Nutritional properties of quinoa (*Chenopodium quinoa* Willd.) and its effects on cardiometabolic risk factors and gut microbiota

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By

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List of abbreviations

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid
ACS-ACOD	Acyl-CoA synthetase Acyl-CoA oxidase
ALT	Alanine transaminase
AST	Aspartate transaminase
AUC	Area under the curve
BMI	Body mass index
BP	Blood pressure
BW	Buckwheat
BWP	Buckwheat protein
CDI	*Clostridium difficile* infection
Chol	Cholesterol
CRP	C-reactive protein
CVD	Cardiovascular disease
DBS	Dried blood spots
DCI	D-Chiro-Inositol
DGGE	Denaturing Gradient Gel Electrophoresis
DBP	Diastolic blood pressure
DPPH	2,2-diphenyl-1-picrylhydrazyl
DTBE	Tartary buckwheat extract
DW	Dry weight
ELISA	Enzyme-linked immunosorbent assay
FAO	Food and Agricultural Organization of the United Nations
FC	Folin-Ciocalteu
Fe3+TPTZ	Ferric 2,4,6-Tripyridyl-s-Triazine
FFQ	Food frequency questionnaire
FGM	Flash Glucose Monitoring
FISH	Fluorescence In Situ Hybridization
FMT	Fecal microbiota transplantation
FRAP	Ferric reducing antioxidant power
GA	Gallic acid
GAE	Gallic acid equivalent
GI	Glycemic index
HbA1c	Hemoglobin A1c
HDL	High-density lipoprotein
HFQ	High fat diet with added quinoa
HMG-CoA	3-hydroxy-3-methyl-glutaryl-coenzyme A
LDL	Low-density lipoprotein
LOA	Limits of agreement

NEFAs	Free fatty acids
NSP	Non-starch polysaccharides
OTU	Operational Taxonomic Units
PBF	Protein buckwheat flour
PBS	phosphate buffer solution
pGI	Predicted glycaemic indexes
PCoA	Principal Coordinate Analysis
QF	Quinoa flakes
QP	Quinoa protein
QPF	Quinoa pericarp fraction
RS1	Physically inaccessible starch
RS2	Native granules
RS3	Retrograded starch
RS4	Chemically modified starch
RCT	Randomized, controlled trials
SCFA	Short-chain fatty acids
sPLA2	Secretory Phopholipase Type A2
SBP	Systolic blood pressure
TBBE	Tartary buckwheat bran extract
TC	Total cholesterol
T2DM	Type 2 diabetes
TE	Trolox Equivalents
TEAC	Trolox equivalent antioxidant capacity
TG	Triglycerides
USPS	United States Post Office
VLDL	Very low-density lipoprotein
WMD	Weighted mean differences
20E	20-hydroxyecdysone

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Abstract

Across the globe, cardiovascular disease (CVD) is the leading cause of cause of death, including in China, where mortality from CVD has increased rapidly in the past few decades. There is a growing body of epidemiological studies to suggest that diets rich in whole grains are linked to a lower risk of CVD and mortality, with similar results found across diverse populations. Quinoa, as an example of a pseudo-cereal, is included in the ''whole grain'' class in terms of nutritional value, suggesting that quinoa is a possible alternative to common cereals, such as wheat, rice and corn. In this study, a total of thirteen commonly used commercial quinoa accessions and nine buckwheat accessions were sourced from various regions, including Peru, Ecuador, Bolivia, USA, UK, Netherlands and China. In order to select the quinoa accession with 'optimal' nutrition for use in the future human intervention study, chemical analysis of these thirteen quinoa accessions was conducted including phytochemical and dietary fibre content. There was considerable variation in the proximate composition but on average the quinoa seed samples had a higher content of protein, fat, fibre, phenolics and apparent antioxidant capacity, as well as well-balance amino-acids compared with other cereals. For the buckwheat accessions, only phenolic content and antioxidant activity were analysed, which also showed a wide range.

The effects of quinoa on humans has rarely been investigated with just three small interventions published, with inconsistent results; moreover, their possible effects on gut microbiota are totally unknown. An exploratory study was carried out to compare the effects of a quinoa-enriched bread as part of the usual diet with refined wheat bread on CVD risk markers and the gut microbiota. Thirty healthy obese men (35-70 years, BMI>25kg/m^2) completed a 4-week cross-over intervention, separated by a washout period of at least 4 weeks. The intervention diet was 1 quinoa roll/day weighing approximately 162 g that included 20g quinoa seed flour and 80 g refined wheat flour compared with an equivalent sized 100% refined wheat roll. Fasting blood sample, 24h urine and gut microbiota samples were collected at the beginning and end of each intervention period, as well as dried blood spots after standard breakfast (100 g quinoa or refined roll with 10 g strawberry jam).

After 4 weeks of quinoa roll consumption, there was a significant decrease in glucose by 4.5% and LDL cholesterol by 5.7% compared with the corresponding baseline, but the changes between the two treatments did not reach significance level. Moreover, anthropometric variables, other blood variables and plasma antioxidant capacity also did not significantly differ between two treatments. Continuous glucose monitoring was applied for 4 days before and after sampling at the end of each intervention period. The AUC for glucose over the four days at the end of the quinoa treatment period was borderline significantly lower than the following four days when quinoa consumption

ceased (p=0.054). As for the postprandial glucose changes, although some important differences in glucose responses between quinoa and refined wheat rolls breakfast, such as significantly different AUC values over 4 hours, were not observed in capillary blood samples, the glucose response curves were relatively similar; there was a more rapid fall approximately after 60 mins after the quinoa roll breakfast. Although there were some changes in the relative abundance of gut microbiota within treatment like Firmicutes and Bacteroidetes, no significantly differences in diversity measures (alpha and beta) and relative abundance of gut microbiota were observed between two treatments. The presence of many 'trend' results, such as glucose, insulin and LDL cholesterol values, in the present study indicates that significant results might have been obtained with prolonged duration, higher dose and larger numbers of subjects. In conclusion, a specific quinoa diet improves cardiometabolic risk-associated biomarkers and gut microbiota in a healthy cohort, indicating potential value as a healthy gluten-free alternative to common cereals.

Chapter 1 Introduction

Across the world, cereal-based foods form an integral part of the human diet, with approximately 30-70% of their daily energy derived from this source (FAO, 2014). The grains comprise the cereal grains, such as rice, wheat, corn and rye as well as pseudo-cereals like quinoa, amaranth and buckwheat. Whole grains are composed of three component parts, including bran, germ and endosperm (Van der Kamp *et al.*, 2014). There is a growing body of epidemiological studies supporting an inverse association between whole grain consumption and risk of several chronic diseases, including cardiovascular disease (CVD), cancer, type 2 diabetes and obesity, with similar results found across diverse populations (He *et al.*, 1995; Chatenoud *et al.*, 1998; Jacobs *et al.*, 1998; Liu *et al.*, 1999b; Jacobs *et al.*, 2000; Mellen *et al.*, 2008a; O'Neil *et al.*, 2010; Ye *et al.*, 2012). CVD continues to be the leading cause of morbidity and death across the globe and including China, and accounts for approximately one third of all deaths around the world (WHO, 2003). Excess body weight, hypertension and dyslipidemia are clinically considered as the most potent established risk factors for CVD. In China, recently obvious changes to traditional diets, including a dramatic decrease in amounts of whole grains consumed from 104 g/d in 1982 to 24 g/d in 2002 may be responsible for the elevated CVD mortality (Ge, 2011).

Quinoa, which belongs to *Polygonaceae* family of plant species, is included in ''whole grain'' class even though it is not a member of the grass family, in terms of its similar nutrient composition to grass seeds (McKeown *et al.*, 2013). In addition to the high content of carbohydrates as an energy source, quinoa is a good source of high quality protein, with a well-balanced amino acids profile, lipids which are rich in unsaturated fats, dietary fibre, minerals as well as other important components such as vitamin C and phenolic compounds, which together promote quinoa to be a potential gluten-free alternative to common cereals (Ando *et al.*, 2002; Konishi *et al.*, 2004; Bhargava *et al.*, 2006; Alvarez-Jubete *et al.*, 2009; Tang *et al.*, 2015). With respect to the effect on markers of CVD risk, quinoa is much less studied either in human or animal studies compared with other whole grains like wheat, oat and barley. Moreover, up to date, there is no human intervention study that has reported the effects of increased quinoa consumption on the composition and population of the gut microbiota. Although there is some evidence that regular consumption of quinoa-based foods eaten daily for around one month promotes a significant reduction in the concentrations of circulating blood lipids in a few studies, features that may be useful in relation to the dietary management of metabolic risk; others report unclear results, which has led to some discussion about the acute impact of a quinoa-containing diet (Jenkins *et al.*, 2008; Farinazzi-Machado *et al.*, 2012; De Carvalho *et al.*, 2014; Zevallos *et al.*, 2014). In addition to inconsistent results from intervention studies, knowledge of what mechanisms which lie behind these observed effects also

remains weak, but bioactive components present in quinoa such as dietary fibre, protein, compounds which exhibit high antioxidant capacity, and other phytochemicals may be the potential contributors (Konishi *et al.*, 1999; Takao *et al.*, 2005; Paśko *et al.*, 2010; De Carvalho *et al.*, 2014).

Therefore, the aim of this thesis is to evaluate the health potential of quinoa in comparison with buckwheat in terms of their nutrient composition, in particular their polyphenolic content. Buckwheat was chosen as a comparator to quinoa as another gluten-free cereal which has been promoted recently as a healthy alternative to wheat in the diet.

The nutritional qualities of quinoa are further evaluated in a human intervention study to assess the impact of regular consumption of quinoa for 4 weeks on plasma biomarkers associated with CVD risk including plasma glucose, lipids and markers of systemic inflammation. The effects of quinoa consumption on the profile of the gut microbiota was further evaluated as a possible mechanism through which quinoa may exert its claimed beneficial effects.

Chapter 2 Literature Review

2.1 Whole grain

Whole grains are defined as intact, cracked, ground or flaked fruit of grains in which all three integral parts of the kernel, including bran, germ and endosperm; in contrast to refined grains, which retain only the endosperm after the refining process (Van der Kamp *et al.*, 2014) as shown in Figure 2.1.

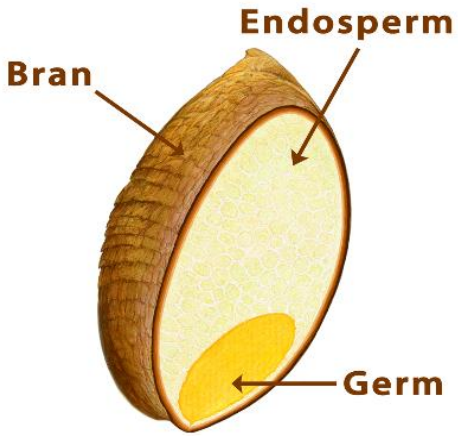


Figure 2.1. The three main parts of whole grains, the bran, the endosperm and the germ (https://wholegrainscouncil.org/).

Whole grain foods as an important part of the human diet are not a new invention, but instead date back to around 10,000 years ago with the advent of agriculture in history (Spiller, 2002). For the last 3000-4000 years whole grains have played a main role as a staple food in the human diet. It is only within the past 100 years, since the industrial revolution and the invention of the roller mill, that refined grain products as a strong competitor have largely replaced whole grain products in the habitual diets of a majority of the global population. Initially, gristmills, used for grinding grains, did not completely separate the bran and germ from the white endosperm to produce a semi-refined refined flour (Slavin, 2004). In 1897, the introduction of roller mill made the separation of the bran and germ from the endosperm more efficient than before. Since that time broad applications of the roller mill have met the rapidly increasing demand for refined grain products from consumers, especially in Western industrialised countries, thereby resulting in a dramatic decline in the consumption of whole grain products (Spiller, 2002). However, since the 1970s, intake of whole grains has increased slightly due to the 'fibre hypothesis' promoted by scientists, which proposed the potential health benefits of whole foods, including those derived from whole grains, fruits and vegetables (Trowell, 1972). Since this time whole grains have gained much more attention, including improvements in food possessing technologies, recognition of the nutrient benefits of whole grain

and human health benefits of whole grain consumption against chronic diseases, and recently with their strong recommended as a part of a healthful diet.

The cereal grains consumed by people are the seeds that come from the *Gramineae* family of grasses (Van der Kamp *et al.*, 2014). The most commonly consumed whole grains in the Western diet are wheat, rice and corn, as well as oats, barley, millet and sorghum in some countries. In other countries such as China, the most commonly consumed grains are rice, wheat, corn, sorghum, millet and buckwheat, with rice constituting around 65% of the total. Pseudocereals as non-grasses, including wild rice, buckwheat, amaranth and quinoa are not botanically true grass grains, but are also typically classified as whole grains due to their similar nutrient composition. On the other hand soya was declined 'whole grain' status on the basis that its nutrient profile (with a higher oil and protein content) was not similar to other cereals (FDA, 2006). The relative proportions of the three naturally-occurring structural parts (bran, germ and endosperm) in grains vary from one species to another, but endosperm in all grains accounts for the largest part followed by the bran and the germ is the smallest component. For example, corn has a relatively low endosperm content (approximately 75-80 g/100g) compared with wheat (83 g/100g) and rye (86 g/100g) (Wei *et al.*, 2004). It is well known that whole grains are a good source of a wide range of nutrient and phytonutrient compounds, which are identified in the three main parts of the grain.

Bran: the course, multi-layered outer skin of the edible kernel that includes considerable amounts of some nutrients, particularly:

1. Dietary fibre
2. Protein
3. Essential fatty acids (omega-3 fatty acids)
4. B vitamins (thiamine, riboflavin, niacin and folic acid)
5. Minerals (constitute 50-80% in grains, such as iron, copper, zinc, magnesium, selenium)
6. 'Antioxidant' compounds
7. Phytochemicals (natural chemical compounds in plants that have potential health benefits)

Endosperm: the largest portion of kernel (the middle layer) serves as the main energy store for the seed, including:

1. Carbohydrates (account for the largest part of grains)
2. Protein (found mainly in the intracellular matrix)
3. Small amounts of B vitamins and minerals

Germ: it is the true embryo of the seed that germinates to grow into a plant. As the smallest part of the grain, it also contains a rich nutritional profile including:

1. Protein
2. Healthy fat (omega-6 fatty acids)
3. B vitamins (thiamine and folate)
4. Minerals (phosphorus, magnesium)
5. Antioxidants (e.g. vitamin E and vitamin E precursors)
6. Phytochemicals (as above, potentially beneficial chemical compounds)

The bran and germ are removed from the starchy endosperm during the refining process depending on the extraction rate. This process reduces the concentration of many of nutrients in refined flours compared with wholemeal/whole grain flours because they are concentrated in the bran and germ and are 'lost' during the refining process, as illustrated in Table 2.1. A number of studies have suggested that some of these nutrients are associated with a lower risk of several chronic diseases (Slavin *et al.*, 2001).

Table 2.1. Nutrients (per 100 g) compared between wholemeal and white flours (Holland *et al.*, 1991)

	Wholemeal flour	White flour	Retained % after refining
Protein (g)	12.7	9.4	74%
Fat (g)	2.2	1.3	59%
Carbohydrates (g)	63.9	77.7	122%
Fibre (g)	8.6	3.6	42%
Sodium (mg)	3*	3*	100%
Potassium (mg)	340	150	44%
Magnesium (mg)	120	20	17%
Phosphorus (mg)	320	110	34%
Iron (mg)	3.9	1.5	38%
Zinc (mg)	2.9	0.6	21%
Manganese (mg)	3.1	0.6	19%
Selenium (mg)	53	4	8%
Thiamin (mg)	0.46	0.10	22%
Riboflavin (mg)	0.09	0.03	33%
Niacin (mg)	5.7	1.7	30%
Vitamin B-6 (mg)	0.50	0.15	30%
Folate (mg)	57	22	39%
Vitamin E (mg)	1.4	0.3	21%

*Before processing

2.2 General aspects on quinoa

Chenopodium quinoa Willd., commonly known as quinoa, is a native grain-like crop grown originally in the Andean region of South America including Peru, Bolivia, Ecuador, Colombia and Chile. Quinoa was domesticated some 3,000 to 4,000 years ago for human consumption and for livestock feed (Vega-Galvez *et al.*, 2010). Due to increasing popularity of the grain, it has been introduced in non-indigenous regions in recent years, such as Europe, North America, Australia, China and Japan. The quinoa plant has a broad genetic diversity that allows it to be highly resistant to cold, salt and drought conditions with ecotypes growing well at high altitudes and poor soils, but which are generally not suitable for the cultivation of common cereals, such as wheat, rice and corn (Zhang *et al.*, 2006b; Martínez *et al.*, 2009; Fuentes and Bhargava, 2011; Li and Yuan, 2012). It is also one of the few crops that grows on high salinity level soils in Southern Bolivia and Northern Chile (Jacobsen *et al.*, 2000). Quinoa is a hardy, drought-tolerant plant with a combined precipitation and irrigation requirement of 25-38 cm per year, which was obviously less than water requirements of other cereals like wheat and rice (Shrestha *et al.*, 2013). As long as the soil is naturally moist, plants should not be irrigated until the seedlings show two or three leaves. On the other hand, over-watering quinoa during the seedling stages can cause damping off and severe stunting off and severe stunting, and excessive irrigation after quinoa is established usually translates into tall, leggy plants with low yield. In the Andean region, quinoa is usually cultivated in rotation with potato or cereals, without the use of fertilizer or manures. In other countries, quinoa responds well to Nitrogen fertilizer, and yields increase with increasing use of fertilizer but to avoid over-fertilisation (Liu and Fan, 2011; Choukr-Allah *et al.*, 2016). When compared with common cereals like wheat and corn, it seems that quinoa cultivation probably requires less water, fertilizer and care, but which also largely depend on growing conditions (Liu and Fan, 2011; Choukr-Allah *et al.*, 2016). Despite the relatively lower quinoa seed yields ranging from 0.23 t/ha in Mauritania to 7.5 t/ha in Lebanon than common cereals, including wheat, rice and corn, quinoa production has intensified quickly in recent years due to the increasing prices on the international market (Jacobsen, 2011; Dost, 2015). The price of quinoa sold by farmers have almost tripled from 2004 to 2012, which is three times the price of soybean and five times the price of wheat (Stevens, 2017). The higher economic profits compared with common cereals drive the farmers to expand the existing plant scale (Liu and Fan, 2011; Choukr-Allah *et al.*, 2016). It has been suggested that high quinoa price will be sustained (including China) because the production of quinoa cannot meet the demand in a short-term period from increasing number of people who intend to include quinoa in their diets (Li *et al.*, 2016).

Quinoa is a member of the family *Amaranthaceae* rather than Gramineae, but it produces seeds that can be milled into flour and used as a cereal crop, thus it is referred to as a pseudo-cereal. The edible parts of the plant include leaves and seeds, the latter being the principle form for human consumption of this species and the form that has been the most economically and scientifically explored. Three main storage compartments can be distinguished within the mature quinoa seed (from centre to edge): a large central perisperm, a peripheral embryo and a one to two cell layered endosperm only in the micropylar region surrounding the hypocotyl-radicle axis of the embryo (Prego *et al*., 1998) (Figure 2.2). In cereals such as wheat, corn and maize, the main starch reserves for embryo development are stored in the endosperm tissue, but in quinoa, the living endosperm tissue is reduced to one or two layers. Instead, the starch is principally stored in the non-living perisperm that occupies around 40% of the volume of quinoa seed, although small amounts also exist in embryo, but not endosperm (Prego *et al.*, 1998; Ruales, 1998). However, mineral nutrients, lipid and protein reserves are mostly found in the embryo and endosperm. The quinoa seed is enveloped in a dry, very thin, two layered pericarp (seed outer coats).

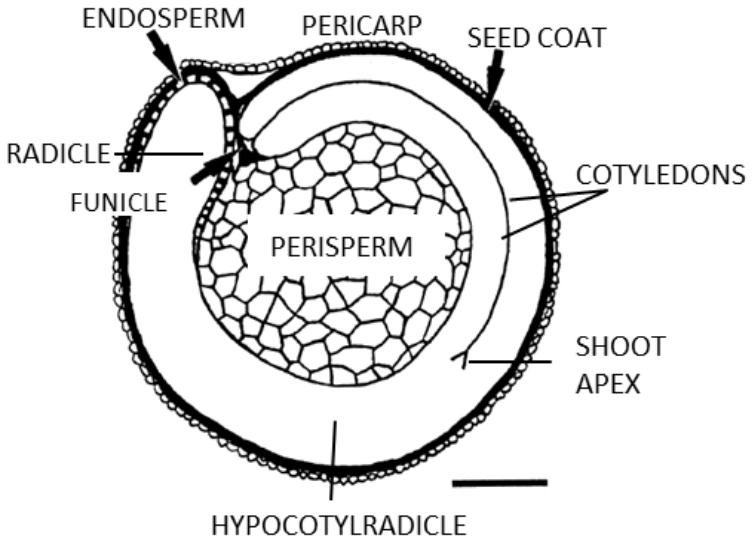


Figure 2.2. Median longitudinal section of quinoa seed (Prego *et al*., 1998)

The seeds are round and flattened, about 1.5-4.0 mm in diameter and 0.5 mm in thickness; around 350 seeds weigh 1 gram, and their color ranges from white to grey and black, potentially having tones of yellow, rose, red, purple and violet depending on their phytochemical content. Up to date, there are approximately 250 quinoa varieties identified, of which classification is based on the colour of the plant and seeds, or on plant morphology (Jancurova *et al.*, 2009; Vega-Galvez *et al.*, 2010). Quinoa seeds have been consumed by incorporation into salads or cooked and used in a similar way to rice, prepared in soup, puffed to make breakfast cereal, or milled into flour to produce various toasted and baked goods as a staple food, such as breads, noodles, cakes, cookies, biscuits, flakes,

pancakes and tortillas (Popenoe *et al.*, 1989; Bhargava *et al.*, 2006). Furthermore, quinoa seeds can be fermented to make beer, or a traditional ceremonial alcoholic beverage from South America called “chicha” (Healy, 2001; FAO, 2011).

Recently, quinoa has attracted much attention for its high nutritional profile, being rich in protein, lipids, dietary fibre, vitamins, minerals and phenolic compounds, with an extraordinary balance of essential amino acids (Ando *et al.*, 2002; Konishi *et al.*, 2004; Bhargava *et al.*, 2006; Alvarez-Jubete *et al.*, 2009; Tang *et al.*, 2015). Thus, the Food and Agricultural Organization of the United Nations (FAO) had officially announced that the year 2013 was "The International Year of the Quinoa" in order to raise the profile of the food and encourage its use. The Table 2.2 describing the nutritional composition of quinoa seed was listed below, together with buckwheat and wheat flour as a comparison (Wijngaard and Arendt, 2006; Hager *et al.*, 2012; Stikic *et al.*, 2012; Collar and Angioloni, 2014; Filho *et al.*, 2017). It should be mentioned that composition of essential amino acids in quinoa was similar to amino acid requirement pattern recommended by FAO/WHO/UNU (1985), which were higher than whole grain and refined wheat. Besides, quinoa also contains a great number of minor compounds. Of these, phenolic compounds have attracted much attention due to their properties as antioxidant and their antiallergic, antiviral, anti-inflammatory, cardiovascular protective, and anticarcinogenic activity (Kehrer and Smith, 1994; Scalbert *et al.*, 2005).

Table 2.2. Nutritional composition of quinoa, buckwheat and wheat flours (dry basis)

Nutrient	Quinoa	Buckwheat	Wholegrain wheat	Refined wheat
Energy (kcal/100g)	385	368	366	361
Ash (g/100g)	2.43	1.65	1.32	0.92
Protein (g/100g)	13.48	12.19	11.97	9.89
Total starch (g/100g)	48.88	61.35	56.84	68.08
Fat (g/100g)	8.59	4.21	3.63	1.81
Saturated fatty acids (g/100g)	0.99	0.90	0.69	0.70
Unsaturated fatty acids (g/100g)	7.34	2.53	2.93	1.09
Total dietary fibre (g/100g)	14.5	11.94	11.42	2.19
Soluble dietary fibre (g/100g)	5.37	6.12	1.60	0.91
Insoluble dietary fibre (g/100g)	9.13	5.81	9.82	1.28
Polyphenols (mg/100g)	78.24	465.47	82.20	13.04
Amino acid (g aa/100g protein)				
Histidine	3.2	2.7	2.3	2.0
Iso-Leucine	4.4	3.8	3.7	3.4
Leucine	6.6	6.4	6.8	6.5
Lysine	6.1	6.1	2.8	2.7
Methionine	2.3	2.5	1.4	1.5
Phenylalanine	4.7	4.8	4.7	4.1
Threonine	3.8	3.9	2.9	2.7
Tryptophan	1.1	2.4	1.5	1.2
Valine	4.5	5.1	4.5	4.0
Minerals (mg/kg)				
Calcium	497.3	148.2	307.7	1797.7
Magnesium	2299.0	1736	782.7	244.0
Sodium	37.0	10.8	19.9	38.1
Potassium	5537.7	4022.7	3997.7	1520.3
Iron	53.5	28.5	26.9	13.4
Copper	7.71	5.1	4.0	1.51
Manganese	13.5	11.8	23.4	8.25
Zinc	32.77	18.8	17.5	7.59
Chloride	433.8	144.0	998.0	825.6
Phosphorus	4415.7	2787.0	2040.7	908.7

Another important family of compounds present in quinoa seeds is the family of saponins. Saponins occur constitutively in a large number of plant species, in both wild plants and cultivated crops. Steroid saponins are common in plants used as herbs probably due to health-promoting properties, while in cultivated crops the triterpenoid saponins are generally predominant (Fenwick *et al.*, 1991). Saponins consist of a sugar moiety usually containing glucose, galactose, glucuronic acid, xylose, rhamnose or methylpentose, glycosidically linked to a hydrophobic aglycone which may be triterpenoid (Figure 2.3. (a)) or steroid (Figure 2.3. (b)) in nature. Steroid saponins have been identified in oats, tomato seed, capsicum peppers, aubergine, allium, yam, yucca and ginseng. Triterpenoid saponins are found in a great many legumes such as soya beans, beans, peas, and also

in tea, alliums, spinach, sugar beet, sunflower, quinoa and ginseng. These compounds are of interest because of their physiological functionalities, such as antioxidant, antitumor, cholesterol-lowering and antifungal activity (Estrada *et al.*, 1998; Woldemichael and Wink, 2001; Li *et al.*, 2002; Gulcin *et al.*, 2006). Triterpenoid saponins are synthesised via the isoprenoid pathway by cyclisation of 2,3-oxidosqualene to give primarily oleanane (β amyrin) or dammarane triterpenoid skeletons. Despite the increasing commercial interest in this group of natural products, the genetic machinery required for the elaboration of this family of plant secondary metabolites have largely not characterised due partly to complexity molecules and lack of commercial availably pathways.

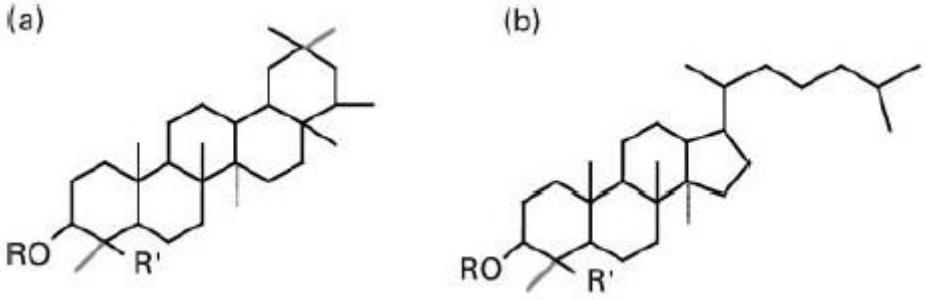


Figure 2.3. Basic structure of saponins: a triterpenoid (a) and a steroid (b) (Friess *et al.*, 1968)

These compounds, primarily located in the pericarp layer, give quinoa seeds an unpleasant bitter taste, making the removal of saponins via washing or mechanical abrasion before human consumption necessary (Johnson and Croissant, 1985; Prego *et al.*, 1998; Vega-Galvez *et al.*, 2010). Saponins from quinoa seeds are a complex of triterpene glycosides which differ in aglicon moiety, in the saccharide moieties and also in the substitution pattern of sugars of the sapogenins (Mizui *et al.*, 1988; Ma *et al.*, 1989; Mizui *et al.*, 1990) (Figure 2.4). Up to date, 10 to 16 saponins from quinoa seeds have been determined in numbers of studies (Woldemichael and Wink, 2001; Gomez-Caravaca *et al.*, 2011; Verza *et al.*, 2012; Yao *et al.*, 2014). Four main structures of sapogenins have been identified in quinoa: oleanolic acid, hederagenin, phytolaccagenic acid and 30-*O*-methyl-espergulagenate. The saponins content in seeds of different genotypes varied from 0.02% to 2.3% (dry matter), which are higher than those in oat and soybeans (Mastebroek *et al.*, 2000; Guclu-Ustundag and Mazza, 2007). Despite health-promoting properties of saponins from other plants, evidence of reporting the effects of saponins derived from quinoa seeds are not available to date. As the most unique compounds to quinoa relative to any other grains, saponins from quinoa deserve more efforts to explore their potential possible beneficial effects.

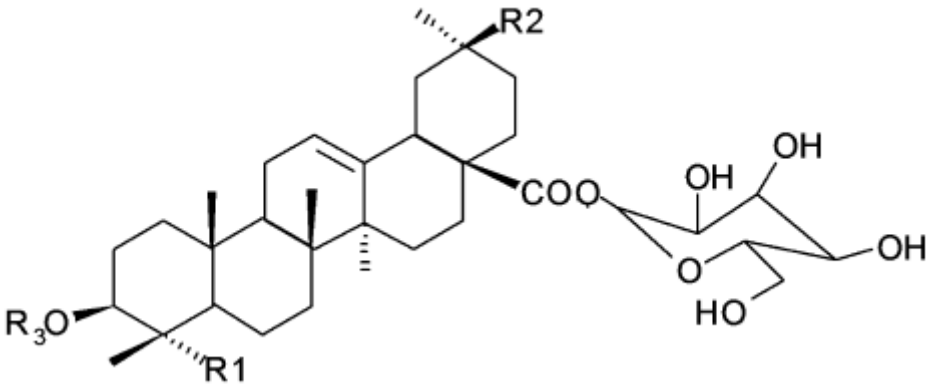


Figure 2.4. The structure of saponins from quinoa seeds (Verza *et al.*, 2012)

In addition to presenting a high nutritional quality, it is also characterized by being gluten-free, a characteristic that enables it to offer a greater variety of more nutritious and suitable food products for individuals with celiac disease. Celiac disease is an autoimmune disorder primarily affecting the small intestine that occurs in genetically predisposed individuals subsequent to the ingestion of gluten–containing grains in the diets, such as wheat, barley, and rye (Catassi and Fasano, 2008). The resultant inflammatory response in the intestine results in autoantibody production, villous atrophy and systemic effects. Classic symptoms include gastrointestinal problems such as weight loss, chronic diarrhoea, abdominal distention, malabsorption, and among children failure to grow normally. Coeliac disease was once thought to only happen in childhood, but it is now recognised to be a common lifelong disorders affecting approximately 1% of the population worldwide (Green and Cellier, 2007). The only treatment to date for celiac disease is the strict lifelong adherence to a gluten-free diet (Chand and Mihas, 2006; Rodrigo, 2006; Catassi and Fasano, 2008). Cereals such as quinoa and buckwheat have a key role to play in this regard.

2.3 Cardiovascular disease

Cardiovascular disease (CVD) is a class of diseases that affect the heart or blood vessels. It is usually associated with a build-up of fatty deposits inside the arteries known as atherosclerosis which are associated with an increased risk of blood clots. When fatty materials, such as cholesterol and triglycerides form deposits called plaques on and within the walls of blood vessels, this results in a narrowing of the artery, limiting the flow of oxygen-rich blood to the heart, brain and other parts of body, this is described as atherosclerosis (Figure 2.5). If the atheromatous plaque ruptures the arterial wall, platelets are enrolled to repair the damage, causing a clot to form. Over time, the walls of the blood vessels become hard and narrow, thereby losing their elasticity. CVD encompasses a variety of pathologies, including disease of the arteries supplying oxygen-rich blood to the heart muscle (angina, heart attacks or heart failure), brain (stroke or cerebrovascular disease) and other parts of the body, including legs, arms, and pelvis (peripheral artery disease).

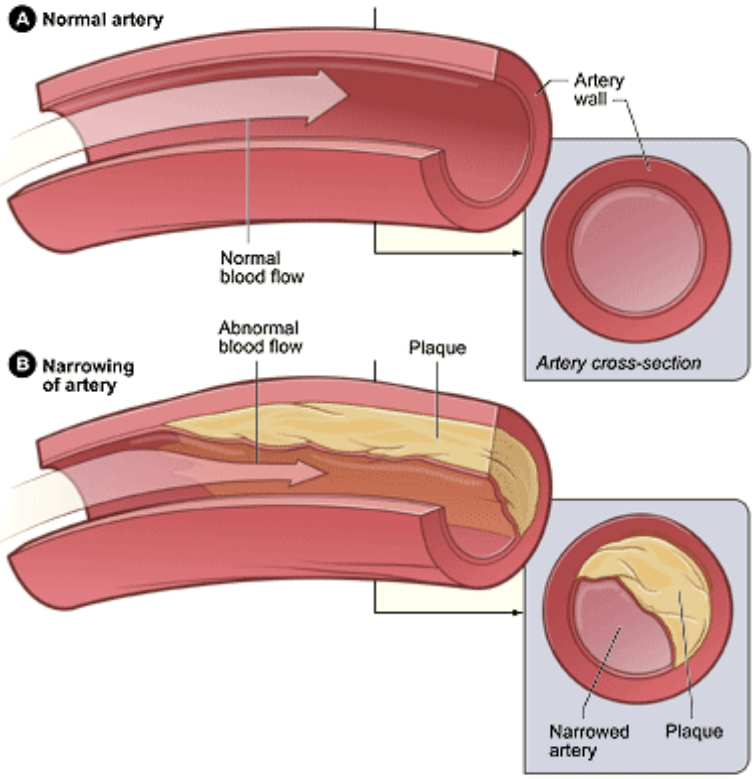


Figure 2.5. The formation of atherosclerosis (https://www.nhlbi.nih.gov/)

There are many risk factors for CVD, including smoking, lack of exercise, excessive alcohol intake, high blood pressure, high blood cholesterol, obesity, diabetes, poor diet. In spite of revolutionary

advancement in medicine over the past decades, CVD continues to be the leading cause of morbidity and mortality globally, together resulting in 17.9 million deaths (32.1%) in 2015 up from 12.3 million (25.8%) in 1990. Likewise, deaths and disability arising from CVD are continuing to increase in China to date, with approximately 230 million patients suffering from CVD. There are 3 million cases of deaths per year associated with CVD, which accounted for nearly 41% of total deaths in China. It has been suggested that CVD events are predicted to rise by 50% from 2010 to 2030 in China based on population aging and growth alone. It has been estimated that up to 90% of CVD may be preventable which involves improving risk factors with behaviour through: avoidance of smoking, increasing exercise, limiting alcohol consumption, losing weight, reduction in blood pressure and bloods lipids values and other, with the healthy diets playing a very important role (McGill *et al.*, 2008; McNeal *et al.*, 2010).

2.4 Markers of CVD risk affected by diets and whole grains

Much research shows that CVD is a life course disease that starts with the evolution of risk markers that in turn contribute to the development of subclinical atherosclerosis (Berenson *et al.*, 1998; Raitakari *et al.*, 2003). Therefore, A number of risk markers have been developed to predict CVD risk including classic hypertension, inflammation and plasma lipoproteins, which are based on confirmed clinical outcomes related to biomolecules, its structure and functions (Upadhyay, 2015). People with high blood pressure are more likely to develop CVD, because high blood pressure puts added force against the artery walls. Over time, this extra pressure can damage the arteries, making them more vulnerable to the narrowing and plaque build-up associated with atherosclerosis. If diagnosed with hypertension, DASH (Dietary Approaches to Stop Hypertension) diet were recommended to patients, including fruits, vegetables, fish, nuts, whole grains, low-fat dairy products and less salt. With respect to whole grains, the possible mechanisms underlying the anti-hypertension effect include increased insulin sensitivity and improved endothelial function (Fukagawa *et al.*, 1990; Katz *et al.*, 2001; Steffen *et al.*, 2003). In specific, consumption of wheat and oat may reduce the vascular reactivity impairment associated with meals high in fat (Katz *et al.*, 2001).

Atherosclerosis has now been clinically accepted as an inflammatory process (Libby, 2006; Golia *et al.*, 2014). Up to date, numerous markers of inflammation have been widely studied, such as C-reactive protein (CRP), plasminogen activator inhibitor-1 (PAI-1) and interleukin-6 (IL-6). Based on the association between markers and clinical cardiovascular risk, the epidemiological observation supports the theory that targeted anti-inflammatory treatment appears to be a promising strategy in reducing cardiovascular risk (Golia *et al.*, 2014). Recently, a few studies have reported that inflammatory protein concentrations such as CRP, IL-6 could be mediated by intake of whole grains, which in turn could beneficially affect CVD, although data on the relationships between intake of whole grains and inflammatory protein concentrations are limited and conflicting (Jensen *et al.*, 2006; Qi *et al.*, 2006; Lutsey *et al.*, 2007; Qi and Hu, 2007; Masters *et al.*, 2010). It has been speculated that the protective effect of whole grain consumption on inflammatory protein concentrations may be the results of lower postprandial glucose responses, weight maintenance and reduced visceral adiposity (Brownlee, 2001; Masters et al., 2010).

Since cholesterol and triglycerides are insoluble in water and therefore these lipids must be transported in association with lipoproteins in the blood stream. Plasma lipoproteins are divided into five main groups based on density/size, including chylomicrons (CM), very-low-density lipoproteins (VLDL), low-density lipoproteins (LDL), intermediate-density lipoprotein (IDL) and high-density lipoproteins (HDL). Of these lipoproteins, LDL and HDL are the most commonly used markers

to predict the CVD risk. LDL particles are often referred to as "bad" cholesterol because they take cholesterol to the arteries which contribute to the formation of plaque build-up in the arteries, known as atherosclerosis. While HDL particles are called "good" cholesterol as they help to remove excess cholesterol from the arteries to the liver for disposal, thereby preventing fatty build up and formation of plaque in the arteries. Hyperlipidema can often be improved by eating a healthy diet high in fruits, vegetables, whole grains, fish and nuts, but low in saturated fat, *trans* fat and sugar. Regarding the whole grains, several possible mechanisms have been proposed to explain the cholesterol-lowering activity, but the soluble is the most likely contributor to this property (Glore *et al.*, 1994; He *et al.*, 1995; Behall *et al.*, 2004; Takao *et al.*, 2005; Ye *et al.*, 2012; Thies *et al.*, 2014). It has been suggested that soluble fibre binds strongly to bile acids in the small intestine and elevates faecal bile acids excretion. The loss of bile acids in the stool stimulates the liver to increase cholesterol uptake from the circulation to replenish the bile acid supply. It also lowers the availability of bile acids for optimal fat digestion and absorption (Gordon *et al.*, 1977; Judd and Truswell, 1981; Story, 1985; Shinnick *et al.*, 1990). There is also emerging evidence that soluble fibre and resistant starch are additionally fermented by some bacteria in the colon, producing short-chain fatty acids (SCFA) perhaps via the inhibition of hepatic cholesterol synthesis in the liver, which helps to lower cholesterol concentrations (Slavin *et al.*, 1999; Escudero *et al.*, 2006). In addition, soluble fibre delays gastric emptying, slowing access of nutrients to digestive enzymes and to absorptive surfaces of the small intestine (Anderson and Siesel, 1990). One other mechanism that contributes to the cholesterol-lowering effects may be due to the protein which possess the bile acid binding activity (Tomotake *et al.*, 2000; Tomotake *et al.*, 2001; Takao *et al.*, 2005).

2.5 Gut Microbiota

2.5.1 General aspects of gut microbiota

The intestine is the largest reservoir of human flora, which consists of a complex microbial community residing in the gut called the microbiota. Estimates suggest that the intestine harbours at least 10^{14} microbial cells, which is 10 times greater than the total number of human cells in the body; thus it is often stated that there are more bacterial cells than human cells in a person's body. Most of the microbes, typically 10^{11}–10^{12} microbes/g, can be found in faeces and from the large intestine (Finegold *et al.*, 1983; Franks *et al.*, 1998; Harmsen *et al.*, 2002). Bacteria make up most of the microbes, and more than 1200 different species altogether reside there, with every individual presenting with their own specific composition of species. It is estimated that on average each individual harbours more than 160 species (Rajilic-Stojanovic *et al.*, 2007; Qin *et al.*, 2010). The most abundant bacteria are *Bacteroides*, which account for around 30% of all bacteria in the gut, followed by *Clostridium*, *Prevotella*, *Eubacterium*, *Ruminococcus*, *Fusobacterium*, *Peptococcus* and *Bifidobacterium*, but their abundance is highly variable across individuals. Escherichia and Lactobacillus are also present, but to a lesser extent. It is estimated that 99% of the bacteria come from about 30 or 40 species (Beaugerie and Petit, 2004). The gut microbiota are significantly affected by various factors, such as host genetics, lifestyle, medical interventions and health status, with diet being a very important factor (Burokas *et al.*, 2015; Lankelma *et al.*, 2015). Indeed, at least 50% of the variation of gut microbiota has been associated with dietary changes, and the microbiota changes and responds to short-term interventions during adulthood (Zhang *et al.*, 2010; David *et al.*, 2014). Under normal circumstances, the gut microbiota of an adult individual remains relatively stable until late age, when marked changes occur (Claesson *et al.*, 2012). The gut microbiota can be categorized as being either beneficial (e.g., *Bifidobacterium* spp. and *Lactobacillus* spp.) or harmful (e.g., *Clostridium* spp., *Shigella* spp., and *Veillonella* spp.) to host health based on its metabolic activities and fermentation end products.

2.5.2 Linking the gut microbiota to human health

The gut microbiota, which has attracted much attention, plays an important, but generally less well understood, role in health and disease in humans; indeed, it is sometimes referred to our ''forgotten organ'' (O'Hara and Shanahan, 2006). It has become increasingly recognised that the gut microbiota plays a critical role in human health through a variety of mechanisms, such as nutrient utilization and absorption by the host, activation of immune response, vitamin synthesis, inhibition of pathogens growth (Saulnier *et al.*, 2009). Indeed, it has been suggested that the gut microbiota is involved in

appetite control, energy balance, immune function, allergies, behavioral perturbations, obesity, diabetes, CVD and cancers like stomach cancer (Flint, 2012).

2.5.2.1 Gut microbiota and Clostridium difficile infection (CDI)

In the intestines, when the numbers of healthy bacteria decreased often due to use of antibiotics, harmful *Clostridium difficile* may proliferate, which caused diarrhea and pseudomembraneous colitis and is responsible for part of hospital-acquired infections (Song *et al.*, 2013). Limited treatment options, increases in failure of conventional treatment and high recurrences following initial cure present serious challenges to human health and economic concern. However, these patients may be helped by a fecal microbiota transplantation (FMT), also known as stool transplantation, which is a procedure in which stool from a healthy donor is placed into another patient's intestine. FMT is very effective therapy for recurrent or refractory DCI, with reported effective rate ranging from 60% to 90% after a single treatment (Kassam *et al.*, 2013; van Nood *et al.*, 2013; Cammarota *et al.*, 2014; Youngster *et al.*, 2014; Kelly *et al.*, 2016). Moreover, FMT has shown therapeutic potential in various conditions, including irritable bowel syndrome, inflammatory bowel diseases, autoimmune diseases, allergic disorders (Russell and Finlay, 2012; Luckey *et al.*, 2013; Ianiro *et al.*, 2014; Pinn *et al.*, 2014). Therefore, FMT has already been recommended as an alternative therapy (Surawicz *et al.*, 2013). FMT can be delivered by diverse modalities, including oral capsules and colonoscopy. But it should be noted that the side effects of FMT may happen, including abdominal discomfort, cramping, bloating, diarrhea or constipation (Lee *et al.*, 2016a).

