrointestinal lumen (182, 184). Although the lipid fraction in whole sorghum is low, this indicates that the wholegrain has similar effects, as this is generally how human consumption would occur. 5-20mg of long-chain fatty alcohols, sourced from wholegrain, can increase HDL and decrease LDL concentrations (384). Wholegrain sorghum contains around 0.23% long chain lipids, of which 37-40% are policosanols (on a dry matter w/w basis) (131). Equivalent human consumption in this model is around 30g per day, which would equate to approximately 25.5mg of policosanol ingestion, well above the 5-20mg requirement.

There have been links established with resistant starch supplementation and the normalisation of plasma cholesterol (385). One way in which sorghum and other sources of resistant starches cause this is by reduction of intestinal cholesterol and bile

absorption (385). Comprehensive reviews of phytochemicals including anthocyanins, phenolics and flavonoids indicated that digestion is required for their release from the matrix (386, 387) and therefore will they will exert some of their effects in the colon (388, 389). Anthocyanin degradation products, specifically protocatechuic acid from cyanidin 3-glucoside, promoted cholesterol efflux by increased expression of two cholesterol efflux regulatory proteins (390). Cyanidin 3-glucoside is present in sorghum at low concentrations (146), and would rely on interaction with the appropriate microbial community. Protocatechuic acid is present in red sorghum as a free phenolic acid (391). Not all studies agreed that sorghum consumption alters cholesterol in a rat model as some cited no changes (232). Sorghum consumption led to increased total plasma cholesterol concentration in a rat model of obesity in one study (392). The lack of changes in triglyceride and NEFA concentration indicated that sorghum cannot fully correct the dyslipidaemia associated with metabolic syndrome.

This study was unable to determine the changes in LDL and HDL cholesterol. Rat models are not ideal for cholesterol-based studies, with a low similarity to lipid profiles seen in humans. Zucker diabetic fatty rats on a high fat diet showed low similarity (393). However, studies in humans showed no improvement in plasma cholesterol in overweight subjects caused by sorghum consumption (394).

Pre-diabetes

Addition of 5% sorghum to this high fat, high simple carbohydrate diet normalised post-prandial glucose tolerance. This outcome is very promising for application to humans for improvement of pre-diabetes; however, in a human study, sorghum did not improve glucose tolerance with 45 grams of flaked sorghum consumption per day (394). Extracts of sorghum showed antidiabetic effects (185, 207, 208). Sorghum consumption reduced glucose concentrations and insulin secretion in overweight cats when compared to a wheat-based diet, which reduces progression of pre-diabetes (395). Wholegrain consumption in general has been associated with reduced diabetes development (396, 397).

There was no change in the pancreas that was associated with sorghum consumption. It was expected that there would be changes associated with the improved glucose

tolerance in HSb and the poor glucose tolerance in HCHF. Impaired insulin release is a major cause of the increased peak blood glucose concentration in a high-fat model, caused by destruction of pancreatic β-cells once disease has progressed (398). Apoptosis of pancreatic β-cells is increased in both lean and obese type 2 diabetic groups, and is generally accompanied by decreased β-cell mass (399). Regeneration of β-cells can restore glucose tolerance (400). In this study, islet cell density was not altered, and no statistical changes in cell count or islet size occurred due to diet or sorghum. It would suggest that a mechanism other than restoration or preservation of β-cell mass is responsible for sorghum's role in improving glucose tolerance. The changes are more likely related to the function of β-cells. Future studies should evaluate this.

Fasting hyperglycaemia was not improved by sorghum, which suggests a lack of change in the pulsatile insulin release, or lack of improvement in sensitivity to pulsatile insulin release (401). Reduced peak glucose concentrations could be affected by improved peripheral sensitivity to insulin, improved release of insulin, or conversion from pro-insulin to insulin (402). The cause of the glucose tolerance improvement have not been elucidated in this study but do not appear to be related to β-cell mass.

The dysfunction of the pancreas in metabolic syndrome is contributed to by inflammation (403). Most recently, the Whitehall Study linked increased pro-inflammatory IL-6 (interleukin-6) and CRP (C-reactive protein) with increased fasting glucose and poor insulin response (368). Interleukin-1 (IL-1) and interferon-γ as pro-inflammatory cytokines are implicated in the dysfunction of β-cells by initiation of nitric oxide production in islets (404). Consumption of anti-inflammatory compounds may reduce the metabolic problems caused by sub-clinical inflammation, however the mechanisms appear complex (405). The addition of multiple components of sorghum might reduce the inflammatory state. Sorghum methanol extracts reduced inflammation by up-regulation of anti-inflammatory PPAR-γ and reduced TNF-α (185), and improved glucose tolerance through reduced hepatic gluconeogenesis (406). Peripheral glucose clearance and hepatic function should be noted as a possible driver in reduced TAUC after glucose challenge in this study, with changes occurring in HSb liver GLUT2 expression. Increased GLUT2 is typically associated with impaired glucose tolerance due to its ability to release glucose into the bloodstream.

This is paradoxical to the improved glucose tolerance seen in the HSb group and the increased GLUT2 expression. In other studies, reduced GLUT2 expression occurred with improvement in diabetes (305, 310, 319, 325) and in diet-induced fatty liver disease (322).

Cardiovascular and liver changes

Few cardiovascular changes occurred due to sorghum consumption. There was reduced hypersensitivity to noradrenaline and acetylcholine with sorghum consumption in aorta from this high fat model. This may be due to a reduction in oxidative stress and a reduction in inflammation, as components of sorghum are widely shown to reduce oxidative stress and inflammation. In diabetes, there have been links correlating high plasma markers of chronic inflammation with macrovascular endothelial dysfunction (407). In obesity and insulin resistance, similar associations with CRP (C-reactive protein) have been demonstrated, which closely correlated with concentrations of pro-inflammatory cytokines, interleukin-6 and TNF-α (408). Consumption of other cereals such as millet reduced CRP concentrations in hyperlipidaemic states (392). Sorghum has not yet been directly linked to this and this study did not study inflammatory cytokine concentrations. Future research assessing the plasma inflammation makers will add weight to this knowledge.

As noted previously, increased PPAR-γ may play a role in improving insulin sensitivity, however this receptor has also been implicated in reducing inflammation, particularly in endothelial cells (409). Studies in mice have shown increased PPAR expression with sorghum extract consumption (185). Future studies would benefit from determining expression and activation of PPAR. Anthocyanin-rich foods in general appear to have beneficial effects on the heart (410, 411), with sorghum being relatively unique as a widely grown commercial cereal crop which contains high concentrations of 3-deoxyanthocyanidins. Sorghum fed to rats showed a better *in vivo* anti-oxidative effect than purple rice or rhubarb rice (412).

Liver function is impaired in metabolic syndrome with deposits of fat, alteration of enzyme activity, and increased glucose release. Sorghum did not change liver weights or amino acid transfer enzyme activities. Liver histology indicated that there was reduced steatosis, ballooning and fat deposits with sorghum consumption in the high

fat diet. Sorghum extracts reduced cholesterol concentrations in the liver of rats (183) and mice (406), and in a hamster model improvements in liver were associated with the lipid fraction of sorghum extract (182). Aqueous sorghum extracts showed no adverse effects in healthy rats and were haematopoeitic at doses of up to 1600mg/kg per day (413). Wholegrain sorghum increased liver weight in overfed ducks more than a maize equivalent (414). Sorghum flour fed to rats on a hyperlipidaemic diet did not change liver enzyme activity (253).

Liver TLR4 expression increased on the HSb diet. Typically, increased expression of TLR4 would result in an increased inflammatory response. Reduced TLR4 expression reduced inflammation and metabolic changes associated with metabolic syndrome (415). However, the effects of sorghum on TLR4 have not been studied. The reason for the increase in this study is unclear, and the lack of difference between the high and low fat diet controls does not shed any light on the cause.

Gastrointestinal function

Gastrointestinal contraction, as assayed by acetylcholine responsiveness, did not produce a clear indication of how sorghum or diet affects colonic reactivity. If anything, there were increased responsiveness in high-fat fed models which were not anticipated. Some data suggest that, in situations such as distal colitis, there was an increased colonic contractility in the proximal colon, and reduced contractility in the distal colon (416). Inflammation upregulated the α7 nicotinic acetylcholine receptor in mouse colon (417) which may explain the increased responses to acetylcholine stimulation. This may be similar to the changes seen in obesity. Studies in humans have suggested that in obese subjects there was a faster colonic transit time (178), and that high force contractions in the colon may be caused by inflammation (418) while others suggest that obesity decreased contraction (419). Compounds isolated by 70% methanol from *Sorghum bicolor* leaf caused relaxation of the isolated ileum of guinea pigs (420) and a reduced intestinal propulsion (421).

One hypothesis was that increased gastrointestinal permeability would occur in a high-fat high-carbohydrate model of obesity, which appeared to be the case. This is consistent with other studies of obesogenic diets (373, 422). Supplementing with sorghum did not improve the gastrointestinal permeability, and therefore the

gastrointestinal benefits at this level of supplementation are not evident. Mannitol is considered an indicator of absorption, while lactulose is an indicator of permeability, being a large molecule not taken up by villi. The increased lactulose recovery in the HSb rats indicated possible poorer gastrointestinal integrity, specifically in the stomach and small intestine.

There may be a decreased expression of tight junction proteins caused by a high fat diet in obese animal models (177, 375, 423, 424). Claudin expression did not alter in any diet or intervention, although these were only studied these in the colon with intent to investigate links between LPS and colonic permeability. Occludin was increased from the CS control with sorghum, with no change in intestinal permeability. This result was not consistent with the intestinal permeability measurements and therefore another mechanism may be influencing the intestinal permeability. The lack of differences in tight junction expression between HCHF and HSb were consistent with the intestinal permeability study. Sorghum can contain mycotoxins which reduce claudin expression (425). In sensitive individuals, prolamin protein may reduce zonulin expression in the gastrointestinal tract (426), while sorghum arabinoxylans restored intestinal barrier function through improving tight junction proteins, mediated by the production of short chain fatty acids (427).

The tight junction protein, zonula occludin-1 (ZO-1), expression was reduced in patients with irritable bowel syndrome, which corresponded to increased permeability (428). ZO-1 is the soluble anchor protein, while occludin and claudin are both transmembrane proteins. The responses between claudin-1 and occludin differed. Occludin appears more susceptible to changes than claudin. Some studies suggest that the changes do not occur in the large intestine with a high fat diet (424), which is where expression was studied in this research. LPS, among other TLR activators, are generated in the large intestine where the vast majority of the microbial population resides. It may be that it is primarily the diet itself, rather than the microbial factors influenced by diet, that caused the changes in a HCHF model (424). As this study was focussed on the interaction of LPS and TLR4, and their influences on intestinal permeability leading in metabolic syndrome, small intestinal changes in tight junctions were not tested, however future research should incorporate this.

Metabolic endotoxaemia

Serum LPS concentrations did not differ between any groups, and therefore its changes could not be linked to intestinal permeability or inflammation which lead to metabolic syndrome. There appears to be no influence of sorghum on the circulating concentrations of LPS.

Studies which implicated LPS in metabolic syndrome development such as that used by Cani*, et al.* (238) used an aggressive approach whereby intraperitoneal injections of LPS induced metabolic changes. Whether this is the case in a "normal" diet-induced development of metabolic syndrome was unclear. This study was unable to find a sound correlation between LPS concentrations and the glucose tolerance, or even an effect of diet on serum concentrations of LPS. TLR4-mediated inflammation is a known mediator of inflammation induced by LPS, and is expressed by β-cells (429). TLR4 mediated insulin resistance in knockout mice populations (430). The complexity of the inflammatory pathways that have been associated with β-cell dysfunction and impaired glucose tolerance is the likely reason that no direct correlation was found. All inflammatory triggers, not only LPS, are important in addition to TLR4-mediated inflammation (431). Lipids can trigger inflammatory mediators including protein kinase C, protein kinase R and TLR4. Many stressors in obesity can imitate the JNK pathway (431). The presence of macrophages, and their inflammatory responses which include TLR2, TLR6 and TLR4 also contribute (432). All mechanisms are at play and addressing a single inflammatory indicator is insufficient to explain the changes in glucose tolerance, particularly where no significant effect of diet was noted. In future, metabolomics will aid to assess all facets of the process simultaneously and will give a much clearer understanding of how sorghum affects glucose tolerance and inflammation. While the expression of TLR4 was studied, and concentrations of LPS were assessed, the study lacked by not addressing TLR4 activation, initiated by circulating free fatty acids which mimic LPS (433, 434).

I hypothesised that LPS concentrations would be altered with both diet and sorghum consumption. LPS is an inflammatory mediator in the gastrointestinal tract, where significant amounts of LPS are excised from Gram-negative bacteria. TLR4-deficient mice appeared resistant to changes is colonic occludin and claudin that occur in a high fat diet (219), implicating its role in the process. It is well known that in inflammatory bowel disease, intestinal permeability increases. In studies, ulcerative colitis and Crohn's disease showed increased TLR4 in epithelial cells of both the colon and ileum (435). Sorghum had no influence on the outcome, therefore is unlikely to influence the status of colonic TLR4 expression when consumed by humans, nor alter serum LPS. Lappi*, et al.* (218) hypothesised the changes in the large intestine were partly responsible for improved type 2 diabetes mellitus with wholegrain consumption. In this study, the two could not be directly associated.

Body composition

Sorghum did not reverse obesity. There were indications that sorghum affects energy intake and body weight gain indicated by two-way ANOVA. In rats, the protein content of the sorghum added to rat diets was a significant source of variance in body weight gain (436). The diet-intervention interactions are of interest, where the indication is that adding small amounts of sorghum into a low-fat diet may increase food intake and feed efficiency, where the opposite is true for supplementing into a high fat, high simple carbohydrate diet. These interactions suggest that the composition of the entire diet is important, even when low amounts of sorghum were consumed. Although the changes were minor, the supplement level is relatively low, equivalent to approximately 30g per day for a 70kg adult human. Recent studies by Stefoska-Needham*, et al.* (394) using 45g of flaked sorghum per day showed no change in weight loss on a restricted diet when comparing with 45g of white wheat flakes. The results we achieved in an animal model were comparable to this and overall, low amounts of sorghum are unlikely to have a significant change on body weight in humans as the key driver of body weight is calorie intake (437). Wholegrain millet (*Sorghum vulgare*), which has a similar composition to sorghum, did not affect body weight in hyperlipidaemic rats (392).

The low digestibility of sorghum compared to other cereal grains is a concern with the use of sorghum in production animal diets where the goal is to maximise weight gain (91, 438). This led to a reduced weight gain in a high sorghum diet (367). Lean mass and organ weight are two major drivers of metabolic rate. Sorghum supplements in rats did not reduce lean mass or organ weights, which would suggest that it is unlikely to have a detrimental effect on overall metabolic rate and energy expenditure. This could be confirmed in the future with the use of metabolic cages such as OxyMax.

The composition of the two diets is likely to impact the nutrient availability *in vivo*. Red sorghum contains tannins which may inhibit amylase activity which will reduce the breakdown of starch and therefore its energy availability (28, 259), although in a rat model it does not appear to be the case. The effects on amylase activity have been well characterised however there may also be effects on lipases and proteases. The formation of tannin-enzyme complexes may have reduced the digestion, as has been shown in other animal models (439), and therefore availability of both fats and proteins to the animal. During the 8 weeks of sorghum addition, there was no loss of lean mass in either group which would suggest that there is minimal risk of reduced amino acid absorption which would affect lean body composition. Bone mineral density showed a diet-sorghum interaction. Chelating properties of tannins reduce biological availability of minerals necessary for bone health, particularly during stages of growth (258, 259). The outcomes are unlikely to be clinically significant.

Other considerations

Sorghum contains components which may affect the digestibility of other components of the diet, and the protein and carbohydrates of sorghum itself (103). There is a strong indication that the effect of sorghum in the diet differs depending on the diet as a whole, indicated by the differing effects in the CS and HCHF diets. The food matrix components contribute to the bioavailability (440). Again, this is a complex response that requires more investigation. The matrix itself, the enzymes induced by the matrix, gut pH, microbial presence, fermentation status and transit time can all influence the uptake of compounds (290) in sorghum with the potential to improve metabolic syndrome and other health parameters. The digestion process altered bioavailability of

hydroxycinnamic acids (441) and the differing digestion from a rat model to human model will result in different outcomes.

Identification and quantification of the bioactive compounds present in the whole grain sorghum used in this study would add considerable weight to the study. Knowledge of the individual components would provide a link between these compounds and the research outcomes. Given the vast differences in sorghum composition due to genetic and environmental factors, this would provide a baseline for further research. In the absence of this information, the effects cannot be well correlated with these components.

High carbohydrate commercial cereal made of wheat, barley, maize and oats, eaten in conjunction with grape seed extract, reduced post-prandial plasma concentrations of dimeric and trimeric procyanidins; however, the concentrations of the monomeric forms, catechin and epicatechin, were increased when compared with sole consumption of grape seed extract (442). The high corn starch diet used in this study might have a similar effect, reducing uptake of bioactive compounds present in sorghum, particularly complex procyanidins however increasing the uptake of monomeric forms. Flavanols had higher post-prandial concentrations when consumed in the presence of both complex and simple carbohydrates (443).

Presence of milk proteins as in the condensed milk of the HCHF diet may also change bioavailability. Milk proteins do not alter uptake of catechins or epicatechins (444, 445). However, the presence of milk can affect the excretion profile over time of epicatechin and its conjugates, in addition to altering the position of conjugates moieties, particularly sulphates (446). This study used whole milk so the interaction of fat and carbohydrates may also play a role. Protein-rich foods did not improve or hinder catechin or epicatechin flavanol uptake (443). The development of a well-designed meal with a combination of foods may optimise uptake of sorghum components, specifically the procyanidin groups.

Increasing the percent of sorghum supplemented may have a more pronounced effect, and this is explored in subsequent chapters.

Longitudinal changes in attitudes and culture toward food have become more important in a society where food is cheap, accessible and choice is relatively unlimited. Examples of changing positive attitudes to cereal foods include chia, quinoa, teff and spelt. Sorghum may follow the same path with the correct evidence to back its health benefits, as has been shown here, particularly for reduction of cholesterol concentrations.

Addition of 5% in the diet in rats, equivalent to approximately 30g intake per day in humans, may not be sufficient to produce changes in some aspects of metabolic syndrome. The average wheat consumption in Australia is 87.7kg per annum per person, equating to approximately 240g per day (447). Replacement of refined wheat products with unrefined sorghum may result in greater benefits than the low level of supplementation in this study. This may be achieved by replacing wheat-based cereals, pasta, breads and bakery products with sorghum substitutes. Higher sorghum doses are studied in Chapter 4.

3.5 Conclusion

Supplementation of sorghum to diet at a level of 5% may be a very simple way to reduce the metabolic syndrome risk especially for correction of cholesterol concentrations, improved liver structure and glucose tolerance. These impacts could be translated to humans. It should be noted that there are multiple varieties of sorghum which differ in tannin, flavonoid and polyphenol content which are some of the functional components of sorghum. Overall, sorghum consumption showed very few negative effects, and had a positive impact on plasma lipid status, glucose tolerance and liver physiology. Furthering these trials to humans will give a more applicable understanding on the possible role of sorghum in human nutrition and as a functional food.

Chapter 4 - 20% Red and black sorghum flour in a rat model of metabolic syndrome

4.1 Introduction

Sorghum and its components have garnered interest for health promotion, due to the composition of both their macro- and micronutrients. Cereal grain products are a large part of the diet, and intakes are expected to remain relatively constant over the next 10 years (447). Calorie consumption globally is increasing (447), which leads to metabolic sydrome (448). There is significant resistance to changing dietary habits that lead to obesity and metabolic syndrome, due to price, time and desire (449). On average, Australians consume approximately 240g of wheat per person per day (447). Can incorporating sorghum flour into the diet as a replacement for wheat flour present an easy and manageable way to alter the risk of metabolic sydrome?

Sorghum contains multiple components which show potential to reduce the risk of, or improve, the symptoms of metabolic syndrome. This is partly due to its phenolic composition (272, 450, 451) and the potential of consumption of the unique sorghum polysaccharides (106). Most studies have investigated isolated components of sorghum, but a few have supported the theory of sorghum as a wholegrain to improve paramenters of metabolic syndrome.

Studies in overweight cats showed that sorghum-based diets reduced post-prandial glucose concentrations, and may be preventative for type 2 diabetes (452). A portion of this may be attributed to the methanol-extractable portion (185) or ethanol-extractable portion which include phenolic acids, 3-deoxyanthocyanidins, flavones, flavanones, catechins and tannins (207). Wholegrain sorghum flour muffins resulted in lower post-prandial glucose fluctuations than wheat muffins, attributed to high concentrations of resistant starches (206).

Decreased cholesterol concentrations in a rat model of diet-induced metabolic syndrome were shown in Chapter 3 of this thesis. Improved serum lipid status is supported by other animal studies, however only with conusmption of sorghum extracts (181-185). Sorghum improved lipid status in human trials with the consumption of 100g per day (187). Effects on central adiposity are expected to be

minimal (186), however long term sorghum consumption improved satiety responses (222) which may reduce calorie consumption. Little research exists on the cardiovascular effects of sorghum consumption, but wholegrain cereal consumption was inversely correlated with cardiovascular disease (397).

The interactions between sorghum and the gastrointestinal tract and the effects of sorghum on metabolic syndrome have not been widely studied and need to be investigated. The addition of sorghum, which includes resistant starches, unique proteins and a combination of bioactive compounds could have similar beneficial effects to other cereal grains such as oats (450). Despite this, some evidence suggests that sorghum may cause problems. Dextran sodium sulphate-induced colitis injury in rat models was exacerbated in black and Sumac sorghum bran-supplemented studies compared with those supplemented with cellulose (453). The same study also showed lower gut microbial population diversity in animals consuming sorghum brans which contained high concentrations of tannins (453). Sorghum cereal consumption altered colonic microflora and improved biomarkers associated with cardiovascular disease such as gamma-glutamyl transferase (454). Circulating endotoxins such as lipopolysaccharides, which originate in the gut, have been linked to the development of metabolic syndrome (237, 455, 456). Improvements in metabolic syndrome with wholegrain intake may be due to reduced gastrointestinal permeability and reduced TLR4 (toll-like receptor 4) mediated inflammation (218). Wholegrains improve colon-mediated inflammation and reduce post-prandial glucose concentrations (457). Wholegrain barley improves inflammation and markers of metabolic syndrome by changes in the colon (458).

Red and black sorghum flours are two types which are commercially available for use. Although both are from the species *Sorghum bicolor*, they differ in colour and also in the composition of polyphenols. The unque combination of phenolics and their concentrations has led to the speculation that sorghum has nutraceutical properties. The 3-deoxyanthocyanidin pigments which result in the colours in sorghum are luteolinidin (orange to brown) and apigeninidin (orange to dark red). Other pigments include 5-methoxyapigeninidin (dark red), 7-methoxyapigeninidin, apigeniniden 5-glycoside, 7-methoxyapigeniniden 5-glucoside, 5-methoxyluteolinidin, 7-methoxyluteolinidin, and 5-methoxyluteolinidin 7-glucoside, apigenin, luteolin,

eriodictyol, eriodictyol 5-glycoside, kaempferol 3-rutinoside-7-glucuronide, naringenin (white), taxifolin (pale yellow (459)), taxifolin 7-glucoside, apiforol, luteoforol, catechin and procyanidin B1 (451). Black sorghum colour is due to higher concentrations of 3-deoxyanthocyanidins in the pericarp than red or tan lines, but also higher concentrations of flavanols and flavones (165). Colours can be affected by light, oxygen, solvent, present of oxidants and pH (460). Condensed tannins also contribute to the red or brown colouring in sorghum, and these are higher in red varieties (165).

Sorghum flour is the most likely way for sorghum to be incorporated into the diet, due to increased reliance on packaged and processed food products (461). While wholegrains are beneficial for health, processing wholegrains alter the bioaccessibility and bioavailability, and therefore biological responses to the grain (88, 160, 272). The macronutrient composition in wholegrain sorghum and sorghum flour may appear similar at the outset but the milling process changes the structure of starch granules. Longer milling times generally result in reduced starches which are resistant to hydrolysis, and higher starch granule damage (462). Both of these properties increase digestibility of starch (463) by reducing the amount of resistant starches. Phenolic compounds are bound to starch granules and proteins, both covalently and by adhesive forces. The milling makes these components more available (159, 160) and as starches and proteins are digested they can release not only differing amounts of the complexed compounds, but also cause their release in different parts of the gastrointestinal tract (272).

In this chapter, two different types of sorghum flour were tested in a rat model of diet-induced metabolic syndrome to determine whether sorghum flour has benficial or detrimental effects on physiology with specific reference to parameters of metabolic syndrome. I propose that the unique combination of phenolics in red and black sorghum will improve metabolic syndrome. I also suggest that part of the mechanism will be alteration of the colon environment which will influence circulating lipoplysachharides, which are known activators of TLR4-mediated inflammation which contributes to metabolic syndrome (Figure 4.41).

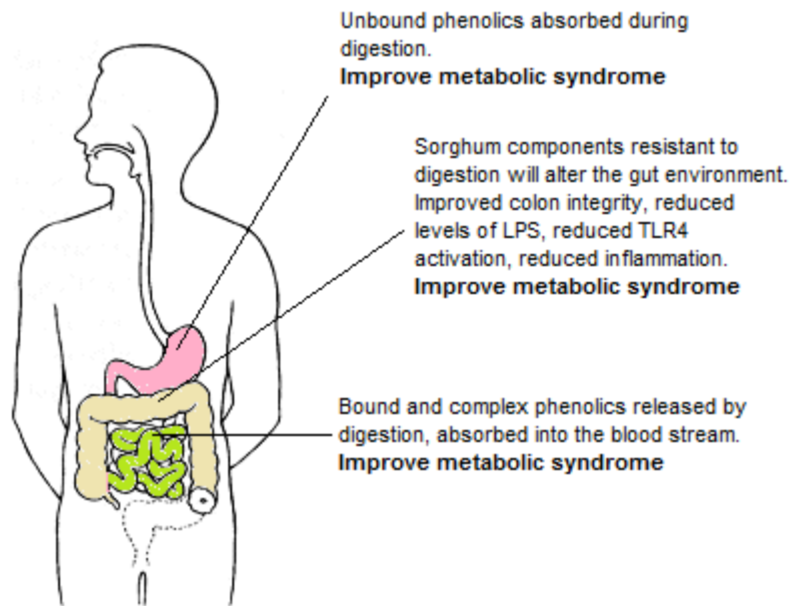


Figure 4.41: Sorghum's potential for improving metabolic syndrome.

4.2 Materials & Methods

4.2.1 Ethics

Approval for experimentation with 20% sorghum was granted under AEC approval number 15REA005 valid from 20th June 2015 to the 20th June 2018 by the Animal Ethics Committee of the University of Southern Queensland. All rats were treated and housed as per the NHMRC (National Health and Medical Research Council) 2014 guidelines for the ethical treatment of animals.

4.2.2 Rat Diet and Experimental Structure

Seventy-two Male Wistar rats were randomly divided into 6 experimental groups of n=12. The groups were mCS (modified corn starch diet), mHCHF (modified high carbohydrate, high fat diet), mCRed (modified corn starch diet + 20% w/w red sorghum flour), mHRed (modified high carbohydrate, high fat diet + 20% w/w red sorghum flour), mCBlack (modified corn starch diet + 20% w/w black sorghum flour) and mHBlack (modified high carbohydrate, high fat diet + 20% w/w black sorghum flour). Partially de-branned red sorghum flour was donated by Maralong Milling Co (Toowoomba, Qld, Australia), and wholegrain black sorghum flour was purchased from Nu Life™ Market (Scott City, Kansas, USA).

For the first 8 weeks of the protocol, all mCS, mCRed and mCBlack rats received the control mCS diet and all mHCHF, mHRed and mHBlack rats received the control mHCHF diet as described in method 2.2. At eight weeks, the mCRed, mHRed, mCBlack and mHBlack groups were transferred to the treatment diet as indicated and remained on these diets until protocol completion.

An additional 3.85 kJ/mL energy was added to calculations for the mHCHF, mHRed and mHBlack diets in fructose water.

At 16 weeks, prior to termination, oral glucose tolerance (method 2.3), body composition (method 2.4) and systolic blood pressure (method 2.5) were determined. Rats had urine collected as per chapter 2.10.1. All rats were starved for 2 hours prior to termination. Rats were euthanised as per method 2.6.1. At termination, rats underwent serum collection (method 2.6.2), plasma collection (method 2.6.3), vascular reactivity (method 2.6.5), ileum and colon contractility (method 2.6.6), organ weights (method 2.6.7) and histological tissue collection (method 2.8.1). 10 rats per group underwent isolated heart perfusion (method 2.6.4), with the remaining two hearts preserved in formalin for histological analysis as per method 2.8.1. Plasma biochemistry was assessed on 8 rats per group for liver enzyme activity (method 2.7.1), and plasma lipid profile (method 2.7.2.). Collected serum was analysed for presence of endotoxins as per method 2.9. Liver and distal colon sections were assessed for expression of TLR4 (method 2.11.3). Distal colon sections were also assessed for occludin and claudin expression using methods 2.11.4. Urine samples collected prior to termination were prepared (method 2.10.2) and analysed as per method 2.10.3.

Histological tissue samples were processed automatically (method 2.8.3) and sectioned (method 2.8.3). Liver, ileum, colon, pancreas and left ventricle were stained with haematoxylin and eosin (method 2.8.5.1). Separate sections of left ventricle were stained with picrosirius red (method 2.8.5.2). Microscopy and imaging were performed as per method 2.8.6.1, 2.8.6.2, 2.8.6.3 and 2.8.6.4.

Statistical tests were performed with one-way ANOVA. Where a one-way ANOVA reported a significant difference between the means, these results are indicated as a significant difference at $p<0.05$, where lower-case letters differ. Further testing was carried out when a two-way ANOVA indicated a significant effect of sorghum or interaction. In this case, Student's *t*-test was performed between the following

comparisons: mCS-mHCHF, mCS-mCRed, mCS-mCBlack, mCS-mHRed, mCS-mHBlack, mHCHF-mCRed, mHCHF-mCBlack, mHCHF-mHRed, and mHCHF-mHBlack.

The following null hypotheses were tested:

1. There is no difference between the mCS and mHCHF diet on the parameter in question.
2. There is no effect of sorghum addition to the base diet, i.e. the addition of sorghum to the mCS diet does not alter the outcome, or the addition of sorghum flour to the mHCHF diet does not alter the outcome.
3. The addition of sorghum to the HCHF does not normalise an outcome to the CS control.
4. The addition of sorghum to the CS diet does not change the outcome to be comparable to the HCHF control.

4.3 Results

4.3.1 Black and red sorghum flour composition

Acidified methanol extracts of red and black sorghum flour show differing profiles. HPLC with detection at 210nm (Figure 4.42) indicated that the hydrophilic compounds were relatively similar between the two. However, red sorghum showed small peaks between 10.5 and 11.5 minutes which were not present in black sorghum flour extracts. Similarly, there were a number of peaks present between 13.5 and 14 minutes which were present in black sorghum flour extracts but not in red sorghum flour extracts.

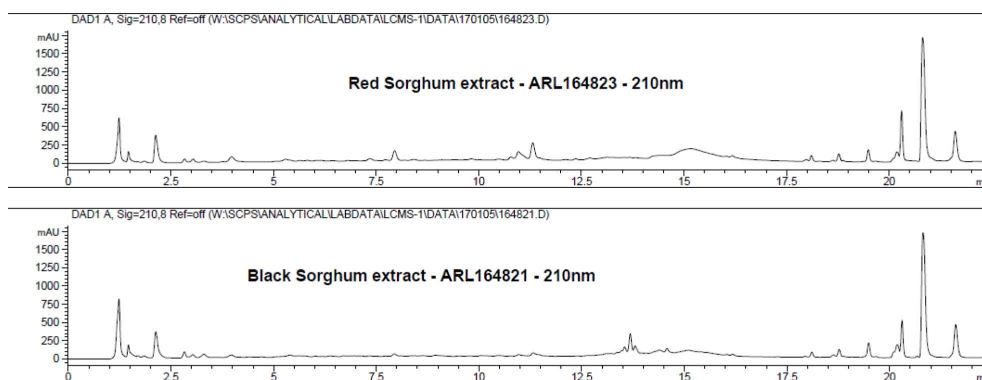


Figure 4.42: 210nm UV detection of HPLC of acidified methanol extracts of red sorghum flour (top) and black sorghum flour (bottom). 210nm detection detects primarily hydrophilic compounds.

Detection of anthocyanins were detected at 510nm. This wavelength will also detect 3-deoxyanthocyanins and their glycosides. There was a marked difference in the compounds in red and black sorghum (Figure 4.43). The peaks at 7.5, 7.0, 6.1 and 5.8 minutes were much higher in the red sorghum extract than that of the black sorghum. Several peaks were present between 13.0 and 15 minutes in black sorghum extract that were not detected in the red sorghum.

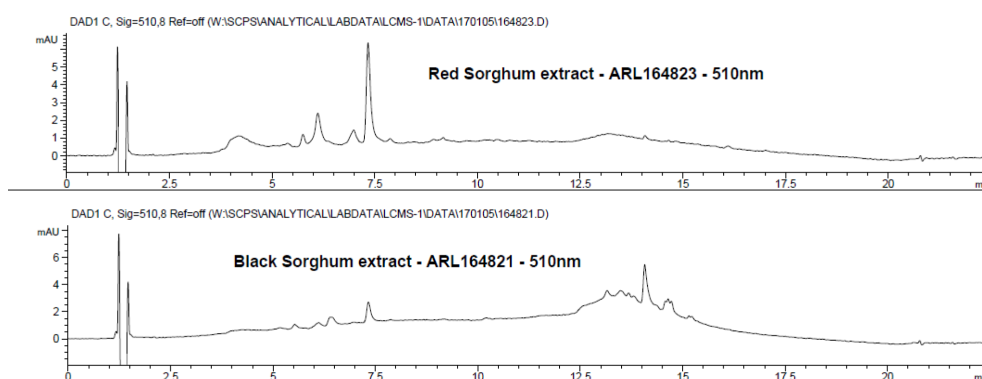


Figure 4.43: 510nm UV detection of HPLC extracts of red sorghum flour (top) and black sorghum flour (bottom). 510nm detection primarily indicates anthocyanin presence.

The 330nm profile (Figure 4.44) primarily indicates phenolic acids. This wavelength is also suitable for detection of flavones (153). These differ considerably between the red and black sorghum extracts. In black sorghum, the majority of phenolic acids eluted between 13.5 minutes and 14 minutes. These 3 peaks were absent, or present at

very low amounts, in the red sorghum extract. There are two clusters of compounds in red sorghum extracts, four peaks between 10.5 and 11 minutes, and two between 12.3 and 12.8 minutes, which are not present in the black sorghum extract.

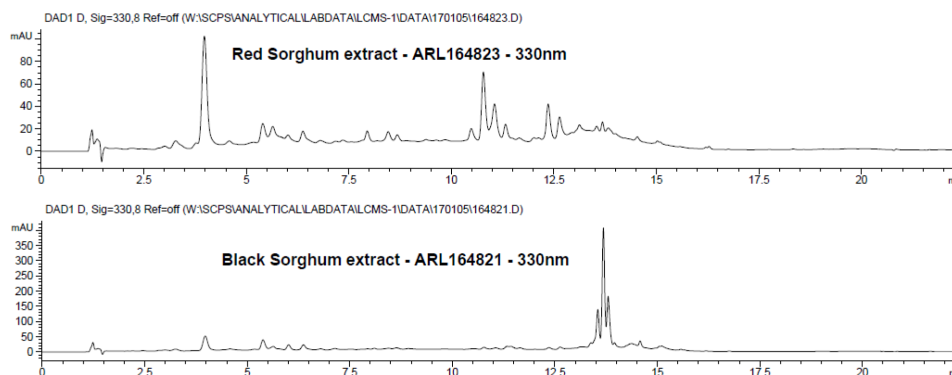


Figure 4.44: 330nm UV detection of HPLC separated acidified methanol extracts of red sorghum flour (top) and black sorghum flour (bottom). 330nm detection primarily indicates presence of phenolic acids.

Despite both types of sorghum having compounds which are potentially bioactive, the composition of these extracts differs markedly and therefore their effect on the body may differ. Macronutrient profiles of the two flours are shown in Table 4.16.

Table 4.16: Macronutrient profiles of red and black sorghum flour used in the dietary protocols.

	Maralong Milling Red Sorghum Flour	**NuLife Black Sorghum Flour**
Energy (kJ)	1500	1463
Protein (g)	9.9	7.5
Fat, Total (g)	2.5	3.75
- Saturated (g)	0.4	0
- Monounsaturated (g)	0.8	0
- Polyunsaturated (g)	1.2	NR
- Trans (g)	<0.01	NR
Fibre - Dietary (g)	5.8	5
Carbohydrates 70.7 (g)	70.7	77.5
- sugars (g)	<0.01	0
Sodium (mg)	<5mg	0

NR indicates not reported.

4.3.2 Physiology and metabolism indicators

The addition of red and black sorghum flour to the diet at 20% increased the caloric value of the mHCHF diet from 14.65 kJ/g to 17.65 kJ/g and 17.78 kJ/g, respectively. Addition to the mCS diet increased caloric value from 9.94 kJ/g to 12.94 kJ/g and 13.08 kJ/g, respectively. At 8 weeks, all mCS groups showed a similar body weight, similarly in all mHCHF groups. The mHCHF, mHRed and mHBlack groups showed higher body weight than all other groups. At 16 weeks, the mCBlack and mCRed groups had higher body weight than mCS (Figure 4.45). The addition of 20% red sorghum flour did not affect body weight in the mHCHF diet, despite the increased calorie density of the food.

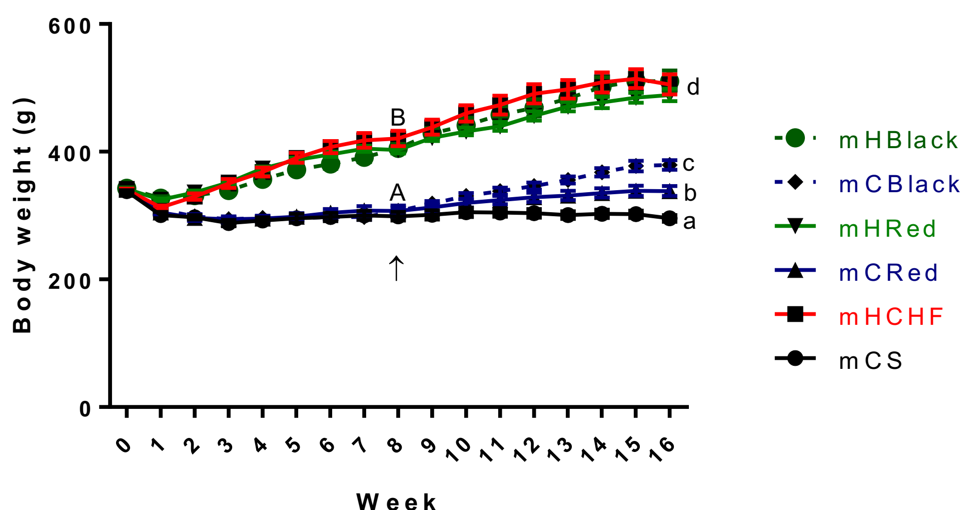


Figure 4.45: Body weight changes over 16 weeks of the dietary protocol. ↑ indicates start of intervention with 20% red or black sorghum flours. Differing lower-case letters indicate a significant difference at 16 weeks ($p<0.05$) and differing upper case letters indicate a difference at 8 weeks ($p<0.05$).

Table 4.17: Physiological parameters of rats fed mCS, mCRed, mCBlack, mHCHF, mHRed, and mHBlack diets.

Parameter	mCS	mCRed	mCBlack	mHCHF	mHRed	mHBlack	Diet	Intervention	Interaction
Food intake (g/day) Week 0-8	36.6 ± 0.5 ^{a}	37.8 ± 1.0 ^{a}	37.0 ± 0.8 ^{a}	30.7 ± 1.1 ^{b}	27.5 ± 0.6 ^{b}	27.7 0.6 ^{b}	0.2391	0.0201	< 0.0001
Food Intake (g/day) Week 8-16	34.5 ± 0.7 ^{a}	26.8 ± 1.0 ^{c}	26.9 ± 0.5 ^{c}	31.7 ± 0.7 ^{b}	23.4 ± 0.6 ^{c}	22.7 ± 0.6 ^{c}	< 0.0001	0.5844	< 0.0001
Water intake (g/day) Week 0-8	34.9 ± 2.7 ^{a}	24.3 ± 1.9 ^{b}	32.1 ± 1.5 ^{a}	21.6 ± 1.1 ^{b}	21.8 ± 1.3 ^{b}	21.4 ± 1.1 ^{b}	0.0568	0.6315	0.5989
Water Intake (g/day) Week 8-16	31.2 ± 3.4 ^{a}	21.1 ± 1.5 ^{b}	31.7 ± 2.3 ^{a}	22.3 ± 1.1 ^{b}	22.9 ± 1.3 ^{b}	25.7 ± 0.9 ^{b}	0.0052	0.0016	0.0142
Energy intake (kj/day) Week 0-8	363.9 ± 5.0 ^{a}	375.6 ± 9.7 ^{a}	368.0 ± 7.9 ^{a}	532.4 ± 16.9 ^{c}	486.6 ± 11.0 ^{b}	405.4 ± 8.9 ^{ab}	< 0.0001	< 0.0001	< 0.0001
Energy intake (kj/day) Week 8-16	343.0 ± 7.2 ^{a}	346.3 ± 13.5 ^{a}	352.1 ± 6.6 ^{a}	550.4 ± 11.6 ^{c}	501.3 ± 9.1 ^{b}	404.7 ± 10.6 ^{b}	< 0.0001	< 0.0001	< 0.0001
Feed efficiency (mg gained. kJ^{-1}) Week 0-8	-2.0 ± 0.3 ^{a}	-1.7 ± 0.3 ^{a}	-1.6 ± 0.5 ^{a}	2.7 ± 0.3 ^{b}	2.2 ± 0.2 ^{b}	2.4 ± 0.2 ^{b}	0.917	0.454	< 0.0001
Feed efficiency (mg gained. kJ^{-1}) Week 8-16	-0.2 ± 0.2 ^{a}	1.7 ± 0.2 ^{b}	3.8 ± 0.7 ^{b}	2.7 ± 0.2 ^{b}	3.1 ± 0.2 ^{b}	3.8 ± 0.3 ^{b}	< 0.0001	0.0006	< 0.0001
Sorghum flour intake (g/day)	0.0 ± 0.0 ^{a}	5.4 ± 0.2 ^{b}	5.4 ± 0.1 ^{b}	0.0 ± 0.0 ^{a}	4.7 ± 0.1 ^{c}	4.5 ± 0.1 ^{c}	< 0.0001	0.0022	< 0.0001
Bone mineral density (g/cm^2)	0.17 ± 3E-3 ^{a}	0.18 ± 3E-3 ^{a}	0.16 ± 3E-3 ^{a}	0.18 ± 2E-3 ^{b}	0.18 ± 3E-3 ^{b}	0.18 ± 2E-3 ^{ab}	0.0003	0.7474	< 0.0001
Bone mineral content (g by DXA)	9.75 ± 0.31 ^{c}	11.39 ± 0.37 ^{c}	12.56 ± 0.54 ^{b}	15.28 ± 0.41 ^{a}	15.07 ± 0.74 ^{a}	15.87 ± 0.71 ^{a}	0.0027	0.1181	< 0.0001
Total body fat mass (g by DXA)	42.2 ± 6.5 ^{a}	59.1 ± 11.9 ^{a}	88.6 ± 14.6 ^{a}	170.2 ± 10.4 ^{b}	151.1 ± 18.2 ^{b}	168.8 ± 21.0 ^{b}	0.0188	0.3008	0.0065
Total body lean mass (g by DXA)	248.2 ± 3.9 ^{a}	274.0 ± 5.5 ^{b}	291.1 ± 14.6 ^{c}	308.4 ± 8.2 ^{c}	327.0 ± 8.5 ^{c}	315.6 ± 7.1 ^{c}	0.0015	0.0742	< 0.0001
Lean:Fat mass ratio	7.4 ± 1.0 ^{a}	5.6 ± 0.8 ^{a}	4.0 ± 0.7 ^{b}	1.9 ± 0.1 ^{b}	2.4 ± 0.4 ^{b}	2.2 ± 0.4 ^{b}	0.012	0.0037	< 0.0001
Retroperitoneal fat (mg/mm tibial length)	150.3 ± 12.3 ^{a}	163.3 ± 16.7 ^{a}	153.8 ± 21.2 ^{a}	394.2 ± 34.4 ^{b}	363.8 ± 23.7 ^{b}	352.2 ± 49.0 ^{b}	0.8035	0.6789	< 0.0001
Epididymal fat (mg/mm tibial length)	67.1 ± 6.8 ^{a}	88.0 ± 9.7 ^{ab}	91.0 ± 13.0 ^{ab}	143.3 ± 16.0 ^{b}	176.7 ± 13.2 ^{b}	197.3 ± 25.4 ^{b}	0.0411	0.6214	< 0.0001
Omental fat (mg/mm tibial length)	97.7 ± 6.6 ^{a}	111.2 ± 11.8 ^{a}	95.9 ± 10.3 ^{a}	209.9 ± 10.9 ^{b}	203.7 ± 15.0 ^{b}	186.9 ± 23.1 ^{b}	0.5045	0.7129	< 0.0001
Total abdominal fat pad (mg/mm tibial length)	315.1 ± 22.7 ^{a}	362.5 ± 35.8 ^{a}	340.7 ± 42.6 ^{a}	747.4 ± 58.3 ^{b}	744.2 ± 49.7 ^{b}	736.4 ± 94.3 ^{b}	0.9227	0.8974	< 0.0001
Total abdominal fat pad (mg/mm tibial length)	315.1 ± 22.7 ^{a}	340.7 ± 42.6 ^{a}	744.2 ± 49.7 ^{b}	362.5 ± 35.8 ^{a}	747.4 ± 58.3 ^{b}	736.4 ± 94.3 ^{b}	0.9227	0.8974	< 0.0001

Differing lower-case superscript letters indicate significant difference at $p<0.05$ determined by one-way ANOVA.

Feed efficiency was higher in the mHCHF than mCS at 8 and 16 weeks. Sorghum did not affect feed efficiency in the HCHF diet. In the mCS diet, red and black sorghum increased feed efficiency (Table 4.17). Feed intake was lower in the mHCHF than mCS in the first 8 weeks. Food intake was decreased from controls in the mCRed, mCBlack, mHRed, and mHBlack. The addition of red or black sorghum flour to the mCS diet resulted in the largest decrease in food intake, with similar decreases between sorghum types. Energy intake was higher in the mHCHF than mCS in the first and second 8 week periods. Energy intake was not changed by adding sorghum flour to the mCS diet, but addition to the mHCHF diet reduced energy intake (Table 4.17).

Despite the decrease of food volume intake with sorghum supplementation in both diets, energy intake did not necessarily reduce, due to increased calorie density of food. For the same energy intake, there was a higher feed efficiency in the mCRed and mCBlack groups (Table 4.17). mCRed group gained 33±5g over weeks 8-16, and mCBlack gained 67.8±7g. For the same energy intake, the mCS group lost an average of 3±3g. This change was from both lean and fat mass increase, as indicated by increased lean mass as shown in Table 4.17. Lean mass changes were not associated with changes in myofibril diameter (Figure 4.46).

Fat mass was increased in the mHCHF group compared with the mCS group. Supplementation with sorghum flour did not affect fat mass in the mHCHF diet. Lean mass was increased with the addition of black and red sorghum flour to the mCS diet, but neither intervention had an effect in the mHCHF diet (Table 4.17).

The ratio of lean:fat mass was higher in mCS than mHCHF groups. The mCBlack group showed a reduced lean:fat mass ratio from the mCS control. The addition of sorghum flour to the mHCHF diet did not alter lean:fat mass ratio from 1.89±0.11 (Figure 4.46).

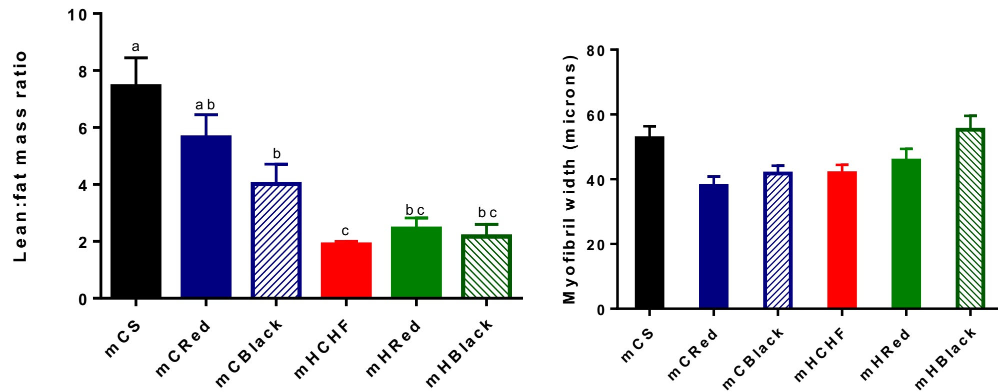


Figure 4.46: Lean:fat mass ratio at 16 weeks (left) and skeletal muscle myofibril width (right). Differing lower-case letters indicate significant differences at $p<0.05$ determined by one-way ANOVA.

Total abdominal fat pad weights, normalised to tibial length did not differ between the mCS control and the mCRed and mCBlack groups. Fat pad mass was increased in all high fat, high carbohydrate diets from mCS. Addition of sorghum flour did not alter the total fat pad mass in either diet as indicated in Table 4.17.

Bone mineral density was higher in the mHCHF group compared with the mCS group, as was bone mineral content. Bone mineral content was higher in all high carbohydrate, high fat based diets (mHCHF, mHRed, mHBlack) compared with the corn starch diets. This is likely a reflection of the elevated bone mineral density and higher overall body size and comparative bone content. Two-way ANOVA indicated that both diet and supplementation were factors contributing to bone mineral content, with p values of <0.0001 and 0.0027 respectively.

4.3.3 Blood glucose regulation, dyslipidaemia and lipopolysaccharides

After 8 weeks of feeding, the glucose TAUC was increased from the mCS control in the mHCHF and mHRed groups. Data shown in Table 4.18 indicate that the mHBlack group did not develop changes in TAUC expected for metabolic syndrome, with no difference from the mCS control. Additionally, the mCBlack group had lower fasting blood glucose concentrations than the mCS controls, and mHBlack showed fasting glucose concentrations similar to mCS. As this occurred prior to intervention, it cannot be attributed to sorghum and suggests an issue with the group of rats used for those

two interventions purchased from ARC, or another environmental problem. For this reason, changes from 8 weeks to 16 weeks were assessed, in addition to changes from the control groups. Red sorghum flour changes appear reliable, as these rats developed the expected metabolic changes, comparable to the control rats by 8 weeks. Changes in fasting glucose concentrations from 8 weeks to 16 weeks showed that mCS, mHCHF and mCBlack groups had an increased fasting glucose concentration at 16 weeks compared with their 8 week tests (Figure 4.47). The mHBlack group showed more impaired glucose tolerance at 16 weeks, than at 8 weeks, while no others changed significantly from their 8 week values.

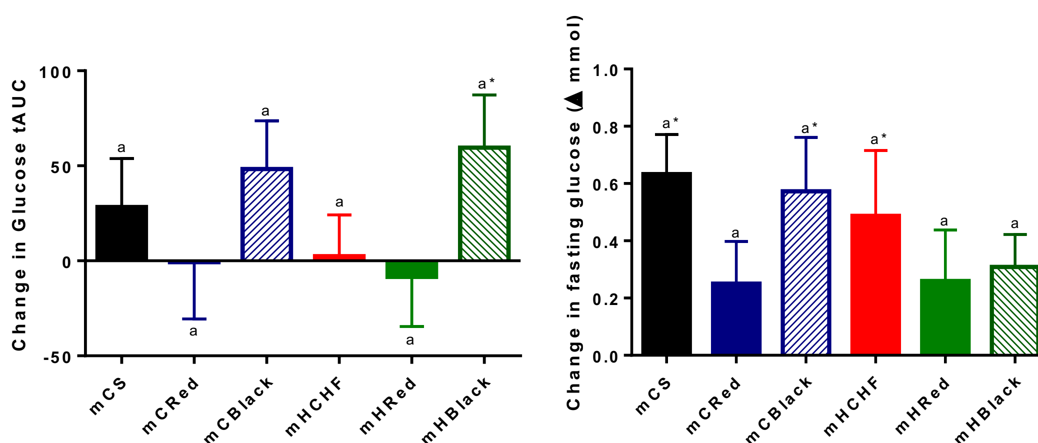


Figure 4.47: Changes in glucose TAUC (area under curve), and fasting glucose blood concentrations between the 8-week test, and 16 week tests. Differing lower-case letters indicate significantly different change from control, determined by one-way ANOVA at p<0.05. * indicates significantly different change from 8-week glucose TAUC, at p<0.05 determined by non-parametric T-test.

Average islet area did not differ between the mHCHF and mCS control groups. The only differences noted were between the mCS and the mHBlack or mHRed groups, which both had lower pancreatic islet size than the mCS control. The cell count did not differ between the mCS and mHCHF groups. mHRed group had lower cell counts per islet than both the mCS and mHCHF control groups. Islet density was higher in the mHCHF than mCS. Red or black sorghum flour increased density in the mCS diet. The only group which did not differ from the mCS control was the mHRed group, however it did not differ from mHCHF (Figure 4.48).

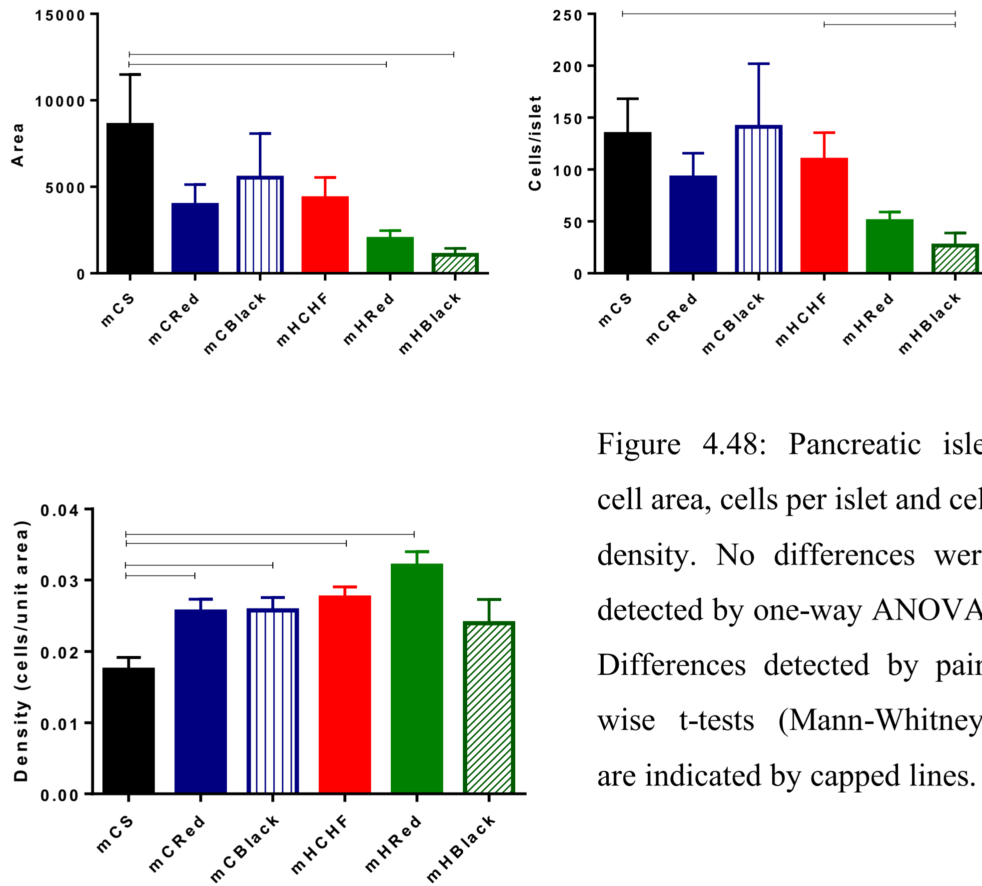


Figure 4.48: Pancreatic islet cell area, cells per islet and cell density. No differences were detected by one-way ANOVA. Differences detected by pair-wise t-tests (Mann-Whitney) are indicated by capped lines.

Table 4.18: Blood glucose and plasma lipids of rats fed mCS, mCRed, mCBlack, mHCHF, mHRed, and mHBlack diets.

Parameter	mCS	mCRed	mCBlack	mHCHF	mHRed	mHBlack	Intervention	Diet	Interaction
Fasting Blood glucose (mM) 8 week	3.3 ± 0.2 ^{a}	4.4 ± 0.2 ^{b}	3.5 ± 0.1 ^{a}	4.4 ± 0.1 ^{b}	5.3 ± 0.2 ^{c}	4.3 ± 0.1 ^{b}	0.5512	< 0.0001	< 0.0001
TAUC Blood Glucose 8 week	695 ± 20 ^{a}	732 ± 29 ^{a}	640 ± 27 ^{a}	838 ± 19 ^{b}	863 ± 17 ^{b}	727 ± 14 ^{a}	0.4127	< 0.0001	< 0.0001
Fasting Blood glucose (mM) 16 week	4.0 ± 0.2 ^{a}	4.6 ± 0.2 ^{ab}	4.0 ± 0.2 ^{a}	4.9 ± 0.1 ^{b}	5.4 ± 0.1 ^{b}	4.6 ± 0.2 ^{ab}	< 0.0001	< 0.0001	< 0.0001
TAUC Blood Glucose 16 week	724 ± 20 ^{a}	740 ± 27 ^{ab}	676 ± 16 ^{a}	841 ± 28 ^{b}	866 ± 48 ^{ab}	785 ± 18 ^{ab}	0.9447	0.024	< 0.0001
Plasma total cholesterol (mM)	1.71 ± 0.11 ^{a}	1.71 ± 0.10 ^{a}	1.78 ± 0.118 ^{a}	1.66 ± 0.13 ^{a}	1.70 ± 0.06 ^{a}	1.65 ± 0.10 ^{a}	0.3099	0.7525	0.0596
Plasma triacylglycerides (mM)	0.48 ± 0.04 ^{a}	0.64 ± 0.06 ^{a}	1.09 ± 0.133 ^{ab}	1.80 ± 0.36 ^{b}	1.49 ± 0.42 ^{ab}	2.44 ± 0.44 ^{b}	0.0599	< 0.0001	< 0.0001
Plasma non-esterified fatty acids (mM)	1.75 ± 0.17 ^{a}	2.44 ± 0.16 ^{a}	2.81 ± 0.198 ^{a}	4.78 ± 0.17 ^{b}	3.68 ± 0.61 ^{b}	4.49 ± 0.44 ^{b}	< 0.0001	0.0003	< 0.0001

Differing lower-case letters indicate significant differences at $p<0.05$ determined by one-way ANOVA.

Cholesterol concentrations did not differ between the mCS and mHCHF groups, nor any other groups as indicated in Table 4.18. Plasma triglyceride concentrations were elevated in the mHCHF group from the mCS control. The addition of red sorghum flour to the mCS diet did not alter triglyceride concentrations, however the mCBlack group showed triglyceride concentrations comparable to both mCS and mHCHF. Triglyceride concentrations in the mHRed group were comparable to mCS and mHCHF. There was a large variability in the concentrations within the mHRed group ranging from 3.51 to 0.51 mM, therefore results should be interpreted with caution. Black sorghum flour did not reduce the triglycerides concentrations in the mHBlack group. Plasma NEFA concentrations were increased in mHCHF from the mCS. Addition of sorghum flour did not alter NEFA concentrations in either diet or group (Table 4.18).

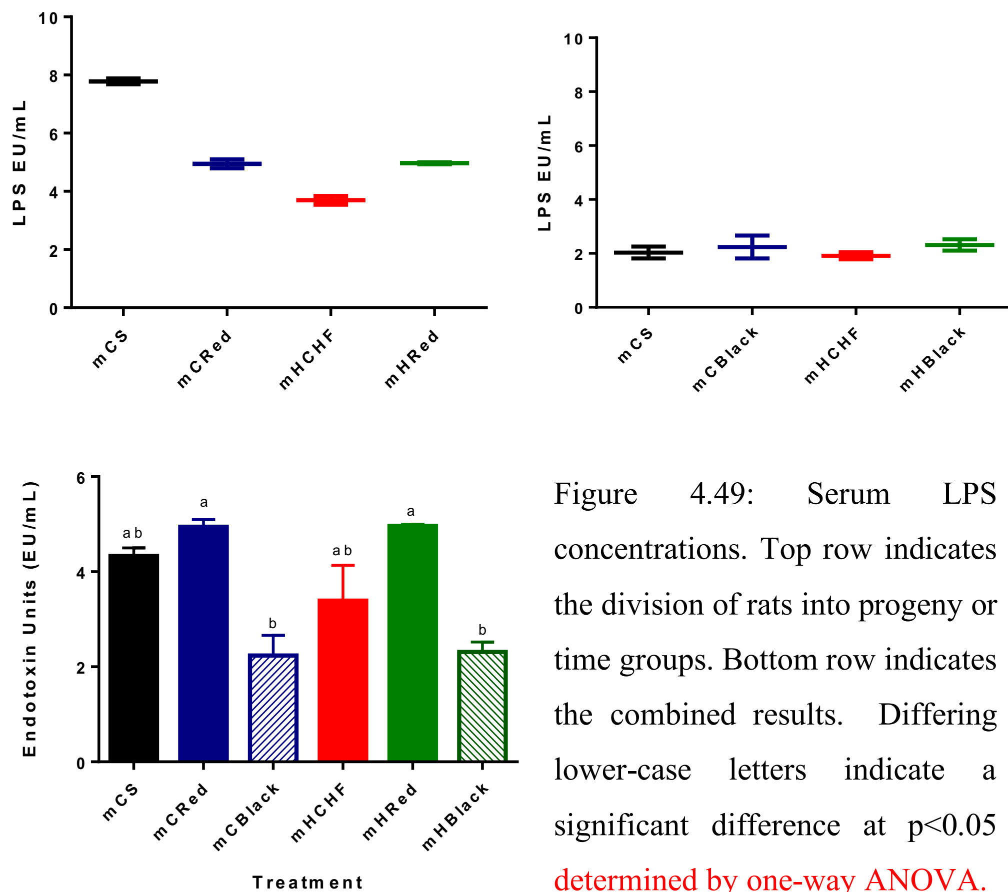


Figure 4.49: Serum LPS concentrations. Top row indicates the division of rats into progeny or time groups. Bottom row indicates the combined results. Differing lower-case letters indicate a significant difference at $p<0.05$ determined by one-way ANOVA.

Serum LPS concentrations did not differ with addition of sorghum flour, red or black. It appears that there were differences between the red sorghum flour supplementation and black sorghum flour supplementation. As these interventions were carried out at different times, it is not clear whether changes were due to the sorghum itself or another time-based factor such as progeny differences in rats. The results shown in Figure 4.49 have been interpreted with caution. There were differences in control groups which were carried out at different times. For controls carried out at the same time as the red sorghum flour intervention, the mCS group had a serum LPS concentration 4 times higher than that of the mCS controls carried at the same time as the black sorghum flour intervention.

4.3.4 Liver and cardiovascular structure and function

Normalised liver wet weight was higher in the mHCHF, mHBlack and mHRed groups than the mCS, mCBlack and mCRed groups. Addition of sorghum did not affect liver weight. Histology indicated that there was ballooning and fat deposition in the mHCHF livers, shown in Figure 4.50. There was a reduction in the ballooning and fat deposition in the mHRed group, which appeared comparable to mCS. In the mHBlack group, there was a reduction in markers of steatosis but these were not normalised to mCS.