2.5.2.2 Gut microbiota and obesity

The worldwide epidemic of obesity and related metabolic diseases are rapidly spreading and has become a serious problem not only for individual health but also for family and society in general (Popkin *et al.*, 2006). As diet consumed is responsible for most of incidence of obesity, recent increasing efforts have focused on whether the gut microbiota has an important role in the development of obesity. For example, Backhed *et al*. (2007) reported that germ-free mice fed on a high-fat, high-sugar 'Western' diet were resistant to obesity, indicating that gut microbiota has played a central role in the development of obesity in mice. In addition, antibiotics, which can non-preferentially deplete all intestinal bacteria, suppressed the development of obesity induced by a high-fat diet in mice, suggesting that essential role of gut microbiota in this process (Cani *et al.*, 2008; Vijay-Kumar *et al.*, 2010). Moreover, it has been confirmed in humans that obese subjects who received a microbiota from lean donors showed significantly improved insulin sensitivity in the serum during a period of 6 weeks (Vrieze *et al.*, 2012). At the phylum level, several studies showed that obese mice and obese humans have a significantly higher ratio of the number of Firmicutes to

that of Bacteroidetes than their lean counterparts (Ley *et al.*, 2005; Turnbaugh *et al.*, 2006b; Peter *et al.*, 2008; Zhang *et al.*, 2009b). Based on these published studies, Zhao (2013) clearly promoted the correlation between gut microbiota and obesity to causality in both rodent models and humans in the review. Furthermore, numbers of studies have demonstrated that changes in plasma and faecal short-chain fatty acid (SCFA) concentrations can be closely linked with overfeeding and obesity, but the mechanisms underlying the associations still unclear (Fernandes *et al.*, 2014; Rahat-Rozenbloom *et al.*, 2014; Murugesan *et al.*, 2015). To answer this question, Perry *et al*. (2016) revealed that increased production of acetate due to a nutrient-gut microbiota interaction in rodents fed on a high-fat diet results in activation of the parasympathetic nervous system, which, in turn, promoted elevated glucose-stimulated insulin secretion, elevated ghrelin secretion, hyperphagia, obesity and related sequelae.

2.5.2.3 Gut microbiota and type 2 diabetes

A human metagenome-wide association study have demonstrated significant correlations of certain bacterial genes, specific gut microbes and metabolic pathways in T2D patients (Qin *et al.*, 2012). After analysis of stool samples from 344 Chinese subjects, the results showed that a moderate dysbiosis was characterized by a reduction in the abundance of butyrate-producing bacteria like *Roseburia intestinalis*. In contrast, another study conducted on postmenopausal female patients with normal, impaired or diabetic glucose regulation in Europe showed different outcomes, probably owning to different sequencing techniques, ethic and dietetic influences (Karlsson *et al.*, 2013). But, the above two studies showed that both Chinese and European diabetics have higher levels of *Lactobacillus gasseri*, *Streptococcus mutans* and *Clostridiales*, but lower concentrations of *R. intestinalis* and *Faecalibacterium prausnittzii*. In addition, compared with non-diabetics, patients with T2D have higher levels of *Lactobacillus* species as reported in a smaller study (Larsen *et al.*, 2010).

2.5.2.4 Gut microbiota and CVD

Mounting evidence in animal models and humans have showed that gut microbiota were associated with CVD (Koren *et al*., 2011). A recent paper analysed microbiota from oral, gut and atherosclerosis plaque in 15 patients with atherosclerosis, suggesting that gut microbiota may correlate with disease markers of atherosclerosis. Furthermore, increased plasma concentration was found to correlate with changes in several bacterial taxa from the gut (Koren *et al*., 2011). Previous studies have shown that gut microbiota metabolizes dietary choline, L-carnitine and phosphatidycholine into trimethylamine (TMA), which is further oxidised into pro-atherogenic molecule trimethylamine N-oxide (TMAO) in the liver(Wang *et al.*, 2011; Koeth *et al.*, 2013; Tang *et al.*, 2013). Interestingly,

dietary supplementation of choline or TMAO blunted reverse cholesterol transport and enhanced the foam cells formation that precede atherosclerosis in mice (Robert *et al.*, 2013). Besides, increased plasma TMAO concentrations were attributable to higher risk of major adverse cardiovascular events in humans (Tang *et al.*, 2013). Thus, prevention of gut microbiota-dependent TMAO seemed to a promising strategy for the treatment of atherosclerosis (Wang *et al.*, 2015).

2.6 Quinoa and markers of cardiovascular risk: a systematic literature review

2.6.1 Introduction

Across the world, cereal-based foods form an integral part of the human diet, currently accounting for 30-70 % of their daily dietary energy (FAO, 2014). The grains comprise the seeds of *Gramineae* family of grasses, such as rice, wheat and rye as well as pseudo-cereals like quinoa, amaranth and buckwheat. These grains all require processing before consumption which involves various degrees of refinement or extraction of bran, germ and endosperm (Van der Kamp *et al.*, 2014). The association between increased consumption of whole grains or whole-grain foods in the diet and a decreased risk of developing diabetes, CVD, weight gain, obesity and some types of cancer has been consistently reported in observational studies (He *et al.*, 1995; Chatenoud *et al.*, 1998; Jacobs *et al.*, 1998; Liu *et al.*, 1999b; Jacobs *et al.*, 2000; Mellen *et al.*, 2008a; O'Neil *et al.*, 2010; Ye *et al.*, 2012; Aune *et al.*, 2016; Chen *et al.*, 2016). CVD is currently a leading cause of death, with an estimated one third of all deaths globally linked to CVD (WHO, 2003). Apart from traditional drug therapies, food can play an important role in disease prevention and treatments by affordable integrative strategies (Bigliardi and Galati, 2013).

Quinoa is an example of a pseudo-cereal which is included in the ‘’whole grain’’ class in terms of nutritional value (McKeown *et al.*, 2013). It is native to Andean region of South America, and was domesticated thousands of years ago for human consumption and for livestock feed (Vega-Galvez *et al.*, 2010). Over the last 20 years, quinoa has become the subject of worldwide attention with respect to its superior nutritional profile and suitability for people suffering from food allergies particularly gluten intolerance and/or have desire to eat healthy diets, as well as its great adaptability to different growing conditions. In addition to offering the starch content as an important energy source, quinoa is rich in good quality protein, with a well-balanced amino acids profile, lipids which are rich in unsaturated fats, dietary fibre, minerals as well as other important components such as vitamin C and phenolic compounds (Ando *et al.*, 2002; Konishi *et al.*, 2004; Bhargava *et al.*, 2006; Alvarez-Jubete *et al.*, 2009; Tang *et al.*, 2015). Thus, the Food and Agricultural Organization of the United Nations (FAO) had officially announced that the year 2013 was "The International Year of the Quinoa".

Some studies have begun to concentrate on the relationship between the intake of quinoa and markers of cardiovascular disease risk, either in human or animal intervention studies, but the number is limited. What is more, results from these studies conducted in free-living individuals are

conflicting in some cases. Thus, a clear and direct correlation between consumption and health benefit of quinoa is difficult to establish. Systematic reviews can provide a wider perspective, but also an evaluation of the validity of the methods of the study and results that can point the direction for future research. The primary aim of this review is to present a comprehensive review and summary of the up-to-date evidence from animal and human intervention studies for exploring the benefits of quinoa consumption in reducing CVD risk.

2.6.2 Method

2.6.2.1 Data sources and literature search

A comprehensive literature search for animal or human studies that had investigated the relationship between quinoa consumption and risk of CVD between 1960 and 2016 was conducted. Figure. 2.3 shows how relevant articles were selected. PubMed, Ovid, Scopus, Web of Science, Compendex, JSTOR, EBSCO, Medline and ProQuest databases were searched using the search terms 'quinoa' AND 'cardiovascular' OR 'cholesterol' AND 'animal' OR 'human', and the same terms were applied in each database during the search phase. CVD was defined to encompass coronary heart disease, stroke, peripheral arterial disease and aortic disease. In addition, the reference lists of retrieved articles were searched manually for all additional potentially relevant articles. The search was limited to studies on animals and humans and included those that were written in different languages such as English and Japanese.

2.6.2.2 Study selection

The studies were included in this review satisfied the following criteria: *1)* study in animals or humans, *2)* quinoa-consumption exposure, *3)* the outcomes included CVD risk markers, such as glucose, insulin, lipid profile. Since cholesterol was the most commonly indicator of CVD response to whole-grain foods, cholesterol was used as a primary outcome marker in this review. The eligibility criteria were set before the start of the research.

2.6.2.3 Data extraction and quality assessment

The following data were extracted from each animal study: lead author, year of publication, animal species, animal age at start, sample size, trial length, control diet, experimental diet and outcomes. The following data were extracted from each human study: lead author, year of publication, trial length, characteristics of subjects, number of subjects, age range, control diet, experimental diet, study design and findings. Missing data are reported as ''Not stated'' if they were not explained in the corresponding articles. The sample size reported in this review was the overall total for the experiment rather than restricting to either control or intervention diet/s.

Data were extracted by a single reviewer.

The included studies were assessed by the 6-item questions of Review Manager Version 5.0 provided by Cochrane Collaboration (Higgins and Green, 2008). Moreover, the scoring criteria was defined by suggestions (yes=1, unclear=0, and no=-1), which would be specified subsequently in the

following Table 2.3. In addition to animal and human data extracted, the quality scores were also added.

Table 2.3. Quality assessment criteria for the included studies in this review

1	Was the allocation sequence adequately generated?
2	Was allocation adequately concealed?
3	Was knowledge of the allocated intervention(s) adequately prevented during the study
4	Were incomplete outcome data adequately addressed?
5	Are reports of the study free of any suggestion of selective outcome reporting?
6	Was the study apparently free of other problems that could place it at risk of bias?

2.6.3 Results

2.6.3.1 Characteristics of studies

The systematic search of the scientific databases resulted in the initial identification of 228 articles for further evaluation. After removing duplicate articles (72) and articles that did not meet the eligibility criteria (144), a total of 12 articles including 8 animal studies and 4 human studies were included in the review. Manual searching of the reference list of the relevant articles yielded 5 additional articles. After applying the inclusion criteria, two of these articles were considered fit to include. Consequently, the combination of electronic and manual reaching resulted in 14 articles which are included in this final review (Figure 2.6). Four animal studies were carried out in Japan, two in France and one each in France, Poland, Brazil and India. With respect to human studies, two were carried out in Brazil and one each in the UK and in Canada.

As shown in Table 2.4 and 2.5, all studies in the review were prospective studies, with follow-up durations ranging from 15 days to 6 weeks in animal studies and from 4 weeks to 6 months in human studies. Overall, quinoa consumption in human studies ranged from 19.5 g to 50 g of quinoa ingredients (median levels of individual series), with two studies the amounts consumed unstated. The methods of these studies were similar, in that a baseline period was normally followed by animals or subjects being offered to consume quinoa/quinoa products (e.g. quinoa meal, quinoa bar or flakes), or placebo diets. Blood samples were obtained at baseline and after the intervention period for comparison of CVD biomarkers. Liver or faeces were only available from animal studies.

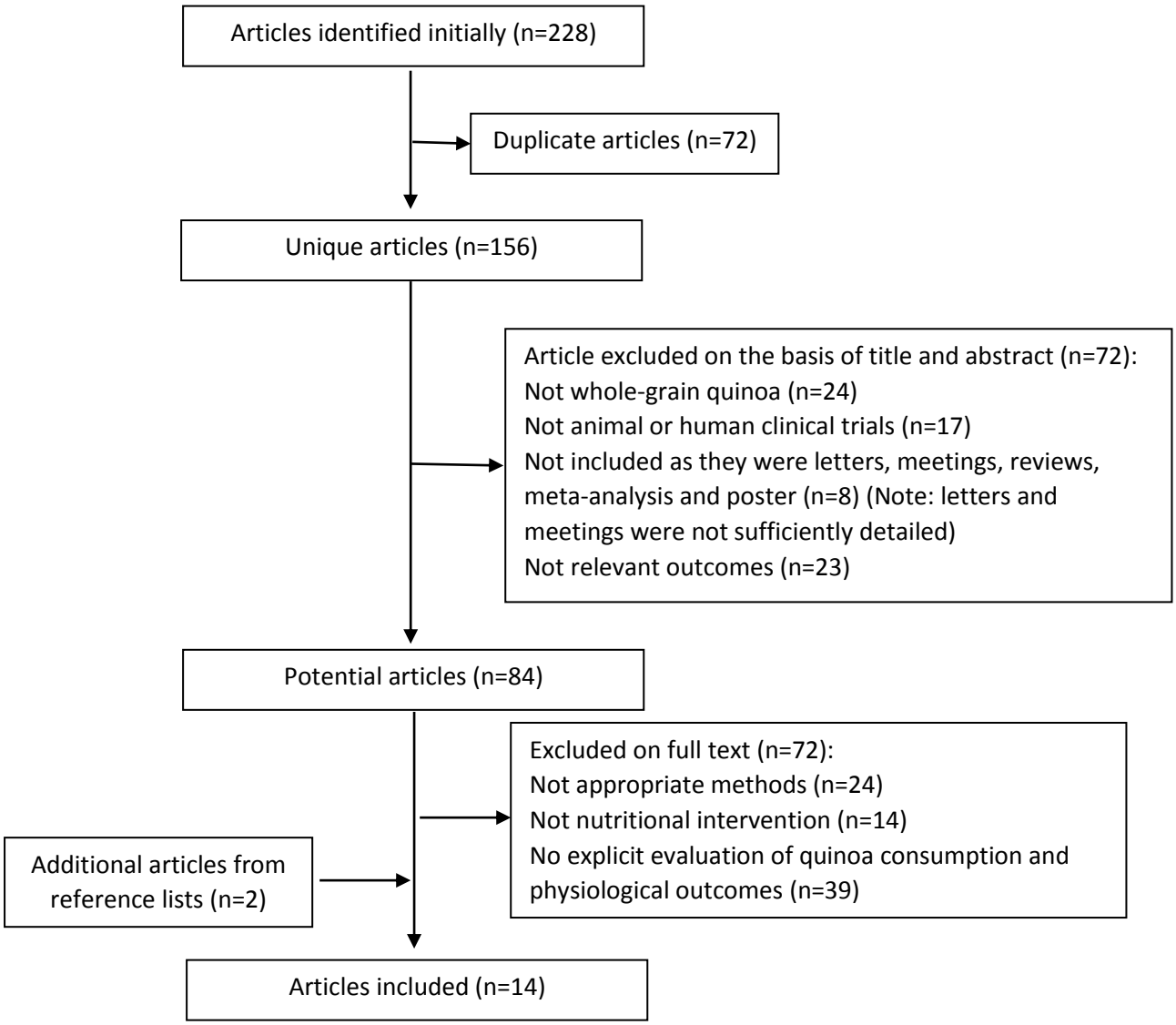


Figure 2.6. Flow diagram of article selection

Table 2.4. Summary of all animal studies reviewed

Reference	Animal species	Animal age at start	Sample size	Trial length	Control diet	Experimental diet	Outcomes	Quality score
Konishi *et al*. 1999	Male Crj: CD-1 (ICR) Mice	7 weeks	23	5 weeks	20% casein, 1% cholesterol	2 groups, control diet with 1.5 % and 3% quinoa pericarp Fraction (QPF), respectively	Food intake significantly higher in 1.5% QPF group, reductions seen in serum and liver Total-Chol concentrations ($p<0.05$) in both QPF group. Non-significant change in body weight gain, plasma HDL-Chol and plasma TG concentrations ($p>0.05$).	3
Ogawa *et al.* 2001	Male spontaneously hypertensive rat	11 weeks	12	6 weeks	High lipid casein diet	High lipid casein diet that included quinoa (20% quinoa powder in diet)	Body weight, blood pressure and liver weight in experimental group lower than control group ($p<0.05$). No significant differences observed in serum Total-Chol, serum TG, serum phospholipids, liver Total-Chol and liver TG as well as enzyme activities related to cholesterol metabolism.	3
Takao *et al*. 2005	Male ICR mice	7 weeks	18	4 weeks	20 % casein, 0.5% cholesterol	2 groups, control diet with casein substituted for 2.5%, 5% quinoa protein (QP) fraction, respectively	The QP supplementation significantly prevented the rise in the plasma and liver Total-Chol level ($p<0.05$), liver weight significantly lower, and the faecal weight and excretion of bile acids increased significantly ($p<0.05$) in 5% QP group. Body weight gain, food intake, plasma HDL-Chol, plasma HDL/Total-Chol, plasma phospholipids, plasma TG, liver TG not significantly affected by both treatment ($p>0.05$).	5
Matsuo, 2005	Male Wistar-ST strain rat	4 weeks	10	17 days	Corn starch and casein meal	Corn starch and casein meal with quinoa (50% quinoa in the diet)	Serum Total-Chol significantly lower and excretion of bile acid in faeces significantly higher in quinoa group. Body weight gain and food intake not significantly affected in quinoa group ($p>0.05$).	2

Paśko *et al*. 2010	Male Wistar rat	Not Stated	24	5 weeks	2 groups, corn or corn starch with 31% fructose	2 groups, corn starch was substituted with 310g/kg of quinoa seed with or without 31% fructose	Glucose, Total-Chol, LDL-Chol and TG in serum were significantly lower ($p<0.05$, $p<0.008$, $p<0.05$ and $p<0.01$, respectively) in quinoa group. Quinoa seeds prevented the decrease of HDL-Chol in fructose diet.	4
Menegue tti *et al*. 2011	Wistar rat	60 days	64	30 days	Rodent chow (Nuvilab®)	(Nuvilab®) with hydrolysed quinoa	Body weight gain, food intake, fat deposition and serum TG significantly lower in quinoa group ($p<0.05$). Serum glucose, serum Total-Chol level not significantly affected by treatment ($p>0.05$).	4
Foucault *et al*. 2012	Male C57BL/6 J mice	6 weeks	48	3 weeks	High fat (HF) diet	High fat diet with added quinoa extract (HFQ) (quinoa powder 6mg/day/kg body weight)	Body weight gain, food intake, plasma glucose, plasma insulin, plasma Total-Chol, plasma TG and liver weight not significantly affected by treatment ($p>0.05$).	5
Foucault *et al*. 2014	Male C57BL/6 J mice	6 weeks	18	3 weeks	High fat (HF) diet	High fat diet with added quinoa (HFQ) diet (quinoa powder 6mg/day/kg body weigth)	Over a 24 h period, faecal lipid content was higher in HFQ group vs control group ($p<0.05$). No significant changes in food intake plasma glucose, plasma insulin, plasma TG and faecal weight between HFQ group and control ($p>0.05$).	3
Mithila & Khanum, 2015	Male Wistar rat	Not stated	24	15 days	Corn starch and 20% casein	Corn starch and 20% of quinoa replacing casein in the diet	Food intake, plasma Total-Chol, plasma HDL-Chol and plasma LDL-Chol declined in the quinoa group compared with control group ($p<0.01$) No significant difference detected in body weight gain, blood glucose and plasma TG levels ($p>0.05$).	5
Maha, 2016	Male Wistar albino rat	Not stated	30	4 weeks	Fat and basal diet	Fat and basal diet substituted with quinoa mill (30, 40, 50 and 60% in the diet)	Body weight gain, food intake, Total-chol, LDL-Chol, HDL-Chol, TG and total lipids in serum reduced in the fat and basal diet with 60% quinoa group, and Aspartate transaminase (AST) and alanine transaminase (ALT) also reduced ($p<0.05$).	4

Table 2.5. Summary of all human studies reviewed

Reference	Length	Characteristics of subjects	N of Subjects	Age	Control diet	Experimental diet	Study design	Findings	Quality score
Farinazzi-Machado *et al*. (2012)	30 days	Healthy student (9M and 13F)	22	18-45	No control diet	Two quinoa bars (9.75g quinoa/bar).	Risk factors related to CVD measured before and after the treatment.	Total-chol, LDL-chol, TG and hepatic enzyme AST reduced when subjects were following the quinoa bar ($p<0.05$). Body weight, glycemic index, blood glucose, blood pressure, HDL-Chol and hepatic enzyme ALT not significantly affected by treatment ($p>0.05$).	1
De Carvalho *et al*. (2014)	4 weeks	Postmenopausal women with weight excess	35	Not stated	25 g, corn flakes (CF)	25 g, quinoa flakes (QF)	A prospective and double-blind study, CVD risk factors measured at the beginning and end of the experiment	Significant reduction in Total-chol, LDL-chol and TG detected in QF groups. Body weight, BMI, waist circumstance, glucose, HDL-Chol unaffected by treatment ($p>0.05$).	3
Zevallos *et al*. 2013	6 weeks	Celiac patients (2M and 17F)	19	Median 19	No control diet	50 g quinoa as part of their gluten-free diet	Detailed histological assessment carried out before and after eating quinoa.	Reduction seen in Total-chol and TG, but only HDL-cholesterol level decreased significantly ($p<0.05$).	1
Jenkins *et al*. 2008	6 months	Patients with type 2 diabetes	210	Not stated	Two slices of Whole wheat bread included in the high-cereal fibre diet	Two slices of quinoa bread included in the low–glycemic index diet	A randomized and parallel study design, glycemic control and cardiovascular risk factor in patients were measured.	HDL-chol increased by 1.7mg/dL significantly ($p<0.05$), fasting glucose and HbA1c decreased significantly ($p=0.02$ and $p<0.001$, respectively) compared with the control. Body weight, blood pressure, Total-Chol, LDL-Chol, Total/ HDL-Chol, TG and C-creative protein not significantly affected by treatment ($p>0.05$).	2

2.6.3.2 Animal Studies

2.6.3.2.1 Effects on Weight Gain and Food Intake

Table 2.6 shows the number of articles with significant increase, no significant effect and significant reduction in CVD risk markers in the 10 animal studies and 4 human studies, respectively. A significant decrease in body weight gain compared with control was detected in three out of eight animal studies. Food intake decreased significantly in one of eight studies, whereas the level increased in three studies ($p< 0.05$), the rest observed no changes with quinoa treatment.

Table 2.6. The number of animal and human intervention studies showing significant increase, no effect and significant reduction on markers of CVD risk

	Number of Studies		
	Significantly higher in quinoa	The effect was not significant	Significantly lower in quinoa
Animal Studies			
Body weight gain	—	5	3
Food intake	1	4	3
Blood glucose	—	3	1
Blood insulin	—	2	—
Total-Chol	—	3	6
LDL-Chol	—	—	3
HDL-Chol	2	2	1
Triglycerides	—	6	3
Liver weight	—	1	3
Liver Total-Chol	2	1	—
Liver triglycerides	—	2	—
Faecal weight	1	1	—
Faecal bile acid	2	—	—
Faecal lipids	1	—	—
Human Studies			
Body weight gain	—	3	—
Total-Chol	—	2	2
LDL-Chol	—	2	2
HDL-Chol	1	—	1
Triglycerides	—	2	2

2.6.3.2.2 Effects on Glucose and Insulin

Four animal studies found that consumption of quinoa-based foods decreased the blood glucose concentrations by 3.4-25.2%, while a significant reduction was only observed in the study conducted by Paśko *et al*. (2010). Insulin concentration in plasma in two studies was higher (5.3% and 18.4%) than in the control group but no significant increase was observed.

2.6.3.2.3 Effects on Lipid Profile

As can be seen in Table 2.6, nine and three animal studies investigated the impact of quinoa consumption on total cholesterol and LDL-cholesterol, and six (66%) and three (100%) of these observed a significant reduction, respectively; the rest of the studies identified no significant response. The largest decreases total cholesterol and LDL-cholesterol were 56.0% and 81.0% in rats fed diets with 60% milled quinoa, respectively (Maha, 2016). HDL-cholesterol concentrations changed significantly in response to quinoa consumption in two out of five animal studies (Table 2.6). The changes in HDL-cholesterol concentrations reported was wide, ranging from an increase of 55.8% relative to the comparison group in one study to a decrease of approximately 31% in another study (Mithila and Khanum, 2015; Maha, 2016). The significant reduction in HDL concentrations induced by fructose was effectively prevented by the addition of quinoa seeds into the diets (Paśko *et al.*, 2010). The other studies found no significant effect on HDL-cholesterol concentration. Three of the nine animal studies identified that quinoa intake significantly reduced plasma triglycerides concentrations by 11.0-54.3% although the majority of the animal studies observed no significant effect of quinoa consumption on triglycerides concentrations.

2.6.3.2.4 Other Outcomes

The liver weight of mice or rats fed with quinoa food decreased significantly by between 8 and 43.3% relative to the comparison group in three out of four studies where these data were reported. Two of the three studies which reported liver cholesterol content showed a significant ($P<0.05$) reduction in this measure, but no significant changes in liver triglycerides were detected. There were significant increases in faecal bile acids by 61.0% and 62.7% in two studies performed by Takao *et al*. (2005) and Matsuo, (2005), respectively, and an increase in faecal lipids in the study of Foucault *et al*. (2014). ($p<0.05$).

2.6.3.3 Human studies

2.6.3.3.1 Effects on Weight Gain

For the human studies, there were no significant changes in body weight response to intake of quinoa-based foods reported.

2.6.3.3.2 Effects on Glucose, Hemoglobin A1c and Insulin

One relatively large study with one hundred and twenty one participants found that consumption of 2 slices of quinoa bread per day significantly decreased fasting blood glucose and haemoglobin A1c (HbA1c) concentrations by 8.0% and 7.0%, respectively, compared with the control group, while another study reported a small reduction in response to quinoa flakes although this was not statistically significant (Jenkins *et al.*, 2008; De Carvalho *et al.*, 2014).

2.6.3.3.3 Effects on inflammatory marker

It has been suggested that inflammation is an important contributor to the development of atherosclerosis. As a frequently used markers of inflammation, fasting plasma C-creative protein failed to show any response to the consumption of 2 slices of quinoa bread (Jenkins *et al.*, 2008).

2.6.3.3.4 Effects on Lipid Profile

All of the four human studies reported that regular consumption of quinoa reduced plasma concentrations of total cholesterol and LDL-cholesterol, although this was only significant in two of the studies. In both of these studies total cholesterol concentrations were within the normal range for healthy subjects before quinoa consumption. This significant reduction ranged from 5.1% to 9.9% for total cholesterol and from 5.9% to 20.5% for LDL-cholesterol. Of the two human studies reporting effects of quinoa intake on HDL-cholesterol, one reported a significant increase of 4.1% (Jenkins *et al.*, 2008), and the other reported a significant decrease of 7.7% (Zevallos *et al.*, 2014). None of the studies reported here reported any significant effect of quinoa consumption on the ratio of HDL-cholesterol: total cholesterol. Two human studies of four found a statically significant reduction in triglycerides concentrations (12.3% and 3.9%) following quinoa-based intervention, with the other two human studies showing no significant effect of quinoa consumption on triglycerides concentrations.

2.6.4 Discussion

2.6.4.1 Effects on body weight gain

A number of animal feeding experiments about mainly focusing on the impact of quinoa consumption on weight gain have not been included in this review due to the inclusion criteria, since these studies did not target on blood lipids which are clinically regarded as key indicators of CVD risk. Weight gain is linked to an elevated risk of high blood pressure and hyperlipidaemia (Thies *et al.*, 2014). In order to evaluate the impact of quinoa intake on body weight, the energy and macronutrient content of diets offered should be considered along with the amount of food fed, which is beyond the scope of this review. However, on the basis of the published literature, it seemed overall that there is some evidence of a beneficial inverse association between quinoa consumption and weight gain, for studies in mice, rats and chickens with a range of dietary levels of quinoa (Konishi *et al.*, 1999; Improta and Kellems, 2001; Ogawa *et al.*, 2001; Meneguetti *et al.*, 2011).

The presence of saponins in quinoa seeds might be the cause of weight loss, but the effect was not seen in piglets probably due to the low dose in the test diets (Carlson *et al*., 2012; Foucault *et al*., 2014). One study conducted by Carlson *et al*. (2012) using the Ussing chamber technique demonstrated that saponins derived from quinoa increased the conductance of pig jejunum. This finding is in line with previous studies suggesting that there was an increased mucosal permeability in the intestine, which is believed to inhibit the absorption of other nutrients in the gut for animal growth (Gee *et al*., 1993; Önning *et al*., 1996). A further explanation may be due to the bitter taste of saponins present in the quinoa seed coats, which adversely affects the palatability of food products containing the seed, leading to decreased food intake (Johnson and Croissant, 1985). A reduction in weight gain was not detected in human intervention studies listed in the Table 2.5, perhaps because the saponins are normally removed before human consumption, either by abrasive dehulling or vigorous washing in water (Jacobsen *et al*., 1997).

20-Hydroxyecdysone (20E) is a naturally occurring ecdysteroid hormone which controls moulting and production of arthropods. Thus, it is one of the most common moulting hormones in insects and crabs. Although 20E is not a mammalian hormone, but it may also display some pharmacological effects on mammals. Interestingly, quinoa is one of the rare food plants that contains 20E (plant steroid), which play a role in the control of glucose homeostasis and also in the prevention of diet-induced obesity in mice (Chen *et al*., 2006; Kizelsztein *et al*., 2009; Foucault *et al*., 2012; Foucault *et al*., 2014). For example, the effects of either quinoa or an equivalent dose of 20E were investigated in mice to prevent the early onset of diet-induced obesity (Foucault *et al*., 2012). Even though the decrease in weight gain was not found in mice when exposed to a high- fat diet with added quinoa

or 20E, there was a slight reduction in adipose tissue mass and the expression of lipid storage genes. The authors suggested that the decrease was probably due to the presence of 20E, which possesses a similar structure to Vitamin D influencing lipid accumulation in adipose tissue. It was suggested that Vitamin D receptors have suitable binding sites for 20E, which enabled it to affect the expression of genes related to lipid storage. Further studies are necessary to explore this proposed mechanism.

2.6.4.2 Effects on glucose and insulin

Hyperglycemia and insulin resistance are associated with risk of developing CVD (Coutinho *et al.*, 1999b; Uwaifo and Ratner, 2003). There is considerable evidence showing that whole grain intake is associated with lower glucose concentrations and is inversely associated with insulin resistance (Hallfrisch and Behall, 2000; Liese *et al.*, 2003; Steffen *et al.*, 2003). In accordance with this, one study using male Wistar rats by Paśko *et al*. (2010) demonstrated that quinoa seed effectively brought down the glucose concentration by 10% ($p<0.01$) compared with the control group, and a similar response was detected in obese mice fed with an extract from quinoa seeds (Graf *et al.*, 2014). Moreover, a relatively large human study demonstrated a reduction in fasting glucose after consuming two slices of quinoa bread per day (Jenkins *et al.*, 2008). The beneficial impacts of whole grain intake on glucose and insulin homeostasis are likely attributable to the presence of fibre, resistance starch and oligosaccharides in the whole grain (Bjorck *et al.*, 1994; McIntyre *et al.*, 1997; Hallfrisch and Behall, 2000; Ylönen *et al.*, 2003; Seal, 2006). By contrast, neither glucose nor insulin concentrations were affected by quinoa supplementation in two studies conducted by the same group (Foucault *et al.*, 2012; Foucault *et al.*, 2014). The majority of the studies from both animals and humans listed earlier, however, reported no significant impact of quinoa on markers of glucose homeostasis, suggesting that the effects of quinoa are inconclusive and require further investigation.

Postprandial glucose and insulin responses were measured after intake of quinoa bread with and without gluten, gluten-free pasta and traditional bread, and the results showed that no improvement in insulin responses to all products tested. However, the glycemic index (GI) for quinoa bread was lower than that of gluten-free pasta and traditional bread (Berti *et al.*, 2004).

2.6.4.3 Effects on Lipid Profile and bile acid excretion

Cholesterol, produced in the liver and absorbed though the diet, is essential for all animal life in normal metabolic process. However, high concentrations of total and LDL cholesterol are associated with an elevated risk of CVD (Nishikura *et al.*, 2014). The present systematic review, which included both animal and humans supports the outcomes of intervention studies indicating that inclusion of

quinoa in the diet has a beneficial effect on plasma cholesterol concentration (Mithila and Khanum, 2015).

As previously suggested, quinoa is a good source of soluble fibre, which helps to lower total and LDL cholesterol concentrations (Glore *et al.*, 1994; Truswell, 1995; Repo-Carasco-Valencia and Serna, 2011). It has been suggested that some soluble fibres can inhibit the absorption of dietary cholesterol and bind cholesterol or bile acids during the intraluminal formation of micelles. The resulting decrease in cholesterol concentration of liver cells results in an up-regulation of LDL cholesterol receptors, thereby increasing clearance of LDL cholesterol (Anderson and Tietyen-Clark, 1986). There is also emerging evidence that resistant starch and soluble fibre are additionally fermented by some bacteria in the colon, producing the short-chain fatty acid propionate which may have a direct effect on the enzyme HGM-CoA-reductase inhibiting hepatic cholesterol synthesis lowering cholesterol concentrations (Slavin *et al.*, 1999; Escudero *et al.*, 2006). Other proposed mechanisms include enhanced satiety, resulting in decreased overall energy intake (Blundell and Burley, 1987); and slowing absorption of macronutrients due to the high viscosity of soluble fibre in the gut, resulting in increased insulin sensitivity (Schneeman, 1987) and accelerated excretion of bile acids by soluble dietary fibre (Gee *et al.*, 1993).

The high content of protein in quinoa seeds may also confer potential lipid lowering properties. Takao *et al*. (2005) reported that protein isolated from quinoa seeds significantly prevented the increase plasma and liver cholesterol concentrations in mice when exposed to fat enriched diets. This is consistent with previous studies showing that some protein from pseudocereals (quinoa, amaranth and buckwheat) can affect serum cholesterol concentration (Berger *et al.*, 2003; Tomotake *et al.*, 2007; Wang *et al.*, 2009a). Furthermore, protein isolates extracted from quinoa seeds showed bile acids binding activity *in vitro* and regulated the expression of 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase in the liver.

It has also been suggested that quinoa pericarp interacts with cholesterol in the intestinal tract, leading to inhibition of cholesterol absorption thereby decreasing serum and liver cholesterol level in mice (Konishi *et al.*, 1999). Saponins present in quinoa may also decrease intestinal absorption of cholesterol (Takao *et al.*, 2005). Squalene has also been shown to inhibit HMG-CoA reductase (Takao *et al.*, 2005; Paśko *et al.*, 2010). However, the mechanism through which component or the combination of these in quinoa seeds exerts hypocholesterolemic impact still remains unclear.

There is insufficient evidence to suggest that quinoa intake has an impact on triglycerides concentration from animal studies, since the benefits were only observed in one third of the studies included in this review. Interestingly, intake of quinoa (19.5 and 25 g) had clear effects on

triglycerides concentrations in two human studies, although one study of nineteen treated celiac patients appeared not to benefit from the consumption of 50 g of quinoa every day for 6 weeks. This may be because the celiac patients had much lower baseline triglycerides concentrations (66.7mg/dL) compared with the other studies (101.8 and 112.3 mg/dL) so the effect was much smaller (Jenkins *et al.*, 2008; Farinazzi-Machado *et al.*, 2012; De Carvalho *et al.*, 2014). Despite the negative outcomes shown in animal studies, quinoa supplementation has the potential to reduce triglycerides concentrations in humans, although more studies are warranted to confirm the benefits.

Increased HDL-cholesterol and favourable changes in the ratio of HDL: total cholesterol are desirable since these are linked to lower CVD risk (COMA, 1994). The impact of quinoa intake on changes in HDL-cholesterol concentration was quite conflicting, since both significant increases and reductions were observed in animal and human studies. This may be because animals or subjects were offered a low fat, high carbohydrate diets with the addition of daily quinoa consumption. High carbohydrate diets have been previously associated with reduction in HDL-cholesterol concentration (Willett, 2006). Overall, this review does not support the theory that intake of quinoa increases HDL-cholesterol concentration and produces a more favourable lipoprotein ratio.

The examination of cardiovascular benefits associated with quinoa seeds should embrace an attempt to define optimal amounts for human consumption. Intake of quinoa foods in successful human intervention studies, which significantly decreased the levels of total cholesterol, LDL-cholesterol and triglycerides, were 19.5 g quinoa in the form of bars and 25 g quinoa in the form of flakes; both for around one month. Therefore, the amount required to achieve an effect appears to be 20g or more per day for at least 4 weeks, but more well designed dose-response trials are needed to demonstrate a minimum amount and duration of exposure.

2.6.4.4 Other outcomes

Other markers that have been associated with an increased CVD risk included blood pressure, AST and ALT. However, the studies that investigated the impact of increased quinoa intake on these markers are scarce. Several studies using quinoa flour or protein hydrolysate have demonstrated that the bioactive properties of quinoa lead to a decrease in blood pressure (Ogawa *et al.*, 2001; Aluko and Monu, 2003). The serum level of the enzymes AST and ALT are commonly measured clinically as biomarkers for liver health. In the study conducted by Maha*,* (2016) inclusion of quinoa in the diet of obese rats resulted in a significant reduction in serum AST and ALT, which the authors suggest may be due to the presence of polyphenols with antioxidant property. Farinazzi-Machado *et al.* (2012) found reductions in AST in men and women but no differences in ALT concentration.

2.6.4.5 Consideration of animal studies translation

Animal models have historically contributed much to our understanding of mechanisms of disease, but whether the effectiveness of clinical treatment strategies can translate to humans has remained controversial, since animal models do not certainly predict what will happen in humans (Hackam and Redelmeier, 2006; Hackam, 2007; Perel *et al.*, 2007). In a review of animal studies published in seven journals with high impact factor, around one-third of the studies translated in humans, but only one-tenth of these studies were subsequently approved for application in patients, not to mention less frequently cited animal studies (Hackam and Redelmeier, 2006). For example, the way that rodents handle fat differs from humans, thus making the extrapolation to humans questionable (McGonigle and Ruggeri, 2014). Besides, a high fat or cholesterol (%) included in the diets consequently resulted in a question of how applicable it is to a 'normal' diet consumed by humans; the foods might be just fed as a flour and not cooked in the diet, digestion and absorption of which was hugely different from cooked foods; the doses consumed by animals were unable to give any instructions for the amounts that can induce any significantly beneficial effects on humans (Konishi *et al.*, 1999; Takao *et al.*, 2005; Alves *et al.*, 2008). Additionally, whether humans consuming quinoa foods during the same period as animals can lead to any significantly beneficial impacts were also needed to confirm (Paśko *et al.*, 2010; Mithila and Khanum, 2015).

2.6.4.6 Reasons for the absence of meta-analysis

A total of four human intervention studies were included in this review, but two of which did not give standard deviation (SD) of variables, including glucose and lipids (No reply after asking authors for SD). In this case, on one hand, it seemed that it was not worth doing a meta-analysis with only two papers; on another hand, the rest two papers still remained to be described in other ways, which would probably add more confusion to the readers.

2.6.4.7 Limitations

Several limitations of this review should be noted. First, the number of human intervention studies which investigated the impact of quinoa consumption on CVD risk markers was limited, leading to only 4 articles being included in this review. In order to support the any theory of health benefits of quinoa consumption adequately, more studies would be required to assess the interventions effects. Secondly, the majority of human studies included in this review were of short duration with small sample sizes and may not have had sufficient power to support the effect. Thus large-scale human intervention studies of longer duration are required. Thirdly, animals in some studies were treated with isolated fractions from quinoa, such as the protein extracts in the study conducted by Takao *et al*. (2005), rather than whole quinoa flours. However, the human studies were based on

consumption of entire quinoa seeds instead of food extracts, making direct comparison of the effects difficult. Finally, the bioactive compounds responsible for quinoa's cardiovascular health still remain uncertain, and the mechanisms underlying the effects were not fully elucidated.

2.6.5 Conclusion

In general, a few studies included in this systematic review (10 animal and 4 human intervention studies) suggest that quinoa consumption have some beneficial effects on cardiovascular health in humans, but overall results still remain highly inconsistent and also supporting evidence, especially from human studies, is still very limited. There is increasing evidence that several decreased risk markers associated with CVD could be due to soluble fibre, protein, pericarp, saponins and other components in the quinoa seeds, but it has not been fully elucidated which bioactive compounds are responsible for underlying effects. Up to date, it seems that providing the people suffering from celiac disease with this nutritionally excellent gluten-free alternative is the only certain benefit, since no negative effects have been reported in previous studies. Further research, especially large-scale human intervention studies, are required to further understand and promote the role that quinoa seeds can play in cardiovascular health.

2.7 Buckwheat and CVD risk markers a systematic review and meta-analysis

2.7.1 Introduction

Across the globe, cardiovascular disease (CVD) is the leading cause of morbidity and death, including in China, where mortality from CVD has increased rapidly in the past few decades (Critchley *et al.*, 2004). Elevated blood pressure, total cholesterol, low density lipoprotein cholesterol (LDL-cholesterol) and high density lipoprotein cholesterol (HDL-cholesterol) are clinically considered as major CVD risk factors. There are increasing epidemiological studies suggesting that diets rich in whole grains are linked to a lower risk of CVD and mortality (He *et al.*, 1995; Liu *et al.*, 1999a; Jacobs *et al.*, 2000; Mellen *et al.*, 2008b; Aune *et al.*, 2016; Chen *et al.*, 2016). In China, recently changes to traditional diets, encompassing a dramatic decrease in the amount of whole grains consumption from 104 g/d to 24 g/d may be a contributory factor for the elevated CVD mortality (Ge, 2011). The pseudo-cereal buckwheat, which belongs to *Polygonaceae* family, is included in the ''whole grain'' category in the terms of nutritional value (Van der Kamp *et al.*, 2014). Buckwheat has been cultivated as a traditional food in China since 1000BC and is found almost everywhere globally, but mainly in the northern hemisphere, such as in Russia and China (Li and Zhang, 2001). Buckwheat grows faster than many of other crops, but require less water and less nutrition from soil (Li and Zhang, 2001).

In recent years, there has been increasing interest in the use of buckwheat as a raw food material owing to the ''re-discovered'' nutritional value and health benefits (Li and Zhang, 2001; Wu *et al.*, 2016). Among the main nine species with agricultural significance, common buckwheat and Tartary buckwheat (also known as bitter buckwheat) are the most widely grown species, where common buckwheat is widely grown in Asia, Europe and America, but Tartary buckwheat is most grown in Asia, including China, India, Nepal (Bonafaccia *et al.*, 2003). Compared with common buckwheat, Tartary buckwheat tends to contain higher concentrations of certain bioactive phytochemical like flavonoids, which give Tartary buckwheat a much bitter taste (Fabjan *et al.*, 2003). For example, the concentration of rutin, a unique flavonoid in buckwheat compared with other common plant foods, is approximately 30-150 times greater in Tartary buckwheat than that of common buckwheat (Kitabayashi H, 1995; Wieslander and Norback, 2001). The yield (t/ha) of common and Tartary buckwheat were similar, which also largely depend on variety, environment, management and others, and the price of common buckwheat was much lower than that of Tartary buckwheat, but prices of both which were higher than that of wheat and rice (Kalinova and Vrchotova, 2011; Xiang *et al.*, 2016). Buckwheat seeds are the principle form for human consumption of this species, and they are mainly consumed as in the form of bakery products (bread, noodles, snacks and cookies)

enriched with buckwheat flour at levels ranging from 0.3-60%, and non-bakery buckwheat products (honey, tea, sprouted grains and tathana) (Gimenez-Bastida *et al.*, 2015a). In addition to a high starch content as an energy source, buckwheat is rich in nutritionally valuable protein with a well-balanced amino acid profile, dietary fibre, lipids and minerals, along with other health-promoting components such as phenolic compounds and sterols, which has attracted growing attention as a potential functional food (Krkošková and Mrázová, 2005). Buckwheat, as a traditional Chinese foodstuff, is well known to contain high concentrations of rutin compared with other common plant foods. In addition, the absence of gluten, makes buckwheat-containing products potential alternatives for patients suffering from celiac disease (Saturni *et al.*, 2010). It has been demonstrated that intake of buckwheat or buckwheat enriched products is associated with a wide range of health benefits, including anticancer, anti-inflammatory, hypoglycaemic and hypocholesterolaemia effects, although the specific bioactive components responsible for buckwheat's beneficial effects remain uncertain (Gimenez-Bastida and Zielinski, 2015b).

To date, relatively few studies have been carried out to investigate the impact of buckwheat intake on human health. Moreover, to the author's knowledge, there has not been any quantitative study to systematically review and summarize the effects of buckwheat consumption on CVD risk markers. With accumulating evidence, the object of this work was to comprehensively review the recent literature and carry out a meta-analysis evaluating the changes in blood glucose and lipid concentrations induced by buckwheat intake. A secondary objective was to explore possible mechanisms underlying any beneficial effects observed.

2.7.2 Methods

2.7.2.1 Data sources and literature search

A comprehensive literature search for animal or human studies that had evaluated the correlation between buckwheat intake and CVD risk between 1960 and 2016 was undertaken. Figure 2.7 shows how relevant papers were chosen. PubMed, Scopus, Ovid, EBSCO, Web of Science, JSTOR, Medline and China National Knowledge Infrastructure were searched using the search terms 'buckwheat' AND 'cardiovascular disease' OR 'cholesterol' AND 'human' OR 'animal', and the same terms were applied in each database during the search phase. CVD was defined to include stroke, aortic disease, peripheral arterial disease and coronary heart disease. In addition the reference lists of retrieved papers were searched manually for all additional potentially relevant papers. The search was restricted to studies on humans and animals and included those that were written in different languages including English or Chinese. Data were extracted by a single reviewer.

2.7.2.2 Study selection

The studies were included in this review satisfied the following criteria: 1) study in animals or humans, 2) buckwheat-consumption exposure, 3) the outcomes included CVD risk markers, such as glucose, insulin, lipid profile. Since cholesterol was the most commonly indicator of CVD response to whole-grain foods, cholesterol was used as a primary outcome marker in this review. The eligibility criteria were set before the start of the research.