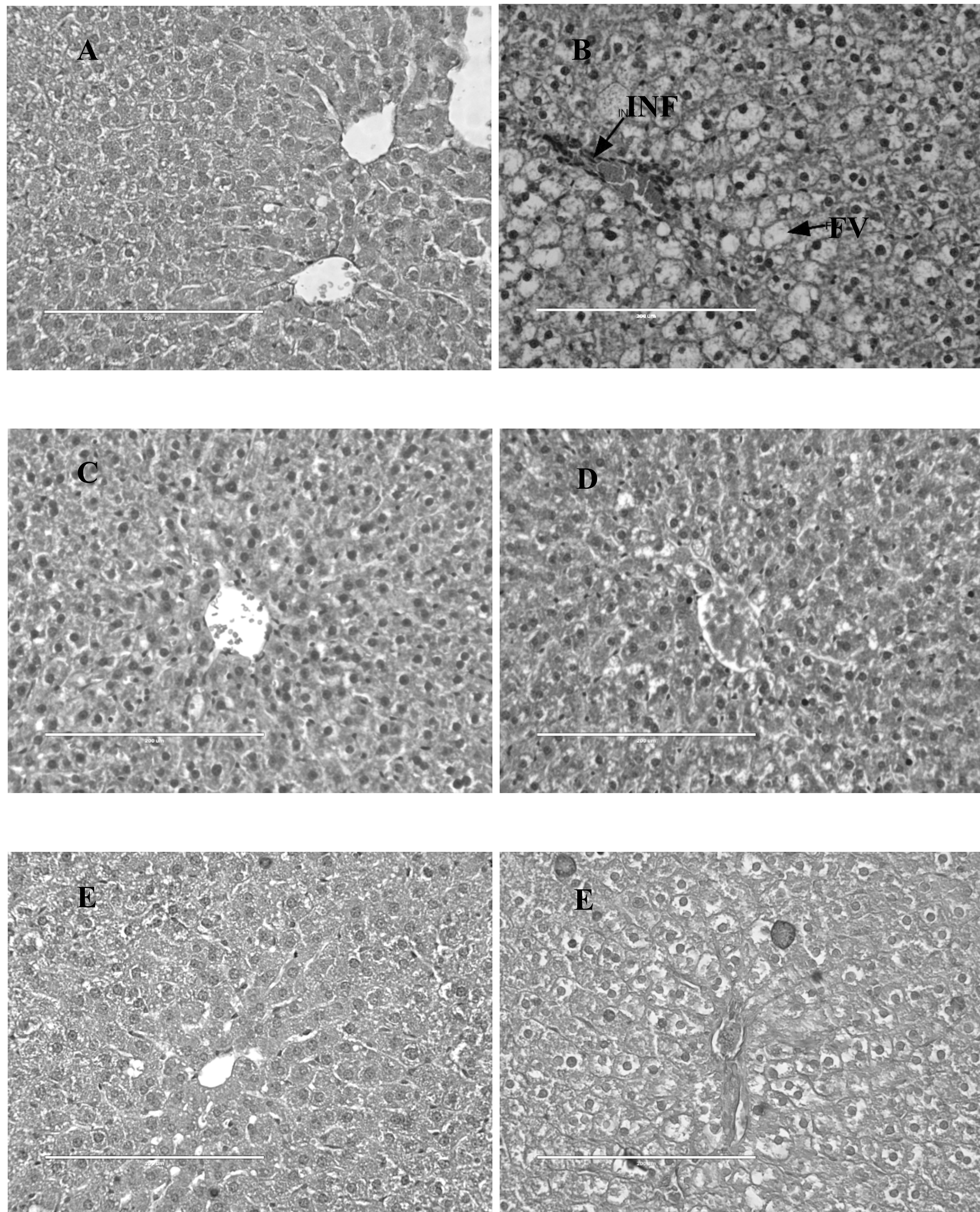


Figure 4.50: Haematoxylin and eosin staining of liver sections (10µm) under 20x magnification. mCS (A), mHCHF (B), mCRed (C), mHRed (D), mCBlack (E), mHBlack (F). “INF” indicates inflammatory cell, “FV” indicates fat vacuole.

Plasma AST activities were higher in the mCS group compared with the mHCHF group. Addition of sorghum to either diet only resulted in a change in the mCBlack group, where AST activity was comparable to mHCHF. Plasma ALT activities were not altered by diet or intervention. The AST/ALT ratio was lower in mHCHF than mCS. All other groups were statistically comparable to the mCS and mHCHF. This indicated a possible increase in mCS sorghum flour supplemented rats and a possible

reduction in the mHRed and mHBlack rats, supported by significant interaction effect. Creatine kinase activities did not change between groups. Lactate dehydrogenase activity was higher in the mCS group than all other groups, as indicated in Table 4.19.

Table 4.19: Cardiovascular and liver structure and function parameters of rats fed mCS, mCRed, mCBlack, mHCHF, mHRed, and mHBlack diets.

Parameter	mCS	mCRed	mCBlack	mHCHF	mHRed	mHBlack	Intervention	Diet	Interaction
Systolic Blood Pressure 16 week (mmHg)	135 ± 0.5 ^{a}	140 ± 1.8 ^{a}	132 ± 1.7 ^{a}	139 ± 0.6 ^{a}	133 ± 2.3 ^{a}	143 ± 4.7 ^{a}	0.1329	0.5739	< 0.0001
Left ventricle + septum (mg/mm tibial length)	17.61 ± 0.91 ^{a}	18.21 ± 0.58 ^{a}	17.07 ± 1.65 ^{a}	23.89 ± 0.97 ^{b}	20.14 ± 0.62 ^{ab}	22.86 ± 1.26 ^{b}	0.087	0.3361	< 0.0001
Right ventricle (mg/mm tibial length)	4.64 ± 0.37 ^{a}	4.30 ± 0.22 ^{a}	3.77 ± 0.43 ^{a}	6.35 ± 0.36 ^{b}	4.90 ± 0.36 ^{ab}	5.24 ± 0.35 ^{ab}	0.3719	0.0096	0.001
Liver wet weight (mg/mm tibial length)	184.10 ± 6.65 ^{a}	216.30 ± 10.98 ^{a}	209.70 ± 20.01 ^{a}	340.60 ± 9.05 ^{b}	309.80 ± 8.61 ^{b}	300.70 ± 27.94 ^{b}	0.0851	0.8693	< 0.0001
Diastolic stiffness constant (κ)	24.9 ± 0.30 ^{a}	25.6 ± 0.30 ^{ab}	29.8 ± 1.0 ^{c}	27.50 ± 0.40 ^{b}	26.9 ± 0.30 ^{ab}	26.6 ± 0.20 ^{ab}	0.0014	0.6076	< 0.0001
Plasma ALT activity (U/L)	31.7 ± 2.2 ^{a}	30.1 ± 2.0 ^{a}	26.4 ± 1.8 ^{a}	32.4 ± 5.3 ^{a}	25.4 ± 1.1 ^{a}	25.8 ± 1.3 ^{a}	0.0116	< 0.0001	0.0414
Plasma AST activity (U'L)	93.3 ± 6.2 ^{a}	89.6 ± 8.4 ^{a}	64.5 ± 3.4 ^{b}	65.3 ± 4.3 ^{b}	70.6 ± 3.1 ^{b}	68.1 ± 3.5 ^{b}	< 0.0001	< 0.0001	< 0.0001
Plasma ALT/AST Ratio	3.0 ± 0.4 ^{a}	3.0 ± 0.2 ^{ab}	2.5 ± 0.1 ^{ab}	2.1 ± 0.3 ^{b}	2.8 ± 0.1 ^{ab}	2.7 ± 0.2 ^{ab}	< 0.0001	0.0004	0.0001
Plasma CK activity (U/L)	99.5 ± 11.6 ^{a}	104.1 ± 10.5 ^{a}	88.1 ± 12.2 ^{a}	117.8 ± 25.6 ^{a}	87.0 ± 17.9 ^{a}	121.0 ± 15.1 ^{a}	0.0005	0.09	0.0241
Plasma LD activity (U/L)	273.8 ± 29.2 ^{a}	188.4 ± 19.9 ^{b}	170.4 ± 16.0 ^{b}	218.0 ± 62.7 ^{b}	186.3 ± 15.3 ^{b}	166.4 ± 11.5 ^{b}	0.0195	< 0.0001	0.0188

Differing lower-case letters indicate significant differences for a given parameter at $p<0.05$ determined by one-way ANOVA.

Abbreviations are as follows: ALT (alanine transferase), AST (aspartate transferase), CK (creatine kinase), LD (lactate dehydrogenase).

GLUT2 expression did not differ between the mCS and mHCHF groups, nor any intervention groups, Figure 4.51.

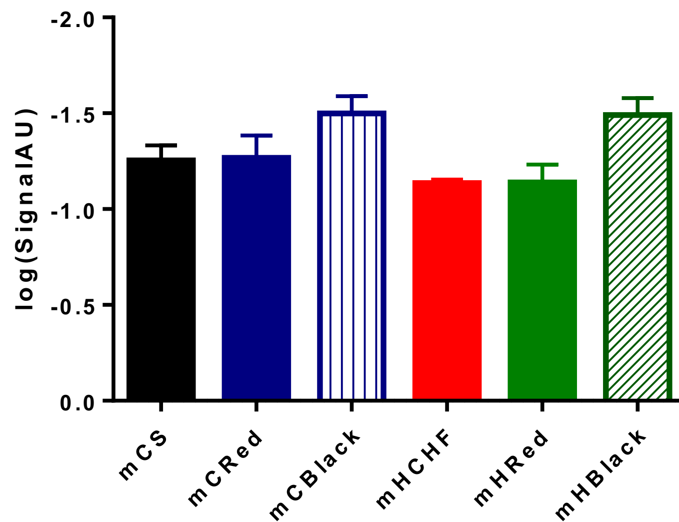


Figure 4.51: GLUT2 expression in liver determined by semi-quantitative ELISA. No significant differences were noted at $p<0.05$ determined by one-way ANOVA.

Liver TLR4 expression did not differ between the mCS and mHCHF. Sorghum addition to the diet did not change expression in either diet.

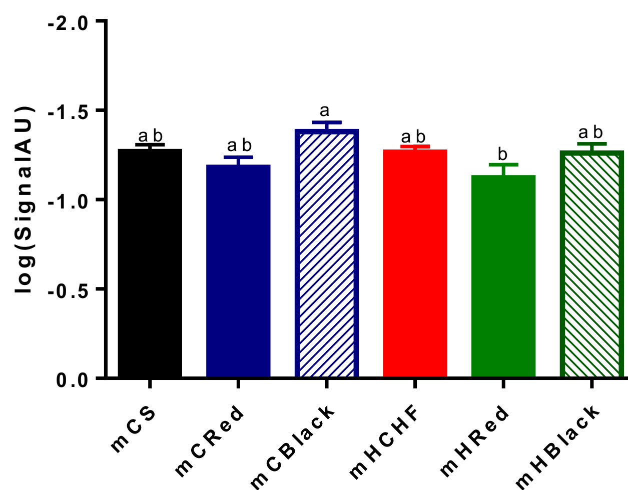


Figure 4.52: Liver TLR4 expression determined by semi-quantitative ELISA. No significant difference between CS and HCHF, using a one-way ANOVA, $p<0.05$ determined by one-way ANOVA.

Normalised left ventricle + septum weight was increased in the mHCHF group from the mCS control. Addition of sorghum partially reduced weight in the mHRed group to comparable to the mCS control (Table 4.19). Intervention had no effect in any other group. Right ventricle weight was higher in the mHCHF group than mCS. The mHRed and mHBlack groups showed a partial reduction in right ventricular weight with no difference from mCS, however not different from mHCHF. Diastolic stiffness was increased in the mHCHF from mCS. Diastolic stiffness of mHRed and mHBlack was comparable to both the mCS and mHCHF. The mCBlack group had a higher diastolic stiffness (κ) than all other groups (Table 4.19).

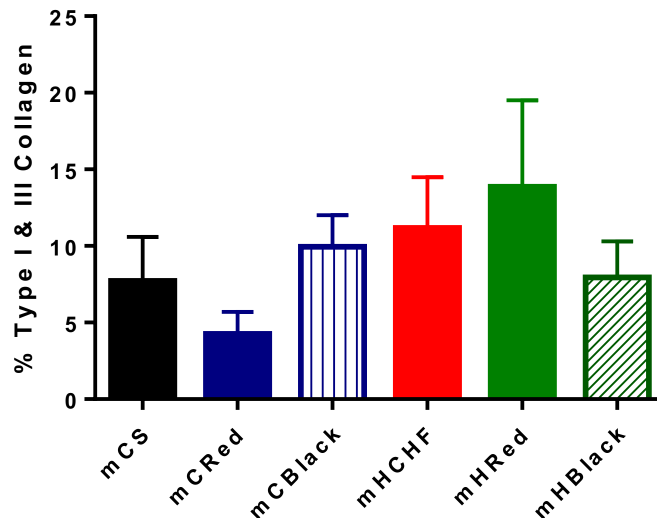


Figure 4.53: Picrosirius red staining indicating the % type I and type III collagen deposition in the left ventricle. No significant difference was found at $p<0.05$ determined by one-way ANOVA.

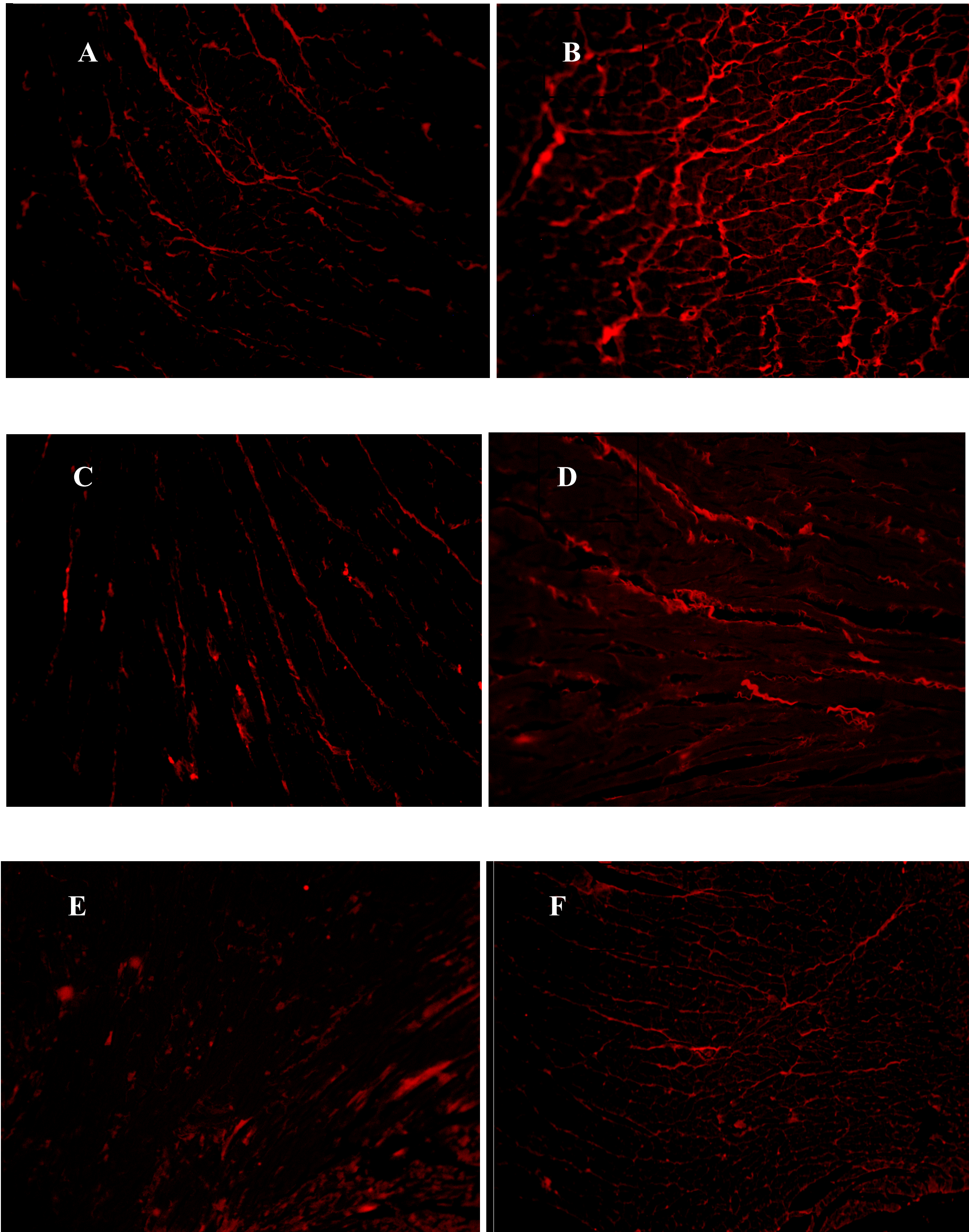


Figure 4. 54: Picrosirius red stained left ventricle sections, 20x magnification. Bright red striations indicate deposition of collagen (Type I and Type III fibres). mCS (A), mHCHF (B), mCRed (C), mHRed (D), mCBlack (E), mHBlack (F).

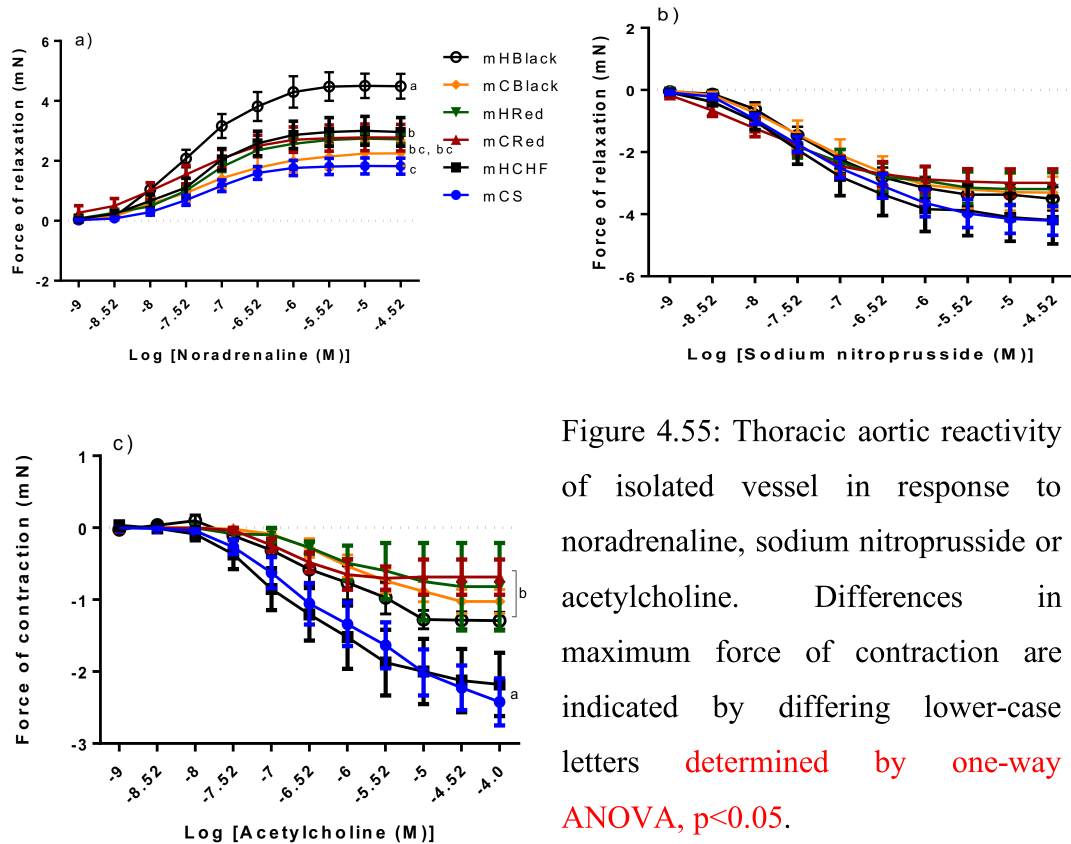


Figure 4.55: Thoracic aortic reactivity of isolated vessel in response to noradrenaline, sodium nitroprusside or acetylcholine. Differences in maximum force of contraction are indicated by differing lower-case letters determined by one-way ANOVA, $p<0.05$.

Thoracic aortic force of contraction with exposure to noradrenaline was greater in the mHCHF group, than mCS. The mHBlack group had higher reactivity than all other groups. There was no significant difference between the mCS group and the mHRed or mHBlack group, yet there was also no significant change from the mHCHF group. Response to sodium nitroprusside did not differ between any group for any concentration as shown in Figure 4.55.

Responses to acetylcholine did not differ between the mCS and mHCHF groups. All treatment groups had a reduced force of contraction.

4.3.5 Gastrointestinal function

No changes in maximum ileal force of contraction to acetylcholine were noted between any diets, as is indicated in Figure 4.56. The minimal concentration required for contraction did not differ between the mCS and mHCHF groups. The minimum concentration required to elicit a response was lower in the mHRed and mHBlack groups than in the mHCHF control. The TAUC of the curves indicated the overall responsiveness of the ileum. These values did not differ between any groups.

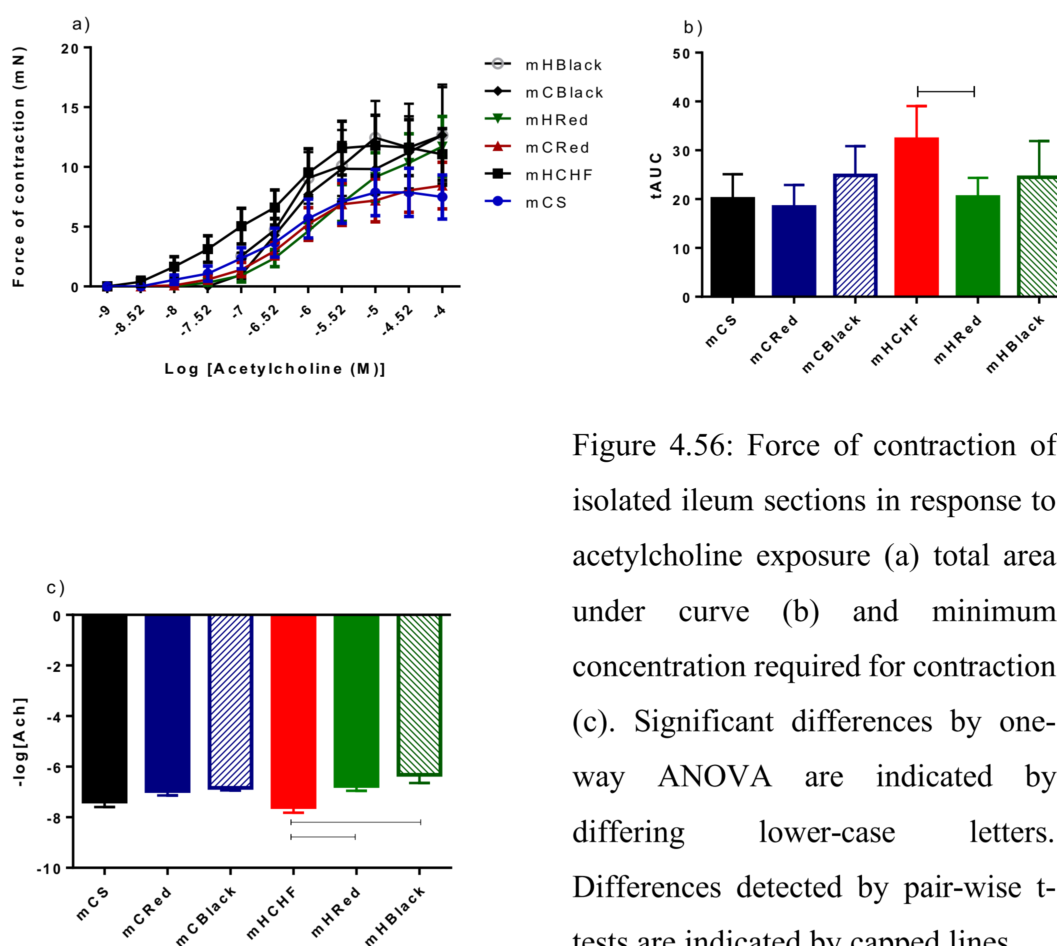


Figure 4.56: Force of contraction of isolated ileum sections in response to acetylcholine exposure (a) total area under curve (b) and minimum concentration required for contraction (c). Significant differences by one-way ANOVA are indicated by differing lower-case letters. Differences detected by pair-wise t-tests are indicated by capped lines.

There were no differences in maximal colonic contraction to acetylcholine in the mCS and mHCHF diets. No differences in minimum concentration of acetylcholine required for contraction, or total dose-response area under curve were found between groups. The overall p value for one-way ANOVA of minimum concentration of acetylcholine

required for response was 0.0464, therefore the null hypothesis cannot be rejected in this case.

Further investigation using *t*-tests indicated that addition of black sorghum flour to the mHCHF diet lowered the minimum concentration required for a response, to a level that was comparable with the mCS group. Minimum acetylcholine concentration required for a response was reduced in mCRed from mCS (Figure 4.57).

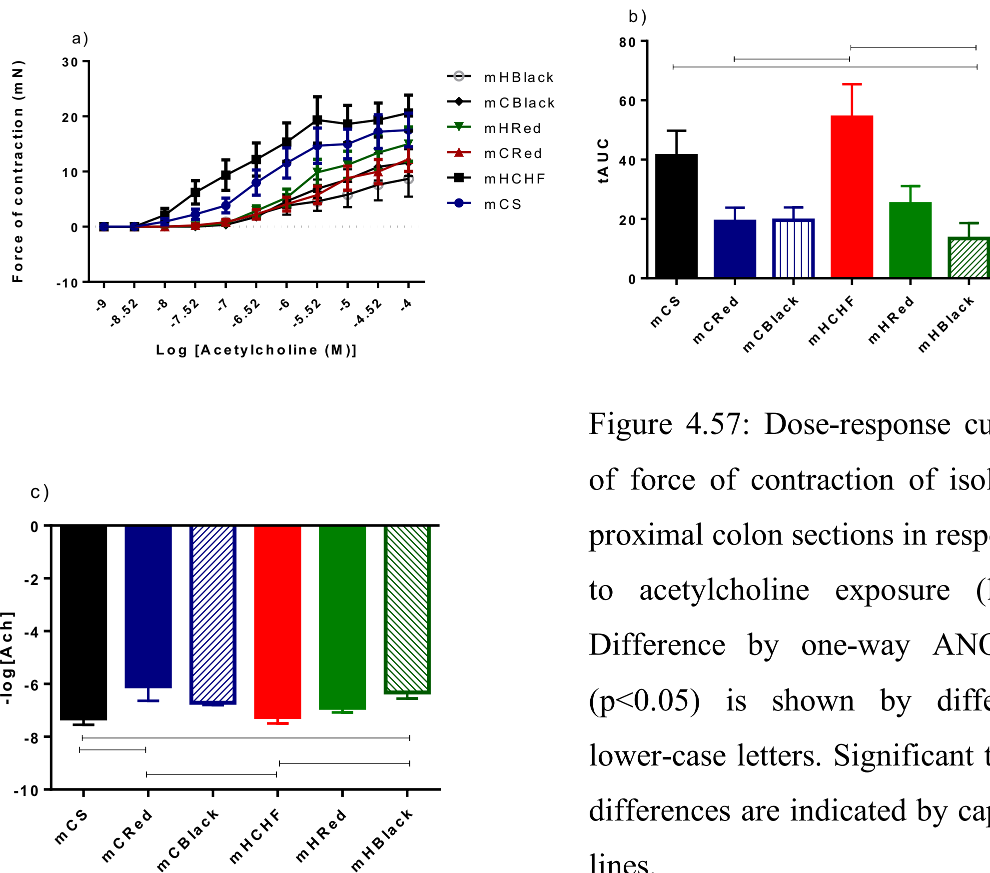


Figure 4.57: Dose-response curves of force of contraction of isolated proximal colon sections in response to acetylcholine exposure (left). Difference by one-way ANOVA ($p<0.05$) is shown by differing lower-case letters. Significant t-test differences are indicated by capped lines.

Colon TLR4 expression did not differ between any experimental or control groups, Figure 4.58.

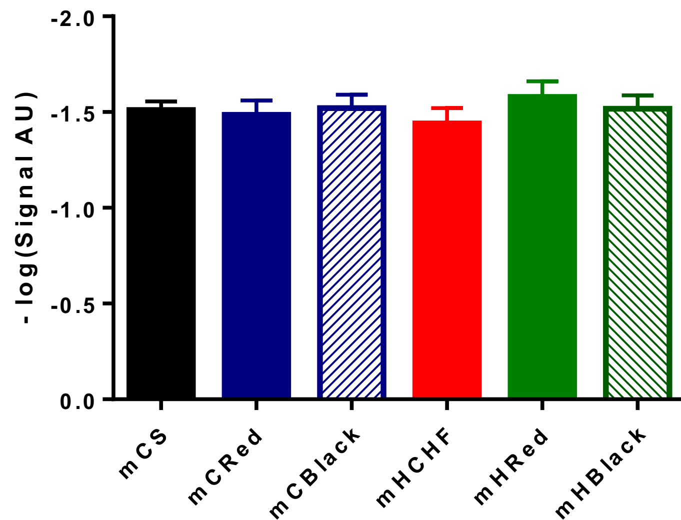


Figure 4.58: Colon TLR4 expression as determined by semi-quantitative ELISA. No significant differences were noted at p<0.05 determined by one-way ANOVA.

Colon claudin expression did not differ between mHCHF and mCS model after 16 weeks, Figure 4.59. No differences were evident after supplementing with either black or red sorghum flour.

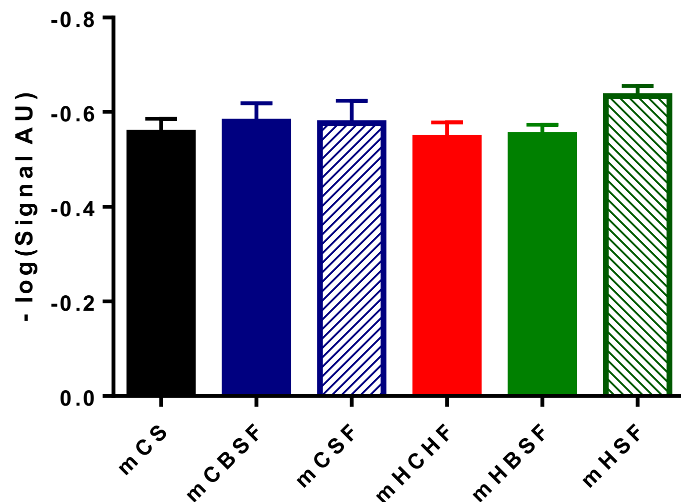


Figure 4.59: Claudin expression as determined by semi-quantitative ELISA. No significant differences were noted at p<0.05 determined by one-way ANOVA.

Colon occludin expression was higher in mCS compared with mHCHF group after 16 weeks. 20% red sorghum flour in the mCS diet did not alter occludin expression from mCS. In both the mHRed and mHBlack groups, addition of sorghum did not result in a difference from mHCHF, nor did it differ from mCS. Results are shown in Figure 4.60.

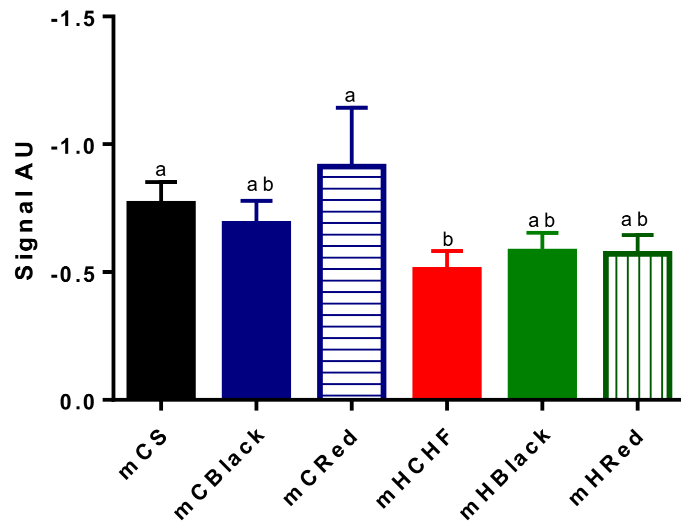


Figure 4.60: Colon occludin expression. Differing lower-case letters indicate significant difference, determined by paired t-tests against the mCS and mHCHF control at p<0.05 determined by one-way ANOVA.

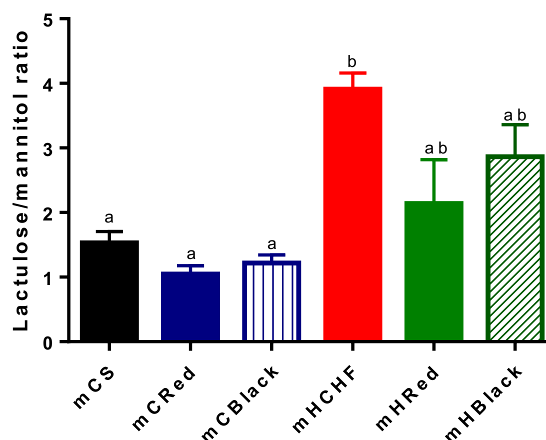


Figure 4.61: Lactulose:mannitol ratio of percent recovery. Differing lower-case letters indicate significant differences by one-way ANOVA at p<0.05.

The lactulose:mannitol ratio based on total percent recovery or orally gavaged sugars was higher in the mHCHF group compared with the mCS group. Addition of sorghum flour to the mCS diet did not alter the ratio of recovered sugars. Addition of either 20% black or red sorghum flour to the mHCHF diet did not alter the ratio of recovery from the mHCHF group. It also did not differ from the mCS control.

Percent recovery of individual sugars only differed between mCS and mHCHF for D-mannitol and sucralose in the 8-20 hour time-period. For both compounds, the percent recovery was higher in mHCHF than mCS. Results discussed are for the 8-20 hour time period. Mannitol recovery was not affected by sorghum in the mCS diet. Both mHBlack and mHRed showed no statistical difference from mCS or mHCHF. Sucralose percent recovery in mCBlack, mHBlack and mHRed did not differ from either the mCS or mHCHF controls. Results are shown in Table 4.20.

Table 4.20: Percent recovery of orally gavaged sugars from urine over a 20-hour period.

	Time (hours)	mCS	mHCHF	mCRed	mHRed	mCBlack	mHBlack
Sucrose	0-3	0.05 ± 0.02 [a]	0.05 ± 0.01 [a]	0.17 ± 0.04 [a]	0.07 ± 0.02 [a]	0.05 ± 0.02 [a]	0.04 ± 0.02 [a]
	3-8	0.20 ± 0.07 [a]	0.18 ± 0.03 [a]	0.32 ± 0.07 [a]	0.11 ± 0.02 [a]	0.17 ± 0.02 [a]	0.19 ± 0.07 [a]
	8-20	0.26 ± 0.06 [a]	0.24 ± 0.09 [ab]	0.05 ± 0.02 [b]	0.34 ± 0.05 [ab]	0.42 ± 0.09 [ab]	0.32 ± 0.07 [ab]
Lactulose	0-3	0.31 ± 0.14 [a]	0.21 ± 0.08 [a]	0.54 ± 0.16 [a]	0.61 ± 0.32 [a]	0.25 ± 0.10 [a]	0.10 ± 0.06 [a]
	3-8	1.29 ± 0.72 [a]	1.26 ± 0.32 [a]	0.80 ± 0.20 [a]	0.90 ± 0.14 [a]	0.88 ± 0.14 [a]	1.19 ± 0.20 [a]
	8-20	0.90 ± 0.30 [a]	1.20 ± 0.40 [ab]	0.20 ± 0.07 [ab]	1.53 ± 0.24 [ab]	2.66 ± 1.24 [b]	1.63 ± 0.16 [ab]
Mannitol	0-3	0.44 ± 0.14 [a]	0.58 ± 0.16 [a]	1.26 ± 0.27 [a]	0.63 ± 0.34 [a]	0.33 ± 0.15 [a]	0.39 ± 0.25 [a]
	3-8	1.28 ± 0.32 [a]	2.51 ± 0.67 [a]	2.36 ± 0.63 [a]	1.06 ± 0.50 [a]	1.81 ± 0.28 [a]	2.65 ± 0.40 [a]
	8-20	1.51 ± 0.47 [a]	4.07 ± 0.87 [b]	0.53 ± 0.17 [a]	2.98 ± 0.34 [ab]	2.03 ± 0.62 [a]	2.70 ± 0.50 [ab]
Sucralose	0-3	0.54 ± 0.22 [a]	0.70 ± 0.23 [a]	1.31 ± 0.40 [a]	0.80 ± 0.54 [a]	0.39 ± 0.26 [a]	0.29 ± 0.29 [a]
	3-8	3.08 ± 1.07 [a]	2.86 ± 0.59 [a]	2.36 ± 0.96 [a]	1.40 ± 0.45 [a]	1.80 ± 0.31 [a]	1.71 ± 0.46 [a]
	8-20	1.80 ± 0.56 [a]	4.27 ± 0.90 [b]	0.27 ± 0.17 [a]	2.08 ± 1.36 [ab]	3.39 ± 1.16 [ab]	3.73 ± 0.97 [ab]

Differing superscript lower-case letters indicate a significant difference at $p<0.05$ determined by one-way ANOVA.

4.4 Discussion

This study quantified the responses following supplementation of 20% red or black sorghum flour into the diet of rats to alter metabolic syndrome. Overall, red and black sorghum altered metabolic syndrome minimally. There were different effects of red or black sorghum flour. More changes occurred with sorghum addition to the mCS diet, than to the mHCHF diet. These implications are also discussed. Red sorghum in a corn starch-based diet increased feed efficiency, increased lean mass, increased epididymal fat, increased body weight, reduced feed intake, increased pancreatic β-cell density, reduced vascular responses to acetylcholine *ex vivo* and decreased urinary recovery of sucrose given orally. Changes caused by black sorghum in a corn starch-based diet were increased feed efficiency, increased lean mass, increased epididymal fat, increased body weight, reduced feed intake, increased pancreatic β-cell density, increased cholesterol, increased left ventricular diastolic stiffness, reduced vascular responses to acetylcholine *ex vivo* and increased urinary recover of lactulose given orally. Red sorghum flour in the mHCHF diet improved liver morphology, reduced vascular responses to acetylcholine and noradrenaline, reduced minimum concentration of acetylcholine needed to induce ileum contraction. It also showed indications of reduced cholesterol, reduced right ventricular weight, reduced diastolic stiffness, increased colon occluding expression, and reduced mannitol and sucralose recovery in urine after an oral challenge. Black sorghum addition to the mHCHF diet resulted in reduced β-cell density in islets, improved liver structure, reduced vascular reactivity to acetylcholine and noradrenaline exposure, increased sensitivity to acetylcholine in the ileum and colon, and reduced mannitol recovery in urine after an oral challenge, indicating improved small intestinal integrity.

Body composition and dietary intake

The reduced food intake caused by adding sorghum flour to the mHCHF diet shows that there is a satiety effect of sorghum that is not simply caused by increased energy density. In humans, increased concentrations of post-prandial satiety hormones, GLP-1 (glucagon like peptide-1), PYY (peptide YY/peptide-tyrosine-tyrosine) and GIP (gastric inhibitory peptide) occurred after consumption of sorghum-based foods (222). The same study suggested higher subjective satiety than with wheat consumption.

Resistant starches present in sorghum increased satiety in other studies (464). This may be in part due to delayed gastric emptying (465). The increased protein, energy density and carbohydrates are all likely contributors to the change in food volume consumption. Further, the presence of tannins, 3-deoxyanthocyanidins and phenolic acids slow the digestion, primarily of starch (56, 103). Extruded whole white or red sorghum consumption by rats showed satiety effects (466).

The reduced feed intake, improved feed efficiency and increased lean mass following sorghum consumption in the mCS diet suggests that the mCS diet may have been protein-deficient. "Low protein diets" in Wistar rats contain protein contents of 10% (467) or 9% (468-470), and some as low as 6% (471-473). The percent protein in the diet was only 1.58%; the supplemented diets had protein contents of approximately 5%. In the mHCHF rats, the diet had a protein content of 4.31%, and the mHBlack and mHRed rats were consuming diets with 7.3% protein. Protein in the diet is the primary driver of food consumption (474). In this case, the increased energy and protein content of the mCS and mHCHF diets when sorghum flour was added is likely to have caused the reduction in food consumption.

Low protein diets result in lower cross-sectional area of muscle fibres, and reduction in body weight (471). These results suggest that sorghum is a suitable nutritional source of protein and calories in protein-deficient diets. Furthermore, higher protein diets were more effective in reduction of bodyweight and correcting metabolic syndrome than low protein counterparts (224, 475-477).

Changes in body composition can be explained, in part, by increased calorie intake and increased protein intake. Protein-deficient diets led to reduced muscle synthesis, and with the addition of protein to a suitable level, repair can occur. This is typically simultaneous with some fat gain, as was seen in this study. Rats given diets with 6.4% protein showed no body weight gain, and when changed to a diet of 18% after 28 days showed large increases in body weight and overall body protein content (lean mass) (478). Other studies showed similar results where switching from protein-malnourished (8% protein diet) to a high-protein (22%) diet led to considerable weight gain (479). That study also reported that, if malnutrition occurs post-weaning, adipocyte differentiation is reduced and may not recover with refeeding (479). Others

note the importance of leucine intake in protein malnutrition recovery (480), and this has been suggested as a treatment of obesity and metabolic syndrome (475).

The amino acid profile of sorghum is favourable for leucine intake. Leucine is the second highest amino acid by weight in sorghum, after glutamic acid (481). Although it can vary by type and treatment (482), the typical amount of leucine is 0.643g of leucine per 100g of raw flour (32). The amount of leucine required to stimulate muscle synthesis does not appear to be exceptionally high. There was a linear trend to increased protein synthesis in rat skeletal muscle with doses of leucine from 0.068g/kg to 1.35g/kg body weight (483). A 20% sorghum supplement in the diet is approximately equivalent to 0.045g leucine intake per day, based on 0.643g leucine per 100g of flour, and intake of 35g per day. For a rat of 300g, this is approximately 0.15g of leucine per kilogram per day. From this data, it appears that leucine derived from sorghum may be a driver of increased lean mass, even though diets were still “low protein” in the mCS and sorghum-supplemented mCS models. The assessment of myofibril diameter showed no differences between rats, yet the DXA results showed increased muscle mass. The increase can thus be attributed to increased myofibril number rather than size. This outcome is particularly beneficial as there was an inverse correlation between skeletal muscle mass and metabolic syndrome in a Korean cohort (484, 485), and in the general population (486-488).

Pre-diabetes

An improvement in the glucose tolerance with the addition of sorghum was expected due to the increased concentration of phenolic acids, flavanols and anthocyanidins. In chapter 3, there was normalisation of glucose tolerance with 5% whole sorghum. The higher dose of sorghum was expected to improve glucose tolerance. No improvements in fasting glucose concentrations nor glucose tolerance tests were found in this study. These outcomes are comparable to what was seen in supplementing decorticated sorghum flour or sorghum bran into an obesogenic diet of rats (489). That study showed that only whole sorghum flour without decortication corrected glucose tolerance in a high-fat diet, despite having a lower phenolic content (489). This outcome suggests that the effects on glucose tolerance when consuming sorghum flour are due to another component, possibly the starch, lipid or protein. Short-term

ingestion of whole sorghum flour muffins lowered insulin fluxes more than a wheat-based counterpart and marginally reduced fluctuations in glucose (206), and whole sorghum resulted in a smaller increase in plasma glucose than with rice and wheat foodstuffs (204). Methanol extracts of sorghum reduced fasting glucose in a mouse model of diet-induced obesity (185). Ethanol extracts of sorghum showed insulin-independent normalisation of glucose responses in diabetic rats (207).

The different outcomes suggest that the matrix by which sorghum is delivered in the diet, not the amount, is important as supported by Moraes*, et al.* (489). Whole sorghum has a greater effect at a lower dose than sorghum flour. Particle size of flour does not affect glucose responses in wholemeal flours (490). Wholegrains and wholemeal flours, although similar in a macronutrient sense, have very different effects on blood sugar and post-prandial responses, whereby wholegrains are more slowly digested and show lower insulin responses (491). The structure and digestibility differ largely, although macronutrients do not generally differ markedly between the wholegrain and the wholemeal flour (492).

Dyslipidaemia

Blood lipid profiles were expected to differ between the mCS and mHCHF groups (353). For cholesterol, no difference was noted between any groups. This was unexpected as typically diets with high levels of beef tallow resulted in high circulating cholesterol concentrations (353). Diet-induced obesity in rats showed differences in HDL and LDL concentrations, even when total cholesterol did not differ, but sorghum flour had no effect (489). I could not link sorghum flour consumption to changes in serum cholesterol, however LDL and HDL were not assessed separately. Sorghum extracts decreased plasma cholesterol concentrations in hamster models (183, 184), while in hyperlipidaemic rats increased total cholesterol (392). Unrefined sorghum consumed at a rate of 100g per day in human studies improved serum lipid profiles (187).

The outcomes with respect to triglycerides reflected the outcomes of another sorghum flour study, where no change in triglycerides was found (489). Sorghum extract reduced plasma triglycerides in mice (185) and rats (181). Resistant starches may reduce the concentrations of serum triglycerides in rat models (493). Phenolic

compounds including gallic acid, vanillic acid, coumaric acid, caffeic acid, ferulic acid, chlorogenic acid and naringenin reduced triglyceride synthesis *in vitro*, while protocatechuic acid, hydroxybenzoic acid and syringic acid were ineffective (494).

Cardiovascular changes

Very few changes occurred with respect to cardiovascular parameters. The lack of differences between the two control diets was of concern. The raised systolic blood pressure, above the expected healthy 120mmHg, in all groups may be caused by the low protein in the mCS, mCBlack and mCRed groups. Rats maintained on a low protein (8%) diet after *in utero* protein restriction showed increased blood pressure (495). A few studies have linked undernutrition to hypertension in children and adolescent humans (496). Post-weaning protein malnutrition in male Wistar rats has been linked to increased systolic and diastolic blood pressure, with contributions from increased superoxide production, and increased nitric oxide release (497).

As this model appears to have insufficient protein, the lack of change in systolic blood pressure cannot be directly related to metabolic syndrome. I cannot make reasonable assumptions about the effects of sorghum on hypertension in metabolic syndrome. Red or black sorghum flour at 20% supplemented to the diet did not correct hypertension, although the cause in this study remains unclear. Additionally, I observed no change in diastolic stiffness, which is consistent with the lack of changes in systolic blood pressure. Evidence of sorghum's direct effect on blood pressure is scarce. Wholegrain consumption reduced blood pressure in adult individuals (498-500).

It was expected that an obesogenic diet would impair endothelium-dependent vasoconstriction (353) and that overweight rats would have lower reactivity to acetylcholine. This has been shown on studies of high-fat diet-induced obesity in mice (501) and rats (353). In our model this did not occur. This is similar to the increased vasoconstriction sensitivity to noradrenaline in diabetic dogs (502), and diabetes has been linked to hypertension in rats (503). Therefore, the marked increase in response with black sorghum flour to noradrenaline is potentially detrimental, as sensitivity to vasoconstrictors may be a contributor to the development of hypertension, possibly caused by a diabetic state (504).

The reduced vasodilation to acetylcholine in all sorghum-treated groups is of concern. Impaired vasodilation indicates vascular dysfunction and is associated with type 2 diabetes (505, 506), hypercholesterolaemia (507) and hypertension (508). No research on the effects of sorghum flour on vasodilatation and vasoconstriction sensitivity have been published. Our outcomes suggest that sorghum flour does not improve these symptoms of metabolic syndrome.

Impaired vasodilatation is associated with high concentrations of circulating free fatty acids, such as occurs in dyslipidaemia of metabolic syndrome (509), which is consistent with the lack of changes seen in plasma lipids. Wholegrain cereals can improve endothelial-dependent vasodilation (510). Flavonoids reduced blood pressure in rat models and *ex vivo* vascular reactivity to acetylcholine (230). As both sorghum flours were produced for human consumption and subject to quality testing, we assume that there was no effect of mycotoxins which commonly cause vasoconstriction (511).

Liver changes

Supplementing with sorghum flour showed that there are benefits to liver function and to structure. Plasma AST and ALT activities are increased in liver disease, as is LDH to a lesser extent. The increased AST in the "healthy diet" is concerning and makes interpretation of sorghum's effects difficult. Median reference values for male Wistar rats are 33 U/l and 66 U/l for ALT and AST respectively (512). The "healthy" controls had AST activities in the upper range of normal, while the mHCHF had "healthier" activities comparable to the median reference range. Sorghum did not affect these enzymes in the obesogenic diet, but black sorghum flour reduced and likely improved AST activity in the corn starch diet. Similarly, lactate dehydrogenase activity appeared improved with sorghum consumption in the mCS diet. The elevated activities of lactate dehydrogenase and AST show higher liver damage in the mCS model, yet histopathology data indicates that steatosis was more pronounced in the mHCHF model.

Low protein diets in Wistar rats can result in increased amino acid transferase activity, and increased lactate dehydrogenase activity (513). The suggested mechanism is an adaptive increase in nitrogen efficiency to minimise loss (513). Other studies of low protein diets also indicate that there may be increased AST with low protein intake, in

studies where protein was 4.13% of the total diet. Additionally, the raised ratio of AST:ALT can be indicative of hepatocellular damage. ALT is considered more specific to liver damage than AST, therefore this change may not simply be a reflection of the liver damage.

Values of approximately 204U/L lactate dehydrogenase indicate a healthy liver function in rat models (512). All sorghum-supplemented groups showed a lactate dehydrogenase activity comparable to the mHCHF group, which is comparable to the CS diet in other studies (353), which suggests poor liver function in the healthy control which is improved by sorghum consumption.

Despite the apparently healthy liver enzymes, histological analyses indicated cellular ballooning and fat deposition in hepatocytes due to a high fat diet, which improved with sorghum flour, but the damage was not completely absent. Chronic consumption of diets high in fatty acids can lead to fat deposition and dysregulation of liver function as seen in metabolic syndrome (514). A low protein diet of 4.13% exacerbated development of fatty liver in high simple carbohydrate models (515). The improved liver structure was consistent with other studies using 51% sorghum in a high fat diet (412).

Expression of TLR4 and GLUT2 in the liver were unchanged by diet or intervention in this study. The expression of GLUT2 in the liver was increased in metabolic syndrome and diabetes (310, 322, 516). In type 1 diabetes, there was increased TLR4 expression in monocytes (517). Insulin-resistant individuals showed higher TLR4 expression in muscle tissue (518). As the liver plays such a major role in peripheral glucose control, as does muscle tissue, similar changes might occur in the liver and be involved in progression or reversal of metabolic syndrome. This was not the case in our model.

Gastrointestinal changes

Effects on the gastrointestinal tract were variable. Most changes that were noted could be attributed to sorghum, rather than the type of diet it was added to. Very few differences were evident between the mCS and mHCHF controls in ileum and colon reactivity. The diet can affect different parts of the gastrointestinal tract in different

ways. High fat diets lead to increased gastric emptying (176, 519), while some research showed slowed gastric emptying (520). Most studies suggest that high-fat diets resulted in increased upper gastrointestinal motility (179) and delayed transit in the colon (179, 521). Reduced cholinergic (acetylcholine-stimulated) contraction was associated with slow colonic transit (522).

More research needs to be conducted as there are problems associated with both weak and strong contractions of the gastrointestinal tract. The optimal gastrointestinal contraction rate or force has not been fully studied. The controls in this research were subject to very low fibre intake. The increased contractions may have been due to the low fibre content. It was noted that the faecal pellets of the mCS and mHCHF rats were much harder and less prevalent in cages than those which received either type of sorghum flour. It is difficult to say whether the changes caused by sorghum were "good" or "bad".

Isolated sorghum compounds caused relaxation of pig ileum *ex vivo* (420). In mice, increasing sorghum leaf extract concentration in the diet increased transit time and reduced intestinal motility and doses of 200mg/kg or more showed anti-diarrhoeal activities (421). Sorghum extracts dose-dependently increased relaxation in the rabbit jejunum and rat stomach (421). Likewise, methanol extracts of red sorghum seeds showed anti-diarrhoeal activity and prolonged gastrointestinal transit time (523).

The expression of occludin in the colon was altered by the diet, however no changes in TLR4 or claudin-1 expression were noted. Claudin and occludin were chosen because they share similar properties with respect to their cellular location but have very different roles (compared with other tight junction proteins), as shown in Figure 4.62.

Given that expression of claudin did not change, I can assume that structurally, the tight junctions are still intact. However, the key change is more likely to be the regulation of the function of these tight junctions. Depletion of occludin resulted in increased selective macromolecule movement across the epithelial barrier, without altering the overall permeability or epithelial resistance (524). One of the molecules studied was mannitol, which had increased flux where occludin was silenced (524).

This is consistent with the increased mannitol recovery in the intestinal permeability tests.

The improvement (reduction) of intestinal permeability in the sorghum treated rats was promising for gastrointestinal health, although permeability was not completely normalised. The increased urinary mannitol recovery in the obesogenic diet indicates that malabsorption is occurring, and this is improved with the addition of sorghum. Because the sorghum consuming groups did not differ from either control for lactulose/mannitol ratio, we cannot reject the hypothesis that sorghum flour improved the GI tract integrity in the small intestine. Dextran sodium sulphate-induced colitis in a rat model was exacerbated by black and Sumac sorghum bran compared with cellulose (453), while sorghum lipids reduced intestinal stress and damage (525).

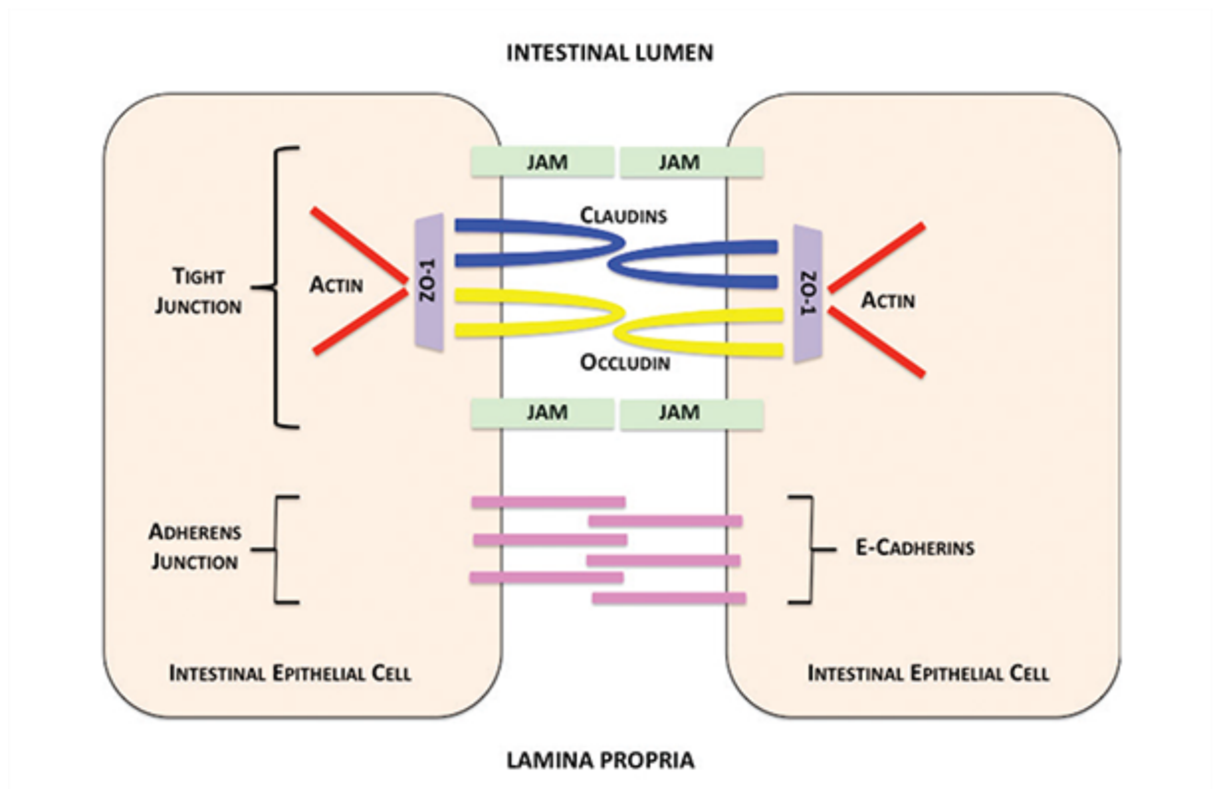


Figure 4.62: Cellular location of tight-junction proteins (526).

Other considerations

While this study compared black and red sorghum flour, it should be noted that “black” and “red” varieties can show major differences within a group with respect to the content of phenolic acids, procyanidins, 3-deoxyanthocyanidins, anthocyanins, flavones and flavanones. Not all sources of “red” or “black” sorghum will give the same effects. While this research is a starting point to promote sorghum as a food source, this information must be taken into consideration. Even within “red” sorghum varieties, there can be marked differences in the composition. The 3-deoxyanthocyanidins, luteolinidin, apigeniniden, 5-methoxyluteolinidin and 7-methoxyluteolinidin, can vary from undetectable to 282.6, 166.2, 120.6 and 104.9µg/g (153). The concentrations of flavones such as luteolin and apigenin can also differ considerably from undetectable to 182.2 and 203.7µg/g similar to the flavanones such as eriodictyol and naringenin which can vary from undetectable to 12.9 and 48.4µg/g (153). The coloured compounds are often biologically active, hence changes in these compounds may confer different health benefits (527). Other compounds including tannins and phenolic acids also vary depending on whether the source is “red” or “black” and also markedly differ within the “red” group of sorghum types (528). In Australia, a number of “red” grain sorghum varieities are grown. Depending on seasonal conditions and the genotype, these will vary in their composition (529).

When comparing black and red sorghum, the true genetic makeup of the plants and breeds that were used in the process is unclear. There are a number of known genes that affect the plant pericarp colour (153). At least two genes control pericarp colour, two affect the testa colour, and 2 affect secondary colour which includes the kernel, sheath and glume (153). These genes may be present in a number of combinations, depending on the variety, and will affect the secondary plant metabolites that affect colour, phenolic contant and therefore biological activty.

Identification and quantification of the bioactive compounds present in the sorghum flours used in this study would further the understanding of how sorghum composition affects health outcomes. This would also provide a baseline for future comparative studies.

Additionally, even though macronutrient profiles of the two sorghums were similar, other studies have shown that the concentrations of resistant starches can differ in

addition to the way they interact in the rat (530), and their health effects (489). The preparation of sorghum will alter its ability to affect metabolic syndrome context, and for human consumption. Major differences in biological effects of products from a single grain source can be due to processing. Hydrolysable starch and glycaemic index vary considerably, since decortication increases hydrolysable starch, as does making a bread, and the hydrolysable starch is more easily digested (531). One of the most significant differences between the whole sorghum and the sorghum flour, although these are marketed as “wholemeal”, is that there is a large reduction in resistant starch, both soluble and insoluble fibre, and in phenolic compounds (531). Most of these components are present in the bran layer, and the degree of decortication will inevitably change their concentrations (531). Processing sorghum alters the starch granule structure, reducing resistant starch amounts.

4.5 Conclusion

There are differing effect of red and black sorghum flour on metabolic syndrome. Red sorghum flour improved appearance of the liver remarkably, while black sorghum flour showed moderate improvements. No changes were noted in blood lipids with either flour. Both red and black sorghum flour were efficient in helping gain lean mass and improve feed efficiency in a low protein, high complex carbohydrate diet. Occludin expression in the colon appeared more susceptible to changes with sorghum consumption than claudin, however this did not translate to reduced concentrations of circulating lipopolysaccharides.

Red or black sorghum flour has shown benefits with its consumption, however there was indications that it may alter vascular reactivity in a way that could be detrimental. Following this project, human trials will provide more accurate information on its effects. Studying sorghum replacements for wheat flour-based diets should be considered, as incorporation into human diets will likely be by replacement rather than addition as in this study. Overall the changes were minimal, but should not be discounted as sorghum can be integrated into a whole diet approach to improving metabolic syndrome.

Chapter 5 – 20% wet cake sorghum in a dietary model of metabolic syndrome

5.1 Introduction

Wet cake sorghum (WS), also called dry distillers grain sorghum, is the by-product of ethanol production. It is produced on a commercial scale and has typically been used for livestock feed. The interest in this sorghum product for human health and especially metabolic syndrome is due to concentrated compounds that have been identified as having nutraceutical benefits. As an example, the concentration of policosanols, long chain alcohols (CH_3-$(CH_2)_n$-CH_2OH $_{n=24\text{-}34}$) derived from plant waxes, are 3 times higher in WS than in grain sorghum (123). The two major long chain policosanols in WS with approximately 90% of the total content are the C30:0 and C28:0 alcohols (131). Treatment of hamsters with WS lipid extracts resulted in reduced plasma and liver cholesterol concentrations (182, 183). Further, plant sterols are approximately 4 times more concentrated in WS than in sorghum grains (123). Plant sterols reduced plasma concentrations of LDL cholesterol and triglycerides (532). Conversely, tocopherols are decreased in WS to 0.2mg/g of lipid (126) compared with 4mg/g of lipid from whole sorghum grains (182).

The fermentation process concentrates phenolic acids to approximately 3 times higher than the raw grain equivalent (533), although some studies suggest phenolic acids are present at around half the amount in whole sorghum, and tannins are also lower at around 80% of that found in whole sorghum (534). Phenolic acids are recognised as having potential to reduce plasma lipids, weight (535) and inflammation (140), and are hypoglycaemic (216, 536). Sorghum distillery waste also contains increased concentrations of flavonoids and anthocyanins compared with the raw sorghum starting product (537). While flavonoid glycoside concentrations decrease, flavonoid aglycones increase, bound phenolic acids and flavonoids are released, and depending on the specific fermentation process, new metabolites are generated (150). Increased intake of dietary flavonoids is associated with reduced risk of developing type-2 diabetes (538), reduced arterial stiffness (539) and reduced systemic inflammation (540) which are all pathologies of metabolic syndrome.

Recent studies on the sorghum waste from biorefinery sources showed that the fermentation process resulted in peptides that have antimicrobial activity, and that the waste contains phenolics with anti-inflammatory properties including ferulic acid,

cinnamic acid, 4-hydroxybenzioc acid, taxifolin, protocatechuic acid and p-coumaric acid (541). Anti-microbial activity is also noted in other studies which has implications for gastrointestinal function (542).

Sorghum contains a unique profile of proteins, most notably kafirin proteins. These have been studied as an oral delivery model for tannins (43), and catechins (543). Their resistance to digestion prevents the breakdown of encapsulated compounds within the kafirin micro-particles, allowing for effective delivery of nutraceuticals to the intestine (40, 43) (543). The proteins themselves have not been widely studied in relation to their potential health benefits, although kafirin fractions can inhibit ACE (angiotensin converting enzyme), which may reduce hypertension and cardiovascular disease risk (544).

These features suggest that sorghum waste from bioethanol production is a possibly untapped resource for nutraceuticals or functional foods. The potential of this sorghum waste product is explored in a rat model of metabolic syndrome and its implications for human health are discussed.

5.2 Materials and Methods

5.2.1 Ethics

Approval for experimentation with 20% sorghum was granted under AEC approval number 15REA005 valid from 20th June 2015-20th June 2018 by the Animal Ethics Committee of the University of Southern Queensland. All rats were treated and housed as per the NHMRC (National Health and Medical Research Council) 2014 guidelines for the ethical treatment of animals.

5.2.2 Rat Diet and Experimental Structure

Forty-eight male Wistar rats were randomly divided into 4 experimental groups of n=12; mCS (corn starch diet), mHCHF (high carbohydrate, high fat diet), mCWS (corn starch diet + 20% w/w dehydrated wet cake sorghum), mHWS (high carbohydrate, high fat diet + 20% w/w dehydrated wet cake sorghum). Wet cake sorghum was kindly donated by the United Dalby Bio-refinery (United Petroleum).

For the first 8 weeks of the protocol, all mCS and mCWS rats received the control mCS diet and all mHCHF and mHWS rats received the control mHCHF diet as

described in Methods method 2.2. Energy and nutrient profile of the wet cake sorghum (WS) was tested by Agrifood Technology (Werribee, Victoria), NATA (National Australian Testing Authority) accreditation number 2726. Energy was determined by Atwater metabolizable energy, protein by method TP/026 (Agrifood test method), fibre by method TP/098, carbohydrate by method TP/110 and fat by method TP/050.

At eight weeks, the mCWS and mHWS groups were transferred to the treatment diet as indicated and remained on these diets until protocol completion. Macronutrient and energy profiles of the diets are outlined in Table 5.1. An additional 3.85 kJ/mL energy was added to calculations for the mHCHF and mHWS diets in fructose water (8).