2.7.2.3 Data extraction and quality assessment

The following data were extracted from each human study: lead author, year of publication, characteristics of subjects, number of subjects, control diet, experimental diet, trial length and findings. The sample size reported in Table 2.7 was the overall total for the experiment rather than restricting to either control or intervention diet/s. The following data were extracted from each animal study: lead author, year of publication, animal species, control diet, experimental diet, trial length and outcomes. Extracted data from the human and animal studies are shown as the column headings of Tables 2.7 and 2.8 respectively. Missing data are reported as ''Not stated'' if they were not explained in the corresponding articles. The methods of quality assessment for studies included in this review were as described in Section 2.5, and the quality scores of the studies included in this review were also listed in Tables 2.7 and 2.8.

2.7.2.4 Statistical analysis

All statistical analyses were performed with STATA 12.0 (Stata Corp); $P<0.05$ was considered significant. Heterogeneity across studies was quantified by using the I^2 statistic to consider each

study design, as a quantitative evaluation of inconsistency among studies (Higgins *et al.*, 2003). To pool the results of studies of the acute impacts on blood glucose, lipid profiles, a fixed effects models was used when heterogeneity was absent or low (I^2 < 20%); when heterogeneity was greater, a random effects model was used. In this review, weighted mean differences (WMD) between treatment (buckwheat diet) and control groups (normal or refined diet) or before and after treatment were combined via a random effects model to evaluate the size of treatment impacts on CVD risk markers, including blood concentrations of glucose, total, HDL and LDL cholesterol and triglycerides. To examine whether a single study exerted undue impact on the overall results, sensitivity analyses were performed in which each individual study was excluded from the meta-analysis and the effect size recalculated with the remaining studies. For all outcomes, *a priori* subgroup analyses were planned to be conducted with meta-regression models, if there were ≥ 10 studies. Results of the studies reported in mg/dL were converted to mmol/L using standard conversion factors, with 1 mg/dL = 0.02586mmol/L for cholesterol, 1 mg/dL = 0.01129 mmol/L for triglycerides. These values were obtained as mean± SD. For continuous results, summary estimates of WMD with 95% CI were assessed for net changes between each treatment and control groups.

Furthermore, potential publication bias of the studies were also evaluated by visual inspection of Funnel plots and quantitatively assessed using Begg's and Egger's tests, where $P < 0.05$ was deemed statistically significant (Egger *et al.*, 1997).

2.7.3 Results

2.7.3.1 Characteristics of studies

The systematic search of the scientific databases led to the initial identification of 674 articles for further evaluation. After removing duplicate articles (239) and articles that did not meet the eligibility criteria (408), a total of 27 articles including 11 human studies and 16 animal studies were included in the review. It was noteworthy that five trials performed by Zhang *et al*. (2001), Lu *et al*. (2002), Tong *et al*. (2002), Zhang *et al*. (2003) and Zhang *et al*. (2007) were reported in the same population; thus, this current review combined the informative data and retained only the latest paper to avoid information duplication. Manual searching of the reference list of the relevant articles yielded 18 additional articles. After applying the inclusion criteria, 8 of these articles were considered fit to include. Consequently, the combination of electronic and manual reaching resulted in 35 articles which are included in this final review (Figure 2.7). To be specific, this review pooled the results of 15 human studies, consisting of 13 short-term randomized, controlled trials (RCT) and 2 cross-sectional studies, which had the assessed lipid-lowering effects of buckwheat in free-living subjects, and 20 animal studies. Nine human studies were conducted in China, two in India and one each in Sweden, Canada, Italy and Serbia. Nine animal studies were carried out in Japan, seven in China and one each in Spain, Poland, Egypt and South Korea.

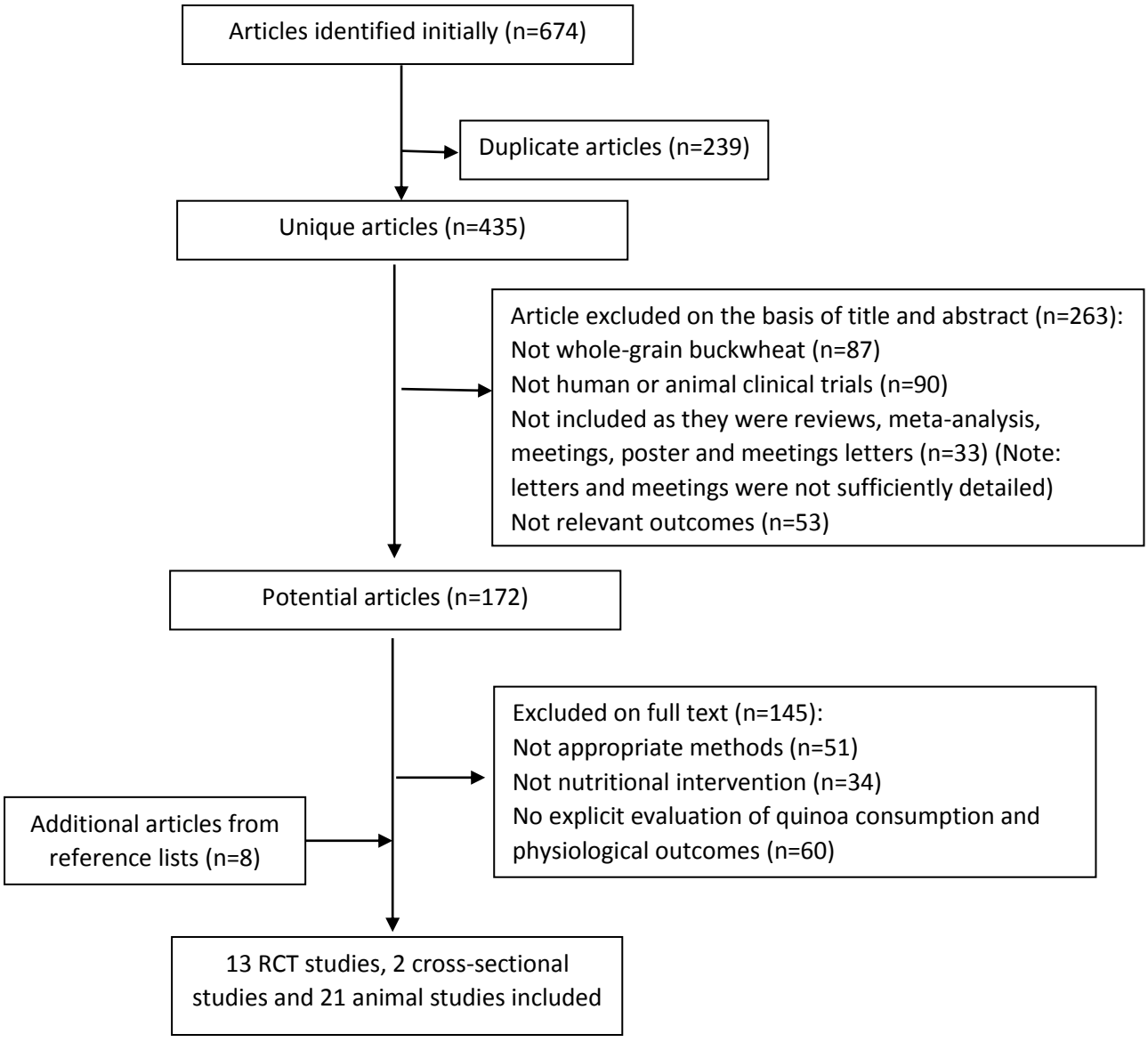


Figure 2.7. Flow diagram of article selection

Table 2.7. Summary of all human studies reviewed

Human studies								
Reference	Population of study	Control diet	Experimental diet	Duration	Outcomes 95% CI			Quality score
					Significant		Insignificant	
Bijlani *et al*. (1985)	healthy (n = 8 ♂)	No control diet	A preparation made from 100 g whole buckwheat (BW) flour	12 weeks	serum: VLDL	↓	body weight serum: TC serum: LDL serum: HDL serum: HDL/TC serum: LDL_{TG} serum: $VLDL_{TG}$ serum: HDL_{TG} serum: TG	1
	healthy (n = 9 ♂)	No control diet	A preparation made from 100 g whole BW flour	4 weeks	serum: HDL/TC serum: LDL_{TG} serum: $VLDL_{TG}$ serum: HDL_{TG}	↑ ↑ ↑ ↓	body weight fasting blood glucose serum: TC serum: LDL serum: VLDL serum: HDL serum: TG	1
Bijlani *et al*. (1984)	healthy (n = 12 ♂)	No control diet	A preparation made from 100 g sieved BW flour	4 weeks	serum: HDL serum: HDL/TC	↑ ↑	fasting blood glucose serum: TC serum: LDL serum: VLDL	0
Lu *et al*. (1990)	patients with diabetes and hyperlipidemia (n=23,13 and 18)	No control diet	BW flour BW flour BW flour	1 month 1 month 1 month	fasting blood sugar serum: TC serum: TG	↓ ↓ ↓		-1
Zheng *et a*l. (1991)	NIDDM patients (n=10 ♂, 9 ♀)	No control diet	Tartary BW flour; 50g	3 months	serum: TG	↓	fasting blood glucose insulin serum: TC	-1
Liu and Fu, (1996)	patients (n=60)	No control diet	Tartary BW flour; 40g/day	4 weeks	body weight systolic BP diastolic BP serum: TC serum: LDL serum: HDL serum: TG	↓ ↓ ↓ ↓ ↓ ↑ ↓		1
Lin *et al*. (1998)	Type 2 diabetes (T2DM) (n=32)	Habitual diet	100g of Tartary BW flour	5 weeks	fasting blood serum: TG	↓ ↓	serum: TC	1
Zhao and Guan, (2003)	T2DM (n=30 ♂, 30 ♀)	No control diet	BW flour (amounts not stated)	8 weeks	fasting blood systolic BP serum: TC serum: HDL	↓ ↓ ↓ ↓	body weight diastolic BP serum: LDL serum: TG	
Huang *et al*. (2009)	patients with diabetes (n=18 ♂, 17 ♀)	No control diet	Tartary BW mixture (amounts not stated)	2 months	fasting blood HbA_1 c/% serum: TC serum: LDL serum: HDL serum: TG	↓ ↓ ↓ ↓ ↓ ↓		1

Wieslander *et al*. (2011)	healthy (n = 62 ♀)	No control diet	group 1: four common BW cookies (daily). group 2: four Tartary BW cookies (daily) (cross-over study) 100 g of sieved BW preparation	6 weeks	serum: TC serum: HDL	↓ ↓	$sPLA_2$	0
Stringer *et al*. (2013)	healthy (n=23)	Rice cracker; 66 g	BW cracker; 76 g	7 days			plasma glucose plasma: TC plasma: LDL plasma: HDL plasma: TG liver enzyme: AST liver enzyme: ALT	3
	T2DM (n=24)	Rice cracker; 66 g	BW cracker; 76 g	7 days			plasma glucose plasma: TC plasma: LDL plasma: HDL plasma: TG liver enzyme: AST liver enzyme: ALT	3
Stokić *et al*. (2015)	Patients (n=7 ♂, 13 ♀)	No control diet	BW-enriched wheat bread; 300g /day	1 month	serum: TC serum: LDL liver enzyme AST liver enzyme ALT	↓ ↓	BMI systolic BP diastolic BP serum: HDL serum: TG	1
Yu, (2015)	patients with hyperlipidemia (n=36 ♂, 24 ♀)	Water	Tartary BW tea, 15g	60 days	serum: TC serum: LDL serum: TG	↓ ↓ ↓	systolic BP diastolic BP blood glucose serum: HDL	3
Qiu *et al*. (2016)	165 patients with T2D(n=67 ♂, 98 ♀)	Refined grans and white wheat flour; 150g	Tartary buckwheat foods; 150g	4 weeks	plasma: Ln insulin plasma: TC plasma: LDL	↓ ↓ ↓	body weight BMI plasma glucose plasma: HbA1c (%) plasma: Ln HOMA-IR plasma: TG plasma: HDL	5
Dinu *et al*. (2017)	participants with high CVD risk (n=10 ♂, 11 ♀)	Wheat products daily (amounts not stated)	BW products daily (amounts not stated) (cross-over study)	24 weeks	fasting blood serum: TC serum: LDL serum: TG	↓ ↓ ↓ ↓	body weight insulin serum: HDL	5
He *et al*. (1995)	healthy (n=857 ♂)		BW; group 1 (n=319), 0 g/day; group 2 (n=207), <40 g/day; group 3 (n=161), 40-200 g/day; group 4 (n=163), >200 g/day	cross-sectional	systolic BP diastolic BP serum: TC serum: LDL serum: HDL/TC	↓ ↓ ↑ ↓ ↓	BMI serum: HDL serum: TG	
Zhang *et al*. (2007)	healthy (n=491 ♂, 470 ♀)		BW (amounts not stated)	cross-sectional	BMI fasting blood serum: TC	↑ ↓ ↓	systolic BP diastolic BP serum: TG	

					serum: LDL	↓			
					serum: HDL	↑			

BW, buckwheat; VLDL, very low-density lipoprotein; TC, total cholesterol; LDL, low-density lipoprotein; HDL, high-density lipoprotein; TG, triglycerides; BP, blood pressure; HbA_1 c, glycated hemoglobin A1c; sPLA2, secretory phospholipase A2; AST, aspartate transaminase; ALT, alanine transaminase.

Table 2.8. Summary of all animal studies reviewed

Animal studies									
Reference	Model	Control diet	Experimental diet	Duration	Outcomes 95% CI				Quality score
					Significant		Insignificant		
Son *et al*. (2008)	♂ Sprague-Dawley rats	other cereals powder; 50% in the diets (diet with 1% cholesterol)	BW powder; 50% in the diets (diet with 1% cholesterol)	4 weeks	plasma: TC plasma: LDL plasma: HDL plasma: TG area of lumen	↓ ↓ ↑ ↓ ↑	food intake body weight gain food efficiency ratio transit time wall thickness		4
Yang *et al*. (2014)	♂ Syrian Golden hamster	casein and corn starch included in diet (fed cholesterol diet)	Tartary BW flour; 24% in diet (fed cholesterol diet)	6 weeks	serum: TC serum: non-HDL liver cholesterol faeces: neutral sterols	↓ ↓ ↓ ↑	food intake body weight gain serum: HDL serum: TG faeces: acidic sterols		5
Prestamo *et al*. (2003)	♀ Wistar Hannover rats	conventional food	cooked BW	30 days	body weight serum: TC serum: HDL HDL phospholipids	↓ ↓ ↓ ↓	blood glucose serum: LDL serum: TG liver weight uric acids		3
Orzel *et al.* (2015)	♂ Wistar rats	wheat starch included in the diet	buckwheat flour ,meal and bran; 200g/kg (normal diet)	4 weeks	body weight gain serum : LDL serum : TG	↑ ↓ ↓	food intake glucose serum: TC serum: HDL		3
Tomotake *et al*. (2006)	♂ Sprague-Dawley rats and ♂ ddY mice	casein in the diet; (rats fed a normal or high-cholesterol diet)	30.7% of BWP extract in the diet (rats fed a normal or high-cholesterol diet); 54.8% of PBF (mice fed a high-cholesterol diet)	10 or 27 days	serum: TC serum : TG serum: phospholipids liver weight liver cholesterol (PBF) faeces: dry weight (PBF) faeces: neutral steroids faeces: bile acids (PBF)	↓ ↓ ↓ ↓ ↓ ↑ ↑ ↑	food intake body weight gain		4
Magdy *et al*. (2014)	♂ albino rats	hypercholesterolemia -induced diet	BW hull extracts; 1000 mg/kg b. wt/day in diet (hypercholesterolemia -induced diet)	8 weeks	blood glucose plasma: TC plasma: LDL plasma: TG plasma: AST plasma: ALT	↓ ↓ ↓ ↓ ↓ ↓	plasma: HDL		4
Wang *et al*. (2009b)	♂pathogen-free Wistar rat	high-fat diet	Tartary BW bran extract; 0.2−1 g /kg body weight (high-fat diet)	6 weeks	serum: TC serum: HDL (medium dose) serum: TG hepatic: TC hepatic: TG	↓ ↓ ↓ ↓ ↓	body weight gain serum: LDL		5

Hosaka *et al*. (2014)	KK-Ay mice	normal chow diet	common BW bran powder; 0.05 mg /g body weight	6 weeks	body weight gain serum: TG liver weight	↓ ↓ ↓	food intake fasting blood glucose insulin resistance serum: TC	2
Yao *et al*. (2008)	♂ C57BL/6 control mice and diabetic KK-Ay	redistilled water	D-Chiro-Inositol (DCI) enriched Tartary BW bran extract (TBBE); 45-182 mg of TBBE/kg in diet	5 weeks	fasting blood glucose level plasma: TG (high dose) insulin immunoreactivity	↓ ↓ ↑	body weight gain plasma: TC	2
Hu *et al*. (2015a)	♂ Kunming mice	high-fructose water	D-Chiro-Inositol (DCI) enriched Tartary BW extract (DTBE); 40, 80 and 160 mg per kg body weight/day (high-fructose water)	8 weeks	body weight gain serum: glucose serum: insulin level serum: TC serum: LDL serum: HDL serum: TG liver weight serum AST and ALT activity	↓ ↓ ↓ ↓ ↓ ↑ ↓ ↓ ↓	all parameters in the group of 40 mg per kg body weight/day showed on significant effect except serum AST activity	5
Tomotake *et al*. (2000)	♂ Golden Syrian hamster	casein; 230g /kg (high-cholesterol diet)	BWP extract; 381g /kg (high-cholesterol diet)	2 weeks	food intake plasma: TC plasma: HDL plasma: HDL/TC plasma: TG plasma: phospholipids liver weight hepatic cholesterol faecal dry weight faeces: neutral steroids faeces: acidic steroids	↑ ↓ ↓ ↑ ↓ ↓ ↑ ↓ ↑ ↑ ↑	body weight gain hepatic TG hepatic phospholipids	5
Tomotake *et al*. (2007)	♂ Sprague–Dawley rats	23.0% casein in the diet (high-cholesterol diet)	Tartary BW flour protein and common BWP extract; 30.7% of common BWP and 43.7% of Tartary BWP in the diet (high-cholesterol diet)	27 days	serum: TC liver weight hepatic cholesterol faecal dry weight faecal excretion: nitrogen faeces: neutral steroids faeces: bile acids apparent protein digestibility	↓ ↓ ↓ ↑ ↑ ↑ ↑ ↓	body weight gain food intake	3
Tomotake *et al*. (2001)	♂ Sprague–Dawley rats	casein; 230 g/kg (normal diet)	BWP extract; 307 g/kg (normal diet)	8 weeks	plasma: TC plasma: HDL faeces: neutral steroids faeces: acidic steroids	↓ ↓ ↑ ↑	body weight gain food intake plasma: HDL/TC plasma: TG plasma: phospholipids fecal dry weight	3
Kayashita *et al*. (1997)	♂ Sprague–Dawley	230 g/kg casein in the diet (high cholesterol diet)	BWP extract; 381 g/kg (high-Cholesterol diet)	3 weeks	plasma: TC plasma: HDL/TC plasma: TG	↓ ↑ ↓	body weight gain food intake plasma: HDL	4

					plasma: phospholipids	↑	hepatic: TG	
					plasma: bile acids	↓	faeces: acidic steroids	
					liver weight	↓		
					hepatic cholesterol	↓		
					hepatic: phospholipids	↓		
					faecal dry weight	↑		
					faeces: neutral steroids	↑		
					apparent protein digestibility	↓		
Kayashita *et al*. (1995a)	♂ Sprague−Dawley	casein; 23.0%	BWP extract; 38.1%	3 weeks	plasma: TC	↓	body weight gain	4
					plasma: HDL/TC	↑	food intake	
					plasma: TG	↓	plasma: HDL	
					plasma: free fatty acid	↓	hepatic cholesterol	
					plasma: phospholipids	↓	hepatic TG	
					liver weight	↓	hepatic:	
					fat pad weights	↓		
Kayashita *et al*. (1996)	♂ Sprague−Dawley	casein; 230 g/kg	BWP extract; 381 g/kg	3 weeks	plasma: TC	↓	body weight gain	4
					hepatic TG	↓	food intake	
					faecal dry weight	↑	insulin	
					fat pad weights	↓	plasma: TG	
							plasma: free fatty acid	
							plasma: phospholipids	
							liver weight	
							hepatic TC	
							hepatic: phospholipids	
Kayashita *et al*. (1995b)	♂ Sprague−Dawley	casein; 240.1 g/kg (high-Cholesterol diet)	BWP extract; 323.1 g/kg (high-Cholesterol diet)	3 weeks	plasma: TC	↓	body weight gain	2
					hepatic: weight	↓	food intake	
					hepatic TC	↓	serum: TG	
					hepatic TG	↑	serum: free fatty acids	
							serum: glucose	
Zhang *et al*. (2017)	♂ Golden Syrian Hypercholesterolemia hamster	corn starch and casein in diet	Tartary BWP extract; 353 g/kg in diet	6 weeks	plasma: TC	↓	body weight	6
					plasma: non-HDL	↓	fatty streak (%)	
					plasma: HDL	↓		
					plasma: TG	↓		
					liver cholesterol	↓		
					total neutral sterols	↑		
					acidic sterols	↑		
Hu *et al*. (2015b)	♂ Kunming mice	high trimethylamine-N-oxide diet (TMAO) diet	Tartary buckwheat flavonoid fraction; 200, 400 and 800 mg per kg bw in diet (TMAO diet)	8 weeks	body weight gain	↓	food intake	3
					serum: TC	↓	water intake	
					serum: LDL	↓		
					serum: HDL	↑		
					serum: TG	↓		
					liver weight	↓		
					hepatosomatic index	↓		
Han *et al*. (2001)	Wister mice	high-fat diet	total flavones of buckwheat seeds; 2g/kg/day (high-fat diet)	10 days	serum: TC	↓	fasting blood glucose	1
					serum: TG	↓		

Qu *et al*. (2013)	♂ Sprague–Dawley	high-fat, high-sucrose diet	high rutin in BW noodles; 980mg/kg in diet (high-fat, high-sucrose diet)	4 weeks	serum: TC liver lipid	↓ ↑	body weight gain feed efficiency serum: HDL serum: TG serum: free fatty liver TC dry weight of feces fecal total lipid	3

BW, buckwheat; TC, total cholesterol; LDL, low-density lipoprotein; HDL, high-density lipoprotein; TG, triglycerides; BWP, buckwheat protein; PBF, protein buckwheat flour; AST, aspartate transaminase; ALT, alanine transaminase.

All except two human cross-sectional studies in the review were RCT studies, with follow-up durations ranging from 7 days to 24 weeks in human studies and 10 days to 8 weeks in animal studies. Overall, buckwheat intake in RCT human studies ranged from 40 g to 300 g of buckwheat ingredients (median levels of individual series), with four studies the amounts consumed unstated. Participants were either healthy or had one or more CVD risk markers, including overweight, hypertension, hyperglycaemia and hyperlipidemia. The methods of the included studies were similar, with a baseline period which was followed by subjects or animals being offered buckwheat or buckwheat-based products (e.g. buckwheat bread, buckwheat flour) for consumption, or placebo diets. Blood samples were obtained at baseline and after the intervention period for comparison of CVD biomarkers. Liver or faeces were only available from animal studies. With respect to the two human cross-sectional studies, since the populations started to consume fairly high amounts of buckwheat seeds as a staple food from an early age, the outcomes obtained were adjudged as representing the long-term impact of buckwheat grain on CVD risk markers.

2.7.3.2 Human Studies

2.7.3.2.1 Effects on Body Weight and BMI

Body weight or BMI changed significantly in response to buckwheat consumption in two out of eight human studies (Table 2.9) but in contrasting ways. Body weight decreased by 3.44 kg among 44 overweight participants in one of the studies by Liu *et al*. (1996), while the BMI level was higher (estimated 3%) in consumers of buckwheat than in non-consumers of buckwheat in the study of Zhang *et al*. (2007). The other studies observed no significant impact of buckwheat consumption on body weight or BMI.

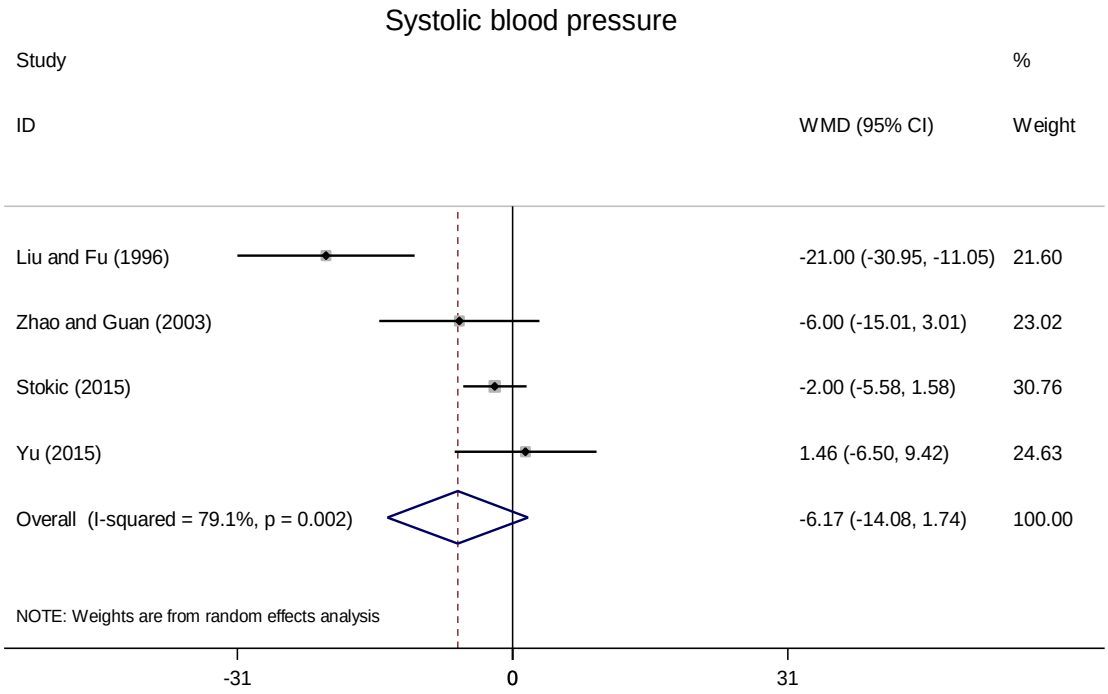
Table 2.9. The number of animal and human intervention studies showing significant increase, no effect and significant reduction on markers of CVD risk

	Number of Studies		
	Significantly higher in buckwheat treatment	The effect is not significant	Significantly lower in buckwheat treatment
Human Studies			
Body weight gain or BMI	1	6	1
Blood pressure	—	3	3
Blood glucose	—	6	6
Blood insulin	—	2	1
Total-Cholesterol	—	5	11
LDL-Cholesterol	—	4	8
HDL-Cholesterol	3	7	3
Triglycerides	—	7	7
Animal Studies			
Body weight gain	1	14	4
Food intake	1	12	—
Blood glucose	—	4	3
Blood insulin	1	2	1
Total-Cholesterol	—	2	19
LDL-Cholesterol	—	2	5
HDL-Cholesterol	4	6	4
Triglycerides	—	6	14
Liver weight	1	2	8
Liver Total-Cholesterol	—	3	8
Faecal weight	5	2	—
Faecal neutral steroids	7	—	—

2.7.3.2.2 Effects on Blood Pressure

Of six human studies which evaluated blood pressure, the association between buckwheat intake and blood pressure yielded inconsistent results. Data on blood pressure was reported in 4 randomised, controlled trials representing 183 participants based on the results of the meta-analysis. Figure 2.8 shows the pooled results from the random-effects model combing the weighted mean difference (WMD) for the impact of buckwheat intake on blood pressure in the total study population. There were no significant effects on systolic blood pressure (WMD, -6.172 mmHg; 95%

CI: -14.079, 1.736; I^2=79.1%, *P*= 0.126) after buckwheat intake, nor on diastolic blood pressure (WMD, -2.703 mmHg; 95% CI: -6.703, 1.297; I^2=62.7%, *P*= 0.185). This finding in the present review is consistent with the result of Zhang *et al*. (2007), who showed that blood pressure of people in a buckwheat-eating region of Mongolia did not differ from that of people in a non-buckwheat-eating region of the country. In contrary, He *et al*. (1995) found that in those who consumed ≥ 40 g buckwheat/day blood pressure was lower compared with those who consumed none or < 40 g/day.



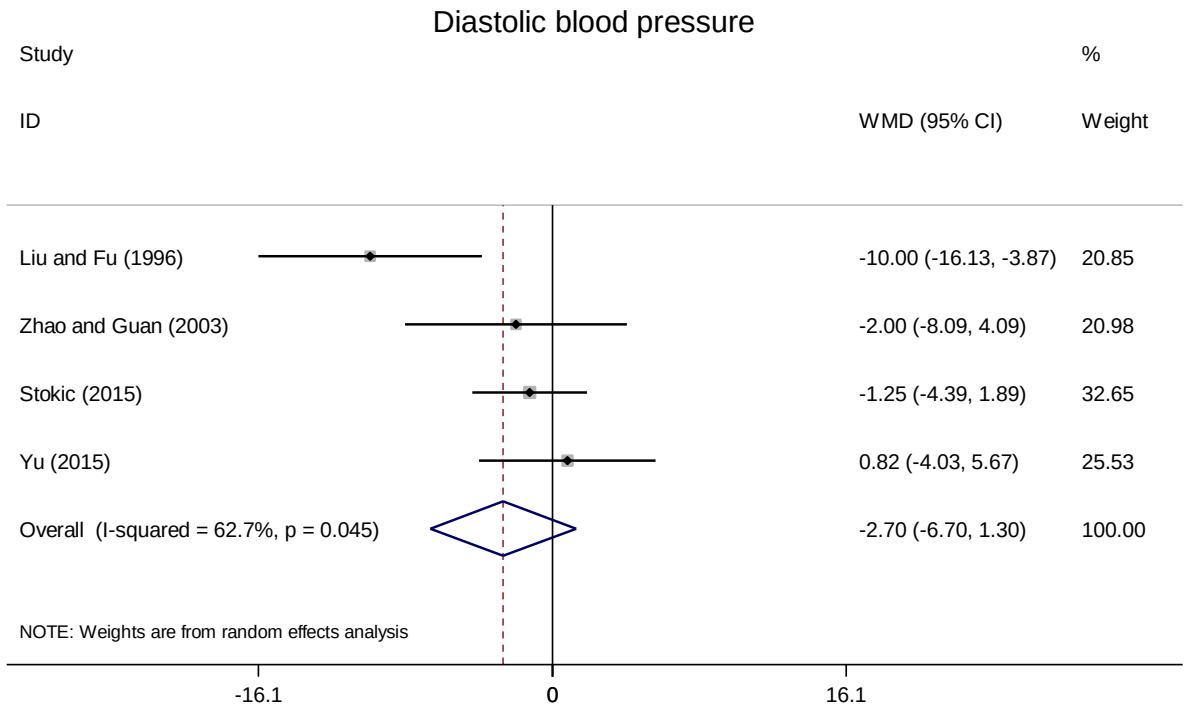


Figure 2.8. Meta-analysis of the effects of buckwheat products intake on systolic and diastolic blood pressure compared with baseline or control groups. Sizes of data markers indicate the weight of each study in the analysis. WMD, weighted mean difference (the results were gained from a random-effects model).

2.7.3.2.3 Effects on Blood Glucose and Insulin

Data on fasting blood glucose concentrations was reported in 9 randomised, controlled trials representing 548 participants based on the results of the meta-analysis (Figure 2.9). The results show that the fasting blood glucose concentration was significantly decreased with buckwheat treatment in comparison with baseline or control group (WMD, -0.85 mmol/L; 95% CI: -1.31, -0.39; P<0.001), with significant heterogeneity in the data (I^2= 94.2%). This finding in the present review is consistent with the result of Zhang *et al*. (2007), who showed that fasting blood glucose concentration of people in a buckwheat-eating region of Mongolia was significantly lower (16.92%) than that of people in a non-buckwheat-eating region of the country. There was no consistent effect of buckwheat on insulin concentrations reported, with a small non-significant reduction and a small non-significant increase in insulin concentrations reported in the studies of Zheng *et al*. (1991) and Dinu *et al*. (2017), respectively.

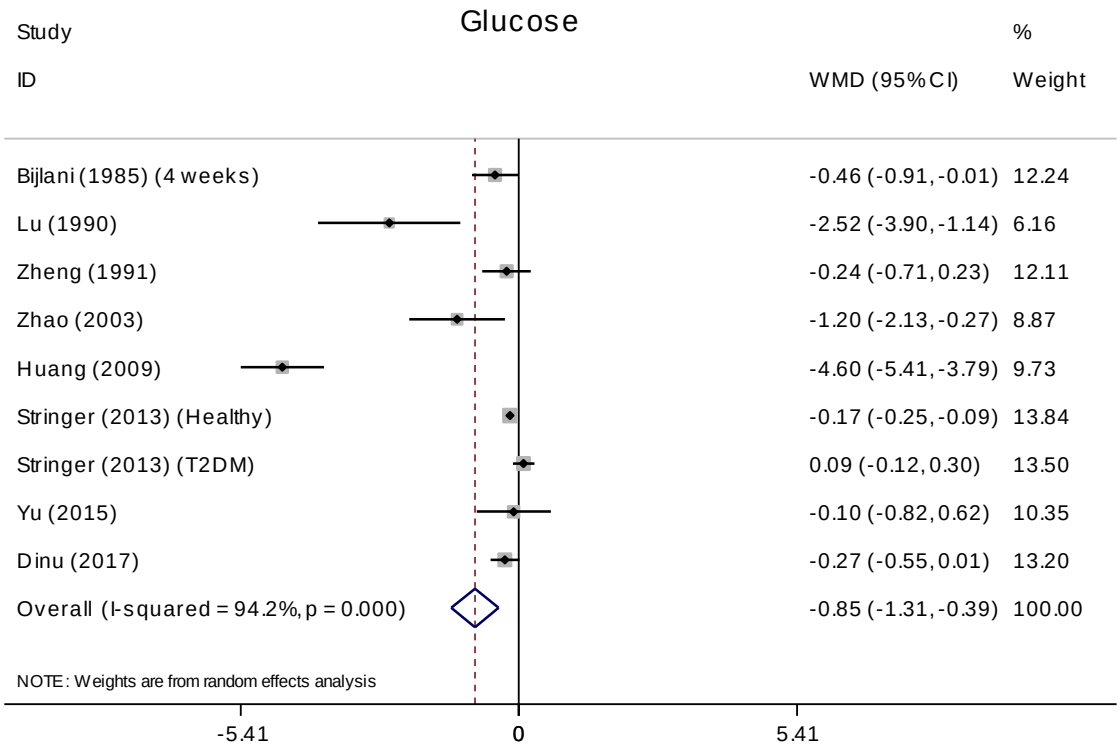


Figure 2.9. Meta-analysis of the effects of buckwheat products intake on blood glucose concentration compared with baseline or control groups. Sizes of data markers indicate the weight of each study in the analysis. WMD, weighted mean difference (the results were gained from a random-effects model).

2.7.3.2.4 Effects on Lipid Profile

Results from the random-effects meta-analysis of the association between buckwheat intake and lipid parameters were shown in Figures 2.10, 2.11, 2.12 and 2.13. Compared with baseline or control arms, buckwheat consumption was associated with statistically significantly lower concentrations of total cholesterol (WMD,-0.50 mmol/L; 95% CI: -0.80, -0.20; 12 trials, 708 participates, I^2=89.5%, *P*= 0.001) and triglycerides (WMD, -0.25 mmol/L; 95% CI: -0.49, -0.02; 11 trials, 592 participates, I^2=92.5%, *P*= 0.034). However, there were no significant effects on LDL-cholesterol (WMD, -0.33mmol/L; 95% CI: -0.66, -0.02; 9 trials, 520 participates, I^2=95.3%, *P*= 0.061) after buckwheat intake, nor on HDL-cholesterol (WMD, -0.09mmol/L; 95% CI: -0.25, -0.07; 10 trials, 642 participates, I^2=94.4%, *P*= 0.282).

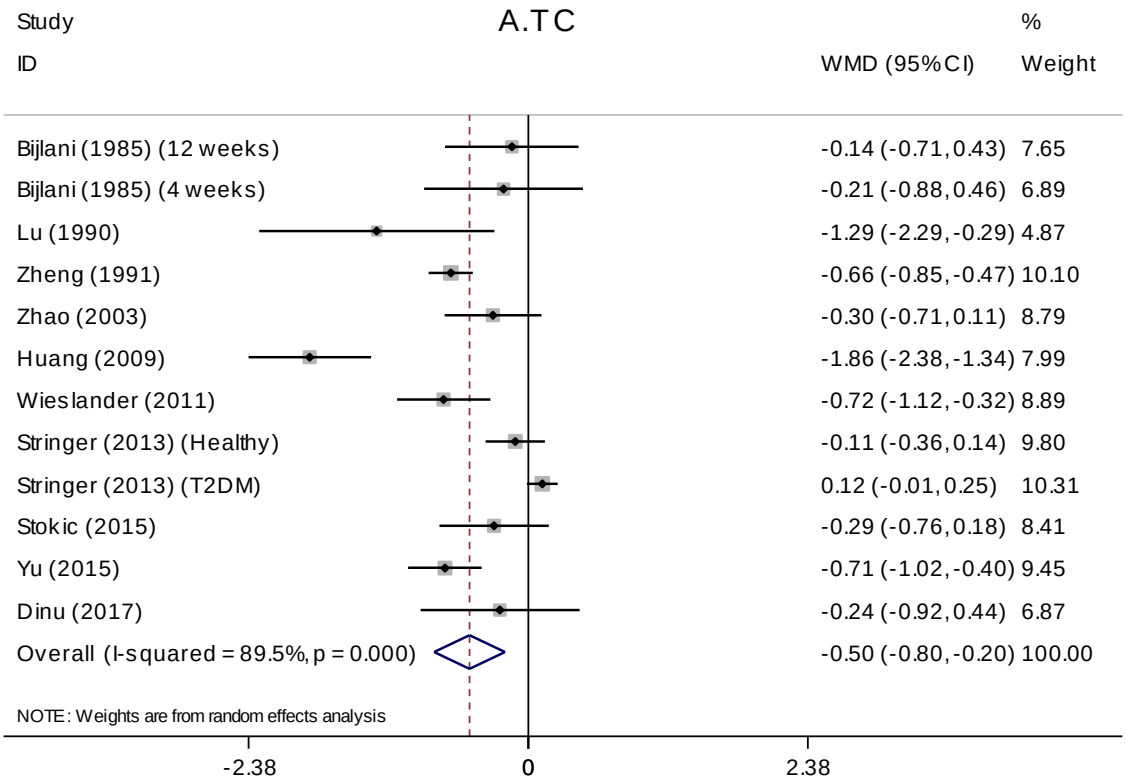


Figure 2.10. Meta-analysis of the effects of buckwheat products intake on blood total cholesterol concentration compared with baseline or control groups. Sizes of data markers indicate the weight of each study in the analysis. WMD, weighted mean difference (the results were gained from a random-effects model).

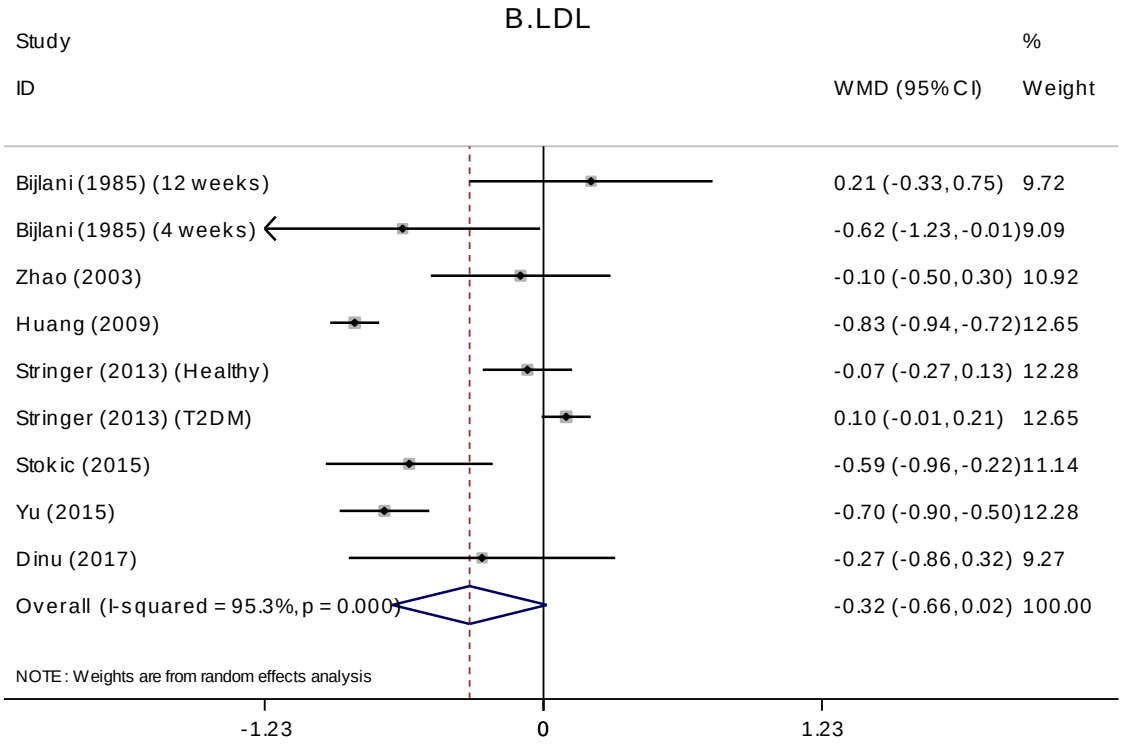


Figure 2.11. Meta-analysis of the effects of buckwheat products intake on blood LDL cholesterol concentration compared with baseline or control groups. Sizes of data markers indicate the weight of each study in the analysis. WMD, weighted mean difference (the results were gained from a random-effects model).

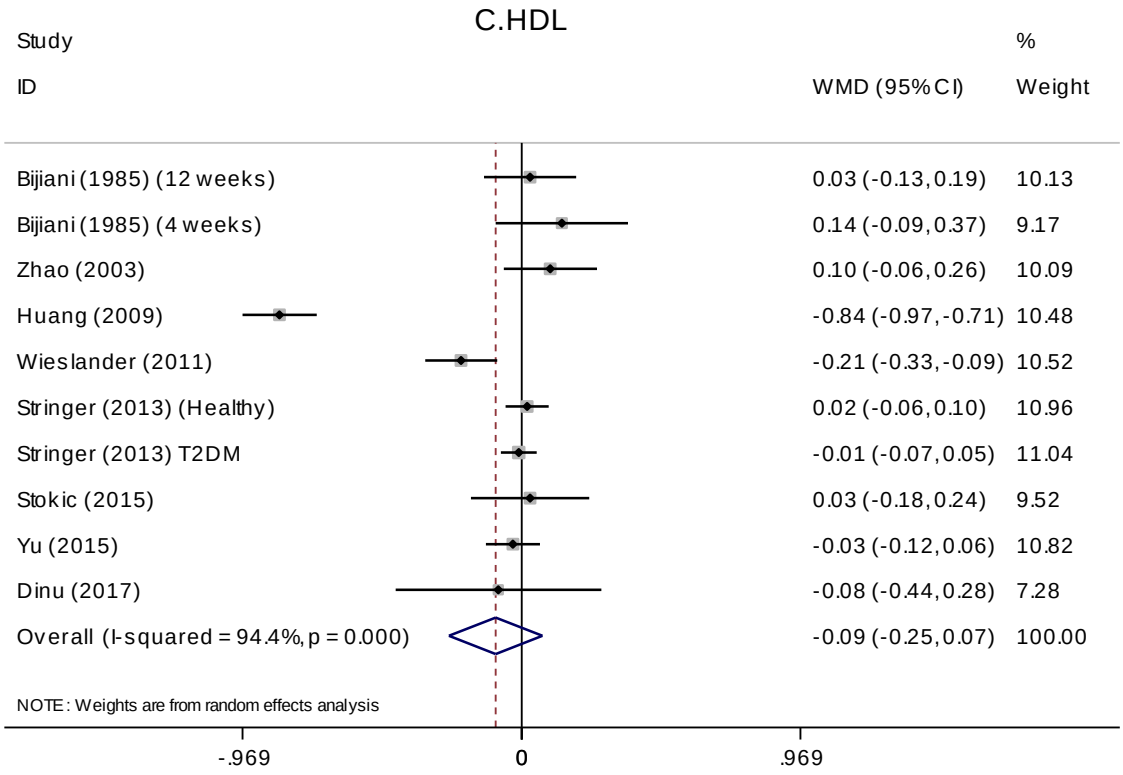


Figure 2.12. Meta-analysis of the effects of buckwheat products intake on blood HDL cholesterol concentration compared with baseline or control groups. Sizes of data markers indicate the weight of each study in the analysis. WMD, weighted mean difference (the results were gained from a random-effects model).