Table 5.21: Macronutrient profiles of diets, per 100g.

	mCS	**mHCHF**	**mCWS**	**mHWS**
Energy (kJ)	992.5	1495.9	2269.5	2772.8
Protein (g)	1.8	5.1	30.6	33.9
Fat, Total (g)	0.5	11.2	9.4	20.0
Fibre - Dietary (g)	0.5	0.0	7.0	6.5
Carbohydrates (g)	54.2	41.9	91.2	78.9

At 16 weeks, prior to termination, oral glucose tolerance (method 2.3), body composition (method 2.4) and systolic blood pressure (method 2.5) were determined. Urine was collected as described in method 2.10.1. All rats were starved for 2 hours prior to termination. Rats were euthanised as described in method 2.6.1. At termination, rats underwent serum collection (method 2.6.2), plasma collection (method 2.6.3), vascular reactivity (method 2.6.5), ileum and colon contractility (method 2.6.6), organ weights (method 2.6.7) and histological tissue collection (method 2.8.1). 10 rats per group underwent isolated heart perfusion (method 2.6.4), with the remaining two hearts preserved in formalin for histological analysis as described in method 2.8.1. Plasma biochemistry was assessed on 8 rats per group for liver enzyme activity (method 2.7.1) and plasma lipid profile (method 2.7.2.). Collected serum was analysed for the presence of endotoxins as described in method 2.9. Liver sections were assessed for expression of GLUT2 and TLR4 (method 2.11.3). Distal colon sections were also assessed for occludin and claudin expression as

described in method 2.11.4, in addition to TLR4. Urine samples collected prior to termination were prepared (method 2.10.2) and analysed (method 2.10.3).

Histological tissue samples were processed automatically and sectioned (method 2.8.3). Liver, ileum, colon, pancreas and left ventricle were stained with haematoxylin and eosin (method 2.8.5.1). Separate sections of left ventricle were stained with picrosirius red (method 2.8.5.2). Microscopy and imaging were performed as described in methods 2.8.6.1, 2.8.6.2, 2.8.6.3 and 2.8.6.4.

5.2.3 Statistical analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Software, California). Data was analysed using a one-way ANOVA or two-way ANOVA were indicated. A test for normality was undertaken using a D'agostino-Pearson test for normality (GraphPad Prism). For analyses where distribution was determined to be normal, one-way ANOVA was used (Tukey's multiple comparison). Where groups were determined not to be of a normal distribution, a non-parametric test was applied. Removal of outliers was based on a Tukey boxplot identification of outliers.

In all cases, the null hypotheses were tested:

1. There is no difference between the mCS or mHCHF diet on the parameter in question.
2. There is no effect of wet cake sorghum addition on the outcomes of the mCS or mHCHF, respectively.
3. Addition of WS to the mHCHF diet does not result in a change which results in comparable outcomes to mCS.
4. Addition of WS to the mCS diet does not result in a change which results in comparable outcomes to mHCHF.

5.3 Results

5.3.1 Phytochemical composition

Extraction profiles of WS supplement are shown. For comparison puposes, profiles of whole red sorghum extract have been included. The red sorghum is not the source from which the WS was produced, as it is made on an industrial scale. It is provided as a guide to indicate that there is alteration in the compounds and their relative abundance which occur during processing.

HPLC with detection at 210nm (Figure 4.42) indicates hydrophilic compounds. There was a change in the real-time abundances of compounds in the WS compred to the whole red sorghum. The peak at 19.5 minutes was increased in the WS, while the peaks between 20.0 and 22.0 minutes were lower than those in the whole red sorghum. There was also increased relative sizes of peaks between 1.0 and 2.0 minutes in the WS comapred to the whole red sorghum extract.

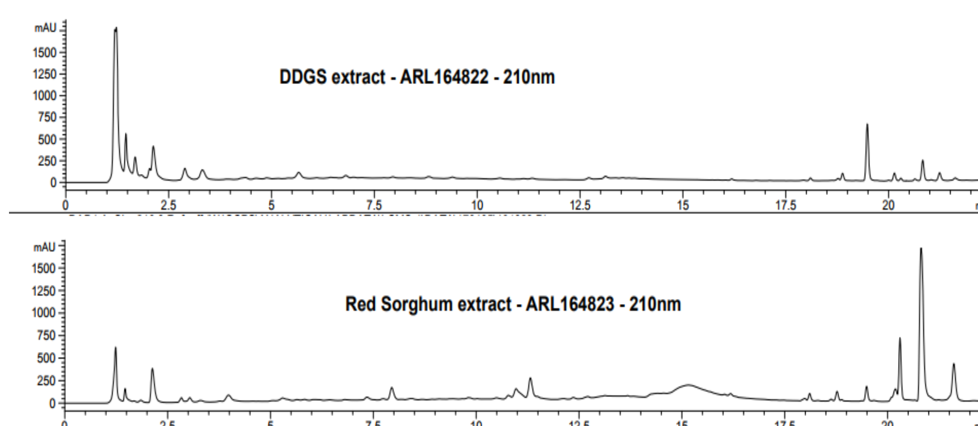
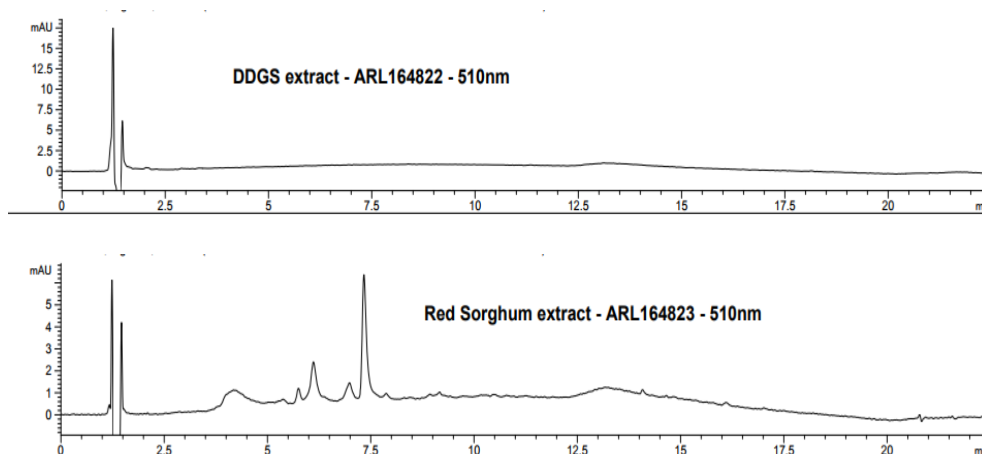


Figure 5.63: 210nm UV detection of HPLC of acidified methanol extract of WS (top) and local whole red sorghum (bottom). 210nm detection detects primarily hydrophilic compounds.

510nm detection is optimal for anthocyanin detection (Figure 4.43). Most peaks seen in the whole red sorghum extract were absent in the WS extract profile.



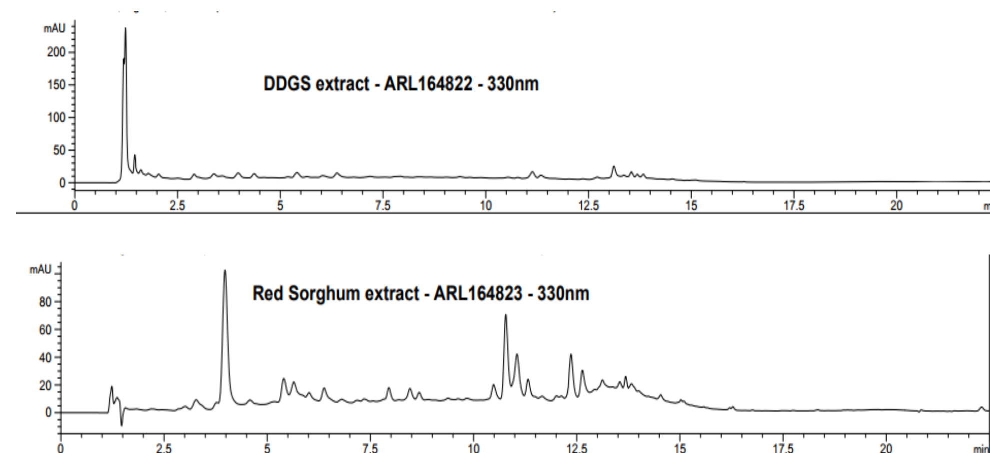


Figure 5.64: 510nm UV detection of acidified methanol extracts separated by HPLC of WS. 510nm detection primarily indicates anthocyanin presence.

The 330nm profile (Figure 4.44) primarily indicates phenolic acids. This wavelength is also suitable for detection of flavones (153). Most of the peaks present in the whole red sorghum extract were absent from the WS extract. Only the peak at 1.0 minutes was larger in the WS profile.

Figure 5.65: 330nm UV detection of HPLC separated acidified methanol extracts of WS. 330nm detection primarily indicates presence of phenolic acids.

5.3.2 Feed intake, energy intake and body composition

All rats began the protocol with the same body weight. At 8 weeks, mHCHF had a higher weight than mCS. There was a lower weight at 8 weeks with the mHWS group prior to intervention, compared to mHCHF, however final body weight did not differ between these groups (Figure 5.66). There was an increase in body weight in the mCWS group compared with the un-supplemented mCS from weeks 8-16.

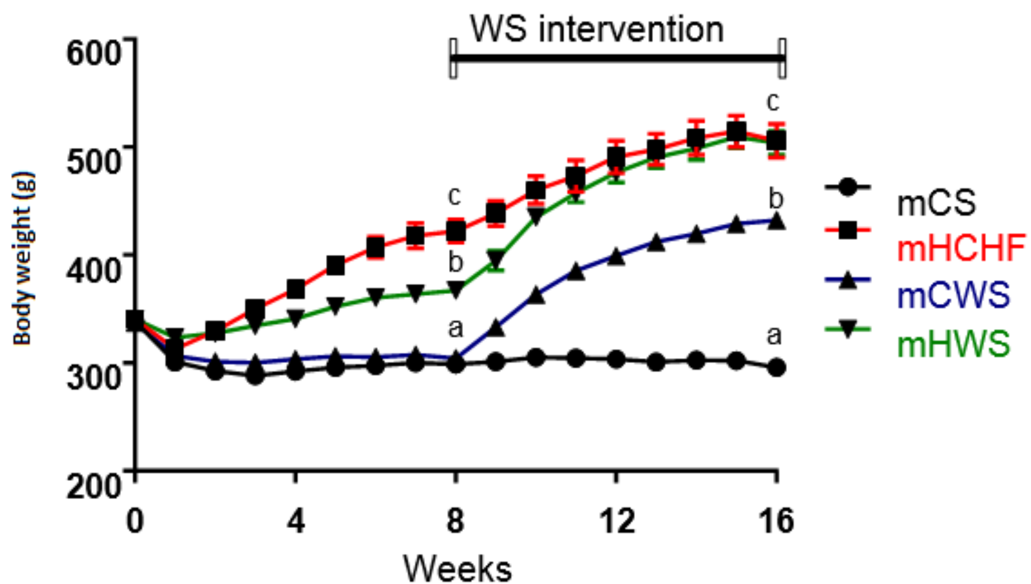


Figure 5.66: Weekly bodyweight gain.

An increase in fat mass was evident in the mCWS group compared with mCS (Table 5.22). No change in fat mass occurred in the mHWS group against the mHCHF control. Abdominal fat pad mass was higher in the mHCHF group than the mCS control. Addition of WS did not alter abdominal fat pad mass (Table 5.22).

The lean mass of mHCHF was higher than that of the mCS group at 16 weeks. Addition of WS resulted in increased lean mass in both diets from their respective controls. An increase in lean mass of 35% in the mCWS and 11.4% in the mHWS was measured, when compared with the CS and HCHF controls respectively (Table 5.22). Bone mineral density was higher in the mHCHF group compared with the mCS group. WS addition to the mHCHF diet reduced bone mineral density. Bone mineral content was again higher in the mHCHF group than the mCS control. Addition of WS to the mCS diet resulted in an increased bone mineral content. Addition of WS to the mHCHF diet did not affect bone mineral content.

Table 5.22: Body composition of rats fed on mCS, mCWS, mHCHF, and mHWS protocols.

Parameter	mCS	mCWS	mHCHF	mHWS	Interaction	Intervention	Diet
Total body fat mass 16 weeks (g)	43.63 ± 6.25 [a]	87.17 ± 11 [b]	166 ± 12.26 [c]	155.7 ± 10.8 [c]	< 0.0001	< 0.0001	0.0456
Total body lean mass 16 weeks (g)	247.2 ± 4.3 [a]	333.8 ± 6.3 [b]	307.6 ± 8.205 [b]	342.6 ± 13.2 [c]	0.0033	< 0.0001	0.0002
Lean:Fat mass ratio 16 weeks	7.10 ± 1.0 [b]	4.31 ± 0.6 [a]	1.98 ± 0.167 [a]	2.33 ± 0.3 [a]	0.0267	0.0807	< 0.0001
Bone mineral density (g/cm^2)	0.167 ± 2E-3 [a]	0.167 ± 2E-3 [a]	0.181 ± 2E-3 [b]	0.172 ± 4E-3 [a]	0.0897	0.0651	0.0005
Bone mineral content (g by DXA)	9.755 ± 0.3 [a]	13.03 ± 0.5 [b]	15.15 ± 0.49 [c]	15.31 ± 0.42 [c]	0.001	0.0004	< 0.0001
Retroperitoneal fat (mg/mm tibial length)	140.4 ± 12.5 [a]	201 ± 14 [a]	380.7 ± 33.92 [b]	331.9 ± 20.1 [b]	0.0157	0.7813	< 0.0001
Epididymal fat (mg/mm tibial length)	69.16 ± 6.73 [a]	103 ± 12 [a]	156 ± 13.06 [b]	185.6 ± 16.6 [b]	0.8678	0.0152	< 0.0001
Omental fat (mg/mm tibial length)	106.4 ± 8.51 [a]	122 ± 13 [a]	206.6 ± 12.23 [b]	186.2 ± 16.8 [b]	0.1648	0.8652	< 0.0001
Total abdominal fat pad (mg/mm tibial length)	316 ± 23 [a]	426.1 ± 29 [a]	743.3 ± 55.6 [b]	703.8 ± 48.7 [b]	0.0766	0.3967	< 0.0001

Values are expressed as mean ± SEM. Differing lower-case letters indicate a significant difference between groups for a parameter determined by one-way ANOVA at $p<0.05$.

Feed intake for the first 8 weeks was not different between the mCS and mCWS groups. The mHCHF groups ate less food than mCS while the mHWS group ate less than mHCHF. It was expected that the mHWS and mHCHF groups would eat the same amount of food prior to intervention. For this reason, changes in intake from the 8 week point are a better indicator of feed intake changes (Figure 5.67). Addition of WS reduced weight of food intake in both diets. Energy intake was increased in the mHWS and mCWS groups (Figure 5.67).

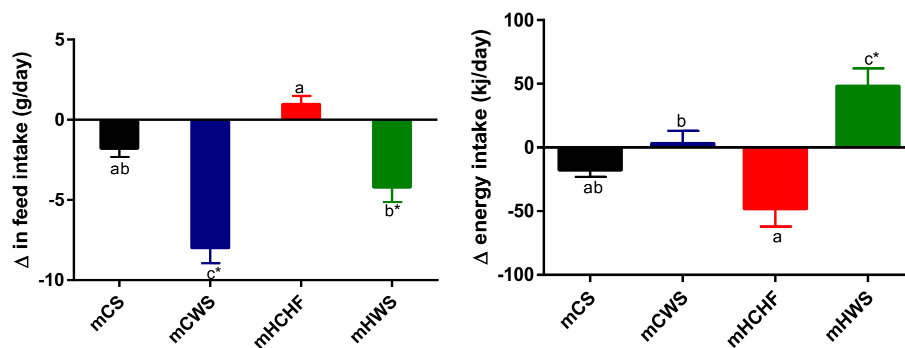


Figure 5.67: Change in feed intake and energy intake. Differing lower-case letters indicate a significant difference between groups, while asterisks indicate a significant change between the 0-8-week period and the 8-16-week period. All data were distributed normally and all analyses were determined using a parametric one-way ANOVA, at significance <0.05.

Feed efficiency was higher in the mHCHF than mCS in weeks 0-8 and 8-16. Addition of WS to the mCS diet or mHWS diet increased feed efficiency (Figure 5.68).

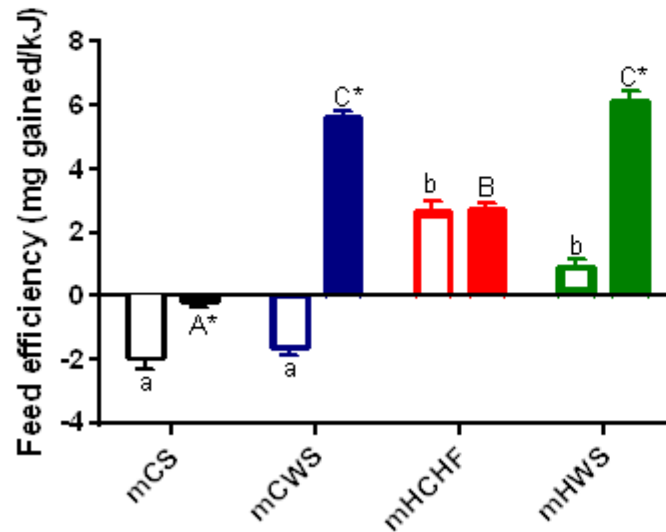


Figure 5.68: Feed efficiency 0-8 weeks (hollow bars) and 8-16 weeks (solid colour). Differing lower-case letters indicate significant difference between groups for the first 8 weeks. Differing upper case letter indicate a significant difference between groups for the second 8 weeks of the protocol. * indicates a significant difference within a group between the first 8 weeks and the second 8 weeks of the protocol. All analyses were determined by one-way ANOVA.

Protein intake in weeks 8-16 was higher in the mHCHF control from the mCS control. Both the mCWS and mHWS groups had higher protein intake in the second 8 weeks than all controls (Figure 5.69).

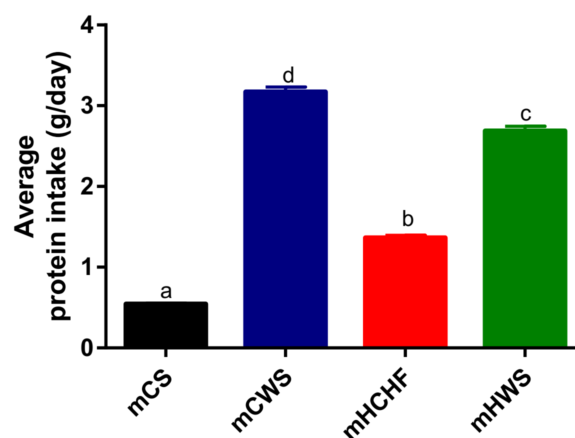


Figure 5.69: Protein intake weeks 8-16. Differing lower-case letters indicate a significant difference at $p<0.05$ determined by one-way ANOVA.

5.3.3 Glucose control and blood lipid profile

Non-esterified fatty acids and triglycerides were higher in the mHCHF and mHWS groups compared with the mCS control. Addition of wet cake to either diet did not alter circulating plasma NEFA or triglycerides (Table 5.23). No changes in cholesterol were found with either diet or intervention.

After 8 and 16 weeks of dietary treatment, mHCHF showed impaired glucose tolerance indicated by higher TAUC than the mCS control. The mHWS group showed improved glucose tolerance, normalised to within the range of the mCS control (Table 5.23).

Fasting plasma glucose concentrations at 8 weeks were increased in mHCHF compared with mCS. The mCWS and mHWS groups did not differ from the mCS control (Table 5.23). At 16 weeks, the mHCHF group again had higher fasting glucose concentrations compared with mCS. The mCWS group was comparable to mCS and the mHWS was not significantly different from either the mHCHF and mCS groups.

Table 5.23: Blood lipids and glucose tolerance.

Parameter	mCS		mCWS		mHCHF		mHWS		Interaction	Intervention	Diet
Fasting blood glucose 8 week	3.5 ± 0.25	a	3.3 ± 0.25	a	4.4 ± 0.13	b	3.6 ± 0.179	a	0.1621	0.0248	0.0044
TAUC blood glucose 8 week	688.8 ± 23.5	a	724 ± 22.6	a	861.4 ± 25.5	b	810 ± 34.77	b	0.1194	0.7775	< 0.0001
Fasting blood glucose 16 week	4.0 ± 0.21	a	3.8 ± 0.14	a	4.7 ± 0.11	b	4.5 ± 0.13	ab	0.8499	0.1253	< 0.0001
TAUC blood glucose 16 week	728.3 ± 20.3	a	761 ± 22.4	a	916.4 ± 46.3	b	791 ± 31.25	a	0.0314	< 0.0001	< 0.0001
Plasma total cholesterol (mM)	1.65 ± 0.08	a	1.79 ± 0.1	a	1.69 ± 0.09	a	1.72 ± 0.065	a	0.5244	0.3287	0.9039
Plasma triacylglycerides (mM)	0.58 ± 0.06	a	0.98 ± 0.09	a	1.77 ± 0.2	b	2.42 ± 0.3216	b	0.4977	0.0076	< 0.0001
Plasma non-esterified fatty acids (mM)	1.96 ± 0.16	a	2.73 ± 0.19	a	4.83 ± 0.19	b	4.54 ± 0.395	b	0.0305	0.3115	< 0.0001

Differing lower-case letters indicate significant difference at $p<0.05$ determined by one-way ANOVA. Fasting blood glucose was measured in mM, TAUC (total area under the curve) is unitless.

Histology sections of the pancreas showed that the cell density per islet was lower in the mCS groups than in all other groups.

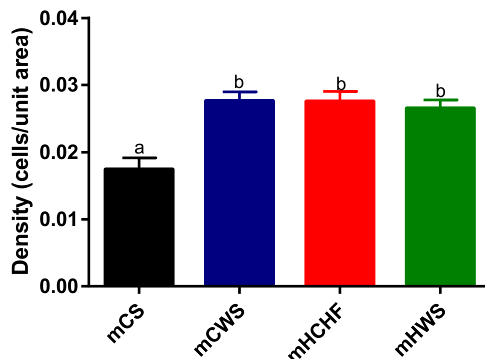


Figure 5.70: Pancreatic islet density. Differing lower-case letters indicate a significant difference at $p<0.05$ determined by one-way ANOVA.

Serum lipopolysaccharide concentrations did not differ between any group as indicated in Figure 5.71.

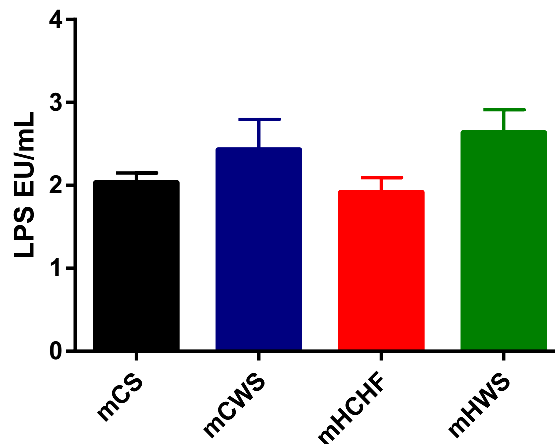


Figure 5.71: Serum lipopolysaccharide concentrations determined by horseradish peroxidase activity. No significant differences were found at $p<0.05$ determined by one-way ANOVA.

5.3.4 Cardiovascular and liver structure and function

Liver wet weight was increased in the mHCHF from mCS. Addition of WS to the mCS diet caused increased liver wet weight. No change resulted from addition to the mHCHF diet (Table 5.24). Histology of livers revealed ballooning and fat deposits in

the mHCHF, which were absent in the mCS rats. In the mHWS rats, these features were all but absent (Figure 5.72).

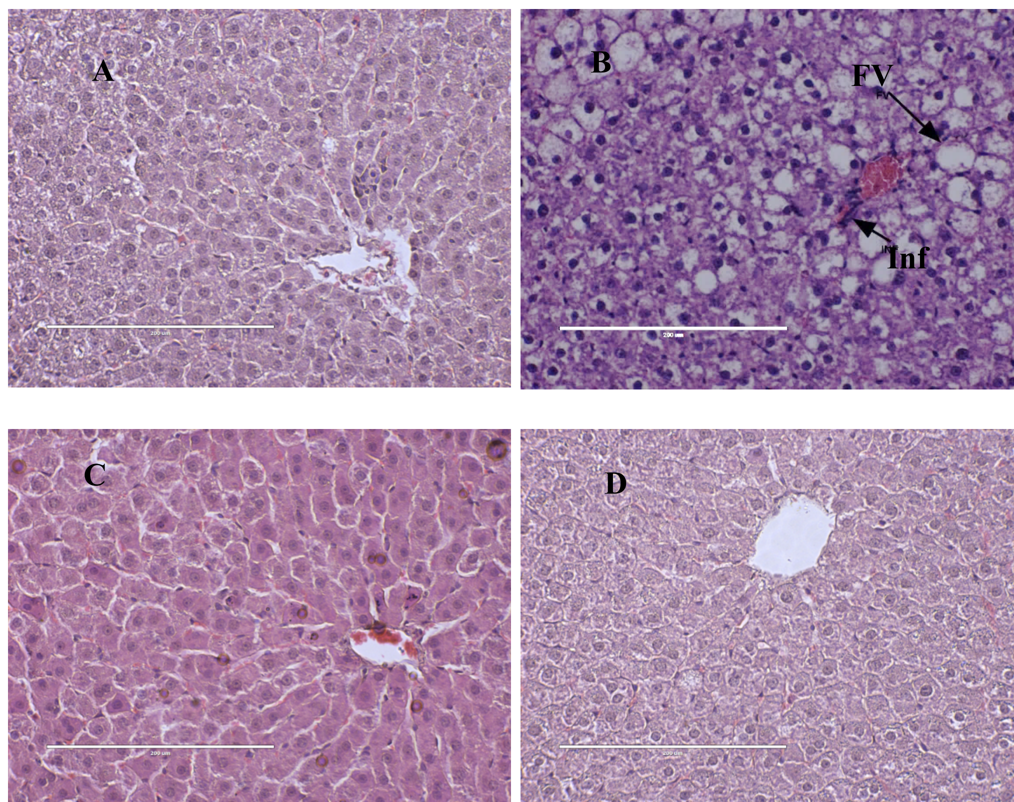


Figure 5.72: Haematoxylin and eosin stain of liver section at magnification 20x. Arrows show indicative cellular ballooning and fat vacuoles (FV) and inflammatory cells (INF). mCS,(A), mHCHF (B),mCWS (C), mHWS (D).

Plasma ALT activity did not differ between any groups. AST activities were lower in the mHCHF than the mCS group. The mCWS and mHWS AST activities were between the two controls, not differing from either (Table 5.4). ALT/AST ratio was lower in the mHCHF group than the mCS. mCWS and mHWS were comparable to both groups. Lactate dehydrogenase was higher in the mCWS model compared with all other groups. Creatinine kinase did not differ between any groups (Table 5.24).

Table 5.24: Cardiovascular and liver structure and function parameters of rats fed on mCS, mCWS, mHCHF, and mHWS protocols.

Parameter	mCS	mCWS	mHCHF	mHWS	Interaction	Intervention	Diet
Systolic blood pressure (mmHg)	134.2 ± 1.6 [a]	138.8 ± 2.1 [a]	128.0 ± 2.5 [a]	127.6 ± 1.4 [a]	0.2158	0.295	0.0004
Left ventricle + septum (mg/mm tibial length)	18.1 ± 1.1 [a]	23.1 ± 1.5 [b]	23.0 ± 1.1 [b]	22.5 ± 0.7 [b]	0.0172	0.051	0.0573
Right ventricle (mg/mm tibial length)	12.4 ± 1.0 [a]	16.7 ± 0.8 [b]	18.2 ± 0.8 [b]	17.5 ± 0.5 [b]	0.0034	0.0273	<0.0001
Liver (mg/mm tibial length)	183.5 ± 6.2 [a]	241.2 ± 6.6 [b]	316.8 ± 14.8 [c]	307.2 ± 6.8 [c]	0.0008	0.0141	< 0.0001
Diastolic stiffness constant (κ)	24.9 ± 0.3 [a]	25.9 ± 1.7 [a]	27.5 ± 0.4 [a]	25.6 ± 0.3 [a]	0.0754	0.5563	0.1732
Plasma ALT activity (U/L)	29.3 ± 1.4 [a]	28.5 ± 2.7 [a]	28.0 ± 2.6 [a]	25.1 ± 2.2 [a]	0.6439	0.4313	0.3165
Plasma AST activity (U/L)	93.3 ± 6.2 [a]	70.8 ± 5.9 [ab]	65.3 ± 4.3 [b]	74.3 ± 5.6 [ab]	0.0095	0.2465	0.0399
Plasma AST/ALT ratio	3.2 ± 0.2 [b]	2.5 ± 0.1 [ab]	2.5 ± 0.2 [a]	3.0 ± 0.2 [ab]	0.0045	0.7153	0.5397
Lactate dehydrogenase (U/L)	273.8 ± 29.2 [b]	124.3 ± 8.8 [a]	159.0 ± 27.2 [a]	159.1 ± 15.1 [a]	0.001	0.001	0.0552
Creatine kinase (U/L)	99.5 ± 11.6 [a]	139.4 ± 30.8 [a]	117.8 ± 25.6 [a]	114.6 ± 19.0 [a]	0.3906	0.4638	0.8962

Differing superscript lower-case letters indicate a significant difference at $p<0.05$ determined by one-way ANOVA.

Liver TLR4 expression did not differ between the mCS and mHCHF controls. Addition of 20% intervention in the mHCHF diet resulted in decreased expression of TLR4 in the liver from the mHCHF control. The addition of 20% intervention to the mCS diet did not alter expression. Liver GLUT2 expression did not differ between the mCS and mHCHF controls. The mCWS group had lower GLUT2 expression in the liver than the mCS control. No change was found in the mHCHF diet group, Figure 5.73.

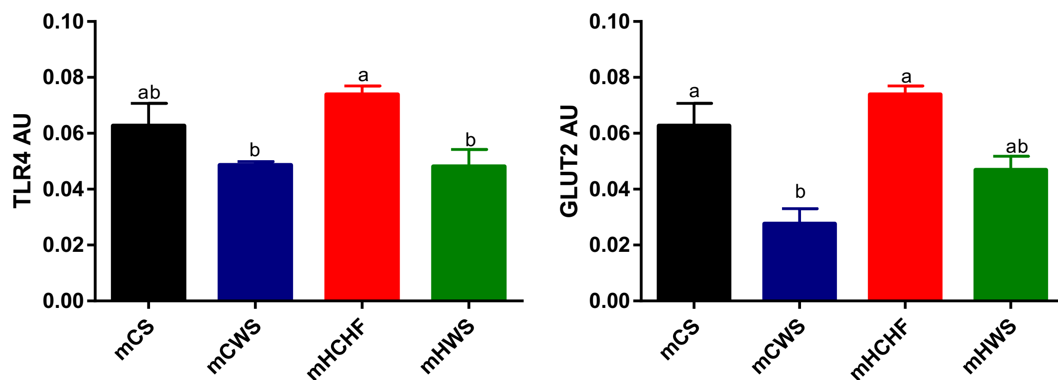


Figure 5.73: Liver TLR4 and GLUT expression determined by semi-quantitative ELISA. Differing lower-case letters indicate significant difference at $p<0.05$ determined by one-way ANOVA. AU indicates absorption units.

Normalised right ventricular weights and left ventricular weights were higher in the mHCHF, mHWS and mCWS groups, compared to the mCS, at termination. Diastolic stiffness was not changed by diet or intervention. Systolic blood pressure was unchanged by addition of WS, and did not differ between mCS and mHCHF.