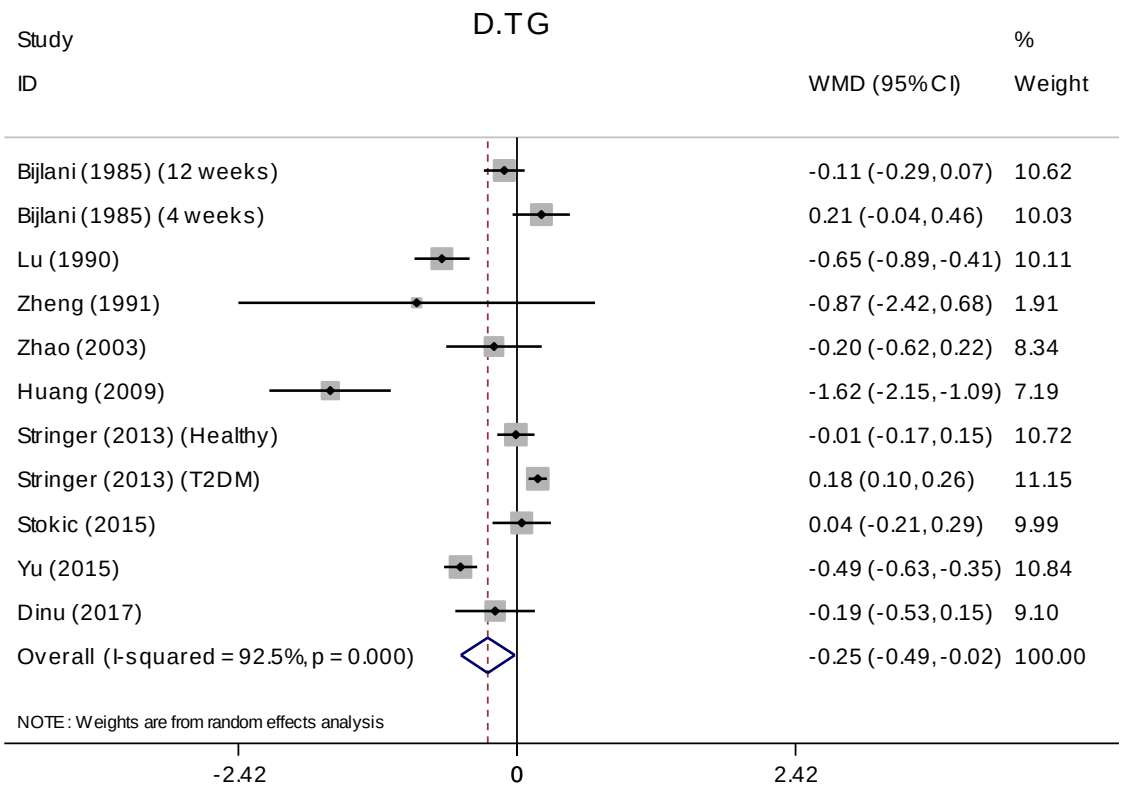


Figure 2.13. Meta-analysis of the effects of buckwheat products intake on blood triglycerides concentration compared with baseline or control groups. Sizes of data markers indicate the weight of each study in the analysis. WMD, weighted mean difference (the results were gained from a random-effects model).

In the cross-sectional study of 857 Yi men conducted by He *et al*. (1995), after multiple-regression analysis, buckwheat intake (100g/day) was associated with significantly lower concentrations of serum total cholesterol (-0.07mmol/L, *P* <0.01), LDL-cholesterol (-0.06mmol/L, *P* <0.05) and a higher ratio of HDL to total cholesterol (0.01, *P* <0.05), with no significant effect on HDL-cholesterol and triglycerides. These findings were in general accordance with the results from the trial by Zhang *et al*. (2007) with 961 participants, which also identified a significant decrease in HDL-cholesterol by 0.10mmol/L (*P* <0.01).

2.7.3.2.5 Sensitivity analyses and subgroups analyses

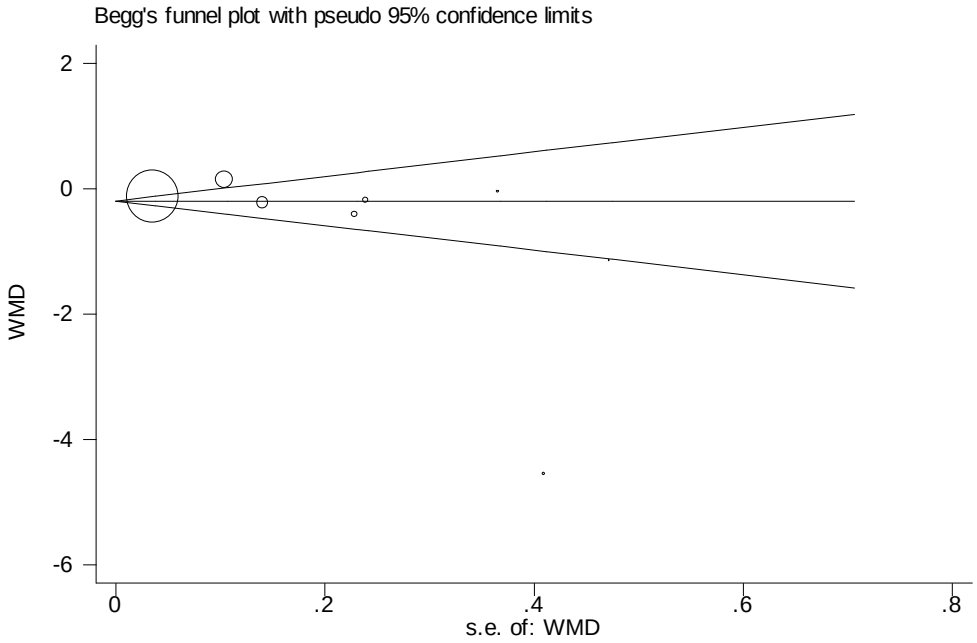
In sensitivity analyses, after systematically removing individual studies the beneficial pooled effects of buckwheat consumption on total cholesterol concentration were retained. However, the effect on triglycerides was no longer significant after removal of the study that had the largest effect on the overall result (Huang *et al.*, 2009). In contrast, the effect on LDL-cholesterol became statistical significant after the study that had the largest negative effects on overall result was excluded (Bijlani *et al.*, 1985) (12 weeks). No effects on glucose and HDL-cholesterol were observed when individual studies were removed (data not shown). Subgroup analyses were planned *a priori* to investigate

whether study duration, buckwheat dose, types of buckwheat and study design altered the effects of buckwheat on glucose and lipid profiles, but the ability to do this was effectively hindered by the small numbers of studies for each trial, and meta-regression requires ≥ 10 studies per factor examined (Higgins and Green, 2009).

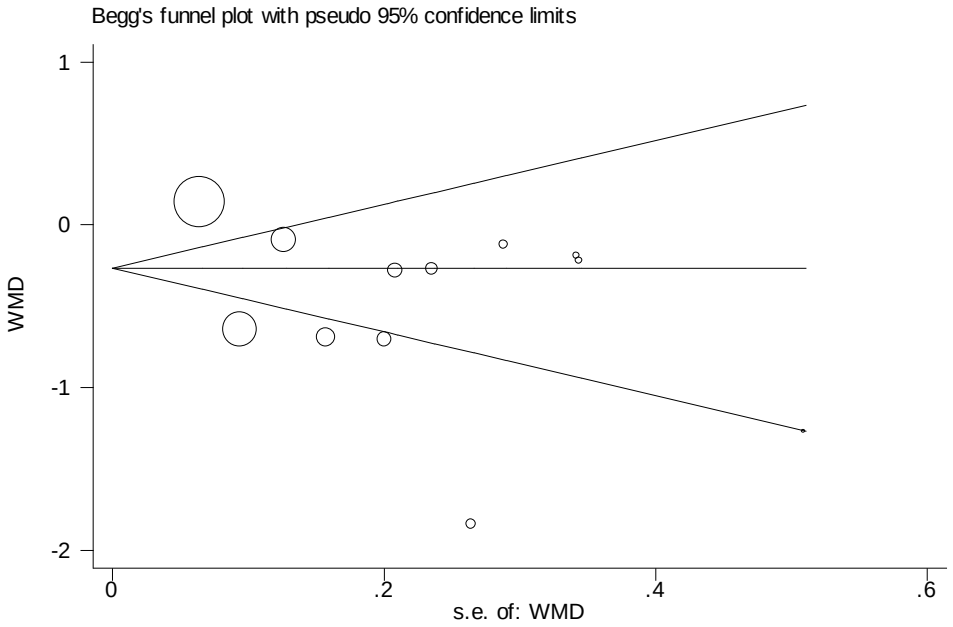
2.7.3.2.6 Publication bias

Funnel plot of the meta-analysis of the effect of buckwheat intake on glucose and lipid concentration were shown in Figure 2.14. Begg's test and Egger's test were not significant ($P > 0.05$), indicating that there was no evidence of publication bias.

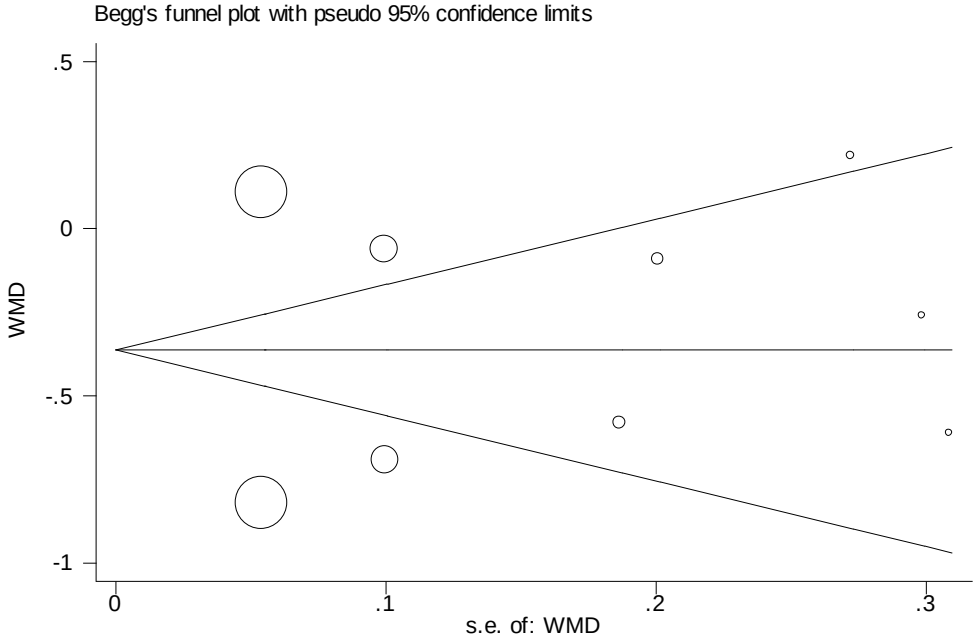
A. Glucose Begg's test $P = 0.058$, Egger's test $P = 0.130$



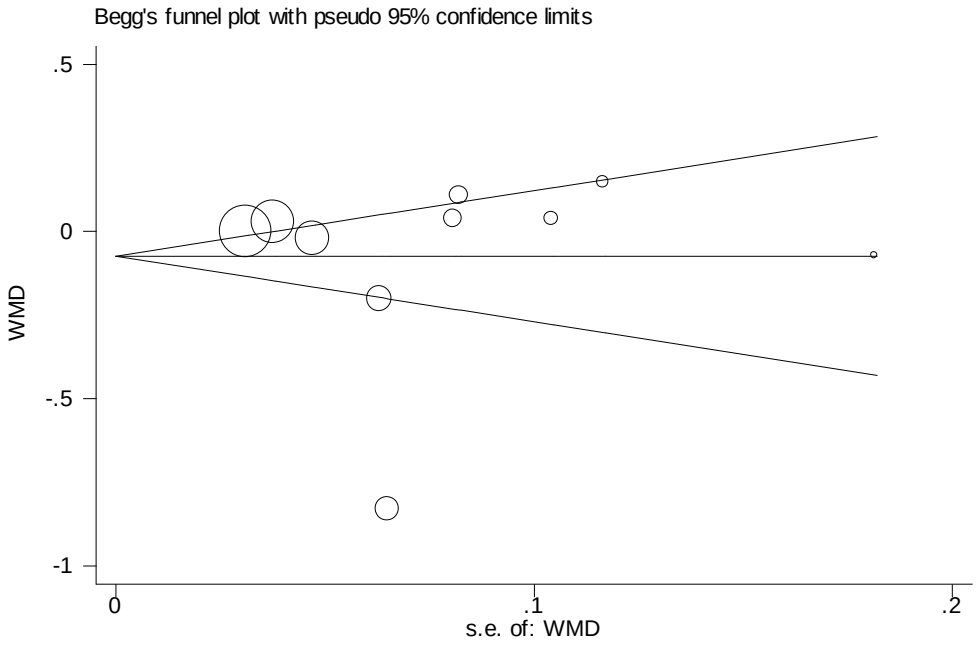
B. TC Begg's test *P* = 1.000, Egger's test *P* = 0.089



C. LDL Begg's test *P* = 1.000, Egger's test *P* = 0.891



D. HDL Begg's test *P* = 0.474, Egger's test *P* = 0.720



E. TG Begg's test *P* = 0.350, Egger's test *P* = 0.0.080

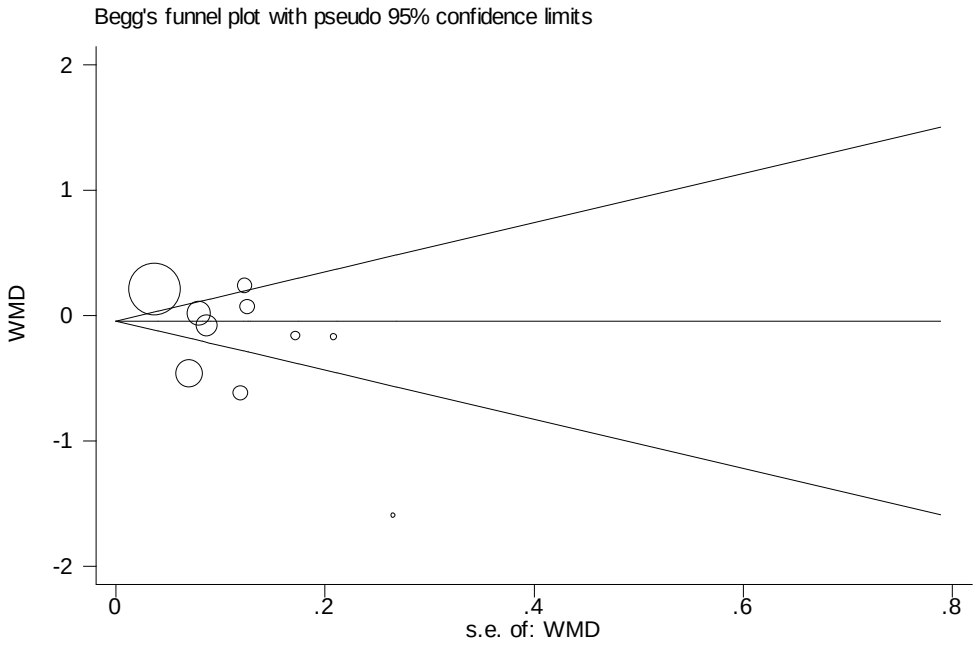


Figure 2.14. Publication bias funnel plots. Tests for publication bias of effects of buckwheat intake on (A) glucose and lipid profile (B, TC; C, LDL; D, HDL; E, TG). The dash lines represent pseudo-95% Cis. *P*-values are derived from quantitative assessment of publication bias by Begg's test and Egger's test.

2.7.3.3 Animal Studies

2.7.3.3.1 Effects on Weight Gain and Food Intake

This review contains 19 animal studies which reported the impact of buckwheat intake on body weight of which only four reported a significant decrease following buckwheat consumption, whereas one found a significant increase in body weight by 21.66% compared with the control (Orzel *et al.*, 2015). With respect to the amounts of food consumed by the animals, food intake did not change significantly compared with that of the control group in 12 out of 13 studies, while a marked increase in food intake was observed in the study by Tomotake *et al*. (2000).

2.7.3.3.2 Effects on Blood Glucose and Insulin

For the studies reported here, three out of seven studies showed a significant reduction in glucose concentration by between 15.20% and 18.44%, with the remaining studies showing that glucose concentration was not affected significantly by buckwheat treatment. With respect to blood insulin, insulin immunoreactivity was enhanced in one study, while a significant reduction in insulin concentration was observed in another study, and the two remaining studies found no significant changes.

2.7.3.3.3 Effects on lipid Profile

Of the twenty-one animal studies reported here, all investigated the impact of buckwheat intake on total cholesterol and seven reported results for LDL-cholesterol. Nineteen (90.5%) of the studies observed a significant reduction in total cholesterol and five (71.4%) of the studies observed a significant reduction in LDL cholesterol; the remainder identified no significant response. The significant decrease ranged from 11.71% to 54.05% for total cholesterol and from 16.20% to 57.75% for LDL-cholesterol. HDL cholesterol level increased from 19.61% to 54.55 in four out of fourteen studies that reported this biomarker, while the level decreased (by between 11.52% and 28.37%) in another four studies. Of twenty animal studies analysing the effect on triglycerides, all studies reported that intake of buckwheat consumption resulted in a fall in the serum concentration of triglycerides, which fell significantly ($p<0.05$) from 2.27% to 73.85% in fourteen of the twenty studies.

2.7.3.3.4 Other Outcomes

The liver weight of animals in this review fed buckwheat food decreased significantly from 8.49 % to 19.15% relative to the comparison group in eight out of eleven studies, while only one showed a significant increase by 5.42%. Eight of eleven studies found a reduction in liver total cholesterol content ($p<0.05$), but no significant changes were detected in the other three studies. There were

significant increase in faecal weight and faecal neutral steroids by 57.58-170.97% and by 68.75-142.37% in five out of seven studies and all seven studies, respectively.

2.7.4 Discussion

2.7.4.1 Effects on Body Weight

Being overweight brings about an elevated risk of health problems such as insulin resistance, type 2 diabetes mellitus, hypertension, hyperlipidemia and cardiovascular disease (Hill and Peters, 1998; Williams, 1999; Goldberg *et al.*, 2000; Kahn and Flier, 2000). In order to evaluate the impact of buckwheat intake on body weight, the overall energy and macronutrient content in diets offered/consumed should be considered, but this was beyond the scope of this study. However, as mentioned above, there were few human and animal studies showing a significant reduction in body weight gain compared with baseline or control in response to consuming buckwheat-based food(s); restricted energy intake or intention to lose weight was not an intention of the studies reported.

Even though a significant reduction was observed in the study of Liu *et al*. (1996), it must be noted that the participants involved in the study were overweight, and so body weight loss would not have been unexpected in an intervention study simply by engaging in a dietary intervention study. Thus, on the basis of the published literature, it seems that the beneficial effects of buckwheat intake were not associated with weight loss, and this lack of association was consistent in both humans and animals with a variety of dietary levels of buckwheat or various forms of buckwheat products provided.

In this review, meta-analysis for body weight or BMI were not conducted, because five out of seven human intervention studies used 'body weight' (no numerical values and/or figures in two studies), but the rest two studies for 'BMI', which were not consistent to do a meta-analysis. Even if some missing information was obtained from authors after contacting them, the overall data still remained to be incomplete.

2.7.4.2 Effects on Blood Pressure

It is well known that hypertension is considered to be an important CVD risk factor, since half of ischemic heart disease and 60% of strokes cases are attributable to increased blood pressure (Lewington *et al.*, 2002; Banach and Aronow, 2012). In a previous study, Tighe *et al*. (2010) revealed that 12 weeks intervention with whole grain (oats or oats plus with wheat) significantly lowered systolic blood pressure compared with a refined cereals group. The effects of whole grain cereals on blood pressure, however, are inconsistent in comparison with observational data as reported by Seal and Brownlee (2015) and the paper from Tighe *et al*. (2010) is the only one to report a reduced

blood pressure in a whole grain intervention that was not based on weight loss. A significant reduction in blood pressure was only observed in one of the human studies reported here conducted by He *et al*. (1995); these authors pointed out that water-soluble fibre, but not total dietary fibre, was independently associated with blood pressure and so an effect of buckwheat which has higher levels of soluble fibre than insoluble fibre is a possibility. However, given the small number of studies carried out to date, this review is not adequately powered to conclude whether or not there are beneficial effects of buckwheat intake on blood pressure.

2.7.4.3 Effects on Blood Glucose and Insulin

Hyperglycaemia and insulin resistance are closely correlated to risk of developing CVD (Coutinho *et al.*, 1999a; Uwaifo and Ratner, 2003). There is considerable evidence showing that whole grain intake is associated with decreased glucose concentrations and is inversely associated with insulin resistance suggesting that it is possible to regulate glucose and insulin homeostasis by cereal foods and their constituents (Hallfrisch and Behall, 2000; Liese *et al.*, 2003; Steffen *et al.*, 2003). Buckwheat is regarded as a low glycaemic index (GI) food, and it has been demonstrated that low-GI diets significantly improved lipid profiles in medium and long-term treatments, particularly with respect to decreasing both total and LDL cholesterol concentrations (Sloth *et al.*, 2004; De Rougemont *et al.*, 2007; Tovar *et al.*, 2014). The results of animal studies with regard to the impact of buckwheat intake on glucose concentration, however, are conflicting, suggesting that results from animal studies do not strongly support the beneficial effects. In contrast, the meta-analysis of 9 clinical trials indicated that diets supplemented with buckwheat were associated with a significant 0.85mmol/L decrease in blood glucose concentration ($p<0.001$). Of the many possible mechanisms in modulating blood glucose concentrations, buckwheat is well known for containing various bioactive phytochemicals (such as various polyphenols and d-chiro-inositol), which have been shown to positively affect either glucose or insulin metabolism in animal models (Fonteles *et al.*, 2000; Johnston *et al.*, 2005; Kwon *et al.*, 2007; Zhang *et al.*, 2012). In addition, Skrabanja *et al*. (2001) showed that the presence of resistant starch in buckwheat and buckwheat products contributed to its low glycaemic index. As for blood insulin, both human and animal studies yielded inconsistent results for the association between buckwheat intake and fasting blood insulin concentrations, indicating that there is no support for a beneficial effect of buckwheat on blood insulin or insulin-mediated glucose responses.

2.7.4.4 Effects on lipid Profile

Cholesterol, produced in the liver and absorbed though the diet, is essential for all animal life in normal metabolic process. However, observational epidemiologic studies reports that risk of heart

attack in subjects with hyperlipidemia is 3 times higher than those in general population with normal lipid status, while a 1% reduction in serum total cholesterol is strongly correlated with a 3% decrease in CVD risk (Yusuf *et al.*, 2004; Lloyd-Jones *et al.*, 2010). Thus, treatments which are aimed at reducing cholesterol concentrations are effective in decreasing death risk from stroke and coronary heart disease. Consistent with two cross-sectional studies, this meta-analysis of the RCT studies indicated that increased intake of buckwheat-based products from 7 days to 27 weeks significantly improved an individual's lipid profile, on average, decreasing total cholesterol by 0.50 moms/L and triglycerides by 0.25 mmol/L. Moreover, the beneficial effects seen in human studies were also supported by strong evidence from animal studies. Even though the change in LDL-cholesterol concentration was not statistically different (p=0.061), the data approached statistical significance, and the mean reduction was 0.33 mmol/L, and significant decreases were also observed in two cross-sectional studies. It has been well known that a 1 mmol/L reduction of LDL-cholesterol lowers the morbidity and mortality of CVD patients by 22% (Cholesterol Treatment Trialists Collaboration, 2010), so a reduction of this magnitude could have significant clinical effects. No effects of HDL-cholesterol were detected in the meta-analysis of RCT studies for buckwheat intake, in combination with inconsistent results from animal studies. The results of the meta-analysis were seen in both healthy and ''at risk'' subjects, but it is not possible within this review to examine differences in response between healthy and ''at risk'' subjects because of lack of power and the limited number of studies available. Nevertheless, it should be noted that the meta-analysis of Ripsin *et al*. (1992) which investigated the effect of oats and oat-based products on lipid biomarkers, demonstrated that greater reductions were observed in studies where subjects initially had higher total cholesterol concentrations (>5.9 mmol/L). Thus, there was an indication that observed effects were generally more marked in subjects with higher CVD risk.

It should be noted that both LDL and HDL cholesterol concentrations were significantly decreased after intake of Tartary buckwheat mixture for 2 months in the study reported by Huang *et al*. (2009). However, the clinical importance of cholesterol reduction can also be seen in the ratio of LDL and HDL cholesterol concentrations, which did not significantly change during the treatment period. HDL cholesterol concentrations, known as 'good cholesterol', are a strong, independent inverse predictor of CVD (Sharrett *et al.*, 2001; Curb *et al.*, 2004). Surprisingly, this relationship was also detected among patients even if whose LDL cholesterol concentrations were substantially decreased below 1.81 mmol/L, indicating that HDL cholesterol still play a protective role against CVD despite the low LDL cholesterol concentration (Barter *et al.*, 2007). In other words, HDL cholesterol concentrations below normal range is not a good sign in people with the LDL cholesterol at low levels.

2.7.4.5 Buckwheat Intake levels

Any evaluation of health benefits associating with food products should include an attempt to define optimal amounts for human consumption. The study of Liu *et al*. (1996), described in Table 2.7, showed that 40 g/day Tartary buckwheat flour for 4 weeks significantly lowered total cholesterol, LDL cholesterol and triglycerides concentrations compared with baseline. The dose needed to reach a significant effect was similar to that of large population-based study by He *et al*. (1995), who found that buckwheat intake (≥ 40 g/day) was inversely related to markedly lower lipid profiles in comparison with those who consumed less than 40 g buckwheat/day. Stringer *et al*. (2013) found that a higher amount of buckwheat cracker (containing buckwheat 76g/day) for a shorter time period (7 days) did not significantly affect lipid profiles when compared with baseline, and similar results were also observed in studies with longer intervention periods (4 and 12 weeks) by Bijlani *et al*. (1984) and Bijlani *et al*. (1985). Studies showing specific amount of buckwheat used are scarce, and more well designed dose-response studies are required to confirm the minimum amounts of buckwheat needed to have a beneficial effect.

2.7.4.6 Bioactive compounds responsible for lipid-lowering activity

The lipid-lowering activity of buckwheat has been ascribed to its nutritional composition including soluble fibre, protein, rutin and quercetin. However, due to complexity of this composition, it is difficult to explore potential mechanisms underlying the beneficial effect of buckwheat on CVD risk. Some have been proposed but not fully explained, and it is possible that a combination of these components have contributed to the effects, instead of a single factor. As remarked previously, buckwheat is a good source of dietary fibre (5-11%), particularly the soluble fraction, which may help lower total cholesterol concentrations in the body (Bonafaccia *et al.*, 2003; Christa and Soral-Smietana, 2008; Dziedzic *et al.*, 2010). The cross-sectional study by He *et al*. (1995) demonstrated that both total dietary and water-soluble fibre from buckwheat were significantly and independently correlated with lower serum total cholesterol concentrations, even though the average cholesterol concentration was low in the study population. This result was in agreement with that of Son *et al*. (2008) showing a similar correlation between water-soluble fibre and serum total cholesterol. The cholesterol-lowering effects of soluble fibre may be accounted for several mechanisms. It has been proposed that soluble fibre binds strongly to bile acids in the small intestine and elevates faecal bile acids excretion. The loss of bile acids in the stool stimulates the liver to increase cholesterol uptake from the circulation to replenish the bile acid supply. It also lowers the availability of bile acids for optimal fat digestion and absorption (Gordon *et al.*, 1977; Judd and Truswell, 1981; Story, 1985; Shinnick *et al.*, 1990). In addition, soluble fibre delays gastric emptying, slowing access of nutrients to digestive enzymes and to absorptive surfaces of the small intestine (Anderson and Siesel, 1990).

In addition, There is also emerging evidence that soluble fibre and resistant starch are additionally fermented by some bacteria in the colon, producing short-chain fatty acids (SCFA) perhaps via the inhibition of hepatic cholesterol synthesis in the liver, which helps to lower cholesterol concentrations (Slavin *et al.*, 1999; Escudero *et al.*, 2006). One other mechanism that contributes to the cholesterol-lowering effects may be due to the low glycaemic index of buckwheat in humans with the presence of resistance starch in the cereal (Liu *et al.*, 2001; Skrabanja *et al.*, 2001). However, the hypocholesterolaemic effect of buckwheat starch, which was extracted from buckwheat flour, was not detected in rats when compared with corn starch (Tomotake *et al.*, 2000).

It has been generally recognised that plant proteins may reduce plasma cholesterol concentrations, and the underling mechanisms of the cholesterol-lowering properties of plant proteins have been extensively analysed (Carroll and Hamilton, 1975; Carroll, 1982; Terpstra *et al.*, 1983). However, in most studies the effect of plant dietary proteins has focused on soybean protein, leading to limited information on the influence of other plant proteins and buckwheat proteins specifically on cholesterol metabolism. Despite having a relatively low digestibility, buckwheat protein, which accounts for 10% to 12.5% of flour weight, is an excellent supplement to other common grains, as it contains a good balance of amino acids with high nutritional value (Pomeranz and Robbins, 1972; Pomeranz, 1983; Ikeda and Kishida, 1993; Li and Zhang, 2001). Previous studies have demonstrated a potent hypocholesterolaemic activity of isolated buckwheat protein products prepared from buckwheat flour in rats or hamsters fed cholesterol-enriched or cholesterol free diets, which appeared to be stronger than that of soy protein isolate (Kayashita *et al.*, 1995a; Kayashita *et al.*, 1995b; Kayashita *et al.*, 1996; Kayashita *et al.*, 1997; Tomotake *et al.*, 2000; Tomotake *et al.*, 2001; Tomotake *et al.*, 2007). In one study by Kayashita *et al*. (1997) further suggested that suppressive effects on cholesterol were mediated by enhanced excretion of faecal neutral sterols and that lower digestibility of buckwheat protein products is at least in part responsible for the effect. The lower digestibility may result in lower gastrointestinal transit time, which in turn leads to a higher stool weight and greater faecal excretion of neutral sterols. It has been observed that faecal excretion of neutral sterols was inversely correlated with serum cholesterol ($r=-0.83$, $P<0.01$) (Tomotake *et al.*, 2007). Taken together, these impacts on rats appear to be similar to the properties of dietary fibre in humans (Kritchevsky, 1988; Eastwood, 1992). To demonstrate this, Kayashita *et al*. (1997) also performed another experiment showing that plasma cholesterol in rats fed intact buckwheat protein products for two weeks was significantly lower than that in rats fed trypsin-digested protein. Moreover, this hypothesis has been confirmed in human body that the digestibility of buckwheat seed proteins was relatively low, owing possibly to the existence of phytic acid, tannins and protease inhibitors (Yiming *et al.*, 2015). However, this seemed to contrast with the results reported by

Tomotake *et al*. (2007) that Tartary buckwheat had a reduced cholesterol-lowering impact on rats compared with common buckwheat, even though digestibility of Tartary buckwheat was lower than that of common buckwheat. It is noteworthy that humans digestion is hugely different from that of rodents, such as rat and hamster, indicating that these results are needed to explain with caution and more studies are required to answer this question (Kararli, 1995). In addition, the strong suppression of cholesterol by buckwheat protein products could be ascribed to its effect on higher bile acid synthesis, and also a greater excretion of faecal bile acids observed in rats, with the possibility that buckwheat protein products could possess some bile acid-binding proteins (Tomotake *et al.*, 2000; Tomotake *et al.*, 2001). It has been further demonstrated *in vitro* that digestion-resistant peptides were largely responsible for bile acid binding activity of buckwheat protein digests and bile acid elimination (Ma and Xiong, 2009; Zhou *et al.*, 2013). In consistent with this, Zhang *et al*. (2017) very recently further suggested that Tartary buckwheat protein was one of the active ingredients to decrease plasma total cholesterol concentration, mainly regulated by improving the excretion of bile acids by its effects on gene expression of hepatic CYP7A1 in a uptrend, but also preventing absorption of dietary cholesterol by its effects on gene expression of intestinal Niemann-Pick C1-like protein 1 (NPC1L1), acyl CoA:cholesterol acyltransferase 2 (ACAT2), and ATP binding cassette transporters 5 and 8 (ABCG5/8) in a downtrend. Moreover, the composition of amino acids in dietary proteins might be another important factor influencing blood cholesterol concentration, especially the ratio of lysine to arginine, which is even lower in buckwheat protein than that of soy protein (Kayashita *et al.*, 1995a). Thus, it has been speculated that cholesterol-lowering effect of buckwheat protein products observed may be ascribed to lower lysine: arginine ratio (Kayashita *et al.*, 1995a). However, this hypothesis did not support the results from Kayashita *et al*. (1997) that plasma cholesterol was unaffected with the addition of arginine in the diets.

It is well known that Tartary buckwheat seeds are a major source of rutin and quercetin (Holasova *et al.*, 2002). Minor amounts of quercetin identified in Tartary buckwheat seeds are the results of rutin degradation (Fabjan *et al.*, 2003; Vogrincic *et al.*, 2010). The possibility of buckwheat rutin being one of the active components responsible for suppressive effect on cholesterol concentrations cannot be eliminated. Rutin has been shown to prevent the increase of plasma total cholesterol and non-HDL cholesterol in rats or mice fed with a high cholesterol or high fat diet (Park *et al.*, 2002; Choi *et al.*, 2006; Kuwabara, 2007; Panchal *et al.*, 2011; Qu *et al.*, 2013). However, in contrast to the results with rats and mice, serum total cholesterol concentrations in day-care staff were found to be lower in response to consuming cookies prepared from common or Tartary buckwheat, but no significant differences were detected between two buckwheat groups, even though the rutin content in Tartary

buckwheat seed was much higher than that in common buckwheat (Kitabayashi H, 1995; Wieslander *et al.*, 2011). It has also been suggested that quercetin may be a contributor to the cholesterol-lowering effects seen in animal models. In animal models (rat, rabbit, and mice) fed a high-cholesterol or high-fat diet, diets with addition of pure quercetin compounds has been shown to lower serum total cholesterol concentration (Igarashi and Ohmuma, 1995; Juzwiak *et al.*, 2005; Odbayar *et al.*, 2006). However, the results regarding the effects of quercetin on cholesterol concentrations are controversial; several studies have reported that quercetin intake had no significant beneficial effects on total, LDL or HDL cholesterol and triglycerides (Yugarani *et al.*, 1992; Hayek *et al.*, 1997; Lauridsen and Mortensen, 1999; Enkhmaa, 2005). The underling mechanisms of the quercetin on lipid metabolisms may be accounted for the inhibition of cholesterol synthesis in hepatocytes and also the enzyme myeloperoxidase which was shown to oxidize lipoproteins (Pincemail *et al.*, 1988; Glasser *et al.*, 2002; Nicholls and Hazen, 2009).

2.7.4.7 Sensitivity analysis

In the sensitivity analyzes, removing individual studies systematically retained the statistical significance of the effects of buckwheat on total cholesterol, supporting the stability of the observed effects, but the effect on triglycerides was no longer significant possibly due to reduced statistical power. This finding was relatively not stable to sensitivity analyze in which individuals studies were removed, thus, such analyses should be interpreted with more caution.

2.7.4.8 Limitations

Several limitations of this review should be noted. Firstly, relatively few long-term randomized and well-controlled human studies have directly investigated the effects of buckwheat intervention on risk markers for CVD, including weight gain, blood pressure, fasting blood glucose, insulin and lipids, and studies up to date have been of short duration with small sample sizes. In order to support the effects, further more large-scale human intervention studies for long-term are required. Secondly, most animal studies performed to date, have analyzed the effect of individual molecular components or various buckwheat extracts on cell lines and animal models. However, human beings consume entire buckwheat seeds (as flour in products) instead of individual extracts, producing the uncertainty whether the efficacy can be extrapolated to human health without further evaluation. Finally, the bioactive compounds responsible for buckwheat's cardiovascular health still remain uncertain, and the mechanisms underlying the effects were not fully elucidated.

2.7.5 Conclusion

In conclusion, even though the literature to date is limited and often inconsistent in study results, this review suggests that increased intake of buckwheat may lower CVD risk markers, including glucose, total cholesterol and triglycerides. Therefore, buckwheat, being a gluten-free alternative to some common whole grains or refined grains, such as wheat, barley and rye, deserves to be a part of our daily diet. However, it still remains unclear whether increased intake of buckwheat has significant impacts on some CVD risk markers like body weight and LDL cholesterol. There is increasing evidence that several lower risk markers associated with CVD could be due to polyphenol, soluble fibre, protein, rutin, quercetin and other components in the buckwheat, but is has not been fully elucidated which bioactive compounds are responsible for the underlying effects. Further research, especially large, well-powered, long-term human intervention studies, are required to further understand and promote the role that buckwheat seeds can play in cardiovascular health.

2.8 Impact of whole grains on the gut microbiota: a systematic literature review

2.8.1 Introduction

Whole grain intake is associated with beneficial health effects and epidemiological studies have consistently shown that diets rich in whole grain foods reduce the risk of many lifestyle-related diseases that plague modern society, such as cardiovascular diseases, diabetes, obesity, the metabolic syndrome and some cancers (He *et al.*, 1995; Chatenoud *et al.*, 1998; Jacobs *et al.*, 1998; Liu *et al.*, 1999b; Jacobs *et al.*, 2000; Mellen *et al.*, 2008a; O'Neil *et al.*, 2010; Ye *et al.*, 2012; Aune *et al.*, 2016; Chen *et al.*, 2016). The observed associations with reduced disease risk have been hypothesized to be due to the modulation of gut microbiota which have co-evolved with the human colon. Whole grains are good source of dietary fibres and other bioactive compounds that may modulate the gut microbiota, thereby conferring benefits to the host' health (Slavin, 2003; Costabile *et al.*, 2008; Carvalho-Wells *et al.*, 2010; Fardet, 2010).

The gut microbiota, which has attracted much attention, plays an important, but generally less well understood, role in health and disease in humans; indeed, it is sometimes referred to our ''forgotten organ'' (O'Hara and Shanahan, 2006). A major function of the gut microbiota is to digest food compounds that are not degraded by human gastrointestinal enzymes. In this manner, compounds like complex polysaccharides and some starches selectively stimulate the growth and/or activity of specific groups of naturally colonizing bacteria, including species now generally seen as beneficial for human health like bifidobacteria and lactobacilli, which in turn provide degradation products for subsequent absorption. Results of a former study in mice indicate that in this way changes in microbiota composition contribute to a higher energy yield, weight gain, and possibly obesity (Turnbaugh *et al.*, 2006a). For example, short-chain fatty acids (SCFA) are derived from the fermentation of fibre, enterodiol and enterolactone from lignans, all of which have been associated with anti-inflammatory, anticancer and other protective effects (Beards *et al.*, 2010; Oozeer *et al.*, 2010).

Currently, some human intervention studies have begun to concentrate on the relationship between whole grains intake and gut microbial species composition and relative abundance, but the number is limited. What is more, results from these studies conducted in humans are conflicting in some cases. Thus, a clear and direct correlation between consumption of whole grain and health benefit cannot be established. Systematic reviews can give us a wider perspective, but also an evaluation of the validity of the methods of the study and the results that can point the direction for future

research. This review aims to present a comprehensive review and summary of the up-to-date evidence from the recent human intervention studies for exploring the effect of whole grains, either as a single cereal grain or as mixed whole grains, on gut microbiota composition and populations, and the mechanisms behind the beneficial effects.

2.8.2 Methods of study search and selection

2.8.2.1 Data sources and literature search

A comprehensive literature search for human studies that had investigated the relationship between whole grain consumption and human gut microbiota between 1960 and 2017 was conducted. Fig. 11 shows how relevant articles were selected. PubMed, Ovid, Scopus, Web of Science, Compendex, JSTOR, EBSCO, Medline and ProQuest databases were searched using the search terms 'whole grains' and 'gut microbiota' OR 'intestinal flora' AND 'human', and the same terms were applied in each database during the search phase. Whole grains were defined to encompass wheat, corn, rice, maize, oat, barley, sorghum and other cereals, as well as pseudo-cereals. In addition, the reference lists of retrieved articles were searched manually for all additional potentially relevant articles. The search was limited to studies on humans and included those that were written in different languages such as English and Chinese.

2.8.2.2 Study selection

The studies were included in this review satisfied the following criteria: *1)* study in humans, *2)* whole grain-consumption exposure, *3)* the outcomes included any changes in intestinal bacterial diversity, relative abundance or population, such as bifidobacteria and lactobacilli. The eligibility criteria were set before the start of the research.

2.8.2.3 Data extraction and quality assessment

The following data were extracted from each human study: lead author, year of publication, number of subjects, age range, BMI range, study design, methods (technique used) and outcomes. Extracted human data are shown as the column headings of Tables 2.10. Missing data are reported as ''Not stated'' if they were not explained in the corresponding articles. The sample size reported in Tables 2.10 was the overall total for the experiment rather than restricting to either control or intervention diet/s. The methods of quality assessment for studies included in this review were as described in Section 2.5, and the quality scores of the studies included in this review were also listed in Tables 2.10.

Data were extracted by a single reviewer.

2.8.3 Whole grain consumption and changes in gut microbiota

The systematic search of the scientific databases resulted in the initial identification of 139 articles for further evaluation. After removing duplicate articles (34) and articles that did not meet the eligibility criteria (104), a total of 11 articles were included in the review. Manual searching of the reference list of the relevant articles yielded 36 additional articles. After applying the inclusion criteria, four of these articles were considered fit to include. Consequently, the combination of electronic and manual reaching resulted in 15 articles which are included in this final review (Figure 2.15). Four studies were carried out in UK, three in USA and Italy, two in Switzerland and one each in Denmark, Finland and Germany.

All studies in the review were randomized controlled human intervention studies, with follow-up durations ranging from 2 to 12 weeks. Overall, a single grain or mixed whole grains consumption ranged from 45 g to 207 g of whole grain ingredients (median levels of individual series), with one and two studies the type of whole grains and the amounts consumed unstated, respectively (Foerster *et al.*, 2014; Cooper *et al.*, 2017). Participants recruited in these studies were all healthy, except for the overweight or obese subjects in one study by Christensen *et al*. (2013) and subjects with metabolic syndrome in two studies by Lappi *et al*. (2013) and Connolly *et al*. (2016). Of the 15 studies, 7 were randomized, controlled crossover studies and 7 were parallel studies; one study did not have a control group (De Angelis *et al.*, 2015).

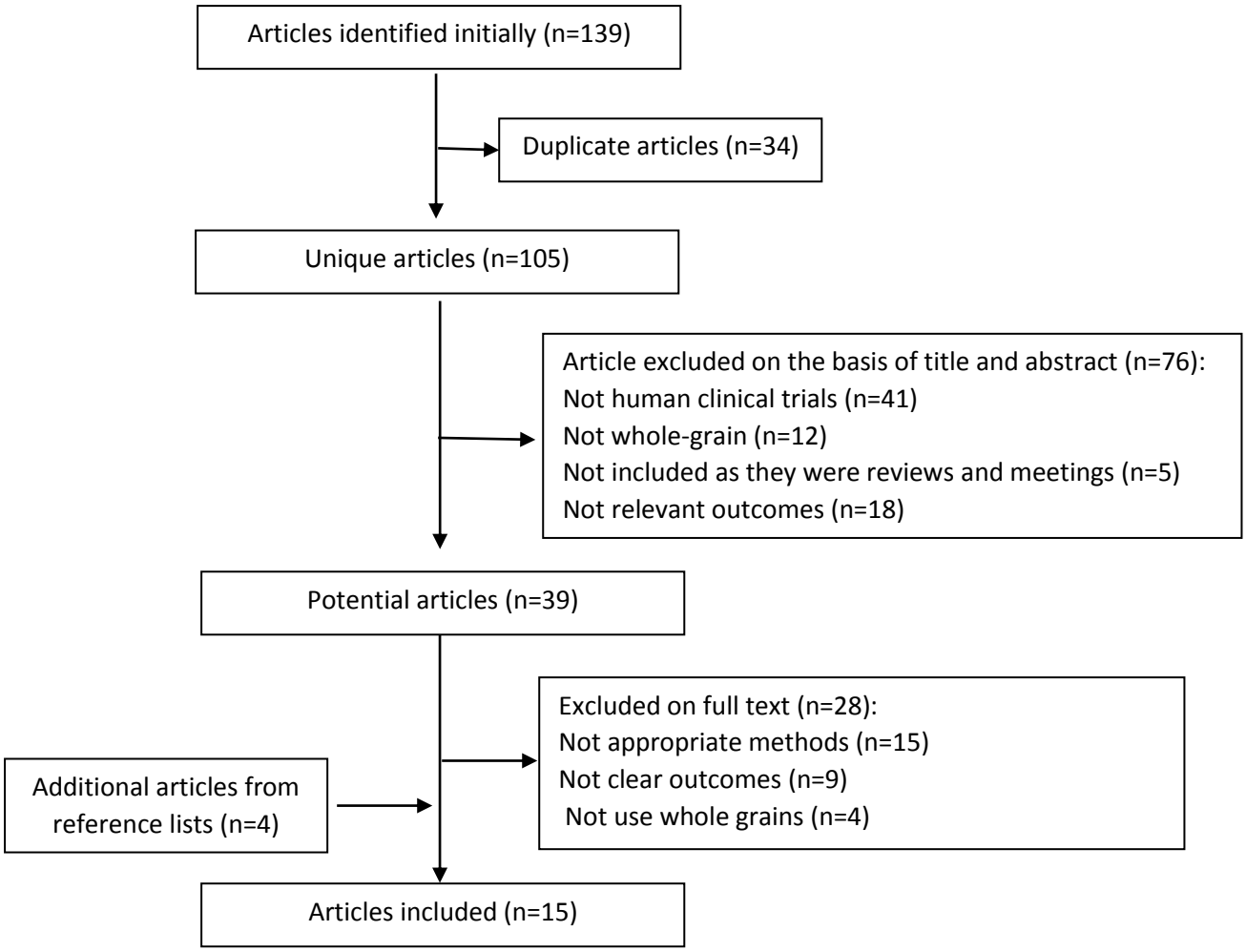


Figure 2.15. Flow diagram of article selection

Table 2.10. Summary of whole grain interventions assessing gut microbiota

Reference	Grain	Subjects	Study design	Methods (Technique used)	Outcomes	Quality score
Ross *et al*. (2011)	Mixed whole grains	6 males and 11 females Age (years) 20-50 BMI (kg/m^2) 19-28	Randomized crossover study 2 arms: ● Whole grains diet 150 g/d dry weight (64% whole grain wheat, 14% barley and rye, 13% WG oats and 9% brown rice) ● Refined grain diet (66% refined wheat, 27% white rice, 8% refined maize) 2 weeks per arm	Quantitative PCR targeting: total bacteria, *Bacteroides*, *Bifidobacterium*, *Clostridium coccoides*, *Clostridium leptum*, *Enterobacteria*, *Enterococcus* and *Lactobacillus* groups	- No overall change in the faecal microbiota population was observed, except for *Clostridium leptum* group, which was slightly higher after the whole grain diet (p=0.02), along with a tendency towards an increase in *Enterococcus* spp. (p=0.06) compared with the refined grain diet.	4
Langkamp-Henken *et al*. (2012)	Mixed whole grains	48 males and 35 females Age (years) 11-15 (Mean: 12.7 ± 0.1) BMI: not stated	Randomized, controlled, parallel-arm study 2 groups ● Whole grain foods (wheat-based foods, also included with oats, rice and corn) ● Refined grain foods (wheat-based foods also included with oats, rice and corn) 80 g for 6 weeks per group	qPCR and Pyrosequencing (only bifidobacteria and lactic acid bacteria)	- There were no significant differences in community diversity measured by Shannon-Weiner and Simpson diversity indices. - Both faecal bifidobacteria and lactic acid bacteria did not significantly differ between groups. - Faecal bifidobacteria increased significantly with both groups, but lactic acid bacteria increased significantly only in the whole grain group compared to baseline.	4
Ampatzoglou *et al*. (2015)	Mixed whole grains	12 males and 21 females, Age (years) 40-65 (Mean: 48.8 ± 1.1) BMI (kg/m^2) 20-35 (Mean: 27.9 ± 0.7)	Randomised, crossover study 2 arms: ● Diet high in whole grains mainly wheat, also included oats, rice, rye, corn and barley (>80 g/d) ● Diet low in whole grains (<16 g/d) 6 weeks per arm	FISH targeting: *Clostridium coccoides /Eubacterium rectale* group, *Bifidobacterium genus*, *Lactobacillus-Enterococcus* group, *Bacteroides-Prevotella* group, *Clostridium histolyticum* group and *Escherichia coli*	- There were no effects of whole grain consumption on the composition of gut microbiota.	3

Copper *et al*. (2017)	Mixed whole grains	21 males and 25 females Age (years) 19-46 (Mean: 25.5 ± 0.9) BMI (kg/m^2) 20-28 (Mean: 23.4 ± 0.6)	Randomised, parallel-group study 2 groups ● Whole grain wheat (75%), corn (15%) and rice (10%) ● Refined grain wheat (75%), corn (15%) and rice (10%) Amounts not stated, 6 weeks per group	Miseq sequencing (Illumina)	- No significant difference in the relative abundance of any particular taxa was observed between and within two groups, although, abundance of order Erysipelotrichales was increased after intake of refined grains (p=0.023).	4
Vanegas *et al*. (2017)	Mixed whole grains	49 males and 32 females Age (years) 40-65 BMI (kg/m^2) 20-34	Randomized, controlled, parallel study 2 groups ● Whole grain group (207 ± 39 g whole grains mainly wheat and 40 ± 5 g fibre) ● Refined grain group (0 g whole grain and 21 ± 3 g fibre) 6 weeks per group	Miseq sequencing (Illumina)	- The bacterial α and β diversity were not significantly different between groups. - There were no differences between and within groups when the relative abundance at the phyla and genera levels were compared, with the exception of a significant increase in abundance of *Lachnospira* in the whole grain group than in the refined grain group.	4
Costabile *et al*. (2008)	Wheat	15 males and 16 females Age (years) 20-42 (Mean 25) BMI (kg/m^2) 20-30	Double-blind, randomised, crossover study. 2 arms: ● Whole wheat breakfast cereal ● Wheat bran breakfast cereal 48 g/d for 3 weeks per arm	FISH targeting: *Atopobium* group, *Bacteroides* spp., *Bifidobacterium* spp., *Eubacterium rectale* group, *Clostridium histolyticum* group and *Lactobacillus*/*Enterococcus*	- Population of faecal Bifidobacterium spp., and lactobacilli/enterococci were significantly increased upon whole grain.	5
Christensen *et al*. (2013)	Wheat	72 post-menopausal females Age (years) 45-70 BMI (kg/m^2) 27-37	Open label parallel study 2 groups: ● Energy-redistricted diet with whole wheat bread, pasta and biscuits ● Energy-redistricted refined diet with refined wheat bread, pasta and biscuits 105 g/d for 12 weeks each group	Quantitative PCR targeting: *Bacteroidetes*, *Firmicutes*, *Bacteroides* spp., *Prevotella* spp., *Lactobacillus* spp., *Enterobacteriaceae*, *Bifidobacterium* spp. *B. bifidum* *B. adolescentis* *B. catenulatum* group *B. longum*	- No significant differences in microbiota composition were detected between two dietary groups. - The whole wheat intervention increased the relative abundance of *Bifidobacterium* (p=0.04), and a decrease in abundance of *Bacteroides* was found in refined wheat group compared with baseline (p=0.04).	3

Saa *et al*. (2014)	Kamut® Khorasan and whole durum wheat	4 males and 26 females Age (years) 25-53 (Mean: 37 ± 7.3) BMI: not stated	Randomised, placebo-controlled, parallel-group study Two groups: ● Whole Kamut® Khorasan cereal-based foods ● Whole durum wheat cereal-based foods 118 g pasta and 88 g baked goods for 3 months per group	The ligase detection reaction–universal array (LDR–UA) platform High Taxonomic Fingerprint (HTF)-Microbi.Array	- There was no significant difference in the microbiota composition between and within dietary groups over time - A trend for a reduction in *Bacteroides*/*Prevotella* and an increase in members of *Clostridium* cluster XIVa was observed after intake of whole Kamut® Khorasan with respect to whole durum wheat cereals	2
Vitaglione *et al*. (2015)	Wheat	23 males and 45 females, Age (years) 19-67 (Mean: 38.6) BMI (kg/m^2) 25-34.9 (Mean:29.8)	Placebo-controlled, randomised, parallel-group study 2 groups: ● Whole grain wheat biscuits (70 g/d) ● Refined wheat crackers (33 g/d) and toasted bread (27 g/d) 8 weeks for per group	Miseq sequencing (Illumina)	- Microbial community structure of subjects consuming whole grain and control foods did not significantly differ, as shown by weighted and unweighted UniFrac phylogenetic metrics. - No significant variation in relative abundance of faecal microbiota was found in relation to treatments. - There was no significant change in whole grain group, excepting individual bacterial taxa, such as *Prevotella, Bifidobacterium*, *Dialister*.	5
Lappi *et al*. (2013)	Rye	25 males and 26 females, Age (years) 40-65 (Mean: 60 ± 6) BMI(kg/m^2): 26-39 (Mean: 31 ± 4)	Randomized, controlled, parallel study Two groups: ● Whole grain rye bread (92 g, a high-fibre content 7-15%), endosperm rye bread (60 g) and whole mean pasta (12 g) ● Refined wheat bread (188 g, a low fibre content 4%) 12 weeks per group	Quantitative qPCR targeting: 1033 distinct phylotypes based on the V1 and V6 hypervariable regions of the 16S rRNA	- The intestinal microbiota composition did not significantly differ between the groups after the intervention. - Within groups, the whole grain group did not change relative abundance of any bacterial taxa. However, a significant decrease of Bacteroidetes, including *Bacteroides vulgatus*, *B.plebeius*, and *Prevotella tannerae* was observed in the group consuming refined wheat bread, while that of bacteria related to *Collinsella* and members of the *Clostridium* clusters IV and XI slightly increased.	4

De Angelis *et al*. (2015)	Barley	11 males and 15 females Age (years) 28-57 (Mean: 39 ± 9) BMI (kg/m^2) 22.6 ± 3	Pre-post study One group: ● Pasta, a mixture of 75% durum wheat flour and 25% whole grain barley flour (minimum recommended daily intake of 3g barley β-glucans) 100 g/d for two months	Pyrosequencing of 16S rRNA gene	- The bacterial diversity measured by Chao 1 and Shannon index were not significantly different before and after 2 months of diet intervention. Also, in weighted and unweighted UniFrac distance principle coordinate plots, the three phylogeny-based β-diversity did not show a clear separation between the microbiota compositions of before and after samples - No significant differences in the relative abundance of the phyla were found in the faecal samples when subject were following the pasta, excepting the phylum Fusobacteria, which was significantly decreased after intervention.	1
Martínez *et al*. (2013a)	Barley and brown rice	11 males and 17 females Age (years) 25.9 ± 5.5 BMI (kg/m^2) 25.1 ± 4.5	Randomised, crossover study 3 arms: ● Whole grain barley flakes (18.7 g total dietary fibre) ● Whole grain brown rice flakes (4.4 g total dietary fibre) ● Equal mixture of the two whole grain barley and brown rice flakes (11.5 g total dietary fibre) 60 g/d for 4 weeks per arm	Pyrosequencing of amplicons by PCR targeting the V1–V3 region of the 16 S rRNA	- All three groups significantly increased the bacterial diversity measured by Shannon's and Simpson's indices (community evenness) but not by Chao1 (total species richness). - All three groups significantly increased and decreased the abundance of Firmicutes and Bacteroidetes compared with baseline, respectively.	1
Carvalho-Wells *et al*. (2010)	Maize	11 males and 21 females Age (years) 20-51 (Mean: 31.6 ± 8) BMI (kg/m^2) 20-30 (Mean: 23.3±0.58)	Double-blind, randomised, placebo-controlled, crossover study. 2 arms: ● Whole grain maize breakfast cereal (29.6% whole grain) ● Non-whole grain breakfast cereal 48g/d for 3 weeks per arm	FISH targeting: *Bacteroides* spp., *Bifidobacterium* spp., *Clostridium histolyticum*/*perfringens* subgroup, *Lactobacillus-Enterococcus* subgroup and total bacteria.	- A significant increase in faecal bifidobacteria was observed in whole grain maize group compared with control group. - With respect to baseline, increases in faecal bifidobacteria and *Atopobium* levels were observed in both groups, but only bifidobacteria in control group did not reach significance level.	5

Connolly *et al*. (2016)	Oat	11 males and 19 females Age (years) 19-60 (Mean: 42) BMI (kg/m^2) 18-30 (Mean: 26.4 ± 5.7)	Double-blind, randomised, controlled, crossover study. 2 arms: ● Whole grain oat Granola breakfast cereal ● Non-whole grain breakfast cereal 45 g/d for 6 weeks per arm	FISH targeting: *Bifidobacterium* spp., *Bacteroides/Prevotella* spp., *Lactobacillus/Enterococcus* spp., *Clostridium coccoides-Eubacterium rectale* group, *Clostridium histolyticum* group, and *Atopobium* cluster including most *Coriobacteriaceae* species	- Significant differences in bifidobacteria and total population at week 6 were observed between groups. - The numbers of faecal bifidobacteria, lactobacilli and total bacteria count significantly elevated compared with the respective baseline. Also, a significant decrease in bifidobacteria and total bacteria population in non-whole grain group were detected after the 6-week feeding time.	4
Foerster *et al*. (2014)	Not stated	10 males and 10 females Age (years) 20-60 (Mean: 40.1 ± 11.6) BMI (kg/m^2) 24.4 ± 2.9	Randomised, crossover study 2 arms: ● Whole grain products with low intake of red meat (amounts not stated, but offer approximately 40 g/d dietary fibre) ● Portions of red meat 200 g/d (fresh weight) with minimal amounts of dietary fibre 3 weeks per arm	PCR-DGGE	- The diet rich in whole grain products increased microbial diversity. - Compared with baseline, 8 bands changed in at least 4 subjects after intake of whole grain diet.	-1

Despite the substantial differences in some certain bacteria within groups, the relative abundance or numbers of faecal microbiota and bacterial diversity did not significantly differ between groups in the seven intervention studies that used wheat as the main source of whole grains or used wheat as the only whole grains, with the exception of individual bacterial taxa (Ross *et al.*, 2011; Langkamp-Henken *et al.*, 2012; Christensen *et al.*, 2013; Ampatzoglou *et al.*, 2015; Vitaglione *et al.*, 2015; Cooper *et al.*, 2017; Vanegas *et al.*, 2017). In agreement with this, inclusion of 152 g whole grain breads and fibre-rich rye bread in their diets for 12 weeks did not significantly change the microbiota composition with respect to refined white breads (Lappi *et al.*, 2013). Noteworthy, the preliminary data demonstrate that the microbiota composition of individuals with metabolic syndrome differed from that of healthy individuals (Munukka *et al.*, 2012). However, in two studies, significantly increased populations of bifidobacteria and lactobacilli in stool samples associated with intake of whole grain oat breakfast cereals (45 g/d) for 6 weeks, as well as total bacterial population relative to non-whole grain group have been reported, and also a bifidogenic effect of whole grain maize breakfast cereal (48 g/d) over 3 weeks has been reported ($p=0.001$) (Carvalho-Wells *et al.*, 2010; Connolly *et al.*, 2016). Interestingly, it has been reported that bifidobacteria levels and the number of bacterial groups returned to near baseline after 3 and 4 weeks, respectively (Carvalho-Wells *et al.*, 2010; Connolly *et al.*, 2016). It is noteworthy that the above mentioned studies all used refined grains as a control to see the effect of intake of various whole grains on human gut microbiota.

Due to the inappropriate (not refined grains) or lack of control in the rest 5 studies, only the changes in gut microbiota before and after the intake of whole grains were shown in this review, which consequently reduced the support of any beneficial effects of whole grain on the gut microbiota. In the two studies reported by Saa *et al*. (2014) and De Angelis *et al*. (2015), the microbiota composition or microbial diversity did not significantly differ within dietary groups following a dietary intervention with whole grain wheat or barley. However, in response to whole grains treatment, microbiota composition or microbial diversity showed a significant change compared with baseline in the faecal samples (Costabile *et al.*, 2008; Martínez *et al.*, 2013a; Foerster *et al.*, 2014). Due to the lack of comparison with refined grain counterparts, relatively limited information can be inferred from these studies to compare whole grains against refined grains to explore if there are differential effects of these whole grain varieties on the faecal microbiota.

There are several reasons for the absence of meta-analysis of gut microbiota: 1) a total of fifteen randomized, controlled studies were included in this review, but eight of them only showed figures without any numerical values, and also most of these figures were not measurable; 2) the rest studies did not describe common intestinal bacteria. For example, some papers focused on phylum level, but others focused on genus level; 3) among these seven studies, five papers used ‘population

or number of bacteria cells', but the rest two studies used 'relative abundance of gut microbiota', which two units were not changeable to demonstrated the bacterial changes.

2.8.4 Food constituents that affect gut microbiota

2.8.4.1 Dietary fiber

The definition of dietary fibre is still being discussed, but according to the CODEX Alimentarius Commission 2009, dietary fibre is defined as carbohydrate polymers with ten or more monomeric units, which are not hydrolyzed by the endogenous enzymes in the human small intestine. The definition includes non-starch polysaccharides (NSP) like arabinoxylans, cellulose, and many other components such as resistant starch, oligosaccharides, beta-glucans, insulin and lignin (Jones, 2014). The definition also allows for the inclusion of smaller oligosaccharides which are also not digested by human digestive enzymes. Dietary fibre intake is thought to have a major impact on the compositional diversity and relative abundance of the gut microbiota in humans, although effects cannot be generalized as they vary depending on the type of fibre.

2.8.4.1.1 Effects of fibre derived from whole grains on gut microbiota

In one strictly controlled intervention study, Langkamp-Henken *et al*. (2012) suggested that small differences in total dietary fibre intake between the two groups was probably responsible for the lack of significant differences in overall microbiota diversity. However, one study compared the effects on the human gut microbiota of 100% whole grain wheat breakfast cereals with a wheat bran breakfast cereal over 3 weeks, and found that the numbers of bifidobacteria and lactobacilli in faecal samples were significantly higher compared with wheat bran treatment, indicating that whole grains are more bifidogenic than wheat bran alone. This study is of particular interest, as it suggested that the different impacts on the gut microbiota between the two intervention groups may be due to other components like fermentable carbohydrates rather than dietary fibre (Costabile *et al.*, 2008). To analyze the effects of fibre from whole grains on human gut microbiota, a few human intervention studies have been conducted up to date. In a randomized crossover study, the effect on the gut microbiota composition of increasing whole grain intake from 28 g/d to 168 g/d ($p<0.001$) for 6 weeks, accompanied by an increase in total fibre intake ($p<0.001$) compared with a refined grain diet, was recently reported by Ampatzoglou *et al*. (2015). Surprisingly, despite the large difference in whole grain and fibre intake, the gut microbiota composition did not differ in the whole grain intervention group. This may have been caused by the reduced content of indigestible, fermentable carbohydrates because of the processing of the foods provided or the use of the Fluorescence in Situ Hybridization (FISH) probes that were not sensitive enough to detect small changes in microbiota composition. Consistent with this, a higher whole grain consumption (207 ± 39 g whole grains containing 40 ± 5 g fibre) over the same time period failed to induce any changes in bacterial composition and diversity, with the exception of a higher abundance of *Lachnospira* in the whole

grain group compared with the refined grain group (Vanegas *et al.*, 2017). Moreover, a randomized parallel study conducted in Finnish individuals with metabolic syndrome and examined whether intake of whole grain and fibre-rich rye breads influenced the intestinal microbiota composition compared with refined wheat breads (Lappi *et al.*, 2013). However, there was no overall change in the microbiota composition between the groups either at the baseline or after the intervention, with the exception of the phylotype *Bryantella formatexigens* in the refined wheat group.

2.8.4.1.2 Observational Studies

The role of dietary fibre in affecting the gut microbiota is well exemplified by comparing the gut microbiota of individuals from different geographical regions that consume rural diets (Africa and South America) which are naturally high in dietary fibre with the gut microbiota of individuals consuming Western diets (Europe and North America) high in animal protein and fat, but low in fibre (De Filippo *et al.*, 2010; Yatsunenko *et al.*, 2012). Rural African children following a fibre-rich diet revealed a significant depletion in Firmicutes and an enrichment in Bacteroidetes, with a unique abundance of bacteria from the *Prevotella* and *Xylanibacter* genera, known to possess bacterial genes for the hydrolysis of cellulose and xylan, which were completely lacking in European children. Additionally, the rural African children had about a 3-fold increase in the level of short-chain fatty acids in stool samples compared with European counterparts. In contract, Enterobacteriaceae species, such as *Shigella* and *Escherichia*, were significantly lower in African than in European children (De Filippo *et al.*, 2010). Another large study involving healthy children and adults also showed pronounced differences in functional gene repertoires and bacterial communities between US subjects from countries with a rural lifestyle (Amazon of Amazonas of Venezuela and Malawi) and those from metropolitan areas. In particular, the genus *Prevotella* was more abundant in humans with a diet rich in corn and cassava and in US children not following a full western diet (Yatsunenko *et al.*, 2012). A recent study including healthy African Americans and rural South Africans, found that the microbial composition was basically different, with a predominance of *Bacteroides* species in most Americans and *Prevotella* in most Africans. Total bacteria, SCFAs and major butyrate-producing groups were markedly more abundant in faecal samples from native Africans, but there were lower levels of faecal secondary bile acids when compared with African Americans. These differences could be explained by the dietary habits in which carbohydrates and fibre (mainly resistant starch) intakes were higher in Africans while animal protein and fat was 2-3 times higher in Americans (Ou *et al.*, 2013). One very recent Dutch population-based study including 1135 subjects from The Netherlands has correlated higher diversity, functional microbiome richness and abundance of Bacteroidetes with higher fruits and vegetables consumption (source of dietary fibre). The total amount of carbohydrate intake in the diet was negatively correlated with *Lactobacillus*, *Streptococcus*,

Roseburia species and microbiome diversity, but positively correlated with bifidobacteria (Zhernakova *et al.*, 2016).

In summary, these observational studies show that long-term intake of fibre-rich diets in their lives boosts the dominance of fibre-degraders of the phyla Bacteroidetes and Actinobacteria (*Bifidobacterium* spp.) and decreases the abundance of Proteobacteria. Notwithstanding these observations, it seems that *Bacteroides* spp. Are adapted to both diets high in animal protein and fat and fibre-rich diets, probably because of their versatile metabolic capabilities. However, it is important to note that these observational data only show associations but not casual relationships between specific dietary intake and composition and number of specific bacterial taxa, which clearly limit their value. Moreover, other relevant environmental factors such as ethnicity, geography, climate and food varieties that may also play a potential role in the gut microbiota profile were not well examined in these above studies.

2.8.4.1.3 Types of dietary fiber

2.8.4.1.3.1 Non-starch polysaccharides

Non-starch polysaccharides (NSPs), the key components of the cell walls of various grains, which together form a major part of the total dietary fibre of grains. The insoluble NSPs in grains have long been known for their faecal bulking properties and decrease in gastrointestinal transit time (Grabitske and Slavin, 2008). These impacts may be especially critical for older adults, who typically include less dietary fibre in their diets and have decreased gastric motility (Bhutto and Morley, 2008). As for the soluble NSPs, it has been suggested that they might interact with gut microbiota in a different way, through a contra-biotic impact, preventing potentially harmfully interaction between the gut epithelium and bacteria that occur upon dysbiosis. For example, giving individuals diets high in NSPs led to significant shifts in the populations of bacteria in the large bowel of humans (Abell *et al*., 2008). The knowledge about effects of various sources of NSPs on the gut microbiota composition and number is still scarce. Nevertheless, the effect of NSPs on the gut microbiota is often overestimated due to their poor utilisation by colonic bacteria. The poorly fermentable dietary fibres in whole grains led to lower distal colonic pH and increased faecal butyrate concentrations (McIntosh *et al.*, 2003; Bird *et al.*, 2004). It is likely that, because of poor fermentation in the upper large intestine, the NSPs pass into the distal colon and then offer some carbohydrate substrate for gut bacterial metabolism in this region, which is irrelevant to most soluble and highly fermentable dietary fibres which are rapidly fermented in the upper large intestine.

2.8.4.1.3.2 Resistant starch

Resistant starch (RS), naturally occurring in foods, is considered a dietary fibre and as such, is defined physiologically as any starch or starch degradation product that escapes from digestion in the small intestine of healthy individuals and persists into the large intestine for fermentation (Asp and Björck, 1992). According to the physical or chemical reasons to be indigestible, resistant starch has been categorized into 4 types: physically inaccessible starch (RS1), native granules (RS2), retrograded starch (RS3), or chemically modified starch (RS4). In recent years, resistant starch has gained much attraction due to its effect on gut microbiota and subsequent impacts on the host (Martinez *et al.*, 2010). Some types of resistant starch including RS1, RS2 and RS3 are fermented by the large intestinal microbiota, resulting in the production of SCFAs, promotion of butyrate-producing bacteria and increased bacterial cell mass, thereby exerting benefits to human health (Brouns *et al.*, 2002). An early study about effect of resistant starch on the composition of the faecal microbial community indicated that bacterial profiles showed changes especially a significant increase in the abundance of the *Ruminococcus bromii* group in response to a diet containing 22 g/day resistant starch when compared with baseline, and also higher levels of total SCFA pools (Abell *et al.*, 2008). The abundance of *Ruminococcus bromii* increased significantly in most volunteers on a RS3 diet over 10 weeks relative to the control group, as well as levels of uncultured *Oscillibacter* and *Eubacterium rectale* (Walker *et al.*, 2011). Similar results were observed in another study on resistant starch when subjects were offered RS2, RS4, or native starch as crackers for 3 weeks. RS4 led to changes in phylum-level, significantly decreasing numbers of Firmicutes while increasing Actinobacteria and Bacteroidetes. At the species level, RS4 raised the proportions of *Bifidobacterium adolescentis* and *Parabacteroides distasonis*, while the proportions of *Ruminococcus bromii* and *Eubacterium rectale* increased with RS2 as compared with RS4 (Martinez *et al.*, 2010). Taken together, the results of these three studies revealed that resistant starch might have a growth-promoting effect on *Ruminococcus bromii*, but mainly depending on the types of resistant starch used.

2.8.4.1.3.3 Beta-glucan

Cereal grains, especially oat and barley, possess a unique NSP in the form of mixed linked beta-(1→4) and (1→3) glucan, also simply known as beta-glucan or β-glucan. In whole grain products, β-glucan is present in both soluble and insoluble forms, the content of which varies depending on the types of grains. Although the dietary fibres are not digested and absorbed in the small intestine, the soluble nature of β-glucan is thought to increase the viscosity of the food bolus, resulting in a slower gastric emptying, improved gut fill and slower nutrients absorption. All these factors mentioned above help

to promote the growth of human microflora. Mixed-linkage β-glucan as a fermentable dietary fibre can selectively contribute to the proliferation of beneficial intestinal microorganisms such as bifidobacteria and lactobacilli as shown *in vitro* studies (Jaskari *et al.*, 1998; Kontula *et al.*, 1998) and animal experiments (Dongowski *et al.*, 2002; Drzikova *et al.*, 2005; Snart *et al.*, 2006). Even though human data from clinical trial regarding the prebiotic impact of β-glucan are rather limited, β-glucan has been shown to actively affect the microbiota according to a few human intervention studies (Mitsou *et al.*, 2010; Chiraphon *et al.*, 2015; De Angelis *et al.*, 2015; Wang *et al.*, 2016). In a study involving 26 healthy subjects, administration of durum wheat and whole grain barley pasta containing a minimum 3 g/day of β-glucan increased the number of *Clostridiaceae* (*Clostridium orbiscindens*, *Clostridium* sp.), *Roseburia hominis* and *Ruminococcus* while decreased other Firmicutes and Fusobacteria in faecal samples after two months of diet intervention with respect to baseline (De Angelis *et al.*, 2015). However, one study by Turunen *et al*. (2011) found that the same amount of β-glucan intake did not induce any significant differences in faecal bacterial viable counts for a longer 3 months compared with the placebo group; also, similar results were detected in healthy subject consuming daily a cake with 0.75 g of barley β-glucan (Mitsou *et al.*, 2010). Taken together, these results suggested that the potential prebiotic effect of β-glucan intake on gut microbiota was still conclusive in humans.

It is important not to forget that physiochemical properties of β-glucan, such as molecular weight and solubility, are thought to be important factors in its physiological properties (Wood, 2004; Wolever *et al.*, 2010). The extent of β-glucan fermentation and type of bacteria in the distal gastrointestinal tract may also depend on its physiochemical structure (Hughes *et al.*, 2008). It has been verified from the study conducted by Wang *et al*. (2016) that high molecular weight barley β-glucan contributed to the variation of gut microbiota composition and number.

2.8.4.2 Fat

Fats are composed of fatty acids which are divided into saturated fatty acids and unsaturated fatty acids. Whole grains are relatively low in fat compared with other food sources like meat, milk and cheese, and fat that they do possess is mostly unsaturated. The impact of fat on the gut microbiota may be partly modulated by indirect mechanisms, since the most of dietary fat is digested and absorbed in the small intestine and does not serve as an energy source for the gut microbiota. It has been suggested that high fat intake may increase the quantities of fat and bile acids that reach the colon, with higher concentrations of secondary bile acids in stool samples (Rafter *et al*., 1987). Because of their selective antimicrobial activity, bile acids, such as deoxycholic acid, could regulate fat-induced intestinal microbiota changes, as recently shown in rats (Islam *et al.*, 2011). Another

study in mice has indicated that diets high in fat over 4 weeks (predominantly TAG) have a detrimental impact on the gut microbiota and host metabolic parameters (Cani *et al.*, 2007). Also, low/moderate-fat intake was shown to increase the abundance of *Bacteroides* spp. and/or *Bifidobacterium* spp., compared with high-fat consumption in human intervention trials (Brinkworth *et al.*, 2009; Fava *et al.*, 2013). In particular, diets high in saturated fat appear to induce a reduction in the relative abundance of beneficial bacteria and reduce microbial diversity (de Wit *et al.*, 2012; Liu *et al.*, 2012). de Wit *et al*. (2012) revealed that saturated fats were especially harmful to the intestinal microbiota if they passed to the distal small intestine conferring an antimicrobial impact, which consequently resulted in reduced diversity. In contrast, no effect on the gut microbiota in the terms of total bacterial counts was reported when subjects were fed high-fat and moderate-fat diets *ad libitum* (66 % energy vs. 35 % energy) for 4 weeks (Duncan *et al.*, 2007). Furthermore, the abundance of *Roseburia* spp. and *Bilophila wadsworthia* was increased after 12 and 16 weeks consumption of high-fat diets, respectively (Schneeberger *et al.*, 2015).

To date, there are only a few *in vivo* studies where the impact of a diet high in in fat derived from whole grains on intestinal microbiota has been examined, especially those involving human subjects. For instance, rats fed high-fat diets supplemented with whole grain barley showed significantly decreased populations of total bacteria and *Lactobacillus*, but increased *Akkermansia* in comparison with low-fat diets. Interestingly, whole grain barley increased the abundance of *Bifidobacterium* only when dietary fat was consumed at a low level, and supplementing high-fat or low-fat diets with whole grain barley increased the total concentration of SCFA in the caecum (Zhong *et al.*, 2015). In a study of Martínez *et al*. (2009), a whole grain sorghum lipid extract incorporated into the diets of hamsters significantly increased the abundance of bifidobacteria and decreased the proportion of members of the Coriobacteriaceae family in the faeces. Furthermore, bifidobacteria concentrations showed a strong association with plasma HDL-cholesterol concentration, whereas Coriobacteriaceae were positively associated with non-HDL-cholesterol concentration. It has been suggested that this may be due to sterol esters which formed an estimated 10% of the total lipids (Martínez *et al.*, 2013b). Cholesterol absorption decreased in hamsters with supplementation of purified steryl esters, resulting in a decrease in Coriobacteriaceae and Erysipelotrichaceae and an increase in the cholesterol pool in the gut. These two bacterial families have been positively correlated with deleterious host lipid parameters (Martinez *et al.*, 2009; Spencer *et al.*, 2011). Whole grains are rich in plant sterol esters compared with other foods (Piironen *et al.*, 2000). In another study, the concentrations of faecal bile acids were significantly greater in mice fed rice bran oil with an accompanying positive association with levels of the family Lactobacillales (Tamura *et al.*, 2012). These studies confirm a link between dietary fat, sources of whole grains, bile acids metabolism and

alternations in gut microbiota. Clearly, more knowledge from controlled human intervention studies are needed to better understand the effects of fat derived from whole grain or the interaction between fat and whole grain on gut microbiota composition, populations and functionality.

2.8.4.3 Protein

The effect of dietary protein on the composition of the human intestinal microbiota has only been investigated to a minor extent. In mice fed a high-protein/low-carbohydrate diet for 2 weeks there was a reduction in the diversity and a change in the microbiota composition relative to a normal diet, which included increases in the relative abundance of the genus *Bacteroides* and *Parabacteroides*, and a decrease in the relative abundance of the family Lachnospiraceae and Ruminococcaceae (Kim *et al.*, 2016). An intervention diet with a high protein and low carbohydrates content was shown to influence the SCFA profiles in faecal samples and gut microbiota in obese men. After a 4 week period, the high-protein diet decreased the *Roseburia*/*Eubacterium* numbers and butyrate, but increased proportions of branched-chain SCFA, concentrations of N-nitroso compounds and phenylacetic acid (Russell *et al.*, 2011). The study of Faith *et al*. (2011), reported that feeding mice with increasing proportions of casein protein changed microbial profiles. Moreover, An *et al*.(2014) investigated the impacts of casein, soy protein and fish protein diets on the gut microbiota in rats after 16 days feeding, finding that microbial diversity in the cecum was markedly higher in rats fed with soy protein than casein. This results were in part consistent with Butteiger *et al*. (2016) who showed that hamsters in an all soy-fed group contained a more diverse gut microbiota than those fed a milk protein isolate diet, with 4 microbial families present at significantly higher abundance in the faecal samples, namely *S24–7*, Bifidobacteriaceae, Clostridiales spp., and Deferribacteraceae. Associations between the gut microbial profiles with serum lipid concentrations and hepatic gene expression were established, suggesting that some of the lipid-lowering properties of soy protein may attribute to alternation in gut microbial profiles.

2.8.4.4 Polyphenols

Dietary polyphenols are natural compounds widely occurring in plants, including foods such as whole grains, fruits, vegetables, coffee, tea and wine, and therefore are an important part of the human diet. Due to the low absorption in the small intestine, as much as 90% of the dietary polyphenols persist intact into the colon (Tuohy *et al.*, 2012). There, they are break down into smaller metabolites via microbial activity, such as simple short-chain fatty acids, phenolic acids and phenols, some of which can be absorbed across the intestinal mucosa (Selma *et al.*, 2009). Microbial metabolites of plant polyphenols may influence biomarkers of disease risk associated with metabolic

syndrome, which may be attributed to the more potent antioxidant and/or anti-inflammatory activities of polyphenol metabolites. A human intervention study conducted with daily consumption of red wine polyphenols for 4 weeks demonstrated a significant increase in populations of *Enterococcus*, *Prevotella*, *Bacteroides*, *Bifidobacterium*, *Bacteroides uniformis*, *Eggerthella lenta* and *Blautia coccoides–Eubacterium rectale* groups ($P < 0.05$) compared with the baseline. This shift was proposed to be partly responsible for the reductions observed in blood pressure, triglycerides, HDL cholesterol, total cholesterol and C-reactive protein concentrations (Queipo-Ortuno *et al.*, 2012).

Dietary polyphenols and their metabolites can also influence the intestinal ecology by mediating microbiota (Selma *et al.*, 2009). In this respect, several phenolic compounds have been considered as potential antibacterial agents with bactericidal or bacteriostatic properties. It has been shown in several *in vitro* studies that phenolic compounds could selectively suppress the growth of different bacterial species inducing alternations in microbiota composition and populations (Lee *et al.*, 2006; Alakomi *et al.*, 2007; Parkar *et al.*, 2008; Romier *et al.*, 2009; Cueva *et al.*, 2010). For example, Lee *et al*. (2006) reported that when bacteria were cultured with phenolic components and metabolites of tea extract, growth of certain pathogenic bacteria such as *Bacteroides* spp. *Clostridium difficile* and *Clostridium perfringens* was significantly habited, whereas commensal anaerobes including *Bifidobacterium* and *Lactobacillus* and non-pathogenic *Clostridium* spp. were less influenced. In addition, *Bacteroides*, *Lactobacillus* and *Bifidobacterium* spp. were predominantly identified in rats administrated with red wine polyphenols for 16 weeks, while *Bacteroides, Clostridium* and *Propionibacterium* spp. appeared to be present in higher concentrations in the faeces of control-fed rats (Dolara *et al.*, 2005). Similarly, administration of resveratrol commonly identified in grape juice significantly increased *Bifidobacterium* spp. and *Lactobacillus* in a rat model after 20 days (Larrosa *et al.*, 2009). In a human study by Tzounis *et al*. (2011), when subjects were provided with a high-cocoa flavanol drink daily for 4 weeks, they had significantly lower bifidobacteria and lactobacilli numbers, and significantly lower Clostridia populations with respect to the control group, accompanied by significant decreases in plasma triacylglycerol and C-reactive protein (CRP) concentrations. Moreover, changes in CRP were associated with alternations in lactobacilli numbers ($P<0.05$, $R^2 = -0.33$ for the model). The effect of polyphenols on bacterial growth and metabolism are largely dependent on polyphenol structure, the dosage used and the microorganism strain (Hervert-Hernandez and Goni, 2011). However, it should be noted that excessive amounts of polyphenols in the diets may also suppress the growth and/or activity of beneficial gut microbiota, which is responsible for the bio-conversion of polyphenols, thus exerting harmful effects on human health. Some recent findings have proposed a range of potential mechanisms of activity of polyphenols on bacterial cells. For instance, polyphenols can bind to membranes of bacterial cells in a dose-dependent manner, which

consequently disturbs membrane function, thereby repressing cell growth (Kemperman *et al.*, 2010). In addition, Vitaglione *et al*. (2008) suggested that the phenolic compounds bound in the dietary fibre complex may also change the intestinal environment which may involve in the gut microbiota population.

2.8.5 Limitations

Although the studies included in this review show that whole grains intake can modulate the gut microbiota, there are serval limitations common to the majority of studies. First, the limited number of studies conducted to date, combined with small sample sizes and short intervention periods, were insufficiently powered to support the effect, thus, limiting the generalizability of shifts in microbiota to larger populations with whole grain consumption. Second, the background diets of many studies included in this review have not been adequately assessed, which may have potentially confounded the influences of whole grains. Third, in many of the presented studies, the gut microbiota characterisation only focus on several targeted bacteria, which suggests that the shifts or changes in non-targeted bacteria may have been missed. Forth, the gut microbiota were characterized at the level of phyla and family, making it too broad to relate specific claims to functionality. Finally, the bioactive compounds responsible for whole grains' modulation in gut microbiota still remain uncertain, and the mechanisms underlying the effects were also not fully elucidated.

2.8.6 Conclusion

To our knowledge, this is the first systematic review to comprehensively assess the overall effects of whole grain foods intake, including wheat, maize, oat, barley and rye, on the human gut microbiota. Noteworthy, numbers of faecal bifidobacteria and lactobacilli were significantly increased upon whole grain ingestion in a few studies; however, human intervention studies have not always found clear results from feeding whole grains on the gut microbiota, which has resulted in some discussion about the acute effect of a whole grain diet. The reasons for the inconsistent results in these human intervention studies is not completely understood, however, several factors such as differences in subject characteristics, the cereal grains used, processing conditions, not having completely controlled for other components of the diets may have been responsible for the divergent results.

Most importantly, based on 15 human intervention studies listed above, it seemed that this systematic review cannot strongly support the hypothesis that intake of whole grains could positively modulate the gut microbiota in humans and thereby exerting impacts on human health, since no significant changes in gut microbiota between groups were observed in most of the studies included in this review. But Copper *et al*. (2017) pointed out that the lack of response in the faecal

microbiota was not necessarily surprising, given that in this human intervention study subjects are 'free-living' and whole grains products only accounted for a minor part of energy intake at the average level of consumption. While dietary fibre may in part explain the changes of gut microbiota, many epidemiological studies focusing on other types of foods have suggested that other bioactive compounds, such as protein, fat and polyphenols may also play a role. Moreover, it has been suggested that the effects of foods on the modulation of human gut microbiota are likely to be due to the combined results of many components with the grain rather than one specific component, like phenolic compounds bound in the dietary fibre complex (Vitaglione *et al.*, 2015). However, these positive outcomes cannot generally extrapolate to whole grains based on the lack of response in fibre-rich whole grain products in a few studies. To further verify the potential prebiotic effect of whole grain products for the modulation of human gut microbiota, as well as the mechanisms underlying the beneficial effects, long-term human controlled intervention trials seem warranted.

2.9 Hypotheses, aims and objectives

2.9.1 Hypotheses

1. Some important nutrients and phytochemicals, such as protein, amino acids, fibre and phenolics, in quinoa seeds and phenolic content and antioxidant activity in buckwheat seeds match better to human nutritional requirements than common cereals and refined grain cereals, including wheat, corn and rice, and also nutrient and phytochemicals in different accessions are different.

2. Quinoa consumption can improve CVD risk via a human intervention study, such as BMI, body fat content, blood pressure, glucose and insulin, inflammatory markers, fasting plasma lipid profile.

3. Quinoa intake can positively modulate the gut microbiota and gastrointestinal health.

2.9.2 Aims

1. To investigate the nutrient composition and then select one accession of quinoa for further human intervention study mainly based on fibre content, and also investigate phenolics and antioxidant activity of buckwheat.

2. To investigate the impact of quinoa intake on CVD risk markers, such as BMI, body fat content, blood pressure, glucose and insulin, inflammatory markers, fasting plasma lipid profile.

3. To investigate the impact of quinoa intake on gut microbiota and gastrointestinal health.

2.9.3 Objectives

1. To quantity nutrients and phytochemicals (protein, amino acids, fibre, fat, phenolics) in different accessions of quinoa sourced from South America, UK, Europe and China, and also quantity phenolics and antioxidant activity of buckwheat.

2. To quantity differences between each accession of quinoa, and choose one quinoa accession for further human intervention study mainly based on fibre content.

3. To conduct a human intervention study to investigate the effects of the quinoa with the highest fibre content identified in chemical analysis, on markers of CVD risk and gut microbiota.

The research project has 3 clear phases in a developing research process.

(a) Identification a characterization of grain materials.

(b) Selection of quinoa accession for the sensory testing.

(c) Selection of quinoa roll for use in human intervention study.

Chapter 3 Composition of quinoa and buckwheat

3.1 Introduction

Emerging interest exists in the use of alternative grains (pseudocereal, such as quinoa, buckwheat and amaranth) for the productions of cereal based foods owing to their high nutritional profile and the dietary need for a large population suffering from coeliac disease. In addition to offering the starch content as an important energy source, quinoa and buckwheat is rich in good quality protein, with a well-balanced amino acids profile, lipids which are rich in unsaturated fats, dietary fibre, as well as other important components such as vitamin C and phenolic compounds (Ando *et al.*, 2002; Konishi *et al.*, 2004; Krkošková and Mrázová, 2005; Bhargava *et al.*, 2006; Alvarez-Jubete *et al.*, 2009; Tang *et al.*, 2015). Quinoa and buckwheat seeds also serve as good source of various essential minerals including K, Mg, Fe, Zn, P and S, which are much higher than those of traditional cereals (Ikeda *et al.*, 1995; Wang *et al.*, 1995; Konishi *et al.*, 2004).

Phenolic compounds (also referred to as polyphenols) are bioactive secondary plant metabolites that are widely present in commonly consumed foods of plant origin (Bravo, 1998). Although data was limited up to date, a few studies have reported that quinoa and buckwheat seeds have higher phenolic content and antioxidant activity than common cereals (Inglett *et al.*, 2015; Tang *et al.*, 2015). These compounds act as powerful antioxidants in food models as well as *in vitro* LDL cholesterol, which might combat oxidative stress in the body by helping to maintain a balance between oxidants or free radicals and antioxidants (Adom and Liu, 2002; Madhujith and Shahidi, 2007; Natella *et al.*, 2007; Brend *et al.*, 2012; de Camargo *et al.*, 2014). The oxidised LDLs, as the products of reaction between LDL and free radicals, are more atherogenic than native LDL, thereby increasing CVD risk (Chu and Liu, 2005; Maiolino *et al.*, 2013). Therefore, dietary phenolic compounds and other antioxidants from fruits, vegetables and whole grains are proposed to reduce CVD risk via prevention of LDL oxidation (Miller *et al.*, 1998; Bruckdorfer, 2008; Mangge *et al.*, 2014; Siti *et al.*, 2015). In contrary, some studies have pointed that antioxidants did not have significant effect on mortality caused by CVD, thus, the property of antioxidants against CVD has been disputed (Kris-Etherton *et al.*, 2004; Miller *et al.*, 2005; Bjelakovic *et al.*, 2008). These three methods (FRAP, TEAC and DPPH assays) are the most commonly used in the literature, so they would provide the most data in this present study in order to compare with other studies.

Although some effort have already been done on the compositional analysis of these two pseudocereals, data is still currently scarce compared with other common cereals, such as wheat, corn and rice. In this study, an accession of quinoa and buckwheat seeds were sourced from

different regions. The potential possible differences attributable to the genetic backgrounds or environmental conditions strongly promoted the need to carry out a compositional analysis (Miranda *et al.*, 2012; Miranda *et al.*, 2013).