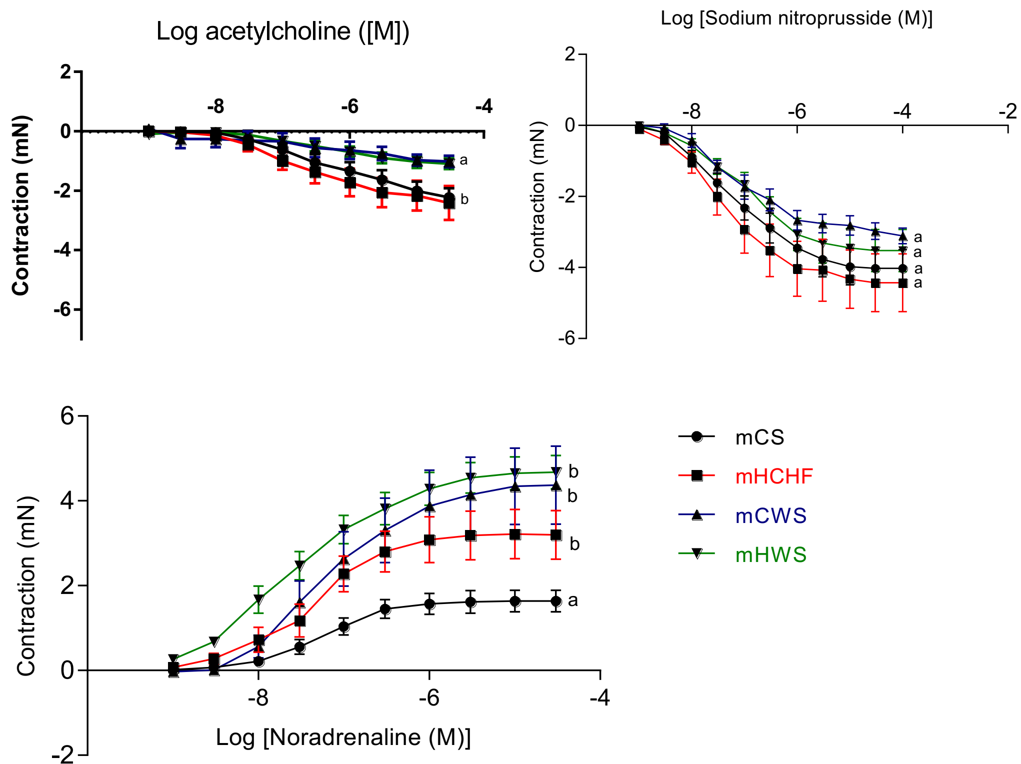


Figure 5.74: Ex vivo thoracic aortic reactivity in response to acetylcholine, sodium nitroprusside and noradrenaline. Differing lower-case letters indicate a significant difference in final maximal force of contraction, $p<0.05$ determined by one-way ANOVA.

Maximum force of relaxation upon acetylcholine exposure did not differ between the mCS and mHCHF groups. Maximum force of relaxation was lower in the mHWS and mCWS groups than both control groups (Figure 5.74). Different forces of contraction were observed at concentrations of $1x10^{-6}$M acetylcholine and above. The minimum concentration required to elicit a response did not differ between any groups. Maximal force of contraction with sodium nitroprusside exposure did not differ between any groups (Figure 5.74). The minimum concentration required to induce relaxation did not differ between any groups.

The maximum force of contraction in response to noradrenaline was higher in the mHCHF group than the mCS group. Both the mHWS and mCWS groups were increased from the mCS control, however not the mHCHF control, Figure 5.74. The minimum concertation required to induce contraction did not differ between the mCS

and mHCHF control groups. The mHWS groups showed contraction at a lower concentration of noradrenaline than the mHCHF control.

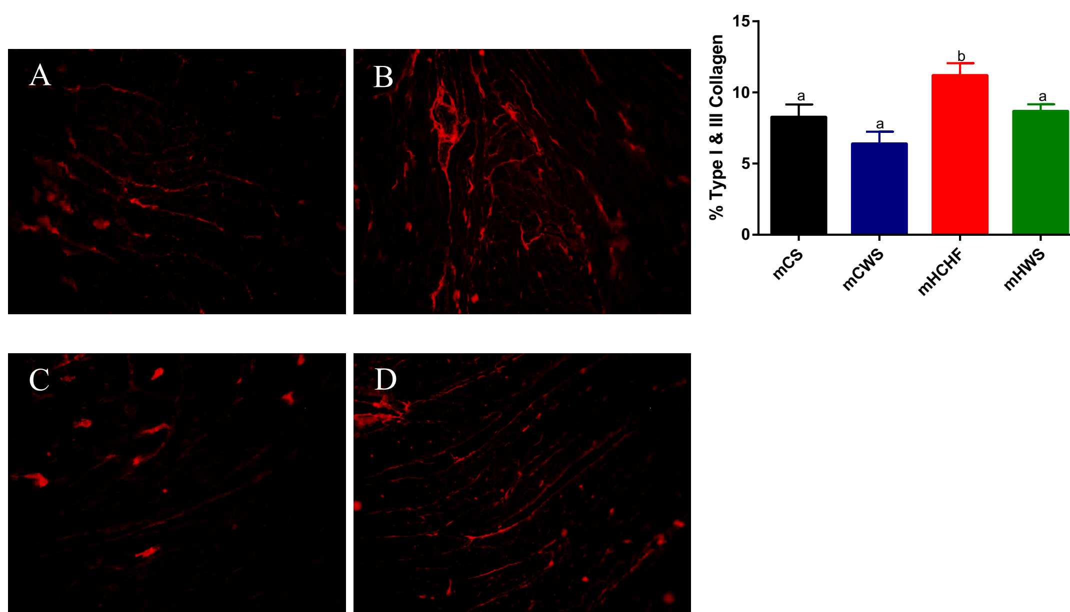


Figure 5.75: Type I and III collagen stained by picrosirius red, determined at % of total cross-sectional area. Differing lower-case letters indicated significant difference as determined by one-way ANOVA, at $p<0.05$. mCS,(A), mHCHF (B),mCWS (C), mHWS (D).

Collagen cross-sectional areas were higher in the mHCHF group than the mCS group. The mHWS group had lower collagen than the mHCHF control, comparable to the mCS group, Figure 5.75.

5.3.5 Gastrointestinal function

No statistical differences were noted in the maximal force of contraction of the ileum between groups. Concentration-contraction comparisons showed no difference in contraction between any groups at any individual concentration. No differences in total area under the curve were noted between any groups, nor was the minimum concentration required for contraction.

There was no difference in the minimum concentration of acetylcholine required to induce contraction between the mCS and mHCHF groups in the colon. The concentrations required in the mHWS and mCWS groups were higher than both

controls. Maximal force of contraction did not differ between the mCS and mHCHF controls. At concentrations of 1x10^{-7}M acetylcholine and above, force of contraction was lower in the mCWS and mHWS than both controls (Figure 5.76).

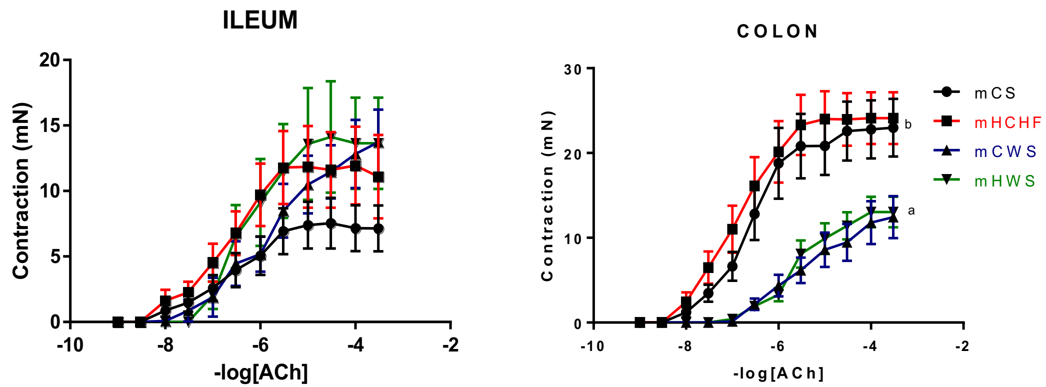


Figure 5.76: Reactivity of isolated ileum and proximal colon sections to acetylcholine. Differing lower-case letters indicate significant difference in maximal force of contraction determined by one-way ANOVA.

Colon TLR4 or claudin-1 expression did not differ between any treatments as shown in Figure 5.77. There was higher occludin expression in the proximal colon of mHCHF rats compared with mCS. Addition of 20% WS to mCS diet increased occludin expression and addition to the mHCHF reduced occludin expression.

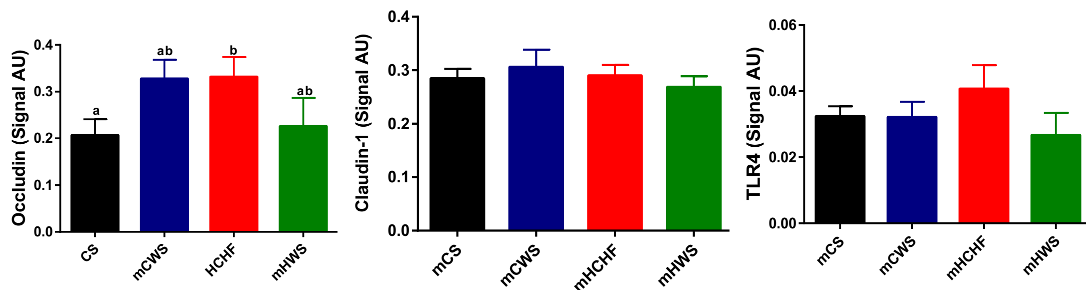


Figure 5.77: Colon occludin, claudin-1 and TLR4 expression. Differing lower-case letters indicate a significant difference at $p<0.05$ determined by one-way ANOVA.

Recovery of sucrose, lactulose and sucralose did not differ between any group, for a given time period. Recovery of mannitol did not differ between any groups for the 0-3 hour time period or the 3-8 hour time period. In the 8-20 hour time period, there was a higher recovery of mannitol in the mHCHF group than the mCS group. The mCWS

recovery did not differ from the mCS control. The recovery in the mHWS was comparable to both the mCS and mHCHF groups (Table 5.25)

Table 5.25: Total percent recovery of sugars from urine.

	Time (hours)	mCS	mCWS	mHCHF	mHWS	Interaction	Time	Treatment
Sucrose	0-3	0.05 ± 0.02 [a]	0.04 ± 0.03 [a]	0.05 ± 0.01 [a]	0.04 ± 0.02 [a]	0.9734	< 0.0001	0.8848
	3-8	0.20 ± 0.07 [a]	0.11 ± 0.03 [a]	0.18 ± 0.03 [a]	0.11 ± 0.05 [a]			
	8-20	0.26 ± 0.06 [a]	0.26 ± 0.05 [a]	0.24 ± 0.09 [a]	0.26 ± 0.10 [a]			
Lactulose	0-3	0.31 ± 0.14 [a]	0.25 ± 0.19 [a]	0.21 ± 0.08 [a]	0.34 ± 0.10 [a]	0.5639	0.0142	0.2600
	3-8	1.29 ± 0.72 [a]	0.54 ± 0.15 [a]	1.26 ± 0.32 [a]	0.90 ± 0.23 [a]			
	8-20	0.90 ± 0.30 [a]	0.45 ± 0.23 [a]	1.20 ± 0.40 [a]	2.02 ± 0.59 [a]			
Mannitol	0-3	0.44 ± 0.14 [a]	0.51 ± 0.27 [a]	0.58 ± 0.16 [a]	0.51 ± 0.17 [a]	0.2940	< 0.0001	0.0007
	3-8	1.28 ± 0.32 [a]	1.03 ± 0.39 [a]	2.51 ± 0.67 [a]	2.77 ± 0.81 [a]			
	8-20	1.51 ± 0.47 [a]	1.59 ± 0.29 [a]	4.07 ± 0.87 [b]	2.94 ± 0.94 [ab]			
Sucralose	0-3	0.49 ± 0.21 [a]	0.59 ± 0.25 [a]	0.70 ± 0.23 [a]	0.89 ± 0.24 [a]	0.7212	0.0001	0.3046
	3-8	3.08 ± 0.95 [a]	1.13 ± 0.34 [a]	2.86 ± 0.59 [a]	3.11 ± 1.15 [a]			
	8-20	2.53 ± 0.89 [a]	2.80 ± 1.22 [a]	4.27 ± 0.90 [a]	3.68 ± 0.96 [a]			

Differing lower-case letters indicate a significant difference at $p<0.05$ determined by one-way ANOVA between groups for a given time point.

Total % recovery over the 24 hour period only differed for mannitol recovery, Figure 5.78. The lactulose/mannitol ratio was higher in mCS than mHCHF. The mCWS had a lower lactulose/mannitol ratio of recovery compared to mCS. No change from the mHCHF control was seen in mHWS (Figure 5.78).

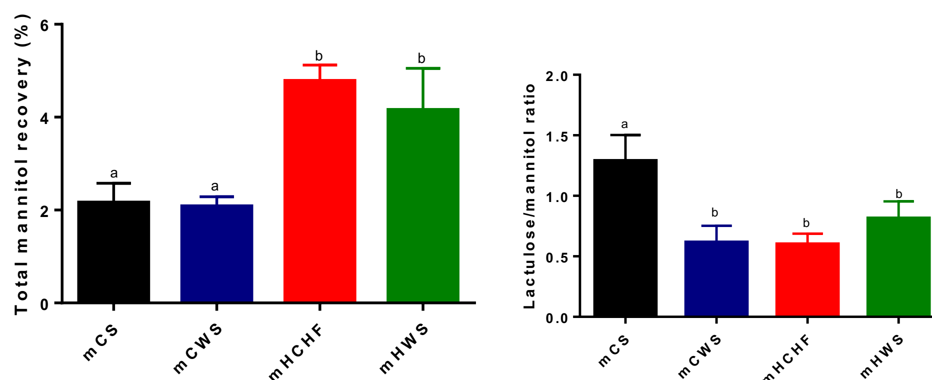


Figure 5.78: Total mannitol recovery and lactulose/mannitol recovery ratio over a 20 hour period. Differing lower-case letters indicate difference at $p<0.05$ determined by one-way ANOVA.

5.4 Discussion

It was hypothesised that supplementation in the mHCHF diet with WS would reverse the symptoms associated with metabolic syndrome. Not all parameters were reversed, however several beneficial outcomes were noted.

Physiology and metabolism

Supplementation resulted in decreased food intake in the mHCHF diet. The reduced intake can be explained by the increased protein and energy densities in the diet. Protein intake is suggested to be the major driver of food intake (474), and higher satiety with protein intake is well documented (545-547). With addition of WS, there was a large increase in protein intake in both diets. The initial mCS diet and mHCHF diets are considered to be low in protein when compared with other studies of rat models (467-470).

Many studies have linked reduced calorie consumption with improvements in metabolic syndrome (548, 549). Therefore, this is especially promising in the mHCHF

model which aimed to mimic metabolic syndrome. High protein, calorie-reduced diets have a wide scientific grounding in improving metabolic syndrome (477, 550-552). In the mCS model, WS consumption increased protein intake and energy intake. In mice, long-term dietary studies of low protein, high carbohydrate intakes indicated good cardio-metabolic health, but with a higher risk of fatty liver, low lean mass and high triglyceride concentrations (474).

Notably, the feed efficiency in the rats which received WS was increased. In the mHWS group, the increased feed efficiency resulted in an increased lean mass, with no apparent change in total body fat mass. In the mCWS diet, the increased efficiency resulted in both lean mass and fat mass increase. Similar results have been seen in other studies. In a cat model, high protein diets resulted in a lean mass increase of 4.2%, with no additional exercise (553). Inverse correlations have been established between lean mass and metabolic syndrome (484-486, 488). Sorghum distillery waste products increased lean mass in feedlot cattle however less efficiently than a corn equivalent (554). In finishing, distillery waste reduced fat free mass (555). Little research has been done on its consumption outside the livestock production field.

Increases in lean mass are driven by protein intake, but also essential amino acid intake (556). Increased leucine intake, even without increased protein intake, resulted in improved muscle mass (483). This has been theorised to be due to inhibition of muscle breakdown (myopathy) rather than increased muscle synthesis (557). Sorghum is high in leucine, and after processing, leucine can represent 9.59% of total crude protein, and up to 2.55% of total dry mass (558).

There is evidence to suggest that epicatechin is a myostatin inhibitor, which may prevent skeletal muscle breakdown (559). WS is known to contain catechin and epicatechin (160). The inhibition of myostatin by epigallocatechin-3-gallate in tea results in inhibition of ubiquitin-mediated protein breakdown, and increased anabolic factors (560). The increased lean mass is also likely to be a contributing factor in the improved glucose tolerance.

Glucose Tolerance

The improved glucose tolerance in the mHWS group was a significant outcome in this study. Sorghum grain extracts improve diabetes by increasing insulin sensitivity in mice fed a high fat diet (185) and have anti-diabetic effects in rats (207, 208).

However, this appears to be the first study that has shown whole spent sorghum from distilleries can have an anti-diabetic effect.

Peripheral glucose clearance dysfunction is one of the contributing factors to the development of impaired glucose tolerance (561, 562). Muscle fibre area correlated with the latter stage of the glucose tolerance test (52) with a correlation of 0.39 between muscle fibre area and glucose tolerance (53). Not all studies agreed, with lean mass amount not related to glucose tolerance in overweight or obese individuals (54). Our results indicate no correlation between lean mass and oral glucose tolerance. Many suggest it is the function of the muscle, rather than the total mass, that dictates skeletal muscle influence on glucose tolerance. This has been linked to mitochondrial dysfunction (563), GLUT4 dysfunction (561) and insulin-like growth factor-1 (564). Procyanidins improved peripheral glucose clearance by correcting GLUT4 translocation (565). Procyanidins are present in sorghum and its distillery waste products (160). Isolated sorghum fractions rich in phenolics showed antidiabetic effects in rats and mice (185, 207, 208, 214)

Liver GLUT2 is involved in control of blood glucose (281). In hyperthyroid rats, impaired glucose tolerance is associated with an increase in GLUT2 expression in the liver (566). Cellular models of fatty liver were associated with increased GLUT2 mRNA expression and glucose output and gluconeogenesis, rather than increased uptake (322). GLUT2 increases are usually associated with impaired glucose tolerance, and correction was seen in improved glucose tolerance (282, 313, 315, 318). In this study, there was a reduction in the GLUT2 expression in mHWS rats which also showed improved glucose tolerance. However, in the corn starch diet, the reduced expression was not coupled with changes in glucose tolerance. There is also a lack of difference between the controls with respect to GLUT2 expression, with vastly different glucose tolerances. Although GLUT2 is involved, explaining the complexity is beyond the scope of this research. The effect of WS on its expression and the mechanism by which it occurs are not clear.

Observations in the pancreas showed that there was no change in islet density accompanying the improved mHWS glucose tolerance. Although progression of diabetes results in reduced β-cell density (567), it has also been shown to be positively correlated with weight in non-diabetic patients (568, 569). The increased body weights

of all these three groups compared to the mCS control may underlie the increased β-cell density in these rats, and not appear to be directly correlated to glucose tolerance. Irrespective of the mechanism, the improvement in glucose tolerance is in line with other studies using sorghum extracts where glucose tolerance was improved (208, 570). This study furthers the work by showing that not only isolated extracts can improve glucose tolerance, but a commercial waste product can do the same as a novel food.

Dyslipidaemia

No changes were noted in the plasma triglyceride, cholesterol or non-esterified fatty acid concentrations. Therefore, sorghum distillery waste does not improve the dyslipidaemia of metabolic syndrome. Other studies indicated that extracts of WS alter plasma lipid profiles, particularly lowering both HDL and LDL concentrations (183). The wax component reduced cholesterol concentration in hamster models (571). These changes may only be noted in certain extracts rather than the whole fraction. Approximately 86% of the lipids present in WS are triacylglycerides (126), so although dietary intake of triglycerides was increased, this did not lead to increased circulating triglycerides.

Previous studies showed that the fatty acid composition of sorghum distillery waste was 53.96-56.53% linoleic acid, 25.25−27.15% oleic acid, 13.25−16.41% palmitic acid, 1.80−2.34% stearic acid and 1.15−1.40% linolenic acids (572). Although these unsaturated fats have been associated with improved lipid profiles, this was not seen in this study. As with cholesterol, plasma triglyceride concentrations and their link to metabolic syndrome are also dependent on the composition whereby unsaturated fatty acids, especially linoleic and oleic acids, are inversely related to metabolic syndrome, and saturated C14 and C16 fatty acids are positively correlated with metabolic syndrome development (573). The intake did not alter plasma lipids, but the dietary lipids may have been the functional compounds which affected other parameters, especially since WS fats comprised 8.8% of the daily diet.

Liver

The mCWS and mHWS showed increased liver weight, which was not associated with observations of steatosis or liver inflammation as indicated by histology. There exists a linear relationship in the body size and organ weight (574). The same increase was

noted in left ventricular weight and right ventricular weight of rats consuming WS. Although these values are normalised to tibial length prior to statistical analysis, the tibial length is set prior to intervention and is not a reflection of changes in lean mass or overall body size. Lean mass is indeed correlated with higher liver size and total body weight (575). Similarly lean mass is correlated with ventricular weight, whereas height or skeletal size is less indicative (576).

Due to the low protein content of the mCS diet, using it as a "healthy" control for liver studies is not ideal. The elevated plasma AST activity in the mCS may be an indication of liver damage, but it can also indicate poor nutrition or low protein intake (513). Other studies of low protein diets using maize meal at 4% protein reported AST increases after just 5 days (513). This would usually be accompanied by an increase in ALT activity but this was not noted, and an increase in lactate dehydrogenase, which was. Median reference values for male Wistar rats are 33 U/l and 66 U/l for ALT and AST respectively (512). Normal ranges for male Wistar rats are 23-50U/l ALT and 49-98U/l AST (512). All rats, irrespective of diet or intervention, had ALT values in the lower range. The mCS rats showed AST activities increased to the upper range of normal, while the mHCHF had "healthier" activities comparable to the median reference range.

Similarly, using this as a control for lactate dehydrogenase may not be optimal. Typical reference ranges for male Wistar rats for lactate dehydrogenase are between 83-298 U/l (512). The rats in this study all had values in the upper range, well above the median 114 U/l value for male Wistar rats (512). All rats that were given an intervention, irrespective of base diet, were closer to the median value than the controls. The addition of 20% WS to the mCS diet markedly reduced the lactate dehydrogenase activity. Again, this is possibly due to the low protein content of the diets. Protein energy malnutrition in humans is associated with low lactate dehydrogenase activity compared to normal protein and energy intake (577).

Despite the lack of change in liver enzyme activity caused by addition of WS to an obesogenic diet, there was improved histology of the liver. The mHCHF group showed steatosis and fat vacuoles, while these were absent in the mHWS group. Aqueous extracts of *Sorghum bicolor* improve liver microstructure in rats (413). *Sorghum bicolor* leaf sheath ethanol extract induced hepatocyte ballooning, however only at

very high doses of over 2000mg/kg per day and prolonged exposure (578). The lack of changes to liver enzyme activity are similar to the outcomes seen with rats ingesting sorghum flour (253, 489). Aqueous sorghum extracts dose-dependently increased ALT and AST activities in rats on a normal diet (579). Methanol extracts of sorghum seed showed no effect on the liver enzyme activity, ALT or AST, but these were also unchanged by a high fat diet (185).

Due to the lack of difference between the mCS and mHCHF for TLR4 expression in the liver, it is difficult to suggest which changes occur with metabolic syndrome, although WS decreased TLR4 expression in the high-fat diet. The implications of this are not clear, and whether the reduction in TLR4 affected other parameters is not clear, but warrants further research. There was no correlation between LPS concentrations and liver TLR4 expression.

TLR4 is a known mediator of hepatic injury in high fat diets, but its research has been focussed on activation and knockouts which have revealed its involvement in metabolic syndrome (219, 370, 580, 581). TLR4 expression was increased in muscle from insulin-resistant patients (518). TLR4 expression was increased in monocytes exposed to oxidised LDL cholesterol (582), which is a risk factor for metabolic syndrome (583). No studies have shown that sorghum altered liver TLR4 expression.

Cardiovascular health

There was no improvement in left ventricular diastolic stiffness or systolic blood pressure with WS consumption. The reduction in collagen deposition may have preceded larger physiological changes such as reductions in diastolic stiffness. There is some evidence to suggest that procyanidins reduced the proliferation of cardiac fibroblasts (584) which are the primary cell type responsible for collagen synthesis in the heart (585).

Increased vascular contraction in response to noradrenaline with WS consumption may indicate hypersensitivity. Increased sensitivity to noradrenaline can lead to increased blood pressure (586). It was expected that the mHCHF group would have lower reactivity than the mCS controls, as has been shown in studies of high fat, diet-induced obesity in mice (501) and rats (353). In this case, all intervention groups showed poorer relaxation when exposed to acetylcholine, which may be detrimental to blood pressure control, as may be the increased contraction to noradrenaline.

Increased vasoconstriction sensitivity to noradrenaline occurs in diabetic dogs (502) and is linked to hypertension in rats (503).

Gastrointestinal health

Generally, reduced gastrointestinal motility is associated with increased satiety in the small intestine, and in the colon with poorer expulsion of faecal pellets (587). Increased sectional contractility resulted in delayed emptying (84). There are still significant knowledge gaps in the role of gastrointestinal contraction and metabolic syndrome. However, this study suggested that there was reduced sectional contractile force with WS consumption which may either reduce risks of constipation, or improve colonic clearing.

This is indeed an area that requires more research. This study indicates that altering the diet by adding WS sorghum will change the gastrointestinal function with respect to contraction, but its implications are only speculative. Although it is observational and data were not collected quantitatively, it was noted that the stool of mCWS rats was softer, bulkier and appeared to be passed more frequently than the mCS control. Isolated sorghum compounds caused relaxation of pig ileum *ex vivo* (420). In mice, increasing sorghum leaf extract concentration in the diet increased transit time and reduced intestinal motility and doses of 200mg/kg or more showed anti-diarrhoeal activities (421). Sorghum extracts dose-dependently increased relaxation in the rabbit jejunum and rat stomach (421). Likewise, methanol extracts of red sorghum seeds showed anti-diarrhoeal activity and prolonged gastrointestinal transit time (523).

There were no improvements in the gastrointestinal permeability with WS consumption. A high fat diet increased gastrointestinal permeability in many rat studies (175, 219, 588, 589). Diet did not affect stomach permeability or colon permeability but the small intestine was affected as indicated by the mannitol recovery. The recovery of mannitol is an indicator of transcellular permeability or absorption, while lactulose is an indicator of paracellular absorption (590). These respectively indicate villi absorptive capacity and intestinal permeability (591). There was a large increase in mannitol recovery in the mHCHF group, which was unchanged by WS. The uptake is proportional to absorptive capacity (591), and the subsequent excretion shows poor absorption in the high fat diet. One study of red and white sorghum showed that colon crypt depth was reduced in the proximal colon with sorghum consumption

(466) and, in fat rats, crypt depth is also decreased and coupled with poor gastrointestinal absorption of sugars (592).

The expression of tight junctions was not studied in the small intestine, which would add weight to this comparison. The expectation based on literature was that changes would occur primarily in the large intestine. Ultimately, addition of WS did not greatly alter colon permeability, nor did there appear to be changes in serum LPS concentrations as was hypothesised.

Metabolic endotoxaemia

No significant differences in serum LPS concentrations existed between any control or treatment groups. LPS can induce symptoms of metabolic syndrome (238) and others suggest it is a direct cause of diet-induced metabolic syndrome (175, 237). While LPS may play a role in the progression of metabolic syndrome by initiating TLR4-mediated inflammation in the pancreas, colon, liver, heart and other tissues (370), this research does not support these changes as being the key factors. It must be considered that there is a complex interaction of multiple factors. LPS is produced by gastrointestinal Gram-negative bacteria (593). Poor gastrointestinal integrity will allow cells and cell components into the blood stream that cause inflammatory responses (237). Other factors will affect the concentrations of LPS, including the microbial composition of the colon (593), detoxification of LPS by intestinal alkaline phosphatase (594), the expression of TLR4 in tissues which is able to be activated (595) and the concentration of serum lipopolysaccharide binding protein (596). A more comprehensive study investigating all of these components will be beneficial. The complexity has not been fully studied, and the high concentration of circulating plasma free fatty acids which mimic LPS cannot be discounted (433, 434).

There are many cellular components that result in inflammation of bacterial origin and these must not be discounted in the process of metabolic syndrome progression. TLR2 is critical in the development of metabolic syndrome (597). TLR5 has a protective effect against metabolic syndrome development, where TLR5-deficient mice develop metabolic syndrome (598), rather than being protected as in TLR4-deficient mice (219). All inflammatory mediators which react to environmental signals are likely to play some role in progression of the disease. There is evidence to show that circulating free fatty acids and other lipids can stimulate TLR4 and TLR2, and exacerbate

inflammation (599). In dyslipidaemia, high concentrations of circulating fatty acid components are surmised to be the cause of excessive inflammation and cellular dysfunction, rather than LPS itself (370). TLR3, typically associated with detection of exogenous double stranded RNA (600), was involved in the progression of type 2 diabetes, where TLR3-deficient mice were resistant to development of whole body insulin sensitivity and somewhat protected from dyslipidaemia (601).

In this study, I approached the expression of TLR4, but I was unable to quantify all the factors which link TLR4 concentrations and the inflammatory process which contribute to metabolic syndrome. A single dietary intervention of WS is not sufficient to change the concentrations of circulating LPS, and changes in colonic permeability did not appear to be involved. Approaching only one inflammatory mediator may have been too simplistic.

5.5 Other considerations and limitations

Identification and quantification of the bioactive compounds present in the distillers grain sorghum used in this study would add considerable weight to the knowledge and outcomes. Determination of the individual components would provide a link between these compounds and the research outcomes. There can be vast differences in sorghum composition due to genetic and environmental factors, and in processing of the grain for ethanol production. This will affect the composition of batches used in other research, and the research outcomes. Knowledge of the bioactives would provide a baseline for further research. In the absence of this information, comparison to future studies will be limited.

5.6 Conclusions

WS sorghum has shown some positive changes in a model of diet-induced metabolic syndrome. One aspect which has become clear is that there is a strong interaction between the diet and the supplement. It appears insufficient to use a single supplement to reverse the symptoms and a focus needs to be made on the whole diet. The most relevant change was the improvement of glucose tolerance, even in a diet high in fat and simple sugars. I could not elucidate the mechanisms that were at play, as changes in GLUT2 in the liver and pancreatic histology revealed little.

The link between gastrointestinal permeability, inflammation and LPS has not been shown in this dietary model. It is most likely that these changes were minor at the molecular level, which did not result in statistical changes, or that the changes are a much smaller component of overall diet-induced metabolic syndrome, which is a very complex disease.

Chapter 6 – Phloretin in a rat model of metabolic syndrome

6.1 Introduction

Glucose transport inhibitors have gained much interest with the theoretical application to the management or amelioration of diabetic progression (602). Many sugar transporters are present, however recent interest has emphasised sodium-dependent glucose transporters 1 and 2 (SGLT1, SGLT2) and Glucose Transporter 2 (GLUT2). Several SGLT2 inhibitors are already on the market including dapagliflozin, canagliflozin and empagliflozin (603).

SGLT1 transports glucose and galactose and is present in the intestine tract, kidney, heart, brain, testis and prostate. SGLT2 is also responsible for glucose and galactose transport, however in the kidney, brain, liver, thyroid, muscle and heart (280). GLUT2 is present in the gastrointestinal tract, pancreas, liver, brain and other parts of the nervous system (281). It is not only responsible for sugar transport of glucose, galactose and fructose (282), but hepatoportal glucose sensing, pancreatic sensing, taste preference and thermoregulation (281).

Several studies have shown potential for SGLT and GLUT inhibitors to ameliorate diabetes (331, 333, 604, 605). Phloretin produces anti-inflammatory responses (349, 350). As many of the signs of metabolic syndrome are initiated by and associated with an inflammatory mechanism (606-608), phloretin has the potential to suppress the inflammatory status of the disease. When exposed to phloretin concentrations greater than 10μM, LPS-induced macrophages had reduced translocation of TNF-α (tumour necrosis factor alpha), PGE-2 (prostaglandin E2), iNOS (inducible nitrogen oxide synthase), COX-2 (cyclooxygenase-2) and NFκ-B (nuclear factor kappa-B) (Chang et al., 2012). Phloretin improved glucose homeostasis (336, 609), reduced glycation which is responsible for diabetic complications (339), was hepatoprotective (341) and improved lipid metabolism (337).

As the targets for phloretin are expressed in multiple tissues, there could be wide-reaching effects including altered glucose uptake in the GI tract, kidneys, altered GLUT2 mediated peripheral uptake and release in the liver, and potentially alterations in the sensing of glucose in the pancreas. In addition, SGLT-mediated glucose re-uptake in the kidney and other tissues may be altered.

Many studies have looked at the cellular responses to phloretin by exposure in cell or tissue culture (339, 343, 349, 351, 610). Very few studies have been conducted to assess the overall physiological changes with ingestion in diet-induced metabolic syndrome. Only one study appears to have combined all of these effects and researched oral ingestion of phloretin in a model of metabolic syndrome, a recently published work by Alsanea*, et al.* (340).