The aim was to investigate the nutrient composition and then select one accession of quinoa for further human intervention study mainly based on fibre content, and also investigate phenolics and antioxidant activity of buckwheat. The objective was to conduct a comprehensive compositional analysis of quinoa and buckwheat seeds (only phenolics and antioxidant activity).

3.2 Materials and methods

3.2.1 Materials

In this study, a total of thirteen commonly used commercial quinoa accessions and nine buckwheat accessions were sourced from various regions, including Peru, Ecuador, Bolivia, USA, UK, Netherlands and China. Samples were either bought directly from the supplier or through commercial retail outlets. All samples were ground to flour (Sieve size, 1 mm) using a laboratory cyclone mill twister (Retsch), then packed in grip seal bags and stored in -20°C freezer until use. In order to select the quinoa accession with 'optimal' nutrition for use in the future human intervention study, chemical analysis of these varieties were conducted, such as phenolic content, antioxidant activity, dietary fibre, fat, protein and amino acids, with the dietary fibre being the determined factor for the selection of quinoa accession for the future human intervention study. For the buckwheat varieties, only phenolic content and antioxidant activity were analysed.

3.2.2 Phenolic content and antioxidant activity of quinoa and buckwheat seeds

3.2.2.1 Phenolic compounds extraction

The free, conjugated and bound phenolic compounds in quinoa and buckwheat flours were isolated according to the method of Li *et al*. (2008) and Adom and Liu, (2002) with slight modifications (See Appendix 1, the flow diagram of phenolic extraction).

3.2.2.1.1 Free Phenolic compounds

25 mg of whole grain flours were blended with 1 mL of 80% chilled ethanol for 5 min using a multi-tube mixer (Stuart SB3), followed by being sonicated for 10 min. The mixtures were then centrifuged at 5000 rpm for 15 minutes (Fisher Scientific accuSpin^{TM}3R; centrifuge radius, 8 cm), and the supernatant was removed into a new 2 mL Eppendorf tube. The extraction was repeated four times under the same conditions. All supernatants were combined, then evaporated to dryness at 45°C under nitrogen gas and finally dissolved with deionised water to a volume of 350 µL. After centrifugation (13200 g, 5 min), the supernatant was transferred to a clean vial. The extracts were stored at −80 °C until future use.

3.2.2.1.2 Conjugated phenolic compounds

10 mg of whole grain flours were mixed with 1 mL of 80% chilled ethanol for 5 min using a multi-tube rotator, followed by being sonicated for 10 min. After centrifugation at 5000 rpm for 15 minutes, the supernatant was removed into a new 2 mL Eppendorf tube. The extraction was

repeated four times under the same conditions. All supernatants were combined, then evaporated to dryness at 45 °C. The dried extracts samples were then hydrolysed with 400 µL of 2 M NaOH at room temperature for 4 h, then acidified to pH 2 with 12 M HCl (80 µL). The solution was extracted four times with ethyl acetate (500 µL). After centrifugation at 13200 rpm for 5 minutes, the upper layer was transferred to a clean Eppendorf tube, and combined ethyl acetate extracts were evaporated to dryness at 45 °C under nitrogen gas. Phenolic compounds were dissolve in 350 µL of water followed by centrifugation (13200 g, 5 min), and the supernatant was transferred to a clean vial, then stored at −80 °C until use.

3.2.2.1.3 Bound phenolic compounds

The residues from free or conjugated phenolics extraction above were digested with 800 µL of 2 M NaOH for 4h before acidification with 12 M HCl (120 µL) to pH 2. The mixture was extracted four times with 800 µL ethyl acetate, the ethyl acetate fraction was evaporated to dryness at 45°C under Nitrogen gas. Phenolic compounds were reconstituted with deionised water to a final volume of 350 µL followed by centrifugation (13200 g, 5 min), and the supernatant was transferred to a clean vial, then stored at −80 °C until use.

In this study, extraction of each sample was repeated four times, so each quinoa or buckwheat accession has four free phenolic extracts, four conjugated phenolic extracts and four bound phenolic extracts.

3.2.2.2 Folin-Ciocalteu phenolic content (FC assay)

Total phenolic content of quinoa and buckwheat extracts were determined using the Folin-Ciocalteu method as described by Zhang *et al*. (2006a) with minor modifications. Before the measurement, commercial Folin-Ciocalteu phenol reagent was diluted 1:10 (v/v) with deionized water. Gallic acid (GA) was adopted as a reference standard against which to assess the total phenolic contents, which were then expressed as GA equivalents (GAE) (Maurya and Singh, 2010). Serial dilutions of GA were carried out accordingly at 500, 250, 125, 62.5, and 31.25 µg/mL in deionised water producing a standard calibration curve. 10 µL of GA standard solutions (0-500 µg/mL) or each extract was added into the 96-well microplate, and then 130 µL of the diluted Folin-Ciocalteu phenol reagent added. Five minutes later, 100 µL of 7.5% sodium carbonate solution was added, and the resulting solution thoroughly mixed. The absorbance values were measured at 765 nm using a spectrophotometer after incubation at 40^{o}C for 30 minutes. Final results were given as mg of GAE per gram of dry weight (dw).

Analysis of each extract of free, conjugated or bound phenolics was repeated four times in this study.

3.2.2.3 Antioxidant assays

3.2.2.3.1 Ferric reducing ability of plasma (FRAP assay)

The FRAP assay was performed according to the method of Benzie and Strain (1996). Briefly, 10 mM of Fe^{3+}TPTZ solution was prepared by dissolving 0.0781 g of ferric 2,4,6-Tripyridyl-s-Triazine (Fe^{3+}TPTZ) into 40 mM hydrochloric acid. A FRAP working solution was prepared by mixing 50mL of 300 mM acetate buffer (pH 3.6), 5 ml of 20mM ferric chloride ($FeCl_3.6H_20$) and 5 ml of TPTZ solution. Standard solutions of ferrous sulphate were obtained at a range of 200 to 1000 µmol/L in deionised water. 10 µL of ferrous sulphate standards or sample extracts were mixed with 300 µL of FRAP working reagent in the 96-well microplate and incubate at 37°C for 4 minutes. The absorbance of samples was measured at 593 nm after incubation. The final results were expressed as µmol Fe^{2+} Equivalent (E)/g sample dw.

3.2.2.3.2 Trolox equivalent antioxidant capacity (TEAC)

A modification of the TEAC assay from Re *et al.* (1999b) was used. The TEAC stock solution was obtained by mixing 7 mM 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) with 2.45 mM potassium persulfate 9:1 (v/v), then stored in a dark cupboard at room temperature overnight prior to use. Before the analysis, the stocking solution was diluted with 5 mM phosphate buffer solution (PBS), pH 7.4, until the absorbance of the mixtures-working solution was 0.7 (± 0.02). The 2.5 mM Trolox standard solution was diluted to the range from 0.1-0.5 mM in ethanol. 10 µL of Trolox standard or sample extracts were mixed with 290 µL of TEAC working solution in the 96-well microplate. The decrease in absorbance was measured at 734 nm. The final results were expressed as µmol Trolox Equivalents (TE)/g sample dw.

3.2.2.3.3 2, 2-diphenyl-1-picrylhydrazyl (DPPH•) assay

A modification of the 2,2-diphenyl-1-picrylhydrazyl (DPPH•) method of Van Hung *et al.* (2009) was applied. Briefly, 10 mg DPPH• powder was dissolved in 100 mL of methanol to the DPPH• stock solution which was stored in the fridge overnight. The DPPH• working solution was prepared by mixing 30 mL of stock solution and 70 mL of methanol to form the final concentration of 0.076 mM. Then 2.5 mM of Trolox standard solution was diluted ranging from 0.25-1.25 mM in methanol. 10 µL of standards, blank or samples was pipetted into the 96-well plate and 390 µL of the DPPH working solution added and mixed well. The absorbance was measured at 517 nm after incubation at 30°C for 30 min. The final results were expressed as µmol Trolox Equivalents (TE)/g sample dw.

When the antioxidant activity analysed by these three assays, each extract of free, conjugated or bound phenolics was repeated four times in this study.

3.2.3 Compositional analysis of quinoa seeds

Thirteen sources of quinoa seeds were estimated for their energy, protein (Dumas method), ash (BS 4401-1:1998), moisture (BS 4401-3:1997), total sugars (N) (Ion Chromatography), sodium (ISO 7485:2000), salt (calculated from sodium), insoluble dietary fibre (AOAC 991.43), soluble dietary fibre (AOAC 991.43), total dietary fibre (AOAC 991.43), fat (based on BS 4401-4:1970), saturated fat (ISO 12966-2:2011), monounsaturated fat (ISO 12966-2:2011) and polyunsaturated fat (ISO 12966-2:2011). Available carbohydrate contents were calculated by difference from the formula: available carbohydrates = 100 − (protein + ash + moisture+ dietary fibre + fat). All of the above analyses were carried out to British Standards by an accredited company [Alex Stewart Agriculture Ltd laboratory] with the milled quinoa flour offered. Quinoa flours were also sent to ALS Food & Pharmaceutical Company for the amino acids analysis (AM/V/206 using HPLC).

3.2.4 Calculations and statistical analyses

In this study, extraction of free, conjugated and bound phenolics was repeated four times (extraction replication), and measurement of each extract by FC assay or three antioxidant activity assays was also repeated four times (analysis replication). After calculating mean value of four measurements per extract (outlier was removed and then the calculation of mean value was based on other three measurements), each type of phenolics (free, conjugated or bound) can get four values (each extract correspond to one value), to express the uncertainty caused by the analysis method. Then value of free, conjugated or bound phenolics per accession was calculated as mean of average of four extracts (extraction replication). Finally, the results as shown in Section 4.3.1 were expressed as means (average of 13 quinoa accession or 9 buckwheat accessions) and standard deviations. The data were analysed by using the SPSS 22.0 for Windows statistical program (SPSS, Inc., Chicago, IL, USA). Normality of the each type of phenolics from quinoa or buckwheat (13 values for quinoa and 9 values for buckwheat, 1 value per accession) was checked with the Shapiro–Wilk test and data that were not normally distributed were transformed (using log10 function) prior to statistical analyses and then back-transformed for presentation of results. To determine the differences between each two types type of phenolics between quinoa and buckwheat (for example free phenolics in quinoa vs free phenolics in buckwheat), an two-sample independent t-test was used with P values less than 0·05 considered to be significantly different. Also, to determine the differences between results in this study, including phenolic content, protein, fiber, fat and values and a reference value from another study, a one-sample t-test was used with P values less than 0·05 considered to be significantly different (for example, 13 quinoa protein values vs 1 reference protein value from another previous study).

3.3 Results and discussion

3.3.1 Phenolic content and antioxidant activity

3.3.1.1 Phenolic content

3.3.1.1.1 Results-Phenolic content of quinoa and buckwheat

A wide range of phenolic contents was found in the quinoa and buckwheat samples as presented in Figure 3.1, expressed as mg of Gallic acid equivalent per 1 g of grain. The free phenolic content of the 13 quinoa samples ranged from 0.89 ± 0.05 mg GAE/g grown in Peru (Peru) to 2.13 ± 0.02 mg GAE/g grown in China Shanxi, with an average of 1.44 mg GAE/g.

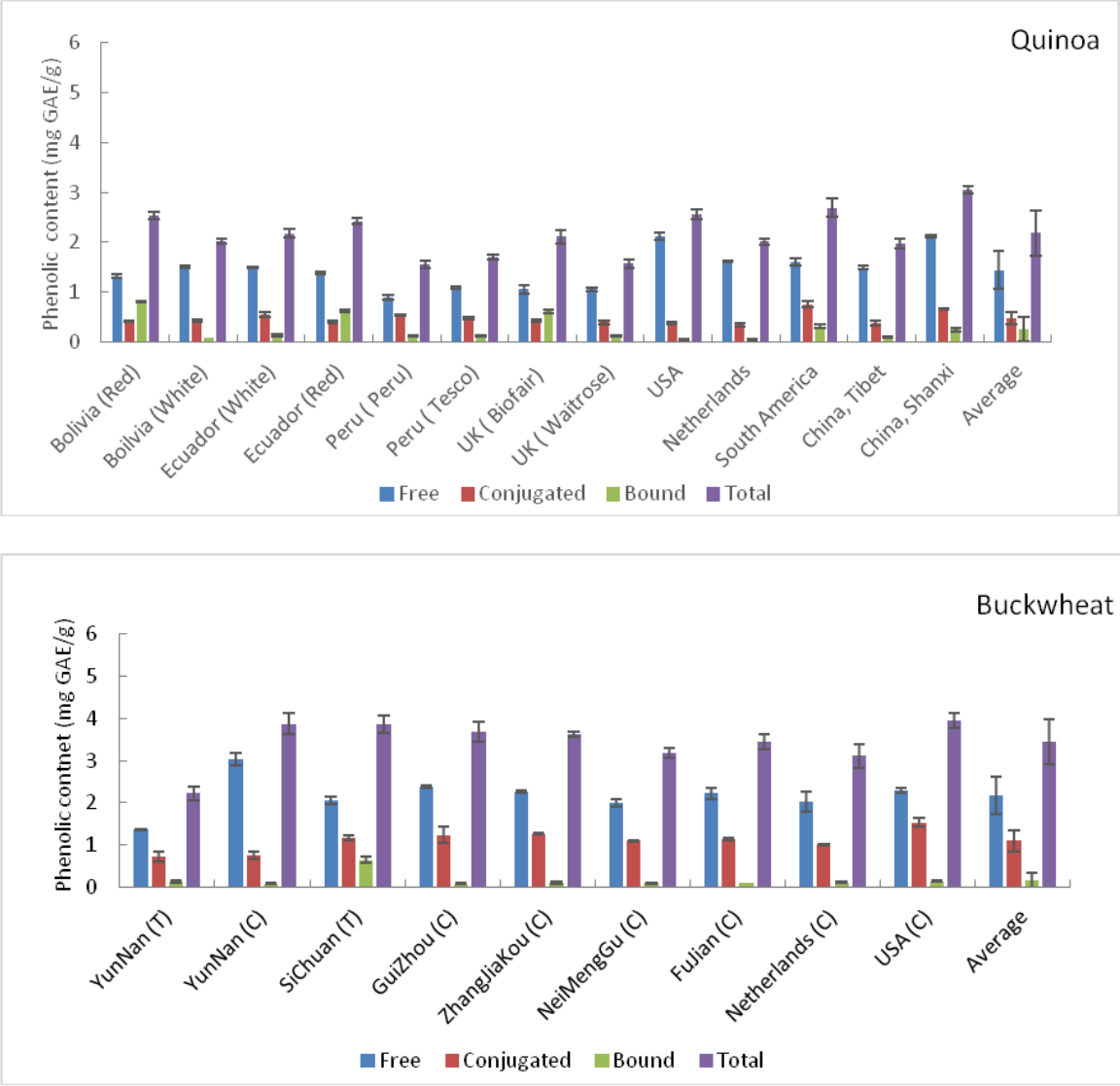


Figure 3.1. Free, conjugated, bound and total phenolic content (mg Gallic acid equivalent/100g) of 80% ethanol: water extracts of different varieties and sources of quinoa and buckwheat. Mena ± Standard deviation.

The quinoa cultivated in China Shanxi exhibited the highest free phenolic content, which was quite similar to the USA quinoa (2.12 ± 0.06 mg GAE/g), but higher than all other varieties. Regarding the

conjugated phenolic content, the values were lower, and ranged from 0.35 ± 0.03 mg GAE/g to 0.76 ± 0.06 mg GAE/g without much variations between the varieties. Interestingly, the bound phenolic contents in the three red quinoa varieties (0.62, 0.63 0.80 mg GAE/g sample) were several times than that of the rest white quinoa varieties, but the values were still much lower than those of 2.97 and 1.99 mg GAE/g in quinoa seeds demonstrated by Gomez-Caravaca *et al*. (2014) and Inglett *et al*. (2015), respectively. China Shanxi quinoa exhibited the highest total phenolic contents (3.05 mg GAE/g), followed by South America quinoa (2.69 mg GAE/g), while the lowest total phenolic contents was found in Peru (Peru) quinoa (1.55 mg GAE/g).

Common and Tartary buckwheat seeds from several regions showed a wide range of free phenolic levels, ranging between 1.36 mg GAE/g in YunNan Tartary buckwheat and 3.03mg GAE/g in YunNan Common buckwheat, with an mean value of 2.18 mg GAE/g. Free phenolic contents of YunNan Common buckwheat was higher than those of the other varieties. In agreement with the quinoa seeds, on average the conjugated phenolic content of the buckwheat varieties was lower than the free phenolic content. The conjugated phenolic content of buckwheat varieties were in the range of 0.72-1.57 mg GAE/g, with the average values of 1.10 mg GAE/g. However, the bound phenolics content was even lower and among all of the buckwheat varieties tested were around 0.10 mg GAE/g except for Sichuan Tartary buckwheat (0.64 mg GAE/g). The total phenolic content in the buckwheat varieties showed a narrow range from 3.11 to 3.96 mg of GAE/g, with the exception of Yunnan Tartary buckwheat (2.22 mg of GAE/g).

As shown in Figure 3.2, the free, conjugated and total phenolic content of quinoa were significantly lower than those of buckwheat, whereas no significant difference in bound phenolic content was observed.

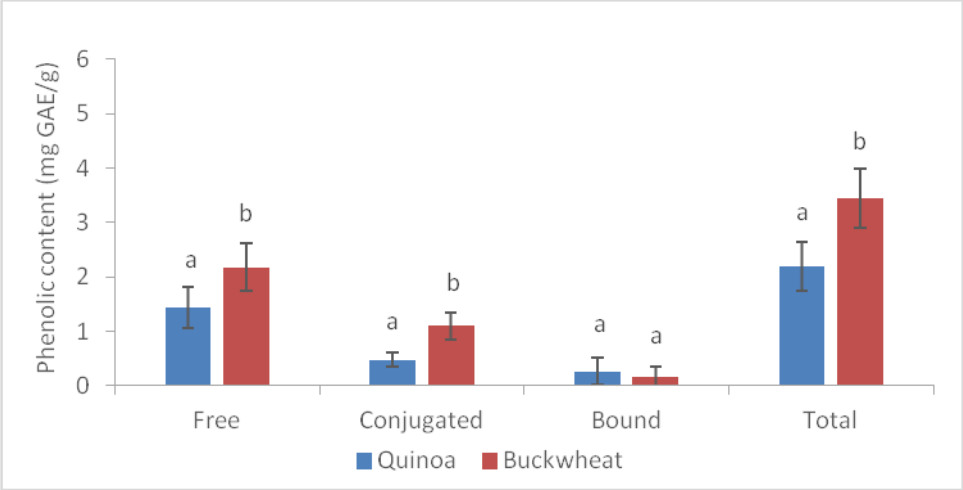


Figure 3.2. Comparison of free, conjugated, bound or total phenolic content (mean of all accessions, mg Gallic acid equivalent/100g) between quinoa and buckwheat. Mean ± Standard deviation.

3.3.1.1.2 Discussion –Phenolic content of quinoa and buckwheat

To the best of our knowledge, this is the first study to show phenolic content of different quinoa and buckwheat accessions sourced from different regions, which can give a comprehensive view to see the variations between them, but almost previous studies only focused on one quinoa or buckwheat accession. Phenolic compounds are secondary metabolites, which in whole grains may exist in three forms: free, soluble conjugate and insoluble bound forms which attached to cell materials (Adom and Liu, 2002). However, little information about the conjugated and bound phenolics was known, since most of the literature in the last decade is mainly focused on the studies of free forms. In this study, free, conjugated, and bound phenolic contents in both the quinoa and buckwheat varieties tested were significantly different ($p<0.01$) from each other, with the ranking order: Free > conjugated > bound phenolic compounds. It is clearly shown in Figure 3.1 that the phenolics in quinoa and buckwheat were mainly found in the free form, which contributed about 50.0-82.7% and 53.3-78.4% of the total phenolic content on a per weight basis across all varieties, respectively. In this regard the results in this study were consistent with those shown by Hung and Morita (2008), but in contrast, the phenolic compounds in corn, rice, wheat, oat and ray are primarily present in bound forms linked to cell wall materials (Sosulski *et al.*, 1982; Adom and Liu, 2002). Even though phenolic compounds are mainly present in free form, this study also indicates that the total phenolic contents of quinoa and buckwheat could be underestimated in the previously published studies without including the conjugated and bound phenolic compounds. Moreover, some studies showed that the free phenolic compounds may be digested in the upper gastrointestinal tract, while the bound fractions could survive stomach and intestinal digestion, allowing them to be released in the colon and, therefore, potentially play a protective role (Andreasen *et al.*, 2001a; Adom and Liu, 2002). For example, Andreasen *et al*. (2001b) demonstrated that diferulic acids can be released from dietary cereal brans by human and rat colonic microflora.

The free phenolic compound levels in this study were significantly lower those (2.53 mg GAE/g) reported by Gomez-Caravaca *et al*. (2014) and (4.2 mg GAE/g sample) by Tang *et al*. (2015). The total phenolic contents of quinoa were on average 2.18 mg GAE/g, which were also significantly lower than those reported by Inglett *et al*. (2015) (3.84 mg GAE/g) and Gomez-Caravaca *et al*. (2014) (5.24 mg GAE/g). The mean concentration of free and total phenolic contents (2.18 and 3.44 mg GAE/g, respectively) for buckwheat in this study were significantly lower than those reported by Inglett *et al*. (2011), Li *et al*. (2013) and Guo *et al*. (2011). The principle of FC assay is based on electron transfer; thus, the results calculated by FC assay were basically antioxidant capacity of total phenolics expressed as gallic acid equivalent, which cannot reflect the real amounts of total phenolics in quinoa and buckwheat. In one study, Lee *et al*. (2016b) have reported that although the amount of rutin in

Tartary buckwheat (10.7 mg/g buckwheat) was 72.8 times greater than that of common buckwheat (0.15 mg/g buckwheat), the difference of phenolic content analysed by FC assay between these two buckwheat species were not huge (20.9 vs 7.0 mg GAE/g, respectively; 3.0 times), which indicated that other minor amounts of phenolics in buckwheat seeds might make a relatively high contribution to phenolic content analysed by FC assay. This was partly in consistent with the present study that phenolic content analysed by FC assay in two Tartary buckwheat accession were similar to those of common buckwheat accessions. The total phenolic content of quinoa was significantly lower than buckwheat ($p<0.001$), but on average, the total phenolic contents in both quinoa and buckwheat seeds were significantly higher than those found in common cereals including barley (0.88 mg/g), wheat (0.56 mg/g) rye (1.03 mg/g) and millet (1.39 mg/g), suggesting that quinoa and buckwheat may serve as an excellent source of phenolic compounds (Ragaee *et al.*, 2006). The wide variations in the level of different phenolics forms with significant differences among quinoa and buckwheat varieties included in this study might be explained by the difference in the genetic background, environmental conditions under which the cereals were grown or location/environment where the crops were grown. The difference between the results presented in this study and the literature may be due to the different extraction methods, especially the extraction solvent differences.

3.3.1.2 Antioxidant activity of quinoa and buckwheat seeds

3.3.1.2.1 Ferric reducing ability of plasma (FRAP) assay

3.3.1.2.1.1 Results-FRAP antioxidant activity

By the FRAP method, the free and conjugated values varied over an approximately three-fold range from 1.57 to 6.44 µmol Fe2+ E/g dw and 0.97 to 3.51 µmol Fe2+ E/g dw, with an average of 3.49 and 1.54 µmol Fe2+ E/g dw, respectively (Figure 3.3). The highest values for both free and conjugated flour extracts were found in the Chinese Shanxi quinoa, which were higher than the values for all the quinoa other grains.

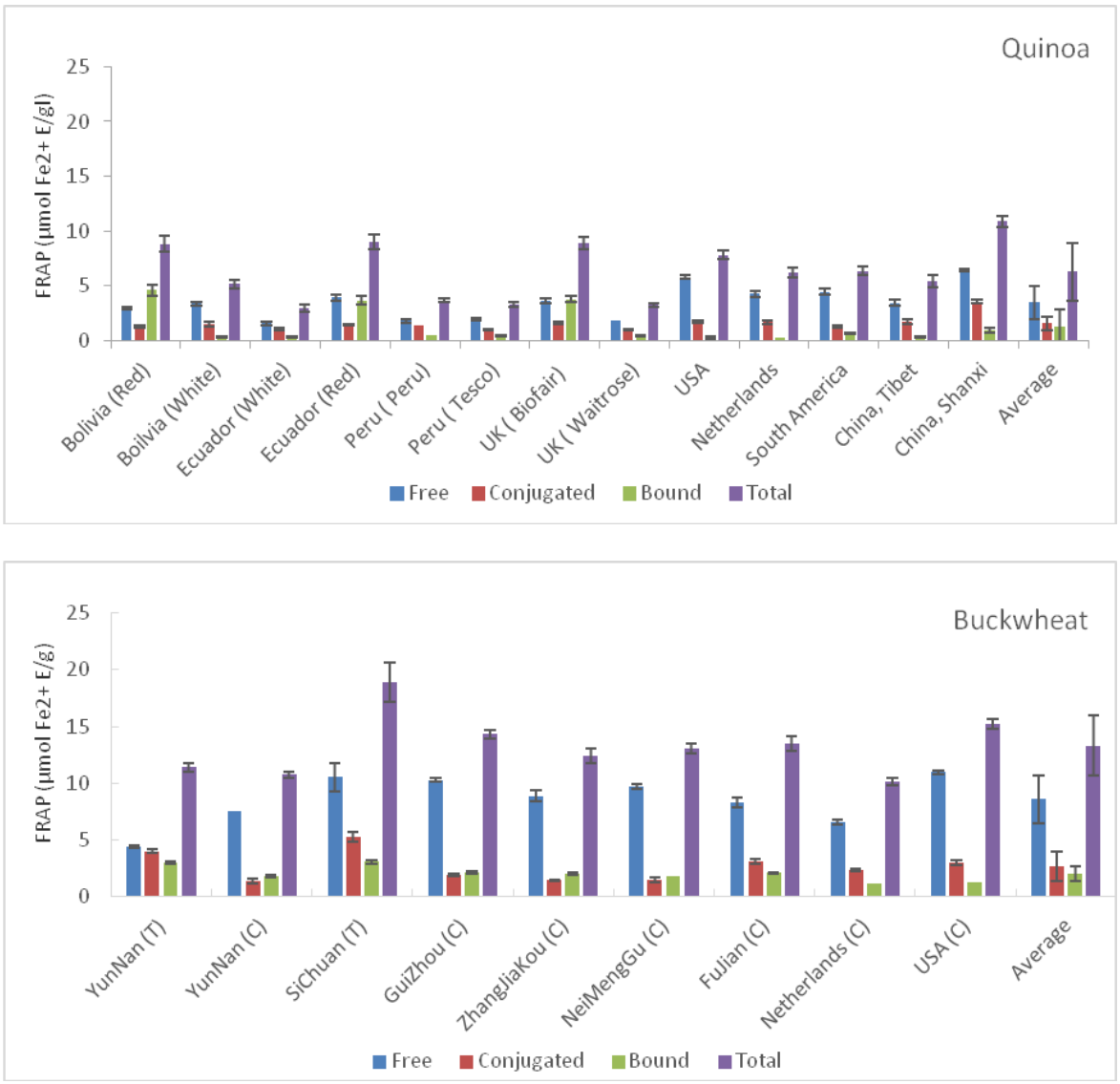


Figure 3.3. Antioxidant activity of free, conjugated, bound and total phenolic fractions (µmol Fe^{2+} E/g) of 80% ethanol: water extracts of quinoa and buckwheat from different varieties and sources measured by the FRAP assay.

The highest FRAP antioxidant activities of bound phenolic extracts was observed in the Bolivia (red) quinoa at 4.58 µmol Fe2+ E/g dw, followed by UK Biofair (3.74 µmol Fe2+ E/g dw) and Ecuador (red) quinoa (3.63 µmol Fe2+ E/g dw), which were higher than the other 10 varieties which ranged from 0.26-0.94 µmol Fe2+ E/g dw. The FRAP antioxidant activities of free flour extract were significantly higher than those of conjugated and bound extracts ($p<0.05$), but no differences were found between conjugated and bound flour extracts ($p>0.05$). The total FRAP antioxidant activities in the 13 quinoa flour samples ranged from the lowest value of 2.90 µmol Fe2+ E/g dw in Ecuador white quinoa and 10.89 µmol Fe2+ E/g dw in the Chinese Shanxi quinoa, with an average value of 6.27 µmol Fe2+ E/g dw.

The FRAP antioxidant activity of free, conjugated and bound extracts from buckwheat varieties ranged from 4.38-10.96 µmol Fe2+ E/g dw, 1.39-5.29 µmol Fe2+ E/g dw and 1.20-3.08 µmol Fe2+ E/g dw, with mean values of 8.58, 2.67 and 2.06 µmol Fe2+ E/g dw, respectively. Significant differences were detected among values of free, conjugated, and bound flour extracts, with the ranking order: Free > conjugated > bound buckwheat flour extracts ($p<0.05$). The highest FRAP value of the 9 buckwheat varieties was observed in Sichuan Tartary buckwheat (18.90 µmol Fe2+ E/g dw), which was higher than all of the other varieties which ranged from 10.16 to 15.23 µmol Fe2+ E/g dw.

As shown in Figure 3.4, the antioxidant activity of free, conjugated and total phenolic fractions of quinoa were significantly lower than those of buckwheat, whereas no significant difference in antioxidant activity of bound phenolic fraction was observed.

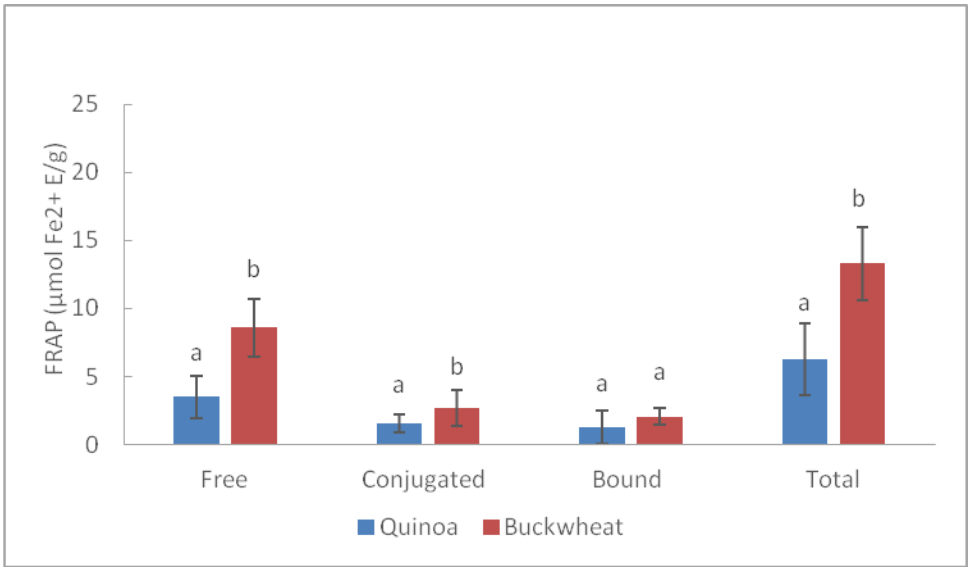


Figure 3.4. Comparison of antioxidant activity of free, conjugated, bound or total phenolic fractions (mean of all accessions, µmol Fe^{2+} E/g) between quinoa and buckwheat measured by the FRAP assay. Mean ± Standard deviation.

3.3.1.2.1.2 Discussion-FRAP antioxidant activity

The FRAP antioxidant activities of free extracts from quinoa was similar to the study reported by Nsimba *et al*. (2008) ($p>0.05$), but significantly higher than what is reported by Tejeda *et al*. (2008) and Brend *et al*. (2012). The FRAP antioxidant activity of free extracts from buckwheat was in line with the results of 8.59 µmol Fe2+ E/g dw reported by Chlopicka *et al*. (2012), but significantly lower than the 17.42 µmol Fe2+ E/g dw. reported by Alvarez-Jubete *et al*. (2010) and more than twice the value of 3.35 µmol Fe^{2+} E/g dw determined by Gorinstein *et al*. (2008) ($p<0.001$). The different extraction methods and extraction solvents between this study and the literature may be the primary contributor to the antioxidant activity variations.

3.3.1.2.2 The Trolox Equivalent Antioxidant Capacity (TEAC) assay

3.3.1.2.2.1 Results-TEAC antioxidant activity

As shown in Figure 3.5, the highest TEAC antioxidant activity of free and conjugated extracts of quinoa flours were observed in USA quinoa (12.87 µmol TE/g dw) and Chinese Shanxi quinoa (8.41 µmol TE/g dw), which were much higher than all other quinoa varieties (6.63 to 10.02, and 3.02 to 5.64 µmol TE/g dw, respectively). The highest TEAC antioxidant activity of bound extracts of flour was found in UK Biofair quinoa at 11.63 µmol TE/g dw, followed by Bolivian red quinoa and Ecuadorian red quinoa, with a similar concentration; values of all other grains were below 4 µmol TE/g dw.

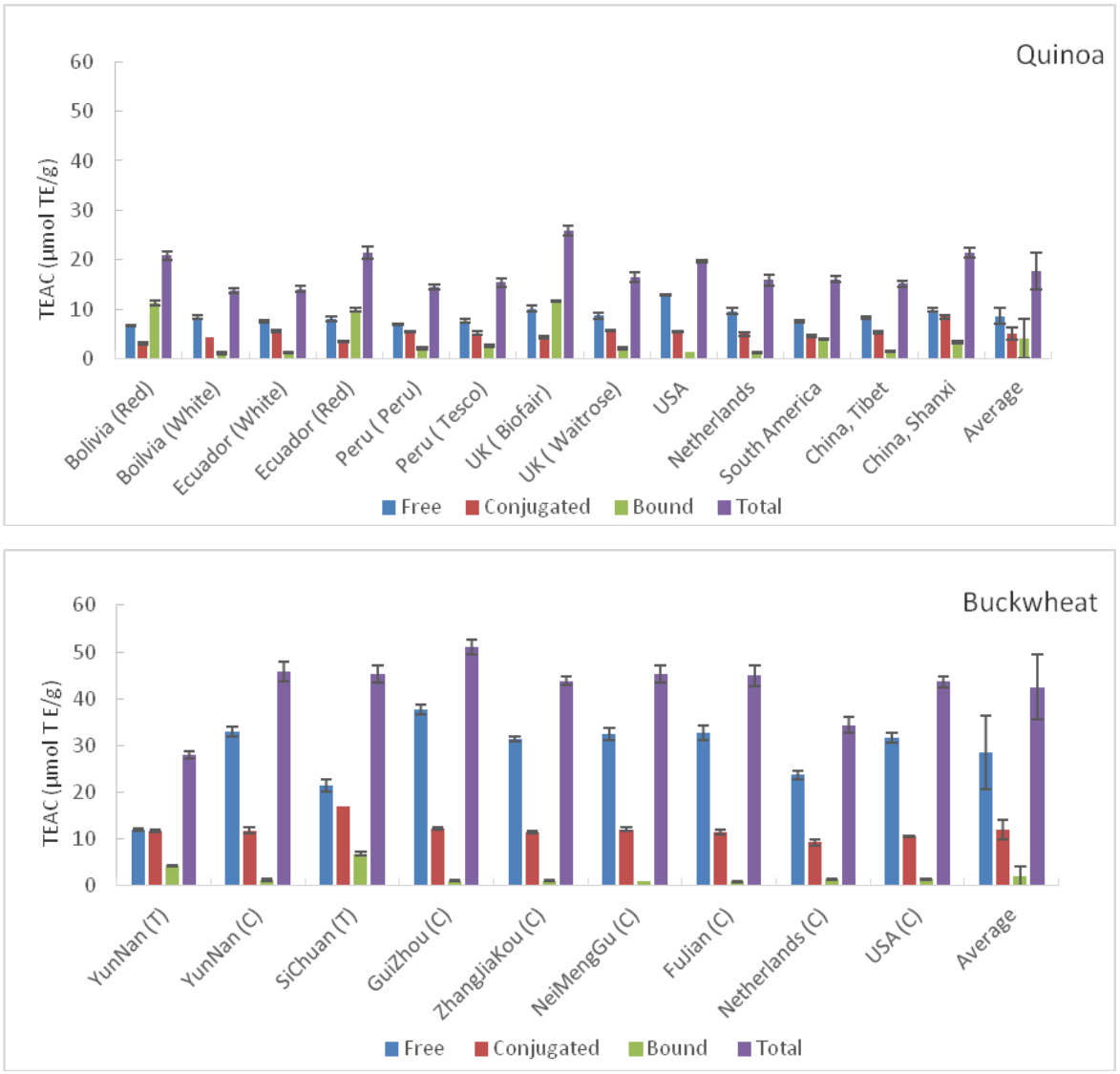


Figure 3.5. Antioxidant activity of free, conjugated, bound and total phenolic fractions (µmol TE/g dw) of 80% ethanol: water extracts of quinoa and buckwheat from different varieties and sources measured by the TEAC assay

The TEAC antioxidant activity of free extracts of quinoa flour was significantly higher than that of conjugated and bound flour extracts, but there were no significant differences between conjugated and bound flour extracts. The TEAC total antioxidant activity in quinoa ranged from 13.72 µmol TE/g dw in Bolivian white quinoa to 25.96 µmol TE/g dw in UK Biofair quinoa, with a mean concentration of 17.71 µmol TE/g dw.

The TEAC antioxidant values of free, conjugated and bound flour extracts from buckwheat were in the range of 12.02 to 37.73 µmol TE/g dw, 9.26 to 16.95 µmol TE/g dw and 0.86 to 6.854 µmol TE/g dw, with an average of 28.48, 11.96 and 2.09 µmol TE/g dw, respectively. There were significant differences among these three flour extracts ($p<0.001$), and TEAC antioxidant activity of free flour extracts was a major contributor, accounting for 67% of total antioxidant activity on average. The total TEAC antioxidant activity in buckwheat extracts varied from 27.94 µmol TE/g dw in Chinese Yunnan Tartary buckwheat to 51.08 µmol TE/g dw in Chines GuiZhou Common buckwheat, with a mean concentration of 42.46 µmol TE/g dw.

As shown in Figure 3.6, the antioxidant activity of free, conjugated and total phenolic fractions of quinoa were significantly lower than those of buckwheat, whereas no significant difference in bound phenolic fraction was observed.

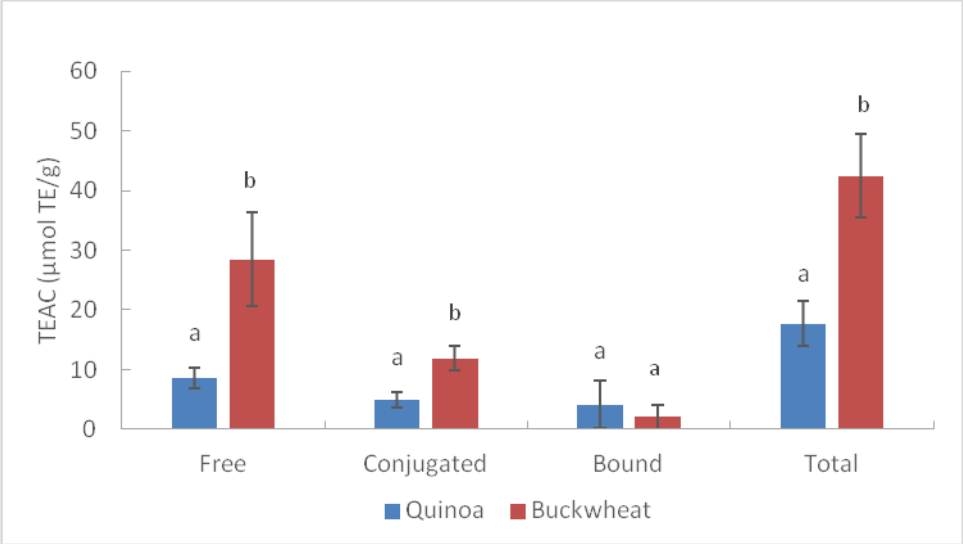


Figure 3.6. Comparison of antioxidant activity of free, conjugated, bound or total phenolic fractions (mean of all accessions, µmol TE/g) between quinoa and buckwheat measured by the TEAC assay. Mean ± Standard deviation.

3.3.1.2.2.2 Discussion-TEAC antioxidant activity

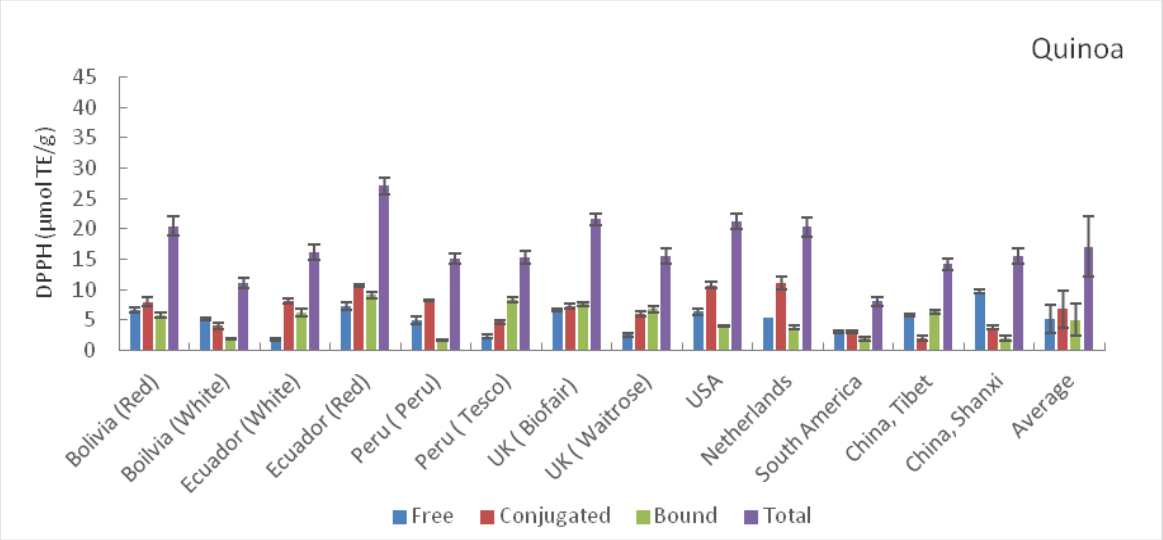
The TEAC values of free quinoa flour extracts in this study was in accordance with values of 9.40-14.74 µmol TE/g dw reported by Repo-Carrasco-Valencia and Serna (2011), but markedly

significantly higher than levels by Tejeda et al. (2008) and Laus *et al*. (2012). For buckwheat the distribution of apparent TEAC antioxidant content between the different fractions was similar to the results of Alvarez-Jubete *et al*. (2010) and Zielinska *et al*. (2007) (p>0.05).

3.3.1.2.3 2, 2-diphenyl-1-picrylhydrazyl (DPPH•) assay

3.3.1.2.3.1 Results-DPPH antioxidant activity

The DPPH antioxidant values of free, conjugated and bound extracts of the 13 quinoa varieties showed a range of 1.81 to 9.72 µmol TE/g dw, 2.07 to 11.15 µmol TE/g dw and 1.76 to 9.12 µmol TE/g dw, with an average of 5.22, 6.77 and 5.05 µmol TE/g dw, respectively (Figure 3.7). The highest DPPH antioxidant activity of free extracts was observed in Chinese Shanxi quinoa, which was higher than the other quinoa varieties. There were no significant differences (P > 0.05) in the DPPH antioxidant activities of free, conjugated and bound extracts in this study between the 13 quinoa varieties, although conjugated extracts had markedly higher DPPH antioxidant values compared with free and bound extracts. The total DPPH antioxidant activity of the quinoa varieties was in was in the range of 8.03 to 27.07 µmol TE/g dw with a mean value of 17.04 µmol TE/g dw.



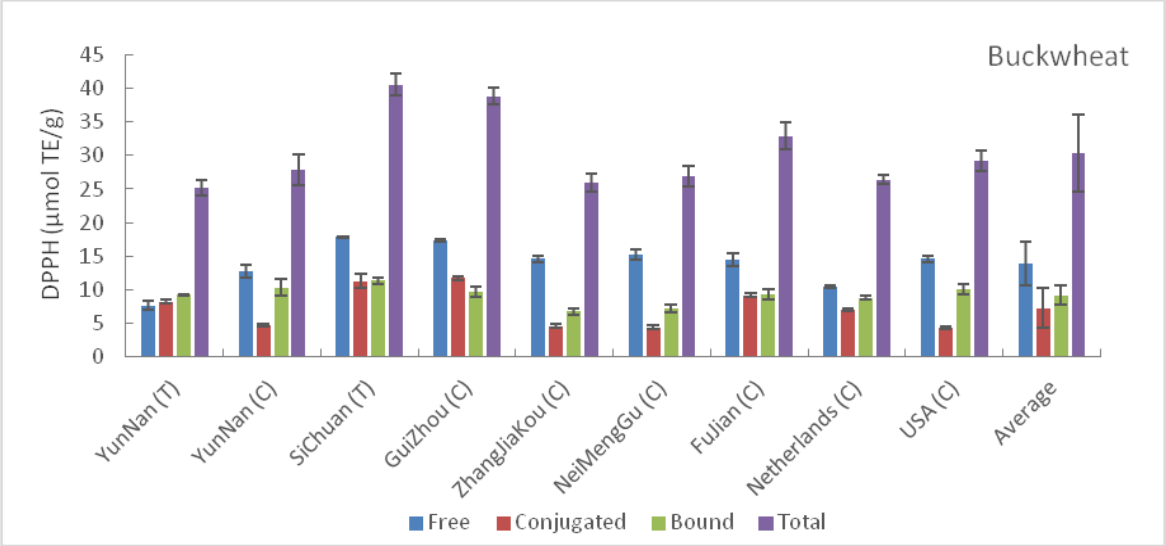


Figure 3.7. Antioxidant activity of free, conjugated, bound and total phenolic fractions (µmol TE/g dw) of 80% ethanol: water extracts of quinoa and buckwheat from different varieties and sources measured by the DPPH assay.

The DPPH antioxidant activity of free, conjugated, bound and total extracts of buckwheat varieties ranged from 7.64 to 17.84 µmol TE/g dw, 4.40 to 11.79 µmol TE/g dw, 6.76 to 11.41 µmol TE/g dw, and 25.19 to 40.54 µmol TE/g dw with the average of 13.89, 7.31, 9.22 and 30.14 µmol TE/g dw, respectively. There are significant differences detected among DPPH antioxidant values of free, conjugated, and bound flour extracts, with the ranking order: Free> bound > conjugated flour extracts ($p<0.05$). In this study, Chinese Sichuan Tartary buckwheat displayed the greatest values of DPPH antioxidant activity of free, bound and total flour extracts, and this accession had the second highest level of DPPH antioxidant activity of the conjugated extracts.

As shown in Figure 3.8, the antioxidant activity of free, bound and total phenolic fractions of quinoa were significantly lower than those of buckwheat, whereas no significant difference in conjugated phenolic fraction was observed.

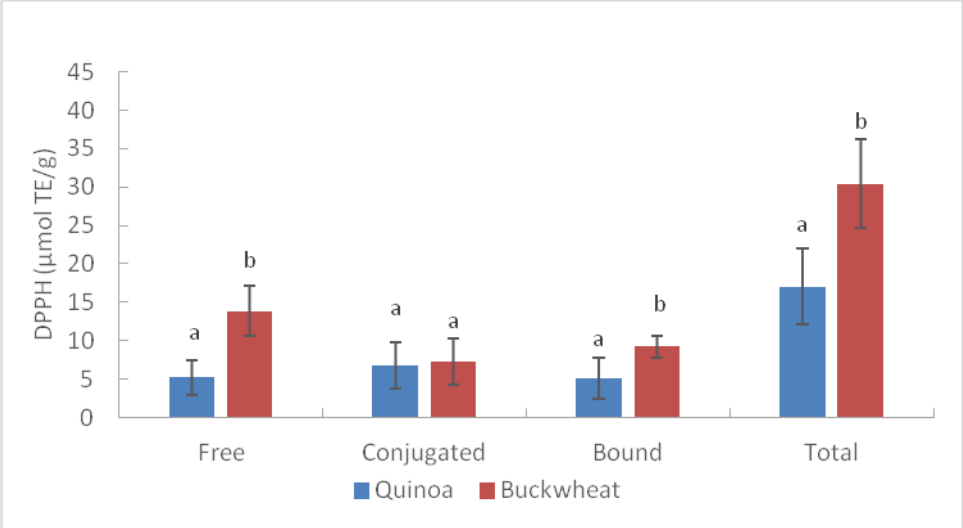


Figure 3.8. Comparison of antioxidant activity of free, conjugated, bound or total phenolic fractions (mean of all accessions, µmol TE/g) between quinoa and buckwheat measured by the DPPH assay. Mean ± Standard deviation.

3.3.1.2.3.2 Discussion-DPPH antioxidant activity

The result for DPPH antioxidant activity were in agreement with the results of Dini et al. (2010), showing that bitter and sweet raw quinoa seeds had values of 6.71 and 2.87 µmol TE/g dw, respectively, and were similar to the range of 0.49-5.08 µmol TE/g dw reported by Inglett *et al*. (2015). Regarding the DPPH antioxidant activity of bound extracts, the results in this study were significantly lower than values shown by Inglett *et al*. (2015). The DPPH antioxidant activity of free, bound and total buckwheat flour extracts in this study were significantly higher than results reported by Inglett et al. (2011) and Inglett et al. (2015), except the values of bound flour extracts in the latter study (10.33µmol TE/g dw)

3.3.1.3 Correlation between phenolic content and antioxidant activity

3.3.1.3.1 Results-Correlation

In this study, possible correlations between the total phenolic content (TPC) and antioxidant activity of quinoa and buckwheat varieties determined using the three different assays were investigated (Table 3.1). The correlation between TPC and antioxidant activity of quinoa measured by FRAP and TEAC methods were statistically positive ($r>0.5$, $p<0.05$), but not when compared against the DPPH method. Additionally, relatively high, and significant positive correlations between FRAP and TEAC methods of measuring antioxidant activity of free, conjugated, bound and total extracts in quinoa were found ($r\geq0.679$, $p<0.05$), but not when compared the antioxidant activity of FRAP and DPPH, and TEAC and DPPH.

Table 3.1. Correlation between total phenolic content (TPC) and antioxidant activity and between different measures of antioxidant activity.

Cereal Species	TPC vs FRAP	TPC vs TEAC	TPC vs DPPH	FRAP vs TEAC	RAFP vs DPPH	TEAC vs DPPH
Quinoa						
Free	0.848**	0.603*	0.457	0.679*	0.625	0.401
Conjugated	0.517*	0.696*	-0.453	0.689**	-0.170	-0.292
Bound	0.979**	0.976**	0.379	0.982**	0.446	0.466
Total	0.797**	0.545*	0.110	0.829**	0.466	0.635
Buckwheat						
Free	0.405	0.748*	0.428	0.654	0.932**	0.641
Conjugated	-0.010	0.020	-0.441	0.605	0.583	0.538
Bound	0.605	0.896**	0.567	0.586	0.297	0.558
Total	0.493	0.830	0.494	0.474	0.494	0.594

P value of linear regression correlation coefficient; *p<0.05, **p<0.01

Regarding the buckwheat measurements, the antioxidant activity determined by the TEAC method showed a significantly positive correlations with Free ($r=0.748$, $p<0.05$) and bound phenolics ($r=0.896$, $p<0.01$). However, the remainder of the correlation coefficients between phenolic content and antioxidant activity tested by FRAP, TEAC and DPPH methods, and also between antioxidant activity tested by FRAP and TEAC, FRAP and DPPH, TEAC and DPPH methods were found to be very weak, less than 0.5, indicating poor correlation between these measures, with the exception of correlation between FRAP and DPPH methods measuring antioxidant activity of free phenolic extracts.

3.3.1.3.2 Discussion- correlation between TPH and antioxidant activity

The high antioxidant activity reported for many natural foods including fruits, vegetable and cereal products is often attributed to the polyphenolic content of the food. The literature is full of such statements. This has then often been translated into supposed associations between 'antioxidant activity' in a food/diet and 'antioxidant status' of an individual consuming that food. It is often suggested that an individual's antioxidant status is, or can be, affected by the amount of high antioxidant activity foods consumed. However, this relationship is being questioned, with more recent studies suggesting that polyphenolics may have beneficial effects through acting as signalling molecules and not as 'antioxidants'. Recently, growing evidence suggests that dietary-derived flavonoids may exert beneficial effects on long-term potentiation (LTP), and consequently memory and cognitive performance, via their interactions with signalling pathways, including the phosphatidylinositol-3 kinase/protein kinase B/Akt, protein kinase A and protein kinase C (Spencer, 2008a; Spencer, 2008b).

The relationship between TPC and antioxidant activity has been widely determined in various foods including fruits and vegetables (Kaur and Kapoor, 2002; Babbar et al., 2011). Previously reported studies showed a liner correlation between TPC and antioxidant activity in fruits and vegetables. However, this cannot be applied to all stuffs analysed for TPC and antioxidant activity (Quettier-Deleu et al., 2000; Holasova et al., 2002; Kaur and Kapoor, 2002; Morishita et al., 2007; Babbar et al., 2011). There are several reasons to explain the ambiguous correlation between TPH and antioxidant activity: (1) Phenolic compounds are not only the components responsible for apparent antioxidant activity in quinoa and buckwheat; other compounds which would have apparent antioxidant activity in the assays used include ascorbic acid, vitamin E, tocopherol and sterols. (2) The antioxidant activity of most pure phenolic content or vitamins were lower than those of the fruits extracts on a weight basis (Vinson et al., 2001). The antioxidant activity are not only dependent on the level of antioxidants, but also synergism, to be specific, the interaction and structures among antioxidants (Sun and Ho, 2005). (3) Different methods to determine antioxidant activity with various analytical mechanisms may lead to different observations (Kähkönen *et al.*, 2001).

3.3.1.4 General discussion – antioxidant measures

In the three antioxidant evaluation systems used FRAP, DPPH and TEAC, antioxidant activities of free, conjugated and bound phenolic extracts accounted for less than 40% of total antioxidant activities determined by TEAC and DPPH methods, with the exception of free phenolic extracts in quinoa by FRAP. The correlations between TPC and antioxidant activity were not always significant. Thus, it is difficult and impractical to define which phenolic component plays a dominant role in antioxidant activity in quinoa varieties. Regarding buckwheat varieties, antioxidant activities of free phenolic extracts were statistically higher than those of conjugated and bound phenolic extracts. Free phenolic extracts accounted for 64%, 67% and 46% of total FRAP, TEAC and DPPH· scavenging activities in an average among buckwheat varieties, respectively. The results were in accordance with studies reported by Huang and Morita (2008) and Guo et al. (2011) that antioxidant compounds mainly existed in free forms, which were contrary to the results of Inglett et al. (2011), as well as that seen for wheat and corn fractions (Adom and Liu, 2002; Liyana-Pathirana and Shahidi, 2006). Among the total antioxidant activities of buckwheat varieties, Chinese Sichuan Tartary buckwheat had the highest FRAP and DPPH values, by contrast, the highest TEAC scavenging activity was found in Chinese GuiZhou Common buckwheat. The antioxidant activity of buckwheat seed extracts, measured by FRAP, TEAC and DPPH assays, was statistically higher than those of quinoa seed extracts ($p<0.05$). In order to comprehensively screen and compare antioxidant activity levels among a wide accession of quinoa and buckwheat samples, three methods were applied in this study.

Comparison of antioxidant activity of quinoa or buckwheat in these three studies was impractical and different, since the different antioxidant activity method has not been standardized.

Up to date, there are a large numbers of methods and strategies that have been proposed and developed to evaluate total antioxidant activity in various samples like foodstuffs and plant tissues (Prior and Cao, 1999). Among them, three methods, FRAP, TEAC and DPPH for assessment of antioxidant activity are the most commonly used spectrophotometric methods based on reaction with electron donating or hydrogen radical (H•) producing antioxidant compounds. In specific, the FRAP method is based on the ability of antioxidant to reduce (electron transfer) Fe^{3+} to Fe^{2+} ions in the presence of TPTZ forming an intense blue Fe^{2+}-TPTZ complex with an absorption maximum at 593 nm. However, this method has been criticized by Prior and Cao, since not all reductants in samples that have the ability to reduce the ferric ion to ferrous ion are necessarily antioxidants. In addition, glutathione, as an important antioxidant *in vivo*, is not included in the analysis. Finally, the use of the ferric ion as a final indicator may also cause problems when an analysed antioxidant, such as ascorbic acid, not only reduces ferric to ferrous but can also react with the ferric ion to generate additional free radicals. The TEAC method is based on the scavenging of the blue-green radical cation $ABTS^{\bullet+}$ converting it into the colourless neutral form through reaction with antioxidants including phenolics, thiols and Vitamin C. As for DPPH method, it is based on the ability of antioxidant to give hydrogen radical to synthetic long-lived nitrogen radical compounds DPPH• having a radical localized on the N-atom. Although the principles of the three analytical methods in the this study were different, the comparison between methods is desirable to give a comprehensive measure of antioxidant activity of quinoa and buckwheat varieties and also compare with the results of other large numbers of studies. For example, the DPPH method gives relatively lower values for extracs than TEAC, but both of which were significantly higher than that of FRAP methods. This finding was partly consistent with the results of study that analysed grains, as reported by Stratil *et al*. (2007). The differences between DPPH and TEAC may be accounted for the a relatively higher stability and lower reactivity of the DPPH radical, which only reacts with more reactive reducing substances (phenolics). However, it is not appropriate to directly compare the results of two methods for same samples due to differences between compounds measured by different methods. For example, the TEAC method can be used to evaluate both lipophilic and hydrophilic antioxidants, whereas the FRAP method is not appropriate for the evaluation of lipophilic antioxidants (Re *et al.*, 1999a; Arnao *et al.*, 2001; Cano *et al.*, 2002).

The solvent extraction using wide range of polarity of solvents has been the major method used to obtain grain extracts rich in antioxidants or isolate cereal antioxidants. Up to date, several methods have been suggested: different solvents including water, methanol, ethanol, acetone and chloroform

have been applied individually or in combination to achieve maximum extraction amounts from grains (Duh *et al.*, 1992; Zielinski and Kozlowska, 2000; Bryngelsson *et al.*, 2002; Inglett *et al.*, 2015). Unfortunately, since the extraction method has not been standardised so far, thus, comparison among independent studies using different extraction procedures or solvents is often problematic. Previous studies have showed that the yield of free extracts increased with the increasing polarity of the solvent used (Duh *et al.*, 1992; Bałłasiriska and Troszyńska, 1998; Przybylski *et al.*, 1998). For example, the efficiency of the solvents on extraction decreased in the following order: methanol > ethanol > acetone > chloroform > *n*-hexane, as reported by Duh *et al*. (1992). When using water as an solvent, Inglett *et al.* (2015) have revealed that amounts of free phenolic contents of ancient grains (amaranth, quinoa, buckwheat and teff) increased, but bound phenolic contents experienced an opposite trend as the proportion of water in the extraction solvent increased (0, 50 and 100%). This was partly agreement with the results obtained in our lab that amounts of phenolic contents of quinoa and buckwheat in water extracts determined by FC assay were significantly higher than that of methanol and ethanol extracts. The phenolic compounds extracted these three solvents were subsequently confirmed by HPLC in the lab (Not published) (see Appendix 6). Besides, regarding wheat germ, the higher efficiency of water in extracting phenolic compounds in comparison with methanol has already been revealed by Gallardo *et al*. (2006). The differences mentioned above may be attributed to the high content of water-soluble phenolics or antioxidants in grains like oat and buckwheat (Watanabe *et al.*, 1997; Watanabe, 1998; Emmons *et al.*, 1999; Bryngelsson *et al.*, 2002). However, as compared with the mixture of water and methanol (20:80, v/v) extract, the contents of total phenolic compounds of lyophilizates of water extracts obtained from whole grains especially oat and buckwheat were substantially lower (Zielinski and Kozlowska, 2000). Likewise, the use of the mixture of water and ethanol (50:50, v/v) produced markedly higher antioxidant activity compared with those obtained using only methanol (Serpen *et al.*, 2008). According to these two published papers, it appeared that replacing the solvent with mixture of water and other solvents like ethanol or methanol significantly increased the measured phenolic contents or antioxidant activity of cereal samples. The extraction solvent used in this current study was based on the two previously published papers of Li *et al*. (2008) and Adom and Liu, (2002) that they both used the water and ethanol (20:80, v/v) as the extraction solvent. In contrast, a mixture of water and other solvents was not always associated with higher free or bound antioxidant activity of several grains (Zielinski and Kozlowska, 2000; Inglett *et al.*, 2015). Therefore, the antioxidant activity of free or bound extracts not only dependent on extraction methods but also grain species, thereby making it difficult to define the best extraction solvent of antioxidants from grains. In this study, after the extraction of free phenolics, alkaline hydrolysis has been applied to liberate cell wall-bound phenolics from

residue, followed by four times ethyl acetate extraction. Although hydrolysis could also be carried out under acid or enzymatic conditions, these conditions did not have the ability to classify phenolics into free, conjugated and insoluble-bound forms. However, it has also been pointed by Serpen *et al*. (2007) that alkaline hydrolysis fails to liberate all bound antioxidants present; moreover, the phenolic compounds could be further oxidised or degraded, thus resulting in loss of antioxidants during the hydrolysis treatment.

To the best of our knowledge, this is the first study to demonstrate the free, conjugated and bound phenolic contents in quinoa and buckwheat seeds to date. Since the methods specific to quinoa and buckwheat have not been explored and standardised to date, the phenolic extraction methods in previous studies as well as the present study were mainly based on wheat and other grains, but these methods were not also necessarily suitable for quinoa and buckwheat seeds. What happened in the extraction process of wheat and other grains cannot easily extrapolate to quinoa or buckwheat due to hugely different phenolic compounds among them. For example, Adom and Liu, (2002) have reported that the contribution of bound phenolic to total phenolics was 90, 87, 71 and 58% in wheat, corn, rice and oat, respectively, which were contrary to quinoa and buckwheat seeds. So far, almost all previous authors investigating quinoa and buckwheat phenolic compounds only used one extraction method in their studies, and then focused in phenolic content and/ or analysis of phenolic compounds by HPLC. It was unlikely to compare and discuss how the different phenolic extraction methods can affect subsequent antioxidant activity and phenolic compounds, since quinoa seeds from different studies might be hugely different, which would consequently give different results. Therefore, this would limit the ability of seeing what have been lost by degradation and crystallisation. For the future studies, to ensure that it is feasible to obtain a reasonably accurate value, the phenolic extraction method should have been calibrated by using it on a sample with known content (pure standard content such as gallic acid, rutin). In specific, after preparation of standard solution for analysis, each standard should be divided into two portions, and one portion should be evaporated to dryness and then processed to the whole extraction process (such as extract, centrifugation and hydrolysis) as the biological samples. The recovery in% can be calculated by comparing the values of the processed standard with the unprocessed standard. This is also one of limitations of the present study that have not calibrated the phenolic extraction methods before this study.

3.4 Composition analysis of quinoa seeds

As shown in Table 3.2, there was a variation in the proximate composition between the thirteen quinoa seed samples; the amino acid profile of the quinoa varieties is shown in Table 3.3.

Proteins are major biological macromolecules that participate in the construction and maintenance of tissues, as potential energy source, formation of enzymes, hormones and antibodies, and regulation of metabolic processes in the body. In addition to offering nitrogen, amino acids are a good source of sulphur compounds for the body. In the form of lipoprotein, they also play an important role in the transport of fat-soluble vitamins, cholesterol, triglycerides and phospholipids (Alves *et al*., 2008). Protein intake and protein quality are therefore very important in determining diet quality. However, many people of the world still fail to include sufficient high quality protein in their diets, especially those following vegetarian or vegan diets who can only get them from regular grains or legumes, but rarely/never consume animal protein; therefore, insufficient intake of some essential amino acids may make the prevalence of protein malnutrition worse (Mujica *et al*., 2001; Alves *et al*., 2008).

Table 3.2. Proximate composition of 13 quinoa varieties (dry basis)

	Bolivia (Red)	Bolivia (White)	Ecuador (White)	Ecuador (Red)	Peru (Online)	Peru (Tesco)	UK (Biofair)	UK (Waitrose)	USA	Nether-lands	China (Tibet)	China (Shanxi)	South America	Average ± SD
Energy (Kcal/100g)	358	357	363	356	352	359	362	362	361	365	370	357	349	359 ± 5
(kJ/100g)	1506	1502	1531	1501	1484	1514	1524	1528	1522	1540	1560	1505	1464	1514 ± 25
Protein (g/100g)	11.80	14.52	14.14	12.61	12.78	13.55	12.72	13.22	13.71	14.21	14.12	14.66	13.58	13.51 ± 0.85
Ash (g/100g)	2.25	2.44	2.44	2.43	2.32	2.22	2.31	2.05	2.57	2.51	2.01	3.87	2.30	2.44 ± 0.46
Moisture (g/100g)	10.85	13.38	11.42	10.72	12.15	10.03	9.42	10.19	11.84	10.95	8.94	10.91	10.37	10.86 ± 1.17
Available Carbohydrate (g/100g)	57.58	53.55	56.77	56.57	55.93	61.11	57.68	61.72	55.98	56.85	60.93	54.61	48.76	57.19 ± 2.63
Total sugars (g/100g)	3.77	3.09	3.78	3.54	3.19	5.05	4.30	3.67	5.77	4.12	4.97	6.29	4.45	4.31 ± 0.98
Sodium (g/100g)	<0.1	<0.1	0.16	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	0.14	<0.1	<0.1	<0.1	<0.1
Salt (g/100g)	<0.1	<0.1	0.41	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	0.36	<0.1	<0.1	0.23	<0.1
Insoluble dietary fibre (g/100g)	9.71	7.27	7.24	9.40	9.05	6.92	9.95	6.51	7.41	6.79	7.71	8.57	11.02	8.27 ± 1.43
Soluble dietary fibre (g/100g)	1.37	1.39	0.95	1.53	1.53	1.33	1.56	1.09	1.26	1.52	0.36	0.49	1.57	1.26 ± 0.44
Total dietary fibre (g/100g)	11.08	8.66	8.19	10.93	10.58	8.25	11.51	7.60	8.67	8.31	8.07	9.06	12.59	9.53 ± 1.65
Fat (g/100g)	6.44	7.45	7.04	6.34	6.24	4.84	6.36	5.22	7.23	7.17	5.93	6.89	7.03	6.48 ± 0.79
Saturated fat (g/100g)	0.73	0.84	0.78	0.76	0.71	0.54	0.72	0.58	0.83	0.82	0.63	0.81	0.81	0.74 ± 0.10
Monounsaturated fat (g/100g)	2.07	1.98	1.94	2.04	1.81	1.24	2.02	1.39	1.99	1.98	1.74	1.58	2.00	1.83 ± 0.27
Polyunsaturated fat (g/100g)	3.37	4.31	4.02	3.26	3.46	2.86	3.34	3.04	4.09	4.06	3.31	4.21	3.92	3.63 ± 0.48

SD, standard deviation

Table 3.3. Amino acid composition of 13 quinoa varieties (dry basis)

	Bolivia (Red)	Bolivia (White)	Ecuador (White)	Ecuador (Red)	Peru (Online)	Peru (Tesco)	UK (Biofair)	UK (Waitrose)	USA	Nether-lands	China (Tibet)	China (Shanxi)	South America	Average ± SD
Essential amino acids														
Histidine (g/100g)	0.33	0.39	0.35	0.34	0.35	0.37	0.35	0.37	0.36	0.35	0.39	0.38	0.35	0.36 ± 0.02
Iso-Leucine (g/100g)	0.45	0.5	0.47	0.46	0.48	0.5	0.47	0.49	0.51	0.48	0.54	0.51	0.46	0.49 ± 0.03
Leucine (g/100g)	0.73	0.82	0.75	0.75	0.76	0.79	0.75	0.81	0.82	0.77	0.84	0.83	0.78	0.79 ± 0.04
Lysine (g/100g)	0.71	0.87	0.76	0.76	0.77	0.78	0.73	0.78	0.8	0.75	0.82	0.83	0.81	0.78 ± 0.04
Methionine (g/100g)	0.25	0.27	0.25	0.25	0.26	0.26	0.25	0.25	0.27	0.26	0.28	0.27	0.27	0.26 ± 0.01
Phenylalanine (g/100g)	0.44	0.51	0.46	0.46	0.47	0.49	0.46	0.5	0.5	0.48	0.52	0.52	0.48	0.48 ± 0.03
Threonine (g/100g)	0.45	0.53	0.46	0.47	0.48	0.48	0.46	0.48	0.52	0.48	0.52	0.52	0.5	0.49 ± 0.03
Tryptophan (g/100g)	ND*	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Valine (g/100g)	0.56	0.63	0.58	0.59	0.61	0.62	0.58	0.62	0.64	0.6	0.66	0.64	0.59	0.61 ± 0.03
Non-essential amino acids														
Alanine (g/100g)	0.5	0.61	0.51	0.54	0.54	0.55	0.52	0.53	0.59	0.54	0.57	0.59	0.58	0.55 ± 0.03
Arginine (g/100g)	1.01	1.2	1.06	1.04	1.07	1.2	1.06	1.21	1.16	1.1	1.27	1.17	1.07	1.13 ± 0.08
Aspartic Acid (g/100g)	0.99	1.13	1.02	1.03	1.04	1.12	1.03	1.08	1.08	1.04	1.12	1.12	1.07	1.07 ± 0.05
Cystine (g/100g)	0.19	0.22	0.19	0.19	0.2	0.2	0.19	0.2	0.2	0.2	0.22	0.23	0.2	0.2 ± 0.01
Glutamic Acid (g/100g)	1.62	1.88	1.71	1.65	1.7	1.9	1.67	1.85	1.72	1.71	1.93	1.93	1.7	1.77 ± 0.12
Glycine (g/100g)	0.59	0.74	0.65	0.63	0.67	0.68	0.62	0.68	0.7	0.67	0.72	0.76	0.68	0.68 ± 0.05
Proline (g/100g)	0.45	0.51	0.48	0.45	0.47	0.49	0.46	0.49	0.51	0.48	0.51	0.53	0.49	0.49 ± 0.03
Serine (g/100g)	0.56	0.66	0.58	0.59	0.59	0.63	0.58	0.62	0.62	0.59	0.64	0.66	0.62	0.61 ± 0.03
Tyrosine (g/100g)	0.33	0.4	0.38	0.36	0.38	0.4	0.34	0.38	0.4	0.37	0.41	0.4	0.39	0.38 ± 0.03

*ND, not determined (Analysis of tryptophan was not in the original list of amino acids offered by the commercial company)

To the best of our knowledge, this is the first study to show nutritional composition of different quinoa and buckwheat accessions sourced from different regions, which can give a comprehensive view to see the variations between them, but almost previous studies only focused on one quinoa or buckwheat accession.

One of the most important properties of quinoa seeds is the quantity and quality of protein they contain. China Shanxi quinoa exhibited the highest protein content in the dry matter of 13 quinoa seeds (14.66 g/100g), closely followed by Bolivia (White) quinoa (14.52 g/100g), whereas the lowest protein content was found in Bolivia (Red) quinoa (11.80 g/100g). The total protein content displayed an average of 13.51 g/100g, which is in line with that found for six genotypes of quinoa seeds from 11.32 to 14.72 g/100g from Peru reported by Repo-Carrasco-Valencia *et al.* (2010a) and from 11.31 to 16.18 g/100g from different regions of Chile by Miranda *et al*. (2012). In comparison with the common cereals, these results show that the protein content in quinoa is significantly higher than that in rice, corn, sorghum, barley and rye, and is similar to that of wheat (Kozioł, 1992; Comai *et al.*, 2007; Jancurova *et al.*, 2009; USDA, 2015).

The nutritional quality of protein in various foods is mainly determined by the composition of essential amino acids that cannot be synthesized or created by animals, and hence must be obtained from the diet. Moreover, the lack of only one of these amino acids will largely affect the absorption and metabolism of the others, resulting in the loss of protein in the diet and poor growth. Nine amino acids are strictly essential for humans: histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine (essential for infants and children), which are all identified in quinoa seeds except for tryptophan which was 'lost' and could not determined in the analysis during hydrolysis (Table 3.3), offering a high protein value similar to casein in milk (WHO, 2007; Vega-Galvez *et al.*, 2010). Therefore, quinoa is one of the only plant foods that offers 'complete protein'. This is a term that describes a source of protein that contains an adequate proportion of all nine of the essential amino acids necessary for human life, with an approximate average concentration of 5.75 g/100g of 13 quinoa seeds, as shown in Table 3.3 (Stikic *et al.*, 2012). The composition of amino acid in quinoa seed is very close to those suggested by FAO/WHO/UNU (1985), with a well-balanced amino acids profile, being rich in lysine and sulphur-containing amino acids, and it is accepted as a high quality protein, contrary to the protein content of common cereals, such as wheat and maize, which are especially deficient in lysine (Mujica *et al.*, 2001; WHO, 2007; Alves *et al.*, 2008; Oh *et al.*, 2016). Not only the proportions of amino acids, but also processing can affect protein quality of quinoa seeds. Protein digestibility or bioavailability of amino acids in quinoa, which is superior to that of common cereals, varies according to possessing of quinoa, and it increased considerably with cooking (Kozioł, 1992; Ruales and Nair, 1992; Comai *et al.*, 2007; Alves *et al.*, 2008;

Abugoch James, 2009). Additionally, the protein quality is also partly influenced by antinutritional factors, which used to describe the class of compounds present in various plant foods that reduce their nutritional value, including their digestibility and absorption, probably resulting in harmful impacts if digested in high amounts (Filho *et al.*, 2017). Up to date, a few antinutritional factors, including saponins, phytic acid, tannins, nitrates, oxalates and trypsin inhibitors, were identified in quinoa seeds. For example, one of undesirable biological effects of tannins was to from complex with protein, thus reducing the nutritional value of foods (dos Santos, 2006).

Carbohydrates are one of the largest groups of organic compounds present in quinoa seeds in common with other cereals, providing the main source of physiological energy in the human diet. Available carbohydrate levels in the evaluated quinoa seeds showed values ranging between 48.76 g/100g in South America quinoa and 61.72 g/100g in UK (Waitrose), with an average of 57.19 g/100g, which is significantly lower than the value of 65.60 g/100g mentioned by Koziol (1992) and between 68.84 and 75.82 g/100g reported by Repo-Carrasco-Valencia *et al*. (2011), but it compares favourably with the values of between 56.08 to 62.47 g/100g of Miranda *et al*. (2013). The quinoa cultivated in UK (Waitrose) exhibited the highest available carbohydrate level, which was quite similar to the Peru (Tesco) quinoa (61.11 g/100g) and China (Tibet) quinoa (60.93 g/100g), but higher than all other varieties.

Quinoa has been regarded as a potential alternative to oilseed crops because of the quantity and quality of its lipid content. Quinoa seeds included in this study had a fat content ranging from 4.84 g/100g grown in Peru (Tesco) to 7.45 g/100g grown in Bolivia (White), with an average of 6.48 g/100g, which is in accordance with the value of Filho *et al*. (2017) showing an oil content of around 7%. This is between 2 and 3 times significantly higher than in buckwheat (4.21 g/100g) and other common cereals, such as wheat (1.81 g/100g) and maize (2.48 g/100g), but much lower than soy (18.90 g/100g) ($p<0.001$) (Hager *et al.*, 2012). From the given data it can be observed that the major fatty acids found in the quinoa are desirable unsaturated fats from a nutritional point of view, corresponding to approximately 84% of the total fatty acids present, which coincide in the proportion of 87.2 to 87.8%) reported by Ando *et al*. (2002). Over recent years, dietary polyunsaturated fatty acids have attracted increasing attention due to human health benefits attributed to them, such as beneficial effects on CVD, increased insulin sensitivity, improved immune response, metabolism of prostaglandins, and cell membrane function (Ogungbenle, 2003; Repo-Carrasco *et al.*, 2003; Abugoch James, 2009). As reported by Filho *et al*. (2017), high quality fat was found in quinoa, being rich in the essential fatty acids linoleic acid and α-linoleic acid. Quinoa also contains considerable levels of antioxidant vitamins, such as α- and γ-tocopherol; however, these

analyses were not performed in the current study and so no comparisons are possible from the current study.

Quinoa seeds are also good source of dietary fibre. Values ranged from 7.60 g/100g found in UK (Waitrose) quinoa to 12.59 g/100g found in South America quinoa, total dietary fiber (AOAC fiber) content for the quinoa varieties analyzed, with an average value of 9.53 g/100g. This value is comparable to or lower than those found for red quinoa seeds (8.87 g/100g) grown in Peru reported by Repo-Carrasco-Valencia *et al*. (2010b), between 11.59 to 15.07 g/100g reported by Miranda *et al*. (2013) and 13.56 to 15.99 g/100g reported by Repo-Carrasco-Valencia *et al*. (2011), with similar comparison being found in soluble and insoluble dietary fiber. The level of dietary fiber in quinoa seeds was significantly lower than that in barley (15.6%), rye (15.1%) and similar to wheat (10.7%), but significantly higher than that of rice (2.8%), corn (7.3%) and sorghum (3.7%) (USDA, 2011).

3.5 Selection of quinoa accession for the future study

In this study, the dietary fiber content, especially the soluble fiber content, was one of the determining factors for the choice of quinoa seed for bread making and the future human intervention, based on previously published studies, which have consistently shown soluble fibre to be the most likely contributor to lower total and LDL cholesterol concentrations (Glore *et al.*, 1994; Truswell, 1995; O'Neil *et al.*, 2010). The highest level of insoluble, soluble and total dietary fiber were all identified in the same quinoa seeds (South America) among these included varieties; moreover, the total and insoluble dietary fiber values are much higher than other varieties. Thus, the accession of quinoa seed South America was selected for the bread making and future human intervention study.

Chapter 4 Preparation of test bread

4.1 Introduction

Breads are basic dietary parts in many countries and are mainly prepared from wheat flour containing gluten. It is well known that gluten can cause allergic reactions like celiac disease, but it is also responsible for the bread's texture quality. In specific, bread dough is a viscoelastic system where the gluten network gives elasticity to dough, maintains its shape and helps it rise, thereby consequently affecting the textural characteristics of final products. As a bread material, quinoa has gained in popularity due to its high nutritional value, as well as naturally gluten-free property. However, because of the lack of gluten in quinoa, the use of 100% quinoa flour in bread formulation cannot result in dough with the same viscoelastic properties that can be achieved by using refined wheat flour. Therefore, previous substitution levels of quinoa flour in refined wheat roll formulations were 15% in the study of Milovanović *et al*. (2014), 10% and 20% by Bilgicli and Ibanoglu, (2015) and 50% by Turkut *et al*. (2016).

The planned intervention study was dependent on providing the required dose of quinoa in a form which would be palatable and acceptable to (male) participants. After considering various options it was decided to base the intervention food on bread. The Artisan Bakery, a Newcastle-based Social Enterprise Bakery, was approached to provide expert advice on bread formulation to help with the development of a suitable product. The aim was to prepare and evaluate bread recipes to select a product for use in the dietary intervention study and to analyse their nutritional profile. The objective was to carry out a sensory analysis of breads to inform the selection of the intervention bread, as well as the compositional analysis.

4.2 Materials and methods

4.2.1 Materials

The South America quinoa accession was selected with the highest fibre content identified in composition analysis described in Section 3.5.

4.2.2 Bread making method

The formulations of breads are detailed in Table 4.1. In bread formulation, refined wheat flour was supplemented with quinoa flour at 0%, 20% and 30% levels, substituting for the refined white flour with the rest of the ingredients remaining exactly the same. Briefly, the ingredients were evenly mixed in a mixer (Model 5K5SS, Kitchen Aid, St, Joseph, MI, USA), and then the mixed doughs left in lidded containers to prove for 16 hours at room temperature. The resultant doughs were divided into pieces of 162 g each, manually rounded, placed on the floured baking tray, and subjected to a second proving for 1 h 30 mins at 35 °C. Baking was initiated at 215°C, although the temperature was immediately decreased to 195°, and doughs were baked in the oven for 15 mins. After removal from the oven, the baked rolls were cooled down to room temperature prior to packing in single-serving food & freezer bags (ASDA), and storing in a freezer at -20°C until analysis or for use in the human intervention study.

Table 4.1. Formulations of rolls

Ingredients	Refined wheat roll	20% Quinoa roll	30% Quinoa roll
Organic refined white flour (g)	100	80	70
Organic quinoa flour (g)	0	20	30
Fine sea salt (g)	1.5	1.5	1.5
Fermipan yeast (g)	0.25	0.25	0.25
Water at 45°C (g)	60	60	60
Roll weight prebake (g)	162	162	162

4.2.3 Bread sensory testing

For the bread sensory test, 41 participants (25 women and 16 men, aged between 20 and 55) were recruited for the test. Before starting the sessions, a written information leaflet for bread assessment, including information on the nutritional and health benefits of quinoa were distributed to the participants and they were asked to sign a consent form to participate. Three different types of bread made with different amounts of quinoa (0%, 20% or 30% of the bread) were involved in this test. The sensory testing took place in the NU-Food sensory facility, which contains a well-equipped kitchen, and 10-booth sensory booth facility located in the basement of Newcastle University's Agriculture Building. The bread samples considered in the present testing were prepared using the

recipe in Table 4.1 but for convenience were baked as loaves and sliced immediately prior to testing to ensure the samples were fresh for testing and to reduce the amount of crust to make more comparable samples for sensory analysis.

The bread samples were presented on identical white paper food plates, each coded with a different symbol, with sample presentation counterbalanced over the entire test to avoid order effects. All participants were served the 3 different kinds of bread roll in a randomised order; participants were asked to rinse their mouth with pure (bottled) water between sample ingestion and to score each sample on an anonymous score sheet. The next sample would be presented after the evaluation of each sample was finished. Sensory characteristics of the bread products, including colour, aroma, softness, moistness, chewiness, flavour and enjoyable flavour, were evaluated using an 10 cm unstructured line scale with anchor points 'none' on the left side and 'very much' on the right side (see the file 'bread questionnaire' attached in the Appendix 7). The consumer test was carried out not only to compare its acceptability in comparison with habitually consumed refined wheat bread, but also to choose a preference between 20% and 30% of the quinoa-enriched wheat bread for the human intervention study.

4.2.4 Compositional analysis of bread rolls

The methods of phenolic and antioxidant content and composition analysis of refined wheat and 20% quinoa rolls were as described in Section 3.2 and 3.3, respectively.

4.2.5 Calculations and statistical analyses

The data from bread sensory testing were analysed by using the SPSS 22.0 for Windows statistical program (SPSS, Inc., Chicago, IL, USA), and the results are expressed as means and standard deviations. Normality of the variables was checked with the Shapiro–Wilk test and data that were not normally distributed were transformed (using log10 function) prior to statistical analyses and then back-transformed for presentation of results. To determine the differences in characteristics between refined wheat bread, 20% and 30% quinoa breads , a paired *t*-test was used with P values less than 0·05 considered to be significantly different.

4.3 Results and Discussion

4.3.1 Sensory analysis

Figure 4.1 shows the three bread formulations used in the sensory analysis. The addition of 20% and 30% quinoa to the bread formulation resulted in progressively smaller, denser and darker breads compared with the control refined wheat bread.

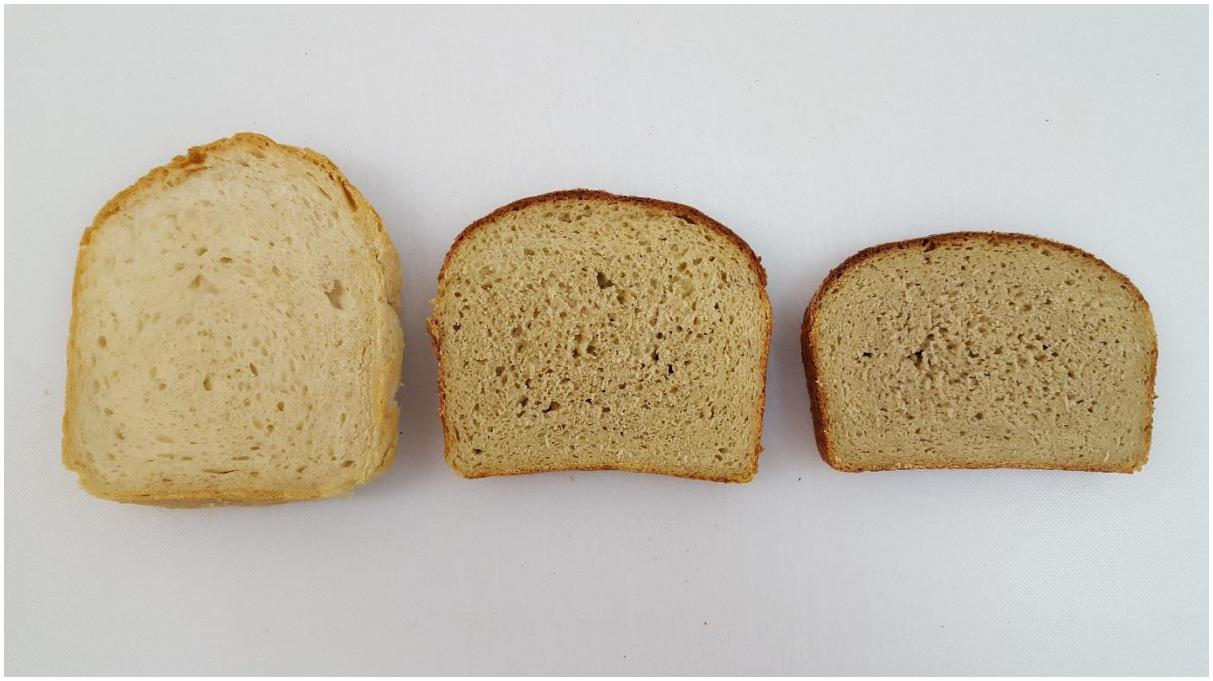


Figure 4.1. Test breads used in sensory analysis (left to right) Control white refined wheat bread, 20% quinoa bread and 30% quinoa bread.

Mean scores for bread sensory characteristics derived from all healthy volunteers are presented in Figure 4.2. According to the scores obtained from each tested attribute by regular consumers, the scores related to the colour and aroma of breads increased significantly ($p<0.05$) with increasing amounts of quinoa (0%, 20% or 30% in the bread). In contrast, the scores for softness showed an opposite trend. Consumer acceptability scores for moistness and chewiness were very similar with no significant difference across the three bread types ($P>0.05$). Comparing the flavour between two quinoa breads and the refined wheat bread, it can be seen that breads with 20% and 30% quinoa flour resulted in breads with a markedly stronger flavour in comparison with the refined wheat bread. However, from an enjoyable flavour prospective, the refined wheat bread and 20% quinoa bread were liked significantly more by the consumers than the 30% quinoa bread which had a significantly lower score ($p<0.05$).

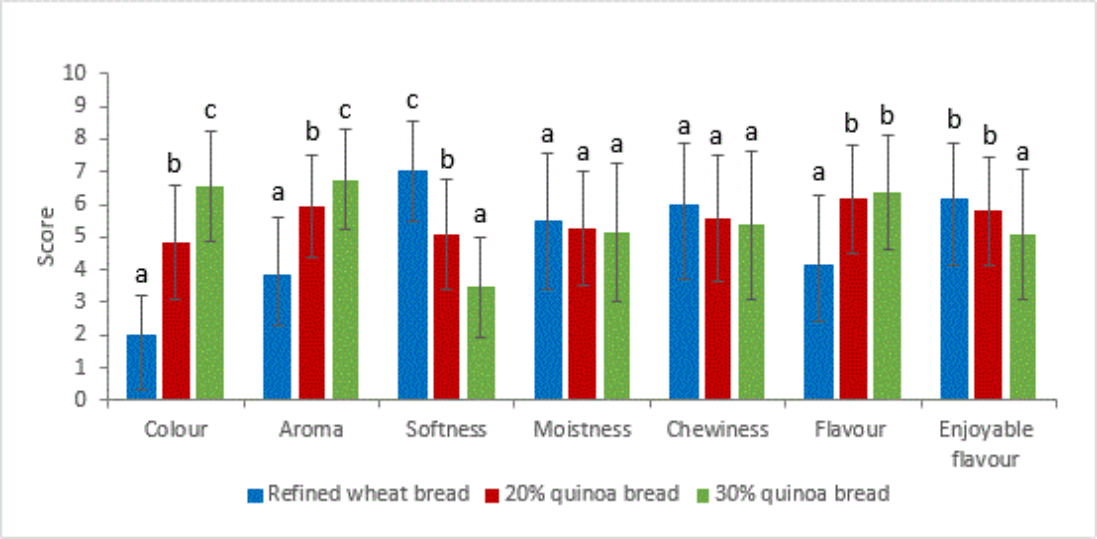


Figure 4.2. Results of the consumer test of refined wheat bread, 20% and 30% of quinoa breads evaluated on the 10 cm unstructured line scale with anchor points 'none' on the left side and 'very much' on the right side by all healthy volunteers. Mean ± Standard deviation.

Mean scores for bread sensory characteristics derived from male healthy volunteers are presented in Figure 4.3. According to the scores obtained from each tested attribute by regular consumers, both quinoa breads (20 and 30%) possessed significantly higher scores related to colour than the refined wheat bread. In the case of softness, it can be seen that refined wheat bread and 20% quinoa bread were softer than 30% quinoa roll ($p<0.05$), without significant differences observed between refined wheat bread and 20% quinoa bread. However, there were no significant differences in the rest of the sensory characteristics including aroma, moistness, chewiness and flavour between refined wheat bread, 20 and 30% quinoa bread. From an enjoyable flavour prospective, the scores obtained from refined wheat bread and 20% quinoa bread were higher than that 30% quinoa bread, but did not reach significance level.