Metabolic syndrome is hypothesised to be both contributed to by gastrointestinal function and as causing gastrointestinal dysfunction (177, 377, 611). Increased permeability of the gastrointestinal tract is linked to high plasma insulin and low HDL cholesterol concentrations (589). The increased permeability has been associated with higher inflammation, resulting from mild endotoxaemia, particularly activation of the TLR4 innate immune receptor (219). TLR4 activation increases the risk of development of fatty liver (612), hepatic dysfunction (613), vascular inflammation and insulin resistance (345). Diet can influence expression of tight junction proteins, responsible for maintaining the gastrointestinal integrity. High fat diets increase small intestinal permeability (424). Lipopolysaccharides reduce tight junction proteins (614), which have origins in the gut induced by a high fat diet (611). Flavonoids studied to date that show improved tight junction barrier protein function in the gastrointestinal tract are epigallocatechin gallate, genistein, myricetin and quercetin (615). Phloretin has not been studied in a gastrointestinal model.

6.2 Materials & Methods

6.2.1 Phloretin analysis

Approximately 1mg of phloretin was dissolved in 10mL of 100% methanol. The sample was analysed using an Agilent 1260 HPLC-MS system. The system consisted of an Agilent 1260 Degassing Unit, 1260 ALS Infinity Autosampler, 1260 TPP quaternary Pump, 260 DAD (Diode Array Detector), connected to an Agilent 6120 Quadrupole LC/MS (Liquid chromatography/ mass spectrometry) (Agilent, Santa Clara, California).

The sample was run isocratically in 90% water, 10% methanol at 0.400mL/min on an Agilent, with a 10μL injection volume. The system was run at 20°C on a 150x10.00mm 5μm Phenomenex Luna 5u C18(2) column (Phenomenex, Lane Cove,

New South Wales). The mass spectrum was analysed using APCI (atmospheric pressure chemical ionisation), in the negative mode. Gas temperature and vaporiser were maintained at 300°C, with a drying gas flow of 9.0L/min, corona current of 20µA, and a nebuliser pressure of 40 pounds per square inch, gage. The mass spectrum peak width was set to 0.1 minutes.

The compound was confirmed to be >99% by peak area, eluting at 0.768 minutes. The mass spectrum confirmed the identity of phloretin with an expected molecular weight of 274.26g/mol. The negative ion mode generated two major peaks at 273.1 (intensity 100, parent peak) and 274.1 (intensity 15.1) as shown in Figure 6.1.

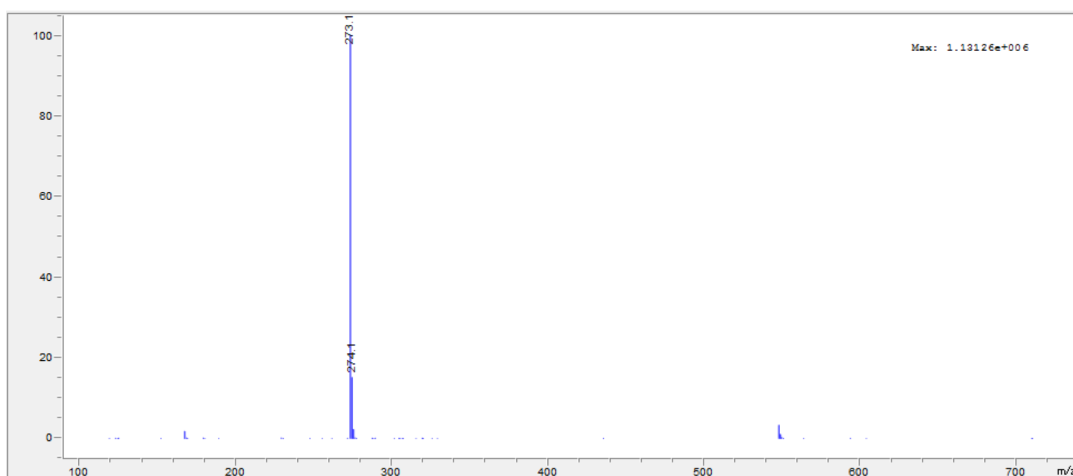


Figure 6.79: Mass spectrum of phloretin.

6.2.2 Rats and diets

Forty-eight male Wistar rats were purchased from ARC (Animal Research Centre, Perth, Western Australia) and divided into 6 experimental groups of n=12. Experimental groups were CS (corn starch diet), HCHF (high carbohydrate, high fat diet), CPh50 (corn starch diet + 50mg/kg/day phloretin), HPh50 (high carbohydrate, high fat diet + 50mg/kg/day phloretin), CPh200 (corn starch diet + 200 mg/kg/day phloretin), HPh200 (high carbohydrate, high fat diet + 200mg/kg/day phloretin).

For the first 8 weeks of the protocol, CS, CPh50, and CPh200 rats received the CS diet and HCHF, HPh50 and HPh200 rats the HCHF diet as described in method 2.2. At eight weeks, treatment groups were changed to their respective intervention diets of CS (corn starch), CPh50 (CS containing 0.1% w/w phloretin), CPh200 (CS + 0.4%

w/w phloretin), HCHF (high carbohydrate, high fat), HPh50 (HCHF + 0.1% w/w phloretin), and HPh200 (HCHF + 0.4% w/w phloretin). Phloretin was considered to have no metabolisable energy.

At 0, 8, and 16 weeks, prior to termination, oral glucose tolerance (method 2.3), body composition (method 2.4) and systolic blood pressure (method 2.5) were determined. Rats were starved for 2 hours prior to termination. Rats were euthanised as per method 2.6.1. At termination, rats underwent plasma collection (method 2.6.3), vascular reactivity (method 2.6.5), ileum and colon contractility (method 2.6.6), organ weights (method 2.6.7) and histological tissue collection (method 2.6.1). 10 rats per group underwent isolated heart perfusion (method 2.6.4), with the remaining two hearts preserved in formalin for histological analysis as per method 2.6.1. Plasma samples were assessed on 8 rats per group for liver enzyme activity (method 2.7.1) and plasma lipid profile (method 2.7.2.). Collected serum was analysed for presence of endotoxins as per method 2.9. Liver sections were assessed for expression GLUT2 and TLR4 (method 2.11.3). Distal colon sections were also assessed for occludin and claudin-1 expression using the method described in method 2.11.4, and for TLR4 expression.

Histological tissue samples were processed manually (method 2.8.2) and sectioned (method 2.8.3). Liver, ileum, colon, pancreas and left ventricle were stained with hematoxylin and eosin (method 2.8.5.1). Separate sections of left ventricle were stained with picrosirius red (method 2.8.5.2). Microscopy and imaging were performed as per method 2.8.6.1, 2.8.6.2, 2.8.6.3 and 2.8.6.4. Statistical analysis was performed on pancreatic islet cell count and density.

6.3 Results

6.3.1 Physiological parameters

Phloretin daily intake, and equivalent human dose is shown in Table 6.1. Calculations have been based on scaling equations by Bachmann*, et al.* (616) for a 70kg adult human.

Table 6.26: Phloretin daily intake of rats and equivalent human doses. Calculations are based on the average rat bodyweight at 16 weeks.

	CS	CPh50	CPh200	HCHF	HPh50	HPh200
Rat phloretin intake (mg)	0	61 ± 0.3	131 ± 0.6	0	26 ± 0.1	100 ± 0.3
Rat bodyweight (g)	379 ± 1.9	397 ± 1.7	378 ± 2.0	505 ± 1.6	543 ± 3.6	511 ± 4.0
Dose (mg/kg)	0	93.4	347	0	47	183
Equivalent human dose (mg)	0	1167	4270	0	657	2543
Equivalent human dose (mg/kg)	0	17	61	0	9	36

Treatment with phloretin did not change body weight gain as indicated in Figure 6.2. Food intake and energy intake did not differ in the first 8 weeks of intervention within a dietary group (Table 6.27). HCHF rats consumed a lower weight of food than the CS groups, however showed higher energy intake (Table 6.2). After intervention, the HCHF, HPh50 and HPh200 rats showed similar food and energy consumption. Similarly, energy intake did not differ between the CS fed rats and CPh50 or CPh200 rats.

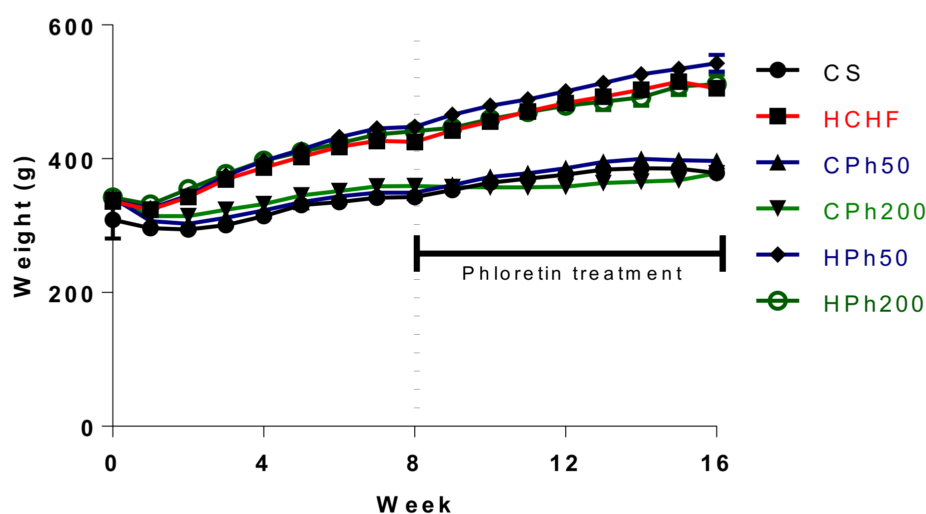


Figure 6.80: Weekly body weight.

Feed efficiency increased in all HCHF rats in the first 8 weeks of feeding. Feed efficiency with phloretin addition was not different from respective diet controls. However, HPh200 feed efficiency was comparable to CS controls, and CPh50 feed efficiency was similar to HCHF diet.

Total body fat mass was affected by both diet and by intervention. Total body fat mass (body composition by DXA) was increased in the HPh50 rats compared to the HCHF group. Addition of phloretin at 200mg/kg/day in the HPh200 rats did not result in any fat mass changes by DXA compared with the HCHF rats.

Addition of phloretin was shown to have an impact on lean body mass, which was increased when a dose of 200mg/kg/day were administered, and decreased at a dose of 50mg/kg/day in the CS diet. The only change was a difference between the CPh200 and CPh50 groups, however neither differed from respective controls (Table 6.27). The ratio of lean:fat mass was increased in the CPh200 rats compared with the CS rats. Lean:fat mass ratio was unchanged by adding phloretin to the HCHF diet.

Both diet and phloretin supplementation had an impact on individual abdominal fat pads and total abdominal fat pads, as determined by two-way ANOVA, shown in Table 6.1. Despite this, statistically significant changes from the respective controls were not evident, based on a one-way ANOVA. The significance of the intervention effect was due to the consistent trend of higher abdominal fat pad mass in the 50mg/kg/day dose, and consistently lower trend of abdominal fat pad mass in rats consuming 200mg/kg/day.

Bone mineral content (BMC) was increased in all HCHF rats compared with CS control, CPh50 and CPh200 rats. There was no difference between HCHF and HPh200 fed rats; however, the HPh50 rats had a significantly higher BMC than HCHF. Bone mineral density (BMD) was higher in the HCHF fed groups. Addition of phloretin did not alter BMD.

Table 6.27: Physiological and metabolic parameters of CS, CPH50, CPh200, HCHF, HPh50, and HPh200 fed rats.

Parameter	CS	CPh50	CPh200	HCHF	HPh50	HPh200	Interaction	Intervention	Diet
Food intake (g/day) Week 0-8	36.6 ± 1.1 [a]	36.8 ± 0.9 [a]	39.7 ± 1.3 [a]	24.4 ± 0.3 [b]	26.3 ± 0.8 [b]	24.4 ± 0.4 [b]	0.0244	0.1883	< 0.0001
Food Intake (g/day) Week 8-16	36.2 ± 0.8 [ab]	37.1 ± 0.6 [a]	32.8 ± 0.9 [b]	24.2 ± 0.6 [c]	25.7 ± 0.9 [c]	24.9 ± 0.7 [c]	0.0238	0.0068	< 0.0001
Energy intake (kJ/day) Week 0-8	410.6 ± 12.1 [b]	412.9 ± 10.1 [b]	445.8 ± 14.2 [b]	514.2 ± 5.6 [a]	548.7 ± 15.4 [a]	530.0 ± 10.8 [a]	0.099	0.0951	< 0.0001
Energy intake (kJ/day) Week 8-16	406.6 ± 9.1 [b]	416.1 ± 6.4 [b]	368.5 ± 10.5 [b]	523.7 ± 10.9 [a]	553.9 ± 17.4 [a]	539.6 ± 14.8 [a]	0.0882	0.0411	< 0.0001
Feed efficiency (g gained. kJ^{-1}) Week 0-8	0.22 ± 0.24 [b]	0.31 ± 0.13 [b]	0.77 ± 0.24 [b]	3.09 ± 0.13 [a]	3.59 ± 0.22 [a]	3.32 ± 0.25 [a]	0.2148	0.1488	< 0.0001
Feed efficiency (g gained. kJ^{-1}) Week 8-16	1.57 ± 0.12	2.03 ± 0.20	0.95 ± 0.38	2.72 ± 0.15	3.06 ± 0.17	2.29 ± 0.25	0.7852	0.0005	< 0.0001
Bone mineral density (g/cm^2)	0.176 ± 0.003 [ab]	0.179 ± 0.003 [ab]	0.174 ± 0.002 [b]	0.183 ± 0.002 [ab]	0.189 ± 0.004 [a]	0.182 ± 0.003 [ab]	0.8616	0.1494	0.0021
Bone mineral content (g by DXA)	11.16 ± 0.35 [c]	12.52 ± 0.40 [c]	11.23 ± 0.32 [c]	15.40 ± 0.38 [b]	17.96 ± 0.48 [a]	15.35 ± 0.39 [b]	0.1835	< 0.0001	< 0.0001
Total body fat mass (g by DXA)	85.3 ± 7.5 [cd]	117.5 ± 11.4 [c]	58.3 ± 4.6 [d]	202.8 ± 9.0 [b]	262.4 ± 14.8 [a]	201.7 ± 16.7 [b]	0.4387	< 0.0001	< 0.0001
Total body lean mass (g by DXA)	284.8 ± 6.8 [ab]	265.2 ± 8.2 [b]	315.1 ± 6.6 [a]	286.8 ± 4.5 [ab]	274.5 ± 10.5 [ab]	294.7 ± 19.0 [ab]	0.4039	0.0133	0.7471
Lean:Mat mass ratio	3.32 ± 0.46 [b]	3.02 ± 0.56 [a]	5.11 ± 0.63 [ab]	1.51 ± 0.09 [c]	1.03 ± 0.09 [a]	1.72 ± 0.27 [ab]	0.1238	0.0053	0.7471
Retroperitoneal fat (mg/mm tibial length)	164.40 ± 15.16 [b]	182.10 ± 14.33 [b]	128.50 ± 7.60 [b]	383.40 ± 40.54 [a]	449.70 ± 23.67 [a]	363.10 ± 30.54 [a]	0.6017	0.0208	< 0.0001
Epididymal fat (mg/mm tibial length)	91.85 ± 8.19 [b]	104.60 ± 8.18 [b]	84.05 ± 2.25 [b]	194.80 ± 18.07 [a]	219.00 ± 10.25 [a]	164.80 ± 21.88 [a]	0.4267	0.0212	< 0.0001
Omental fat (mg/mm tibial length)	97.65 ± 12.91 [b]	123.40 ± 8.21 [b]	88.38 ± 6.00 [b]	206.30 ± 14.86 [a]	240.40 ± 16.54 [a]	195.00 ± 14.23 [a]	0.9084	0.0062	< 0.0001
Total abdominal fat pad (mg/mm tibial length)	338.9 ± 28.5 [c]	410.0 ± 27.5 [c]	300.9 ± 11.5 [c]	784.5 ± 56.5 [ab]	909.1 ± 39.0 [a]	723.0 ± 55.3 [b]	0.6043	0.0014	< 0.0001

Differing superscript letters indicate significant difference at $p<0.05$ as determined by one-way ANOVA. Interaction, intervention, and diet were tested for significant contributions to variation using a two-way ANOVA, with p values reported far right.

6.3.2 Plasma lipids and glucose control

At 8 weeks of dietary feeding, prior to phloretin intervention, the HCHF, HPh50 and HPh200 rats showed increased fasting blood glucose concentrations as indicated in Table 6.28. The CPh200 group also had raised fasting blood glucose concentrations. Glucose area under the curve was raised in the HCHF diets at 8 weeks.

At 16 weeks, the HCHF, HPh50 and HPh200 groups had elevated fasting glucose compared to the CS. Supplementation at 50mg/kg/day did not alter the fasting blood glucose or glucose tolerance in either the CS or HCHF diet. Supplementation at 200mg/kg/day reduced the TAUC in the CPh200 fed rats from the CS control. The HPh200 group showed glucose tolerance normalised to that of the CS control (Table 6.28). The HPh200 glucose tolerance curves show that fasting glucose was not altered from the HCHF model, however peak glucose concentration was reduced, and from 60 minutes onward the curve was comparable to that of the CS control.

Table 6.28: Blood glucose control and plasma lipids of CS, CPH50, CPh200, HCHF, HPh50, and HPh200 fed rats.

Parameter	CS	CPh50	CPh200	HCHF	HPh50	HPh200	Interaction	Intervention	Diet
Fasting blood glucose (mM) 8 week	3.0 ± 0.1 [b]	3.2 ± 0.2 [b]	3.8 ± 0.2 [a]	4.4 ± 0.3 [a]	4.2 ± 0.1 [a]	4.2 ± 0.1 [a]	0.0239	0.1609	< 0.0001
TAUC blood glucose 8 week	686.7 ± 26.5 [a]	689.4 ± 26.8 [a]	692.8 ± 20.3 [a]	724.0 ± 46.8 [ab]	786.1 ± 22.1 [ab]	816.1 ± 26.1 [b]	0.2998	0.2144	0.0004
Fasting blood glucose (mM)16 week	3.5 ± 0.2 [b]	3.7 ± 0.2 [b]	3.7 ± 0.2 [b]	5.0 ± 0.3 [a]	5.1 ± 0.2 [a]	4.5 ± 0.1 [a]	< 0.0001	< 0.0001	< 0.0001
TAUC blood glucose 16 week	723.0 ± 22.4 [bc]	775.9 ± 29.7 [b]	635.9 ± 17.8 [c]	919.1 ± 36.8 [a]	922.4 ± 34.0 [a]	724.0 ± 21.5 [b]	0.1364	< 0.0001	< 0.0001
Plasma total cholesterol (mM/L)	1.66 ± 0.30 [a]	1.67 ± 0.33 [a]	2.08 ± 0.29 [b]	1.62 ± 0.15 [a]	1.85 ± 0.36 [a]	2 ± 0.24 [b]	0.3615	0.0003	0.8255
Plasma triacylglycerides (mM/L)	0.45 ± 0.21 [ab]	0.39 ± 0.11 [a]	0.46 ± 0.26 [ab]	0.98 ± 0.55 [c]	0.7 ± 0.65 [b]	1.24 ± 0.43 [c]	0.2268	0.0946	< 0.0001
Plasma non-esterified fatty acids (mM	1.31 ± 0.43 [a]	1.42 ± 0.41 [a]	1.83 ± 0.95 [a]	3.6 ± 1.60 [b]	2.96 ± 2.42 [ab]	4.23 ± 1.22 [b]	0.5957	0.1826	< 0.0001

Differing superscript letters indicate significant difference at $p<0.05$ as determined by one-way ANOVA. Interaction, intervention, and diet were tested for significant contributions to variation using a two-way ANOVA, with p values reported far right.

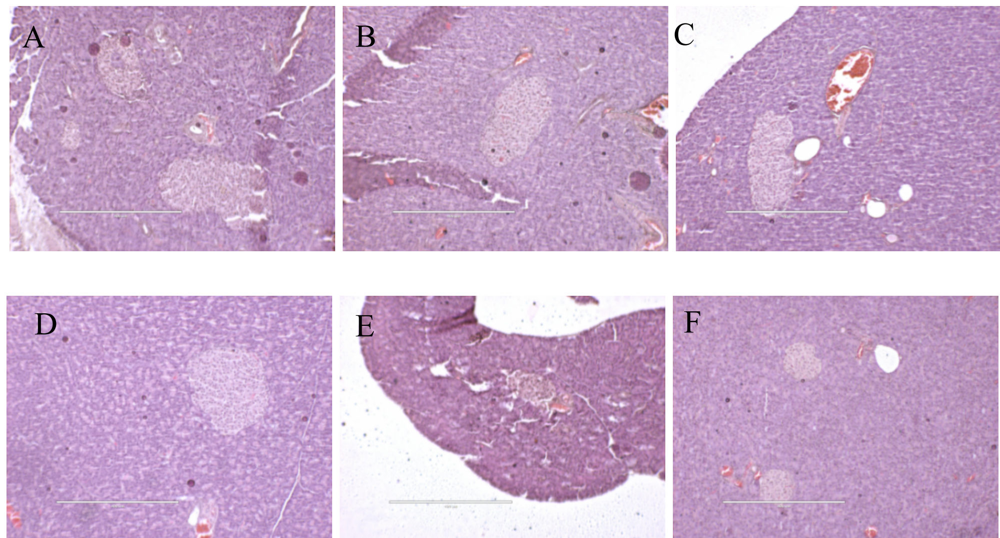


Figure 6.81: Haematoxylin & eosin stain of pancreatic islets at 10x magnification. CS (A), CPh50 (B), CPh200 (C), HCHF (D), HPh50 (E), HPh200 (F).

Pancreatic islet cell density was not changed by diet or phloretin intervention. No indication of β-cell death was noted in any section, as shown in Figure 6.81.

Total cholesterol concentration did not differ between the HCHF and CS control groups. Both the CPh200 and HPh200 groups showed increased total plasma cholesterol concentrations compared to all other groups (Table 6.28).

Plasma triacylglycerides (TAG) were more than doubled in the HCHF diet compared with the CS. No change was evident with phloretin ingestion (Table 6.28).

Plasma NEFA (non-esterified fatty acids) were 2.75 times higher in the HCHF model compared with the CS. No statistical changes were associated with phloretin addition in the CS diet. The HPh50 group had NEFA concentrations comparable to the CS control, but had not changed sufficiently to be different from the HCHF control, as shown in Table 6.3. The HPh200 group did not show the same decrease, indicating that there may be dose-dependent effects.

6.3.3 Cardiovascular and hepatic structure and function

Normalised left ventricular weight did not differ between any groups (Table 6.29). Normalised liver wet weight was increased in HCHF compared to CS diets, and was

not altered by phloretin consumption. The diastolic stiffness (κ) was not altered in any group.

Table 6.29: Cardiovascular and hepatic structure and function of CS, CPH50, CPh200, HCHF, HPh50, and HPh200 fed rats.

Parameter	CS	CPh50	CPh200	HCHF	HPh50	HPh200	Interaction	Intervention	Diet
Left ventricle + septum (mg/mm tibial length)	18.55 ± 0.95 [ab]	18.96 ± 1.04 [ab]	18.10 ± 0.50 [a]	21.83 ± 1.02 [b]	21.55 ± 0.56 [b]	19.99 ± 0.71 [ab]	0.6953	0.2536	0.0003
Right ventricle (mg/mm tibial length)	4.09 ± 0.28 [ab]	4.30 ± 0.44 [ab]	3.87 ± 0.22 [b]	4.97 ± 0.27 [ab]	5.16 ± 0.26 [a]	4.90 ± 0.24 [ab]	0.9502	0.4999	0.0003
Systolic blood pressure (mmHg)	154.4 ± 1.2 [a]	124.1 ± 1.1 [c]	121.4 ± 0.5 [c]	135.6 ± 3.5 [b]	136.5 ± 1.9 [b]	135.4 ± 1.3 [b]	< 0.0001	< 0.0001	0.114
Liver (mg/mm tibial length)	204.40 ± 6.83 [b]	198.70 ± 5.41 [b]	215.80 ± 8.17 [b]	305.00 ± 8.65 [a]	292.40 ± 6.52 [a]	291.10 ± 14.20 [a]	0.3361	0.5296	< 0.0001
Diastolic stiffness constant (κ)	26.45 ± 2.193 [a]	30.94 ± 1.595 [b]	31.51 ± 1.134 [bc]	32.8 ± 1.34 [ab]	27.08 ± 2.301 [ab]	26.66 ± 0.6361 [ab]	0.0097	0.8256	0.1523
Plasma ALT activity (U/L)	33.25 ± 11.57 [a]	28.18 ± 7.236 [a]	30.22 ± 7.085 [a]	28.7 ± 10.6 [a]	29.18 ± 20.33 [a]	31.67 ± 7.599 [a]	0.692	0.7952	0.826
Plasma AST activity (U/L)	83 ± 20.78 [a]	66.82 ± 15.85 [b]	67.67 ± 13.38 [ab]	62.3 ± 14.67 [b]	63.91 ± 23.61 [b]	60.56 ± 10.48 [b]	0.2566	0.2625	0.0284

Differing superscript letters indicate significant difference at $p<0.05$ as determined by one-way ANOVA. Interaction, intervention, and diet were tested for significant contributions to variation using a two-way ANOVA, with p values reported far right.

The deposition of collagen was not altered by treatment with phloretin (Figure 6.82). Deposition of collagen was increased in the HCHF (6.4%) group compared to the CS control (4.8%). Both the CPh50 (14.1%) and CPh200 (9.6%) groups showed increased collagen deposition compared to the CS control. In the HPh200 (6.5%) group, collagen was comparable to the HCHF control.

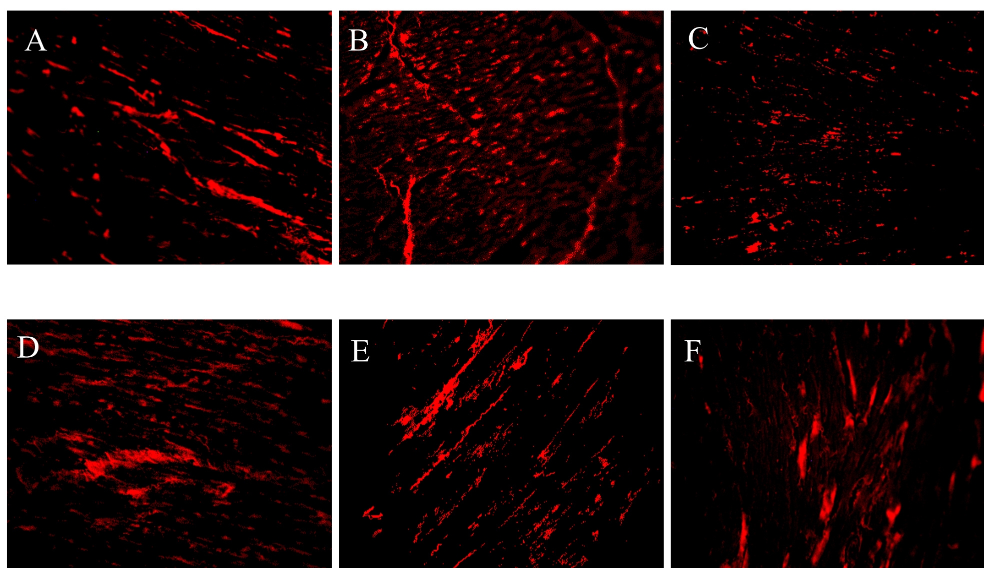


Figure 6.82: Picrosirius red staining indicating deposition of collagen type I and III fibres in the left ventricle. Shown at 20x magnification. CS (A); CPh50 (B), CPh200 (C), HCHF (D); HPh50 (E); HPh200 (F).

HCHF rats had a severely blunted relaxant response to acetylcholine in isolated thoracic aortic rings compared with the CS control. HPh50 and HPh200 rats did not display this. Maximal responses were normalised to the CS control in the HPh50 group. Both the CPh200 and HPh200 rats had comparable responsiveness, which exceeded the CS control, as indicated in Figure 6.5.

Aortic responsiveness to noradrenaline did not differ between CS and HCHF groups. Maximal responses to noradrenaline were markedly increased in the CPh200 group, compared to all other groups, which did not differ, as shown in Figure 6.83. Maximal responses to sodium nitroprusside were higher in the CS, CPh200 and HPh50 groups than the HCHF, CPh50 and HPh200 groups.

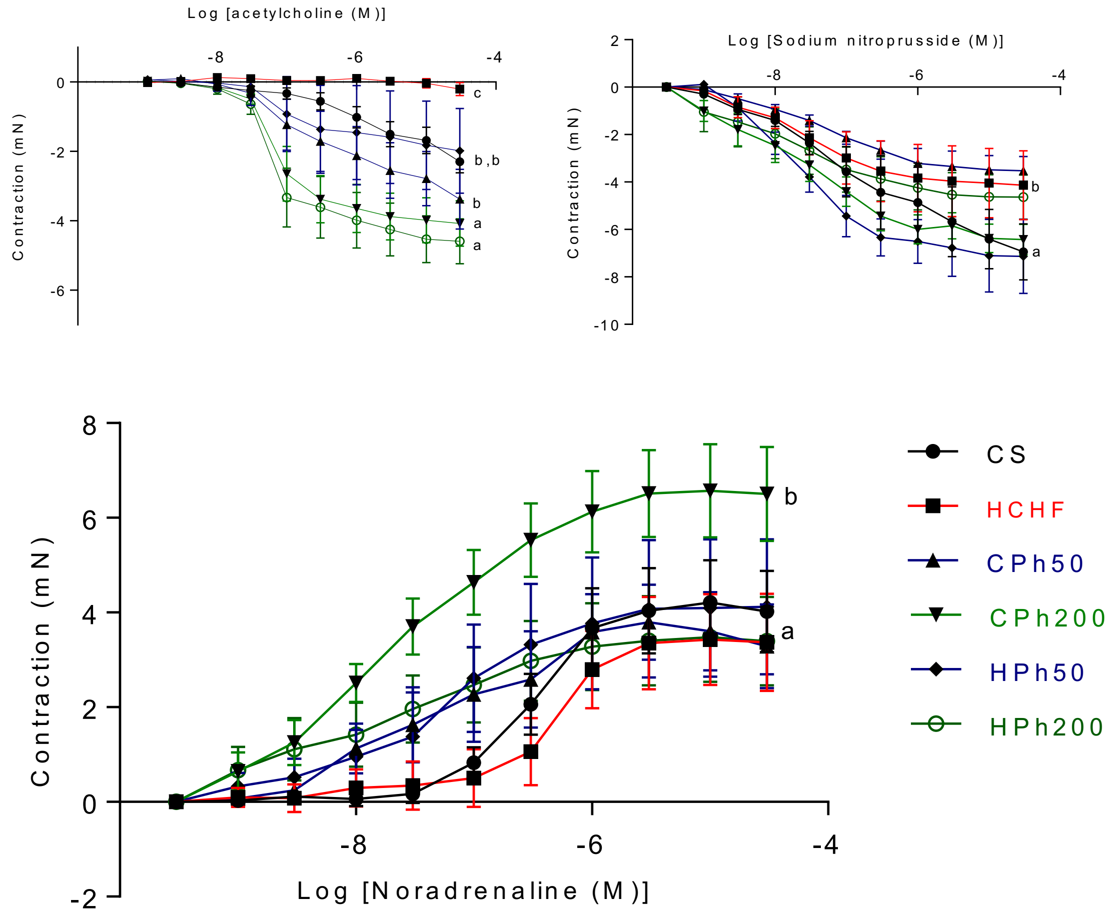


Figure 6.83: Thoracic aortic responses to acetylcholine, sodium nitroprusside and noradrenaline of control and phloretin-treated rats. Lower-case letters indicate a significant difference in final force of contraction (mN) at $p<0.05$ determined by one-way ANOVA.

Plasma alanine aminotransferase (ALT) activity was not affected by diet or intervention. Plasma aspartate aminotransferase (AST) activity was higher in the CS control compared with the HCHF. Phloretin did not change AST activity at either dose in the HCHF model, in the CPh50 group AST was lower than the CS control, comparable to the HCHF control Table 6.4.

Hepatocyte ballooning and fat deposition were evident in the HCHF model, and not in the CS model. This appeared unchanged with phloretin addition as shown in Figure 6.6.

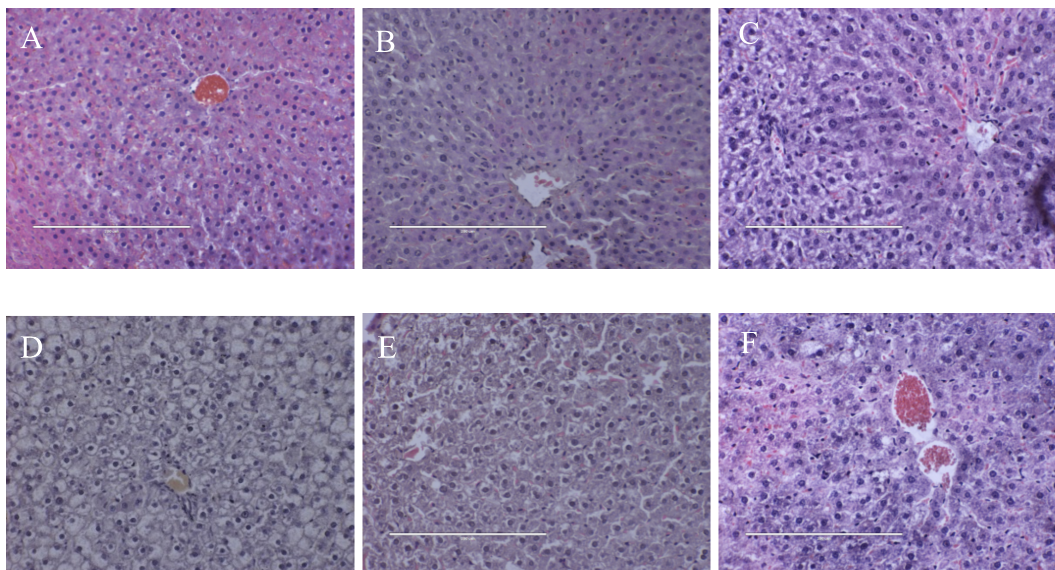


Figure 6.84; Haematoxylin and eosin stains of liver indicating ballooning and fat deposition, at 20x magnification. CS (A); CPh50 (B); CPh200 (C); HCHF (D); HPh50 (E); HPh200 (F). Scale bars indicate 200µm.

GLUT2 and TLR4 expression in the liver were unchanged by diet or intervention, Figure 6.85.

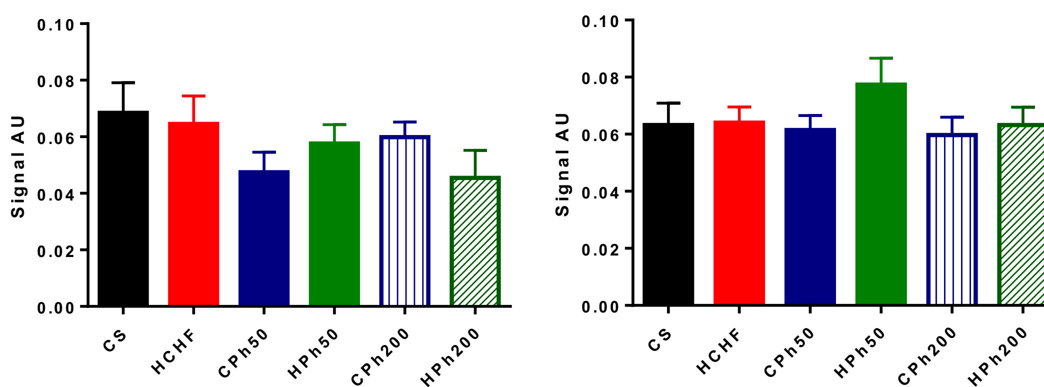


Figure 6.85: Liver GLUT2 expression (left) and liver TLR4 expression (right).

6.3.4 Gastrointestinal parameters

Colonic and ileal reactivity did not differ with dietary groups. Rats on the HPh200 diet had a higher final ileal force of contraction than the CS control, and all other groups as shown in Figure 6.86. No other groups differed. No changes in colonic responses were noted between any groups.

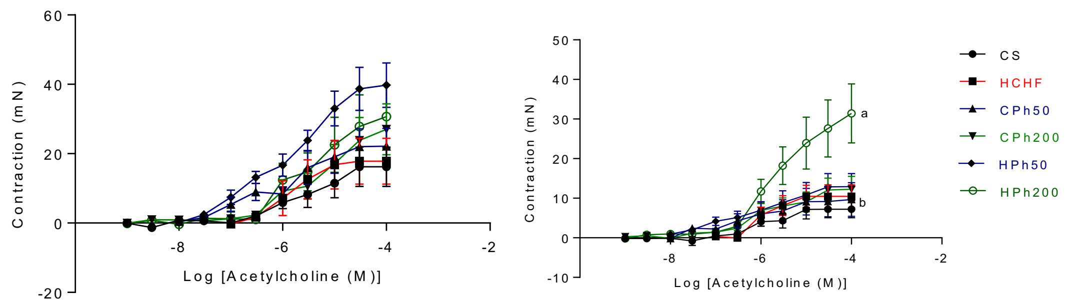


Figure 6.86: Colon (top) and ileum (bottom) reactivity to acetylcholine. Differing lower-case letters indicate a different final maximal force of contraction, determined by one-way ANOVA, p<0.05. Where no letters are indicated, no difference was calculated.

TLR4 expression in the colon did not differ between any groups, Figure 6.87.

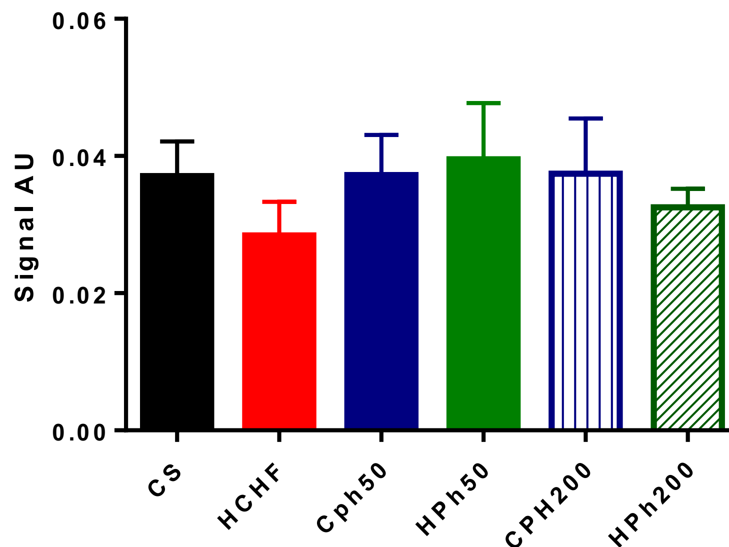


Figure 6.87: Liver TLR4 expression determined by semi-quantitative ELISA. No significant difference was seen between any groups.

Claudin-1 expression was elevated in the HCHF group. The CPh50 and CPh200 groups had increased expression from the CS control. Occludin expression was unchanged by diet or intervention, Figure 6.88.

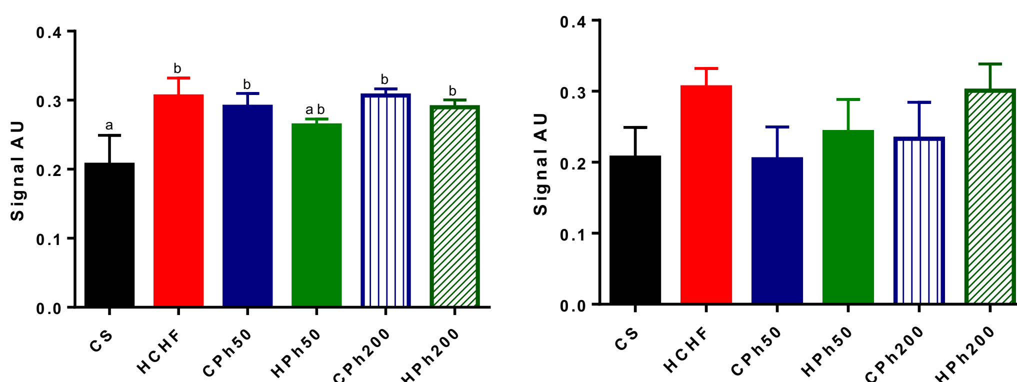


Figure 6.88: Claudin-1 expression in the colon (left) and occludin (right) expression in the colon determined by semi-quantitative ELISA. Differing lower-case letters indicate a significant difference at $p<0.05$.

6.4 Discussion

The aim of this study was to determine whether phloretin was able to ameliorate the physiological and biochemical changes that occur with metabolic syndrome including cardiovascular remodelling, impaired glucose tolerance, adipose tissue presence and dyslipidaemia. Further, the study aimed to determine some of the molecular mechanisms by which phloretin may affect metabolic syndrome including alteration of gastrointestinal integrity reflected in changes in occludin and claudin-1 expression, and expression of TLR4.

It was predicted that phloretin would have the largest impact on glucose tolerance due to its known activity in inhibiting SGLT-1 and GLUT2. Phloretin was also surmised to be highly anti-inflammatory based on *in vitro* studies, which could reverse or prevent progression of metabolic syndrome. Using body weight scaling equations (616), the equivalent human dose for the HPh200 group is 2543mg per day, for an adult of 70kg.

When compared with other studies that use a similar animal model, this high dose is probably excessive, expensive and not more effective than other supplements. Overall, the most promising indication for phloretin intake appears to be improved glucose tolerance.

Body composition

Phloretin did not result in fat loss where obesity is caused by a high fat diet. This is consistent with the data from Alsanea*, et al.* (340), who used intraperitoneal injections in mice. Given the different direction of change in fat mass, lean:fat mass ratio, and abdominal fat pad mass with the two phloretin doses, it is surmised that there is a dose-dependent and dietary interaction. The effect on adiposity may be from altered uptake of sugars and fats, altered gene expression, or changes in metabolic pathways, as the energy intake was unaltered. Lipid accumulation and adipocyte differentiation increased with phloretin exposure in *in vitro* studies using 3T3L-1 adipocytes, and markers of lipid accumulation were increased (609). Adipogenic regulator expression increased on exposure to phloretin at 50μM with increased 3T3L-1 adipocyte differentiation (610). Skeletal muscle palmitate uptake in cell culture was inhibited by phloretin in the presence of 300mmol phloretin (617). Other studies suggest that phloretin inhibited lipid accumulation and reduced the expression of adipogenic transcription factors (351).

Given that the outcomes of fat accumulation differed in magnitude and direction with differing doses, phloretin may be adipogenic or anti-adipogenic depending on the dose. It is also noted that the true dose in the HCHF and CS supplemented diets differ, despite the same dose in food. For this reason, comparison between the interaction with the CS diet and phloretin, and the HCHF diet and phloretin may be affected largely by the dose, rather than simply interaction with the other dietary components.

Energy and food consumption

Phloretin did not affect appetite in high fat diets but reduced food volume intake by approximately 11% in a low-fat diet. To date, very little research has been reported on sugar inhibitors affecting appetite. However, Kuhre*, et al.* (618) showed that inhibition of GLUT2 by phloretin resulted in suppression of neurotensin release, which is surmised to be responsible for glucose-stimulated appetite regulation. Inactivation of

glucose sensors in GLUT2-null mice resulted in over-consumption (619). Administration of 2-deoxyglucose, also a GLUT2 inhibitor, resulted in hypoglycaemia and stimulated food consumption (620). These results contrast with the food intake results in this study. Consumption of a GLUT2-inhibiting compound in the diet did not lead to impaired glucose-controlled feeding.

Pre-diabetes

Phloretin corrected post prandial glucose tolerance but not fasting glucose. There appeared to be no damaging effects of phloretin to glucose tolerance in a normal, healthy diet. The results here are somewhat consistent with other studies. Treatment of obese mice with phloretin by intraperitoneal injection led to reduced insulin concentrations in a rat model of obesity (340). In cell culture models, phloretin improved insulin sensitivity (610). The phloretin glycoside, phloridzin, improved non-fasted glucose concentrations in streptozotocin-diabetic rats at concentrations less than 40mg/kg/day (337). Reduced blood glucose concentrations have been shown with phloretin intake simultaneous to glucose ingestion (621).

The improvement seen could not be attributed to normalisation of β-cell mass. In animal models of obesity, preceding type 2 diabetes mellitus, there is an increase in the β-cell mass (622), whereas after development of type 2 diabetes mellitus there is a distinct decrease in β-cell mass (623). The restoration of glucose tolerance must be due to improved function of the islets, improved peripheral glucose clearance or action of glucose regulating hormones.

Plant-derived flavonoids including marinin and okanin at a dose of 20mg/kg/day restored pancreatic function in diabetic Wistar rats (624). In pre-diabetic rats, there was a loss of GLUT2 from the pancreatic β-cells (302, 303, 625). GLUT2 expression in the pancreas reduced with non-insulin dependent diabetic progression (625) (626). Inhibition of GLUT2 can prevent loss of expression in pancreatic β-cells in rat models of streptozotocin-induced diabetes, exhibiting potential for phloretin (300). In other studies, GLUT2 inhibition was protective and preventative in the progression of insulin resistance and pre-diabetes (627, 628). This was not studied in our research as

humans do not have GLUT2 as their primary glucose sensor in the pancreas (299), limiting the indications for phloretin in human trials.

Peripheral glucose clearance can be improved by normalisation of GLUT2 expression in the liver. The expression of GLUT2 in the liver was unchanged in this study. This is in contradiction to other models of diabetes. In other models, an increase in GLUT2 expression occurred in glucose-impaired hyperthyroid rats (566). Cell models of fatty liver showed increased GLUT2 mRNA expression, glucose output and gluconeogenesis, rather than uptake (322). In an 60 day model of diabetes, Wistar rats had increased GLUT2 mRNA in the liver and increased protein expression (629).

The composition of sugars and complex carbohydrates is likely to be of importance in the situations where sugar transporters are targeted as therapeutic agents. The ability of GLUT2 to transport fructose (630) may be one of the contributing factors that have caused differing effects in the HCHF and CS diets in this study. Other sugar transporters may have a compensatory role when another is inhibited. GLUT2 and GLUT5 work in conjunction to transport fructose, and GLUT5 contributes most of the fructose transport at the brush border (631). GLUT5 increased under high fructose feeding in GLUT2 null mice, therefore this may have a compensatory action (632). Fructose uptake can be reduced by GLUT2 inactivity (632) but cannot be fully stopped. This is likely to explain the lack of weight loss in the high fructose HCHF diet, even though it was expected.

Dyslipidaemia

At high doses, phloretin increased plasma total cholesterol concentrations. The increased total cholesterol concentration suggests an increased risk of atherosclerosis associated with the metabolic syndrome. Low HDL concentrations are a risk factor and diagnostic tool for metabolic syndrome (173). As LDL and HDL were not tested separately, we cannot definitively suggest that phloretin will increase the predisposition of cardiovascular risk due to high total cholesterol. A more significant factor is reduced HDL, and increased LDL cholesterol. Shu, *et al.* (609) showed reduced cholesterol concentrations at low doses of phloretin. Alsanea, *et al.* (340) showed no change in plasma lipids, despite reduced fat deposits in the liver, similar to what was seen in our study. Apple polyphenols, which include phloretin, resulted in an improved plasma cholesterol profile, through altering the ratio of LDL:HDL (633).

In vitro studies have shown that phloretin and GLUT2 inhibition may increase cholesterol secretion rates in intestinal TC7 cells, however it was suggested that this was a non-significant increase (634). In contrast, apple polyphenols were shown by Vidal*, et al.* (635) to reduce cholesterol esterification and secretion by TC7 cells, by the related phloretin metabolite phloretin-2-glucoside. Uptake of cholesterol was inhibited by phloretin *in vitro* (636), which is not supported by this research. If phloretin is able to prevent oxidation of cholesterol molecules, this will reduce the cardiovascular risk (637).

Cardiovascular health and liver health

Phloretin restored efficacy of acetylcholine in eliciting vasodilation in the HCHF diet at both doses, but this was not correlated with improved systolic blood pressure. This may be due to the anti-inflammatory effects with phloretin resulting in reduced aortic stiffness. TNFα antagonists also have these effects (638). Phloretin suppressed changes in endothelial cells when exposed to TNFα (343). In rabbit isolated vessels, phloretin induced vascular relaxation at concentrations as low as 5µM by an endothelium-independent mechanism (639). The same study showed that genestin had similar effects and was transferrable to *in vivo* human trials. Presence of phloretin impaired noradrenaline contractile responses in isolated rabbit vascular sections, and was again suggested to be endothelium-independent, and promote vaso-relaxation (640). There was no reduction in diastolic stiffness or collagen deposition with phloretin consumption. Phloretin does not show promise for cardiovascular health in a model of metabolic syndrome when all factors are accounted for.

The addition of phloretin to the CS diet resulted in reduced plasma AST activity yet the change was not associated with changes in the histology of the liver or liver size. Although no changes in liver enzymes occurred in the high fat diet with phloretin, there was an improvement in the liver histology. Estrogenic studies show no changes in liver enzyme activity with phloretin doses up to 100mg/kg (641). Phloretin delivered by intraperitoneal injection at a dose of 10mg/kg twice weekly reversed fat accumulation and cellular ballooning of hepatocytes in a high-fat diet, mouse model (340).

Gastrointestinal function

Gastrointestinal effects of phloretin include increased colonic and ileal reactivity on exposure to acetylcholine. Within the gastrointestinal tract, there is an ideal contraction strength and frequency which can be altered by multiple disorders. In obese leptin-deficient mice, responses to cholecystokinin were diminished, but responses to acetylcholine were unchanged (642). This may explain the lack of difference between the CS and HCHF diets. Other research indicated increased small intestinal contractility and lowered distal contractility (179). The increase in ileal contractility with phloretin addition, specifically in a high fat diet, may lead to increased smooth muscle contraction, and should be considered a potential contraindication in those with pre-existing gastrointestinal disorders. Phloretin phosphate at 40µg/mL decreased contraction in isolated guinea pig ileum (643).

Gastrointestinal integrity is gaining traction as a contributing factor to overall health, particularly with respect to inflammatory disease such as obesity and metabolic syndrome. In this study, we demonstrated that the HCHF model unexpectedly showed increased claudin expression. The complex of tight junction proteins also contains ZO-1 (zonula occludin-1) and junctional adhesion molecules (644). ZO-1 was not studied, and occludin was not altered. These results appear to contradict other studies that suggest high fat feeding reduces expression of tight junction proteins (237) and in genetically obese and diabetic mice (177, 423).

Increased expression of occludin has been linked to improved gut integrity. Similar to the outcome seen in phloretin ingestion, grapeseed extract containing polyphenols increased colonic occludin expression (645). Although the outcomes in control rats were unexpected, there is a promising indication that phloretin can improve gastrointestinal integrity.

Limitations

A number of shortcomings are evident in this study. Due to the complexity of the system, it was not possible to explore all mechanisms involved in phloretin's improvement of glucose tolerance. Several other aspects such as changes in cholesterol oxidation, oxidative state, GLUT2 and TLR4 expression in more tissues, and the densities of SGLT-1 and other sugar transporters were not assessed. In addition to

determining the pancreatic islet density, it would have been ideal to assess a suite of inflammatory markers that alter insulin release, and determining insulin concentrations in conjunction with blood glucose concentrations. It would also prove beneficial to assess GLUT-4 and 5 expression in muscle tissue, as there appeared to be a much improved peripheral glucose clearance in phloretin-supplemented rats.

6.5 Conclusions

The research provides evidence that phloretin has a therapeutic role with respect to the glucose tolerance aspect of metabolic syndrome, but this is no greater than other more cost-effective examples. There is some evidence to suggest that it may cause gastrointestinal discomfort at high doses, as indicated by the increased ileal contractions. Although phloretin does not seem to be harmful in this rat model of obesity, it does have some contraindications which require consideration. While phloretin impacts glucose uptake and results in lower peak blood glucose concentrations, long-term ingestion with meals does not reduce calorie consumption in *ad libitum* models, nor does it ameliorate weight gain, cardiovascular changes or lipid dysregulation associated with metabolic syndrome.

Chapter 7 – Conclusions

7.1 Conclusions and future directions of sorghum in diets

7.1.1 Summary of key findings and their implications

Sorghum as an addition to diets to improve metabolic syndrome has shown potential in this research as it alters a range of parameters of metabolic syndrome. A key take-away message from the research was that sorghum's impacts were varied, based on the type of intervention.

Whole sorghum showed significant promise for reduction of total cholesterol in a similar way that oats have been touted as a dietary component to improve cholesterol concentrations, and also normalisation of post-prandial glucose. Incorporating sorghum into diets is a simple and feasible way for people to improve cholesterol concentrations at a dose of just 30g per day. Continuing research with clinical trials to further this, and confirm the effects in humans, will be important to promote sorghum products for the consumer market, and reduce the risk of developing type 2 diabetes and cholesterol-related complications.

Black sorghum flour and red sorghum flour improved metabolic parameters in a normal diet, although few changes in high fat-high carbohydrate diets that induce metabolic syndrome. This should not be a deterrent for the consumption of sorghum, as there were indications that sorghum flour can help prevent the changes that lead to metabolic syndrome. A wholegrain flour will have greater functional properties.

Wet cake sorghum consumption resulted in improvements in glucose tolerance, improved liver histology, increased lean mass, and reduced liver GLUT2 and TLR4 expression. Few studies have looked at sorghum products or sorghum-based diets and their effects on metabolic syndrome, and wet cake has not been studied in a human context for health. This presents an important possibility for future research. This research provides a foundation to indicate that this waste product of sorghum fermentation for ethanol production can be used as a functional food product. Sorghum wet cake has been added to chocolate chip cookies, sugar cookies, molasses cookies, bread (646), and spaghetti (647) with no sensory aversion by consumers. These studies

are old, and the idea has not taken off. We may see these fortified food products on the market with added health information backing their use.

Addition in the diet also showed increased lean mass which is of interest for fields other than metabolic syndrome. This could be looked at as an alternate nutrition source in countries where protein is expensive or has low availability, as well as in ageing patients. Further, this is likely to influence nutrition studies where efficient sources of protein are of high interest and components which inhibit muscle breakdown such as catechins, or components which promote muscle growth, are also of interest.

7.1.2 Limitations and considerations for future research

There are limitations to this research, especially considering the physiological differences between rat models of metabolic syndrome and transferring to human consumption. Further, the development of metabolic syndrome was less pronounced in the modified high-carbohydrate, high-fat diet with minimal changes in liver enzymes, diastolic stiffness and cholesterol concentrations. There were inconsistent changes in the expression of GLUT2, TLR4, occludin and claudin between the two diets. This suggests other components may be contributors or that one progresses the molecular changes more quickly. Further, the control diets differed in their macronutrient composition and the rats fed the modified corn starch diet showed indications of liver damage and muscle wasting, possibly due to the low protein or energy content of the diet.

Rat models are inherently complex systems, as are humans. Assessing a single system such as the progression from gastrointestinal tract to tight junctions to intestinal permeability to endotoxaemia and finally to metabolic syndrome is important to understand its contribution and involvement, but does not give a full picture of the processes involved. Because of the complexity of the system, monitoring a wider range of biomarkers as a pilot study might show the weighted average of contributions of the biochemical pathways which lead to metabolic syndrome. Statistically, we did not see changes in many anatomical, pathological or biochemical features of metabolic syndrome. The application of statistics is key in moving forward with metabolic research. Continual improvement of statistics applied to clinical and animal trial needs to be prioritised. In this study, simply adding more "groups" to the mix altered the

outcome when assessing the difference between the corn starch and high-carbohydrate, high-fat diets, hence the application of *t*-tests in the 20% sorghum flour study. Sliding dichotomy models have been proposed (648) to show progressive changes rather than absolute "yes" or "no" statistical changes. Re-assessing this data with new statistical methods will most likely reveal new information.

The situation is further complicated by the presence of responders and non-responders in the population (649). Even within this research, I saw that changes with sorghum consumption varied from individual to individual in a population despite a similar genetic makeup. Not all humans will show the same response to food interventions, as with drugs, and as genomics progresses we may be able to implement "designer" diets based on genetics (650).

Scientifically, the limitations are that not all mechanisms of the changes could be identified. Although I hypothesised that there would be changes in intestinal permeability, occludin and claudin expression, which would translate to a reduction in LPS presence in the blood stream, this was not evident. This linear progression of following the changes in LPS through to implications for metabolic syndrome was not supported by the outcomes. Despite published studies showing that LPS can induce metabolic syndrome, and metabolic syndrome is linked to higher concentrations of circulating endotoxins, this is a very complex system and did not appear to be a driver of metabolic syndrome in this research.

There is also the suggestion that circulating fatty acids can mimic the effect of LPS on TLR4-mediated inflammation that leads to metabolic syndrome. I tested only expression of TLR4, not the activation of TLR4 or the cytokines produced by TLR4 activation. Further to this, I tested it only in the colon and liver. Further testing of expression of TLR4 as well as its activation in cardiac tissue, pancreas, small intestine and kidney would assist understanding whether sorghum alters TLR4-mediated inflammation leading to metabolic syndrome.

The identification of individual compounds in the sorghum and products would have provided understanding of their contribution to health effects. Furthermore a quantitation of these would provide a baseline reference for building upon this research.

Consumption of sorghum likely altered the composition of the gut microbiome, as with consumption of other cereals. Unfortunately, I was unable to assess the changes, however future animal and human studies would greatly benefit from the understanding of changes in the composition of the gut microbiome with sorghum consumption, and how these changes correlate with metabolic and physiological changes. We need to acknowledge that the changes to the gastrointestinal tract in rats may not translate to humans. Notably, humans do not have a caecum while rats do. There is also a discrepancy in the relative surface areas of different gastrointestinal sections between humans and rats, in addition to differences in microbial populations and the ability to extract nutrients (651).

Overall, this research has contributed to the knowledge base of how sorghum can affect health in a single scenario. While it has shown promise, realistically the development of metabolic syndrome is a long-term process which can be best changed by re-education and more comprehensive changes in dietary habits and attitudes, and changes in physical activity. Future human clinical trials should incorporate sorghum into a more thorough diet change and aid in educating participants on making better dietary choices. Animal models do not necessarily translate to humans. Human trials are really the best way forward for researching sorghum as a functional food.

The biggest barrier to inclusion of sorghum into diets is most likely going to be a lack of acceptance by the wider community. Generally, communities are resistant to changing their dietary traditions. Sorghum inclusion into products will be helped by the current trend of “gluten-free” foods, and it has already been incorporated into a number of products. These include bio-fortified sorghum cookies (652), porridges (653), “complete nutrition” foods (654, 655) and breads (656). Wheat flour is the primary grain consumed in Australia. Wheat flour has unique properties and has been bred and highly researched to have improved properties for baking or for flour production in the case of durum wheat.

Sorghum does not possess all of these properties and so there will be a discrepancy in the sensory evaluation of products made with sorghum flour. Likewise, consumers are accustomed to wholegrains such as oats, but steam flaking of sorghum may be able to provide an oat substitute that suits consumer preferences. Research indicates that

wholegrain breakfast cereals made from sorghum show higher sensory acceptance and bioactive content than a wheat counterpart (657).

Sorghum wet-cake is likely incur the most resistance from consumers. Whilst the smell is highly appealing with a “caramel” scent, texturally it may incur resistance from consumers. This will be a challenge to food science technology. However, sorghum wet-cake has been incorporated into chocolate chip cookies, sugar cookies, molasses cookies, bread (646) and spaghetti (647) with little sensory aversion.

7.1.3 Key recommendations for future studies

Future research should focus on human studies. Let’s be honest, people love free food. If you offer them free sorghum products in exchange for a few blood tests, you’ll probably get a number of people on board. Sorghum should not only be researched in the context of metabolic syndrome, it should also be researched in the health and fitness area.

Although it will be difficult to assess levels of expressed proteins in tissues, monitoring plasma indicators of dyslipidaemia, plasma LPS concentrations, and also monitoring intestinal permeability by the lactulose/mannitol test are all possible as they are non-invasive options. In addition, plasma testing for cytokines that are TLR4-dependent will help construct a picture of whether sorghum alters TLR4 activation and alters metabolic syndrome. Metabolomic studies of blood markers where large scale screens are conducted will give a more comprehensive picture of the contributing processes which lead to metabolic syndrome, but also how sorghum consumption affects these biochemical pathways and markers.

Further research should also focus on understanding how to process sorghum to receive a good response from consumers, while retaining the properties of components that produce sorghum’s effects.

Sorghum is in its infancy in terms of breeding. In the future, research on specific effects of genotypes or “specialty” lines of sorghum such as those with high anthocyanidins, high tannins, high phenolics, high amylose and altered proteins for nutraceutical purposes will be possible.

Sorghum is a minor cereal grain for human consumption in Australia, but in Africa and India, it feeds millions of people. This research indicates that sorghum is not detrimental to health, and shows a great deal of promise in improving symptoms in patients with metabolic syndrome and possibly other ailments. The potential is much more far-reaching than simply altering metabolic syndrome. Incorporating sorghum as a human food source will add a market for Australian-produced sorghum which will then feed into plant breeding research, the food processing market and new product development.

Clinical work which supports the findings in humans, and also promoting sorghum as a food, will increase its adoption as an accepted food. If this occurs, I hope that it will contribute to a reduced incidence of diabetes and cardiovascular disease.

7.2 Conclusions and future directions for phloretin in diets

7.2.1 Summary of key findings and their implications

This thesis presents results on different interventions as possible treatments to reverse diet-induced metabolic syndrome. Compounds have been tested using a validated rat model of high-carbohydrate, high-fat diet to induce metabolic, cardiovascular and liver changes mimicking metabolic syndrome in humans. Further, a different rat model was characterised which allowed higher doses of interventions such as cereals which are used at higher amounts in the human diet. Phloretin showed dose-dependent effects, especially a normalisation of post-prandial glucose tolerance with a dose of 200mg/kg/day. Thus, phloretin could be a suitable dietary supplement for patients with insulin resistance that may proceed to diabetes. This should be researched in a clinical trial situation before recommending to patients. The likely mechanism of action of phloretin is by SGLT2 inhibition. Although synthetic SGLT2 inhibitors are on the market with support from clinical trials, their adverse effects including effects on bone (658) need to be tested for phloretin. However, phloretin did not change other cardiovascular parameters and showed improvement in liver structure.

Moving forward to a closely monitored clinical trial with phloretin would increase the knowledge and use of phloretin in metabolic syndrome. Other research in animal models has delivered phloretin by oral gavage or intraperitoneal injection, which are

not appropriate for chronic dosage in humans. A dietary pill will be suitable, such as those in cellulose caps or pressed pills. This will alter the concentration in the gastrointestinal tract and likely alter the uptake of sugars depending on the concentration. This will differ from the chronic low dose in food delivered in this study.

Considering the GLUT2 inhibiting potential of phloretin and SGLT1 inhibiting potential of phloridzin, it is surprising that these natural compounds have not been researched more extensively for their ability to alter insulin resistance. It is possible that more potent compounds such as dapagliflozin, canagliflozin and empagliflozin have taken centre stage (659). It may be time to shift focus and look at the potential of inhibitors of other sugar transport molecules, such as those inhibited by phloretin.

Dysfunction of other sugar transporters including GLUT5 (660) and GLUT4 (661) has been implicated in metabolic syndrome, so the potential for research into preventing and reversing metabolic syndrome is just beginning. Prevention is better than cure, and although this research has focussed on reversing and minimising progression of metabolic syndrome, a healthy diet and exercise is paramount. Integrating whole foods into the diet to limit the risk of metabolic syndrome needs to be stressed, in addition to researching medications and supplements to reverse metabolic syndrome that is already occurring. Sugar transport inhibitors differ between rats and humans. The improvements in glucose tolerance in rats may not translate to humans, especially as human pancreatic glucose sensing is not mediated through GLUT2 as it is in rats.

7.3 Post script

The opportunity to study an underutilised wholegrain is what drew me to this PhD, especially considering sorghum is a crop grown on my family farm. My father introduced me to sorghum as a child, popping it in a similar fashion to popcorn. I had forgotten about this memory until the opportunity to study sorghum as a potential human food source with nutraceutical benefits came up.

I am personally passionate about health, fitness and nutrition. Knowing that sorghum has a great potential and aiding to better the knowledge of its presence in the human food market is of personal satisfaction. I am a big believer that the whole diet and

lifestyle, not just one component, is what influences health. Spreading information and knowledge about this will be my focus in years to come. Having a PhD in health science after a career in agriculture will undoubtedly give rigour to the information and education I am hoping to spread, in addition to helping me make sense of the information and misinformation about nutrition and health that that is rife in the community.

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