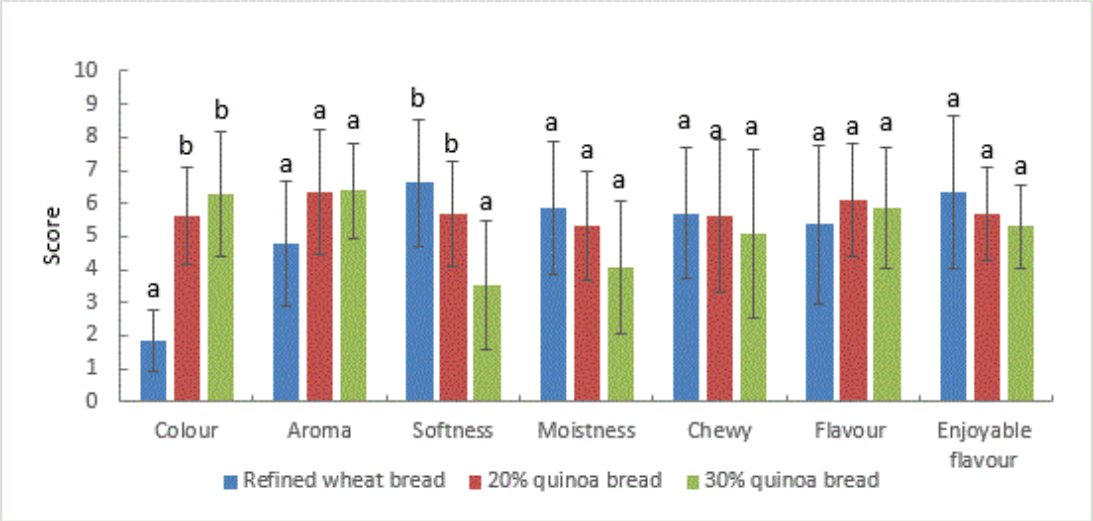


Figure 4.3. Results of the consumer test of refined wheat bread, 20% and 30% of quinoa bread evaluated on the 10 cm unstructured line scale with anchor points 'none' on the left side and 'very much' on the right side by male healthy volunteers. Mean ± Standard deviation.

4.3.2 Composition of refined wheat and 20% quinoa rolls

The phenolic content and antioxidant activity of refined wheat and 20% quinoa rolls are illustrated in Table 4.2. In this study, except for the antioxidant activity of bound extracts measured by FRAP and DPPH methods which were lower in quinoa rolls, the total flavonoid, phenolic content of the free, conjugated, bound and all extracts, as well as antioxidant activity measured by the three antioxidant assays were slightly higher in quinoa rolls compared with refined wheat rolls. The results for compositional analysis are shown in Table 4.3. Refined wheat and 20% quinoa rolls had 61.3% and 56.9% dry matter content, respectively, after drying in the oven overnight at 90 °C. With the same energy content, the proximate composition of 20% quinoa rolls was slightly higher compared with refined wheat roll for ash, moisture, salt, sodium, protein, fat and soluble dietary fibre. The insoluble dietary fibre content of the quinoa rolls was more than double that of refined wheat rolls. As a result, available carbohydrates and total sugar content were lower in quinoa rolls by 4.41% and 0.89 %, respectively, as described in Table 4.3. The amino acids composition of quinoa roll were similar to those of refined wheat roll, and the concentration of total essential amino acids when added in 20% quinoa roll (4.24 g/100g) was slightly higher with respect to the refined wheat roll (3.80 g/100g); surprisingly, the concentration of essential amino acid lysine in quinoa roll was 2.5 times greater than that of refined wheat roll (Table 4.4).

Table 4.2. Total phenolic content and antioxidant activity of refined wheat and quinoa rolls

	Extracts of refined wheat rolls				Extracts of 20% quinoa rolls			
	Free	Conjugated	Bound	Total	Free	Conjugated	Bound	Total
Total phenolic content (mg GAE/g)	0.52 ± 0.04	0.08 ± 0.01	0.17 ± 0.01	0.77	0.76 ± 0.06	0.16 ± 0.01	0.19 ± 0.02	1.11
FRAP value (µmol Fe2+ E/g)	0.66 ± 0.05	0.33 ± 0.09	0.82 ± 0.07	1.81	1.23 ± 0.12	0.51 ± 0.02	0.74 ± 0.02	2.48
TEAC value (µmol Trolox E/g)	1.53 ± 0.10	0.90 ± 0.08	1.60 ± 0.12	4.03	2.11 ± 0.15	1.20 ± 0.06	2.51 ± 0.09	5.82
DPPH value (µmol Trolox E/g)	2.72 ± 0.56	3.07 ± 0.41	4.66 ± 0.43	10.45	4.14 ± 0.11	3.66 ± 0.24	4.63 ± 0.03	12.43

Mean ± standard deviation

Table 4.3. Proximate composition of refined wheat and quinoa rolls (dry basis)

	Refined wheat roll	20% Quinoa roll
Energy (Kcal/100g)	381	381
(kJ/100g)	1615	1612
Protein (g/100g)	12.63	14.04
Ash (g/100g)	2.37	2.87
Moisture (g/100g)	2.69	3.02
Available Carbohydrate (g/100g)	77.05	72.64
Total sugars (g/100g)	6.67	5.78
Sodium (g/100g)	0.51	0.62
Salt (g/100g)	1.30	1.58
Insoluble dietary fibre (g/100g)	2.17	4.99
Soluble dietary fibre (g/100g)	1.43	1.53
Total dietary fibre (g/100g)	3.60	6.52
Fat (g/100g)	1.66	2.73
Saturated fat (g/100g)	0.29	0.37
Monounsaturated fat (g/100g)	0.24	0.68
Polyunsaturated fat (g/100g)	1.06	1.56

Table 4.4. Composition of amino acid of refined wheat and quinoa rolls (dry basis)

	Refined wheat roll	Quinoa roll
Essential amino acids		
Histidine (g/100g)	0.28	0.35
Iso-Leucine (g/100g)	0.49	0.46
Leucine (g/100g)	0.90	0.78
Lysine (g/100g)	0.32	0.81
Methionine (g/100g)	0.18	0.27
Phenylalanine (g/100g)	0.66	0.48
Threonine (g/100g)	0.38	0.50
Tryptophan (g/100g)	ND	ND
Valine (g/100g)	0.59	0.59
Non-essential amino acids		
Alanine (g/100g)	0.41	0.58
Arginine (g/100g)	0.53	1.07
Aspartic Acid (g/100g)	0.56	1.07
Cystine (g/100g)	0.29	0.20
Glutamic Acid (g/100g)	4.55	1.70
Glycine (g/100g)	0.49	0.68
Proline (g/100g)	1.56	0.49
Serine (g/100g)	0.74	0.62
Tyrosine (g/100g)	0.39	0.39

In a separate, informal, palatability study 5 male volunteers were provided with the proposed 20% quinoa test rolls which they consumed daily for one week. All of the participants found that they could consume the rolls as part of their normal dietary routine without any reported side effects. On the basis of this trial and the sensory analysis described above, the 20% quinoa-enriched wheat roll formulation was chosen for the human intervention study, as it provided an acceptable medium for delivering the 20g of quinoa required for the intervention in a quantity of bread which could be consumed readily by participants.

Chapter 5 Human intervention study

5.1 Introduction

Cardiovascular disease is an important cause of adult disability and death across the world. Among treatments for combating these complications, the dietary modulation of CVD have attracted much more attention in recent years. Epidemiological studies have pointed to diets rich in whole grains decreasing the risk of many diet-related diseases, including CVD, cancer, type 2 diabetes and obesity, with similar results found across diverse populations (He *et al.*, 1995; Chatenoud *et al.*, 1998; Jacobs *et al.*, 1998; Liu *et al.*, 1999b; Jacobs *et al.*, 2000; Mellen *et al.*, 2008a; O'Neil *et al.*, 2010; Ye *et al.*, 2012; Aune *et al.*, 2016; Chen *et al.*, 2016). Quinoa, as a pseudocereals, is included in ''whole grain'' class in the terms of similar composition (McKeown *et al.*, 2013). Over the years, quinoa has gained in popularity due to its high nutritional profile, as well as property of the lack of gluten (Ando *et al.*, 2002; Konishi *et al.*, 2004; Bhargava *et al.*, 2006; Alvarez-Jubete *et al.*, 2009; Tang *et al.*, 2015). However, up to date, few studies have focused on the effect of quinoa intake on CVD risk in *vivo*. Moreover, human and animal intervention studies have not always clear results from feeding quinoa-based foods on markers of CVD risk, thereby resulting in some discussion about the impact of a quinoa diet. Besides, the mechanisms responsible for how a quinoa diet may benefit human health still remains unclear.

Therefore, the aim of the current study was to evaluate the effect of substituting refined grain foods for quinoa rolls on markers of CVD risk, including plasma levels of antioxidant activity, glucose, lipids and markers of systemic inflammation, in healthy subjects using a randomised controlled cross-over study. The intervention study was thus designed to explore the mechanisms underlying the potential beneficial effects.

5.2 Materials and Methods

5.2.1 Study design

The study was a randomized, controlled cross-over trial consisting of 2 treatment periods of 4 weeks each separated by a washout period of 4 weeks. Subjects were required to attend the NU-Food Facility at University of Newcastle throughout both dietary periods, and compliance was ensured by the provision of all study foods during the intervention. After the screening, entry into the quinoa treatment arm or refined wheat control arm of the trial was by random allocation using stratification on the basis of age and BMI to ensure that each arm was balanced.

During the first 4-wk period, one group (n=15) consumed one quinoa roll daily, and the other group (n=15) consumed the equivalent placebo (refined wheat roll) daily. After the 4-wk washout period, subjects who consumed the quinoa roll in the first period crossed over to consume refined wheat roll in the second period, and *vice versa* (Figure 5.1).

Volunteers were provided with a 4-wk supply of frozen quinoa or refined wheat rolls packed in an ice bag with ice packs; the study rolls were either collected in person by volunteers or were delivered to volunteers in two 2-wk batches. Due to the characteristics of the food products serving as control (refined wheat rolls) and test (quinoa rolls) products, it was not possible to blind the researchers or the subjects during the study to the treatment group/period. However, samples were randomised during analysis and subject codes were not revealed until analysis was complete.

On the first and last day of each intervention period volunteers came to the NU-Food facility after an overnight fast. Fasting body weight was measured in light clothing to the nearest 0.1 kg, as well as body fat percentage by bio-electrical impedance (Tanita BC-420MA). Blood pressure measurements were conducted on the right upper arm with the volunteer in a sitting position after 5-10 minutes of rest with an automatic Intelli Wrap Cuff (HEM-7321-E, M6 Comfort, Omron). A fasting blood sample was drawn by venepuncture from the antecubital vein into a vacutainer with EDTA anti-coagulant. Blood samples were mixed and immediately chilled then centrifuged at 2,500 rpm at 4°C. Plasma was removed and stored at -80°C until analysis.

Volunteers completed a full urine collection for the 24 hours leading up to the visit, and collected a single spot faecal sample on the morning of the visit using urine sample pots and faecal collection tubes provided.

As changes due to the daily diet may not be evident in fasting blood samples, a postprandial sub-study was conducted as part of the intervention study design. On visit days, after taking all fasting

measurements, a standardized breakfast meal, including 10 g strawberry jam and 100 g quinoa roll or refined wheat roll, appropriate to the diet they were currently following (i.e. quinoa roll before and after the quinoa intervention, and a wheat roll immediately before and after the wheat intervention), was served together with *ad libitum* pure water. The subjects were asked to finish the breakfast meal within 3-5 mins without leaving any rolls or strawberry jam. After the breakfast meal, a selected finger was pricked with a lancet to obtain a small quantity of capillary blood for testing. Capillary blood samples were transferred to a Whatman 903 protein saver card. The Whatman 903 protein saver card is one of generic collection cards that meet the requirements for many sampling programs. The sample collection area of a card contains five half-inch circles, each of which holds 75-80 µl of whole blood sample. The saver card wraparound cover has spaces for name and date of collection and is imprinted with the universal biohazard symbol in accordance with United States Post Office (USPS) regulations. After collection, the back side of the card was inspected, to ensure that it was also completely soaked with blood. The first blood spot sample was taken by the researcher in the NU-Food facility, thereafter volunteers collected the blood samples themselves. The dried blood spots (DBS) were collected at 0, 60, 120, 180 and 240 min, and the card was then packed in a sealable plastic bag containing desiccant. The cards were delivered or posted by subjects for return to the researcher and then immediately stored at -80°C on arrival until analysis.

The Abbott FreeStyle Libre Flash Glucose Monitoring (FGM) System, including a sensor and reader, is designed to be a continuous monitoring system that does not need finger pricks for calibration, and measures interstitial fluid glucose concentrations. The sensor was applied on to the back of the upper arm of subjects using the applicator by trained staff on D_{23} or D_{24} and was worn up until after D_{32}. The sensor records glucose concentrations every 15 minutes providing comprehensive data for a complete glycaemic profile of the wearer. The sensor stores the recorded data which is downloaded to a monitor/reader by the wearer. To obtain a complete glycaemic picture, the sensor must be scanned at least once every eight hours, and the reader can capture data when it is within 1 cm to 4 cm of the sensor. With every scan the reader also provides a current glucose reading, together with (up to) the last 8 hours of glucose data and a trend arrow showing the direction that the blood glucose values are heading. Cumulative data for the period the sensor was worn by the volunteer was downloaded to a PC in a spreadsheet format for analysis.

An outline of the study protocol is shown in Figure 5.1.

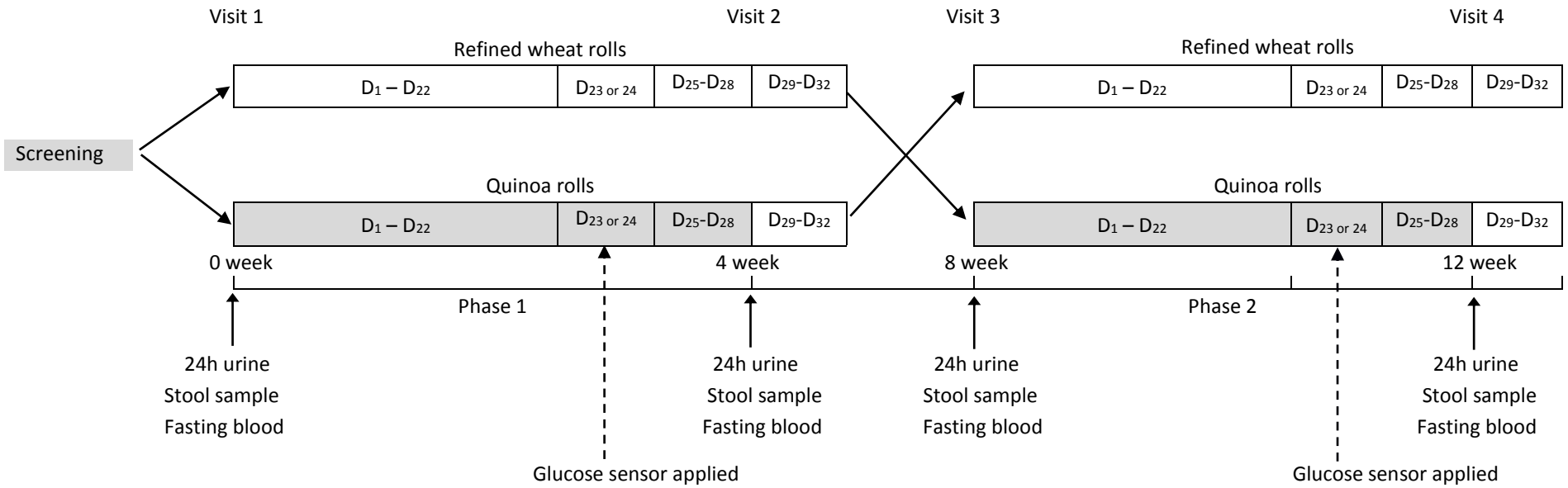


Figure 5.1. Intervention study design. Randomized controlled crossover study in which subjects received either refined wheat rolls or quinoa rolls for a period of 4 weeks. Anthropometric and blood pressure measurements were performed and fasting blood, 24hu urine and stool (fresh and 24 h) samples collected from each volunteer before and after each intervention period as indicated.

5.2.2 Subjects

Sample size was determined using a Paired t Test approach in Statistical Software Minitab 17, based on blood lipid data reported in the two papers describing quinoa interventions by Farinazzi-Machado *et al*. (2012) and De Carvalho *et al*. (2014) that used a similar quinoa dosage, as well as a recent dietary intervention study completed in Newcastle UK. Using levels of significance less than 0.05 and 80% power, a sample size of between ten and twelve subjects per group was required to observe a 10% decrease in LDL cholesterol concentration in the intervention group, assuming that no change would occur in cholesterol concentration in the control group. Also, in order to quantify changes in bacterial numbers and species with this design, even though no data on the effects of quinoa on the gut microbiome was available, generally published studies on the effects of diet on the microbiome have used less than 20 subjects. To account for dropout, sixteen subjects per group were targeted for recruitment.

A total of thirty-seven healthy and non-smoking male volunteers, aged 36-70 years, were recruited for the study in the city of Newcastle upon Tyne between August 2016 and February 2017. The inclusion criteria were as follows:

1) Healthy males >35 years old;

2) Body mass index >25 kg/m^2;

3) Non-smokers with no known previous history of cardiovascular disease or type 2 diabetes;

4) Not receiving any current medication.

Supplement users were included but were asked to stop taking supplements for the duration of the study. Individuals were excluded if they reported or were observed to have diabetes or CVD; to have smoked in the past; to have an allergy to gluten, grain products or any ingredients used in the treatment foods; to have experienced recent weight loss (>10%) or plan to lose weight during the study; or to use any medications.

Volunteers were recruited by advertisements using posters and by sending invitation letters to potential volunteers from the NU-Food volunteer database and by circulating a recruitment email in local organisations such as Voice North and ‘’ion-volunteer’’. ‘’ion-volunteer’’ is a volunteer organisation containing a large number of members who are willing to get involved in research, and it was founded by the Institute of Neuroscience, Newcastle University.

After an initial telephone or email screening on broad criteria to ascertain gender and approximate self-reported BMI, recruitment was completed at a screening visit during which the potential volunteers completed an eligibility questionnaire and confirmation of adherence to inclusion/exclusion criteria. Height and weight were measured to calculate body mass index (BMI) as body weight (kg) / height (m^2). Older, overweight males were selected because they have an elevated risk of CVD compared with younger, normal weight males, but they remain a relatively understudied population.

Subjects received an honorarium in the form of shopping vouchers for their participation in follow-up visits.

Details of study enrolment and completion are presented in Figure 5.2. In this controlled, cross-over designed study, only seven of the 37 subjects randomized to treatments did not complete the study (3 whilst on the quinoa treatment, 4 whilst on the refined wheat treatment, all during the first period of the cross-over study). Reasons for con-completion were difficulty to avoid whole grains (n=2), withdrawn by researcher due to poor compliance (n=1), unwillingness to comply with the regimen (n=1), lost to follow up (n=1), or reasons not given (n=2). A total of 30 men completed the study: 15 per intervention group.

Recruitment and interventions were conducted at the NU-Food Food and Consumer Research Facility at University of Newcastle and the protocol was approved by University of Newcastle Faculty of Science, Agriculture and Engineering Research Ethics Committee (reference 16-LI-034) and written informed consent was obtained from each subject after oral explanation of the study before commencing the study. Prior to commencement, the study was registered on ClinicalTrials.gov, registration number NCT03036618.

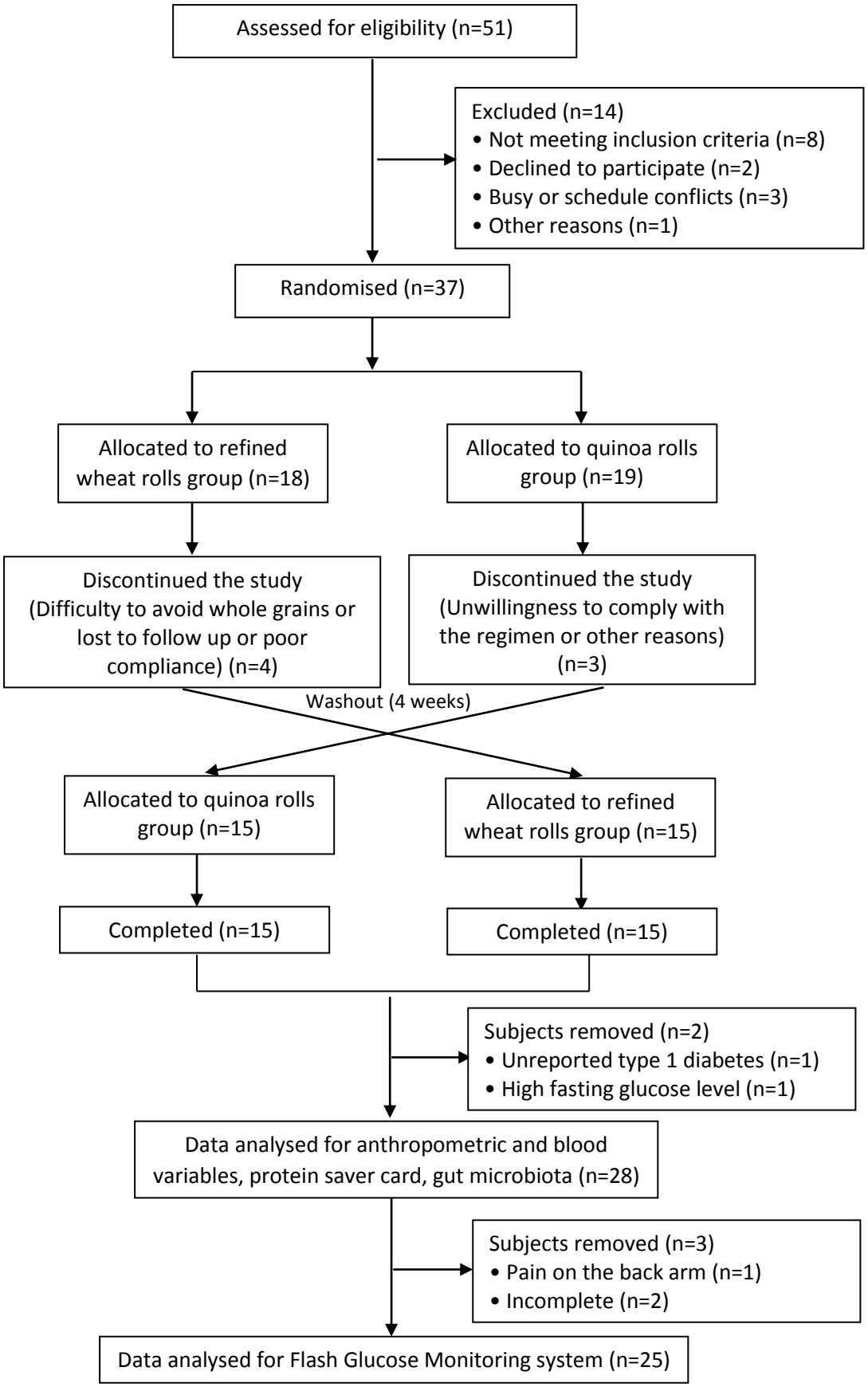


Figure 5.2. Flow diagram of enrolment, random allocation, withdrawals and follow-up of participants through the trial.

5.2.3 Study food

The refined wheat and quinoa rolls used in this study were manufactured and purchased from one bakery (The Artisan Baking Community, Earth Doctors Ltd., Northumberland, UK) as described in Chapter 5. Freshly baked rolls were packaged in single-serving food & freezer bags and then transferred to a -20°C food storage room before dispensing to volunteers.

The subjects were asked to keep the rolls in their home freezers and were instructed to take them out of the freezer to defrost immediately before consuming. To ensure that the subjects ate the correct amounts of test bread, they were advised to consume one roll daily at room temperature, in place of other bread products (e.g. pitas, bagels, dinner rolls) or carbohydrate-rich foods (e.g. rice, pasta), in their diet. They were advised to eat the rolls with their regular portion of different products, such as spreads, cheese, jam and salad.

During the 13-wk trial, subjects were instructed to avoid consuming any other source of whole grains or related products derived from whole grains, such as bran flakes, wholemeal bread and brown rice (a list was provided), and they were also asked not to take any supplements during the intervention, such as vitamin or mineral supplements. In addition, the participants were asked not to change their normal consumption of the following foods that potentially change markers of CVD risk: coffee, tea, oily fish and dark chocolate. A list of foods that could be freely consumed was also offered with instructions to maintain their normal physical activity, dietary and lifestyle patterns while on the study.

Compliance with the diet was checked by 3-day food records and daily records. Three-day food records including two weekdays and one weekend day were kept by the subjects before and at the end of each treatment arm. In daily records, the subjects recorded what time each quinoa or refined wheat roll was consumed as well as amounts if they did not consume the whole roll in a single meal. Additionally, during the intervention periods, the subjects were requested to report if they had experienced any possible side effects related to the study rolls, such as flatulence, stomach problems, abdominal or bloating pain.

Finally, a food frequency questionnaire (FFQ), which recorded food intake during the previous 7 days only, was undertaken by each subject before and at the end of each intervention period. The FFQ also contained questions regarding dietary habits including how much and how often the participants consumed vegetables, eggs, milk and meat, etc.

5.2.4 Analytical methods

5.2.4.1 Plasma and urine

Blood samples were collected in 2 × 10 ml Na_2-EDTA tubes and then centrifuged at 1400 g for 12 mins at 4°C to separate plasma, which was then aliquoted and stored at -80 °C until analysis. The volume of 24-hour urine collections was recorded, the urine thoroughly mixed and then aliquots stored at -80 °C until analysis.

5.2.4.2 Blood metabolites

Blood plasma metabolites were analysed using the ABX Pentra 400 (Horiba Medical), using the following standard enzymic procedures (reference number, procedure): glucose (A11A01668, Peroxidase), total cholesterol (A11A1634, Cholesterol esterase / Cholesterol Oxidase), low-density lipoprotein (LDL) cholesterol (A11A01638, Detergent / Cholesterol Oxidase/ Esterase), high-density lipoprotein (HDL) cholesterol (A11A01636, Polyanions), Triglycerides (A11A01640, p-Chlorophenol + 4-aminoantipyrine), Apo A1 (A11A01687, Turbidometric Immunoassay), Apo B (A11A01688, Turbidometric Immunoassay), C-reactive protein (CRP) (A11A01611, Latex Turbidometric Immunoassay), Aspartate transaminase (AST) (A11A01629, International Federation of Clinical Chemistry), Alanine transaminase (ALT) (A11A01627, International Federation of Clinical Chemistry). Plasma insulin was determined by enzyme-linked immunosorbent assay (ELISA) using the Invitrogen Human Insulin (KAQ 1251) kit. Plasma free fatty acids (NEFAs) were determined by the Acyl-CoA synthetase Acyl-CoA oxidase (ACS-ACOD) method using Wako Chemicals GmbH NEFA-HR(2) kit and antioxidant activity using the FRAP and TEAC assays as described in Section 3.2.

5.2.4.3 Dried blood spots

According to the manufacturer's instructions, each circle (diameter 13 mm, area 133 mm^2) of the Whatman 903 protein saver card holds 75-80 µl of whole blood sample. A 3.2 mm disc (diameter 3.2 mm, area 8.0 mm^2) is 6.0% of the 13 mm circle, which translates into 4.25 µL of whole blood. Based on the median haematocrit of 45% for the general population, a 3.2 mm disc therefore contains approximately 2.34 µL of blood plasma only which is used in viral load determination (Purves *et al.*, 2004). For analysis, protein saver cards were thawed at room temperature, and one 3.2 mm diameter disc equivalent to about 2.34 µL blood plasma was removed in duplicate from the centre of each blood spot using a Harris Uni-core puncher (Pat. No. 7093508, General Electric Company) (O'Neil, 1999; Stene-Johansen *et al.*, 2016). The disc was eluted for 2 hours in 100 µL trichloroacetic acid (5% w/v) in an Eppendorf tube with shaking at 10-minutes intervals. After centrifugation the

supernatant was analysed for glucose using the procedure described in Section 5.1.4.2 above (Rattenbury *et al.*, 1989; Ward *et al.*, 1996).

5.2.4.4 Correlation between venous blood glucose, dried blood spot glucose and glucose monitor measurement

At the end of the intervention periods when the volunteers were wearing the FGM monitors after taking the fasting blood sample and before starting the test breakfast, the subjects were asked to scan the sensor with the reader, and the current glucose values shown on the reader was recorded. The blood glucose values derived from the first blood spots (0 min) on the protein saver cards were noted for the comparison.

5.2.5 Calculations and statistical analyses

The 3-day food records were analysed by Windiets 2015 (Robert Gordon University). The data were analysed by using the SPSS 22.0 for Windows statistical program (SPSS, Inc., Chicago, IL, USA), and the results are expressed as means and standard deviations. Normality of the variables was checked with the Shapiro–Wilk test and data that were not normally distributed were transformed (using log10 function) prior to statistical analyses and then back-transformed for presentation of results. To determine the differences in the measured variables following intake of the refined wheat and quinoa rolls, a paired *t*-test was used with P values less than 0·05 considered to be significantly different. The percentage change was calculated as follows: (value at 4 week – value at baseline)/value at baseline × 100.

Area under the curve (AUC) was determined for blood glucose determined from the FGM by using GraphPad Prism (version 7.01; San Diego, CA, USA), and the results are expressed as means and standard deviations. Data were analysed for the last 4 days (D_{25}-D_{28}), the following 4 days in the washout periods (D_{29}-D_{32}), total 8 days (D_{25}-D_{32}) in the two treatment periods after the sensor had been applied, and the time periods 0-240 min after the test breakfast at the end of each treatment period (for FGM and Protein saver card). The changes within refined wheat or quinoa treatment in the AUC for glucose when expressed as a percentage of initial AUC for glucose: changes (%) = [(D_{29}-D_{32}) - (D_{25}-D_{28})]/ (D_{25}-D_{28})*100. In the acute breakfast measurements of postprandial glucose concentration, fasting (0 min) glucose concentrations were used as baseline for incremental area under the curve (IAUC) calculations.

5.3 Results

5.3.1 Dietary intake and compliance

In this study, nutrient intake did not significantly differ within and between the two treatments, with the exception of a significant higher intake of carbohydrates during the refined wheat rolls period. According to daily records, compliance was very good (Table 5.1). During the intervention, the volunteers reported consuming all the portions of refined wheat and quinoa rolls (1 roll/day) as advised during both treatment periods. Despite the absence of detected effects on metabolic variables, quinoa rolls were well tolerated by the majority of the subjects, without any apparent side effects or complaints. A general positive experience with feelings of well-being was reported after the quinoa rolls period.

Table 5.1. Mean daily intake of nutrients before and at the end of each treatment arm in all subjects

	Refined wheat rolls (n=28)			Quinoa rolls (n=28)			
	Baseline	Week 4	Change (%)[1]	Baseline	Week 4	Change (%)[1]	*p* value for changes[2]
Energy (KJ)	9505 ± 1320	10065 ± 1275	5.9	9600 ± 1630	9956 ± 1685	3.7	0.733
Carbohydrates							
(g/d)	241 ± 50	285 ± 46	18.3*	250 ± 60	270 ± 43.1	8.0	0.623
(% of energy)	50.2 ± 6.1	53.1 ± 5.0	5.8	49.5 ± 7.9	52.2 ± 5.5	5.5	0.799
Protein							
(g/d)	79.2 ± 16.1	87.5 ± 20.3	10.5	81.5 ± 16.9	90.4 ± 17.4	10.9	0.347
(% of energy)	17.0 ± 2.2	15.8 ± 2.4	-7.1	15.7 ± 2.2	16.9 ± 3.5	7.6	0.414
Fat							
(g/d)	64.2 ± 13.1	71.0 ± 18.4	10.6	76.5 ± 20.1	72.2 ± 19.8	-5.6	0.424
(% of energy)	29.8 ± 5.2	28.5 ± 4.5	-4.4	32.6 ± 5.0	28.9 ± 4.2	-11.3	0.396
SFA							
(g/d)	27.5 ± 5.4	29.3 ± 7.9	6.5	32.8 ± 8.9	30.7 ± 9.4	-6.4	0.198
(% of energy)	12.8 ± 2.2	12.0 ± 1.9	-6.25	13.9 ± 2.7	12.6 ± 2.2	-9.4	0.465
MUFA							
(g/d)	23.4 ± 5.8	25.7 ± 6.6	9.8	27.5 ± 6.8	25.0 ±7.6	-9.1	0.785
(% of energy)	10.9 ± 2.0	10.2 ± 1.5	-6.4	11.6 ± 2.1	10.2 ± 1.4	-12.1	0.743
PUFA							
(g/d)	9.3 ± 2.5	10.7 ± 2.4	15.1	10.3 ± 2.8	10.3 ± 2.4	0	0.854
(% of energy)	4.5 ± 0.8	4.4 ± 1.0	2.2	4.8 ± 0.7	4.3 ± 1.0	-10.4	0.782
Alcohol							
(g/d)	5.3 ± 2.3	6.6 ± 4.0	24.5	4.5 ± 1.9	5.2 ± 2.8	15.5	0.587
(% of energy)	1.8 ± 0.8	2.2 ± 1.3	22.2	1.5 ± 0.7	1.5 ± 0.9	0	0.154
Dietary fibre (g/d)	22.1 ± 5.6	20.1 ± 6.4	-9.0	24.7 ± 6.9	22.5 ± 4.8	-8.9	0.687

Mean values with their standard deviation.
Mean values were significantly different from baseline (week 0): *P<0.05.
[1]Changes (%) = (week 4 - baseline)/baseline *100
[2]P for change means the comparison of changes after wheat and quinoa rolls consumption.

5.3.2 Markers of CVD risk and blood plasma antioxidant activity

As indicated in Figure 5.2, 37 subjects were randomized to participate in the intervention study. Of these 28 completed the intervention, providing all fasting blood samples, urine and stool samples. 25 subjects additionally successfully completed the FGM continuous glucose monitoring.

5.3.2.1 Anthropometric variables

Anthropometric data for the study participants are shown in Table 5.2. The mean age of participants was 51.54 years (range 36 to 70). Average BMI was 27.7 kg/m^2 showing that the objective of recruiting overweight volunteers had been achieved. Mean body weight, BMI and body fat percentage did not change throughout the study period. Systolic (SBP) and diastolic (DBP) blood pressure values were mildly elevated (SBP >120 mmHg) but did not change during the intervention period with any of the treatments ($p>0.05$).

5.3.2.2 Blood variables and antioxidant capacity

At the end of the intervention, there were no significant effects of quinoa and refined wheat rolls consumption on fasting plasma concentrations of insulin, total cholesterol, HDL cholesterol, NEFAs, ApoA1, ApoB, AST, ALT and CRP with respect to corresponding baseline measurements. The difference in change in each intervention period was also not significant for any of the parameters measured. Neither treatment affected the ratios of HDL/Total Cholesterol or ApoB/ApoA1. However, after 4 weeks of quinoa roll consumption, there was a significant decrease in glucose by 4.5% and LDL cholesterol by 5.7% compared with the corresponding baseline, but the changes between the two treatments did not reach significance level. Surprisingly, there was an unexpected increase in triglycerides concentration by 14.3% after 4 weeks of consuming the quinoa rolls ($p=0.049$), with no difference after wheat rolls consumption; there was no significant difference in change between the two treatments. Plasma antioxidant capacity analysed by both FRAP and TEAC assays was not affected by the treatments.

Table 5.2. Effect of refined wheat and quinoa rolls on anthropometry, blood pressure, blood parameters and plasma antioxidant capacity measures.

	Refined wheat rolls (n=28)						Quinoa rolls (n=28)						P for change (W vs Q)
	Baseline		Week 4		Change (%)[2]	P[3]	Baseline		Week 4		Change (%)[2]	P[3]	
	Mean	SD	Mean	SD			Mean	SD	Mean	SD			
Age	51.5	10.7	-	-	-		-	-	-	-	-		-
Body weight (kg)	85.8	9.5	86.0	9.3	0.2	0.404	85.9	9.6	85.8	9.5	-0.1	0.508	0.279
BMI (kg/m^2)	27.6	2.3	27.7	2.3	0.3	0.379	27.7	2.38	27.6	2.37	-0.4	0.553	0.281
Body fat percentage (%)	25.4	5.2	25.7	5.0	1.2	0.126	25.4	5.2	25.2	5.0	-0.8	0.253	0.331
Blood pressure (mmHg)													
Systolic	129.5	12.2	130.5	12.2	0.8	0.280	128.8	12.6	128.5	11.0	-0.2	0.869	0.272
Diastolic	85.4	10.2	86.4	14.5	1.2	0.550	84.1	9.7	85.8	10.1	2.0	0.200	0.726
Blood variables													
Glucose (mmol/L)	5.71	0.56	5.64	0.53	-1.2	0.483	5.84	0.63	5.58	0.68	-4.5	0.009	0.103
Insulin (pmol/L)	54.86	21.09	58.41	26.67	6.47	0.516	54.07	25.35	61.50	32.32	13.74	0.187	0.278
Cholesterol (mmol/L)													
Total	5.63	1.20	5.50	0.99	-2.3	0.416	5.64	1.04	5.54	0.78	-1.8	0.396	0.769
LDL	3.53	0.93	3.36	0.76	-4.8	0.168	3.49	0.84	3.29	0.65	-5.7	0.024	0.439
HDL	1.36	0.25	1.38	0.23	2.0	0.470	1.39	0.22	1.39	0.22	0.23	0.939	0.363
Triglyceride (mmol/L)	1.41	0.59	1.53	0.59	8.92	0.102	1.39	0.52	1.59	0.57	14.34	0.049	0.587
NEFA (µmmol/L)	398.09	172.23	358.62	135.28	-9.91	0.130	421.99	173.61	353.72	152.60	-16.18	0.086	0.587
Apo (g/l)													
ApoA1	1.38	0.19	1.38	0.18	0.03	0.985	1.41	0.18	1.39	0.16	-1.50	0.318	0.415
ApoB	1.16	0.25	1.12	0.20	-3.30	0.247	1.15	0.21	1.12	0.16	-2.05	0.318	0.798
Ratios													
HDL/Total	0.25	0.08	0.26	0.05	7.01	0.565	0.25	0.06	0.26	0.05	3.16	0.755	0.295
ApoB/ApoA1	1.25	0.36	1.27	0.27	3.47	0.472	1.26	0.26	1.26	0.26	0.37	0.671	0.203
CRP (mg/l)	1.69	0.71	2.21	0.69	30.8	0.154	1.72	0.41	1.43	0.28	16.9	0.317	0.197
AST (U/L)	41.07	17.79	40.21	16.55	-2.09	0.589	47.18	47.35	42.79	27.97	-9.31	0.296	0.526
ALT (U/L)	34.50	26.80	35.68	26.39	3.42	0.500	60.39	46.78	39.28	38.61	-34.96	0.332	0.277
Antioxidant capacity													
FRAP (µmol Fe^{2+} E/L)	355.63	61.35	344.81	47.38	-6.95	0.238	348.68	45.68	347.21	48.30	-1.47	0.899	0.557

TEAC (µmol Trolox equivalent/L)	0.69	0.07	0.69	0.05	0	0.991	0.70	0.06	0.69	0.07	-0.01	0.387	0.556

[1]BMI, body mass index; CRP, C-reactive protein; AST, aspartate transaminase; ALT, alanine transaminase.
Mean values with their standard deviation.
[2]Changes (%) = (week 4 - baseline)/baseline *100
[3]Mean values were significantly different from baseline (week 0): $P<0.05$.
[4]*P* for change (W vs Q) means the comparison of changes after wheat and quinoa rolls consumption.

5.3.3 Flash glucose monitoring

Of 28 subjects with sensor records, for one subject, the sensor was removed early due to discomfort with the sensor, and three subjects had less than 4 days of data because of accidental ''falling off'' or poor compliance with transferring data to the reader. These subjects were subsequently removed from analysis, thus, full sensor records on 24 subjects remained for analysis (primary sensor failure of 14.3%).

5.3.3.1 Area under curve

Data for AUC the glucose curve determined by FGM are shown in Figure 5.3. For sensor glucose concentrations, although the AUC between subjects consuming refined wheat rolls or quinoa rolls (D_{25}-D_{28}) and without rolls (D_{29}-D_{32}) offered did not differ within treatments, there was a trend for an increase by 2.0% in the AUC for the first 4 wash-out days after quinoa rolls consumption compared with wheat consumption (p=0.054). Moreover, there were significant difference between treatments in changes in the AUC for glucose when expressed as a percentage of initial AUC for glucose (refined wheat rolls -2.2%, quinoa rolls 2.0%, p=0.001). There was no statistical difference in AUC for glucose for the total 8 days between treatments (D_{25}-D_{32} W vs D_{25}-D_{32} Q) although it was numerically higher in the wheat treatment period. However, the AUC for glucose during the last 4 days (D_{25}-D_{28}) of the quinoa treatment was significantly lower than that for the same period of the refined wheat treatment (p=0.039).

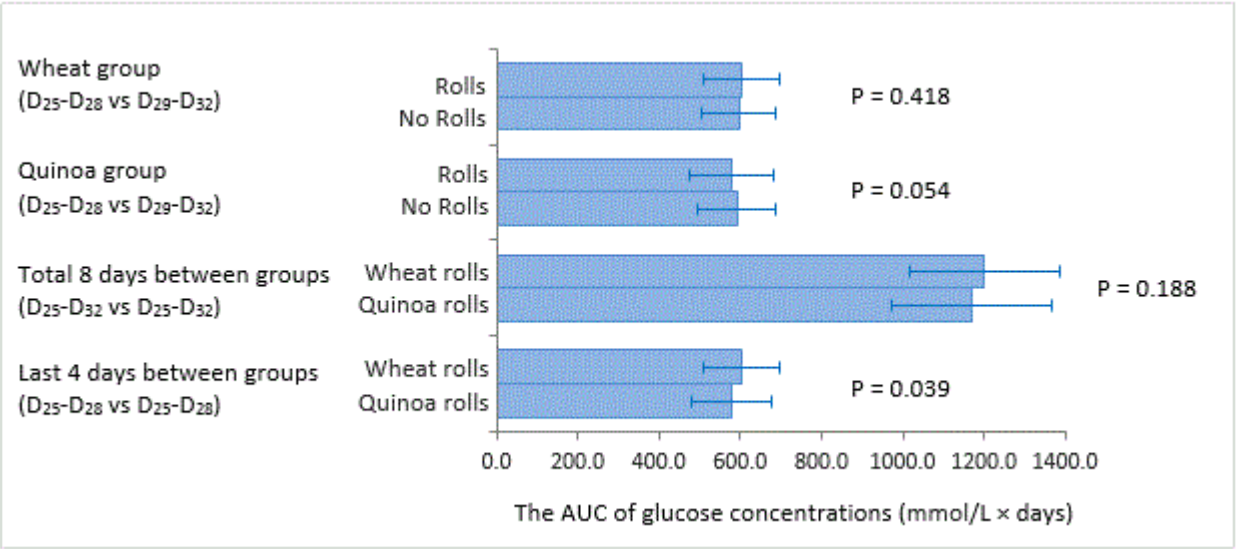


Figure 5.3. The area under curve of glucose concentration responses to intake of quinoa and refined wheat rolls during the 8 days (D_{25}- D_{32}). Values are means with their standard deviations represented by horizontal bars (n=24). Rolls means 4-day period whilst consuming the rolls at the end of the treatment period (D_{25}-D_{28}); No Rolls means the first 4 days of the washout period (D_{29}-D_{32}). Difference was consider significant if P<0.05 (paired-samples *t* test).

5.3.3.2 Postprandial glucose

The measurements of postprandial glucose concentrations derived from the FGM System over the 4 hour postprandial period are presented in Figure 5.4. No effects of the order of quinoa or refined wheat rolls consumption were detected in glucose responses. At baseline, the glucose concentration was slightly lower in the quinoa treatment than in the wheat treatment but this was not statistically significant ($p>0.05$). After the test meals plasma glucose concentrations increased significantly from the baseline concentration for both treatments, and remained above baseline up to 4 h post-meal. Following the quinoa roll treatment, the glucose responses at 105, 120 and 135 min were significantly lower than those after the control meal ($p<0.05$, $p<0.01$ and $p<0.05$, respectively). The IAUC for glucose for the 4 hour glucose responses was, on average, 5.6% ($p<0.05$) lower after consumption of the quinoa rolls compared with the control meal. The glucose response curve following the refined wheat meal was at its highest at about 60 min and remained at approximately the same concentration up to 120 min. The peak glucose concentration was also observed at 60min after the ingestion of the quinoa meal, but then decreased more rapidly until 120 min and then more slowly between 120 and 240 minutes. The difference in peak glucose response or time to peak glucose response on the two meals did not reach statistical significance ($p=0.177$ and $p=0.235$, respectively) due to highly variability in individual responses.

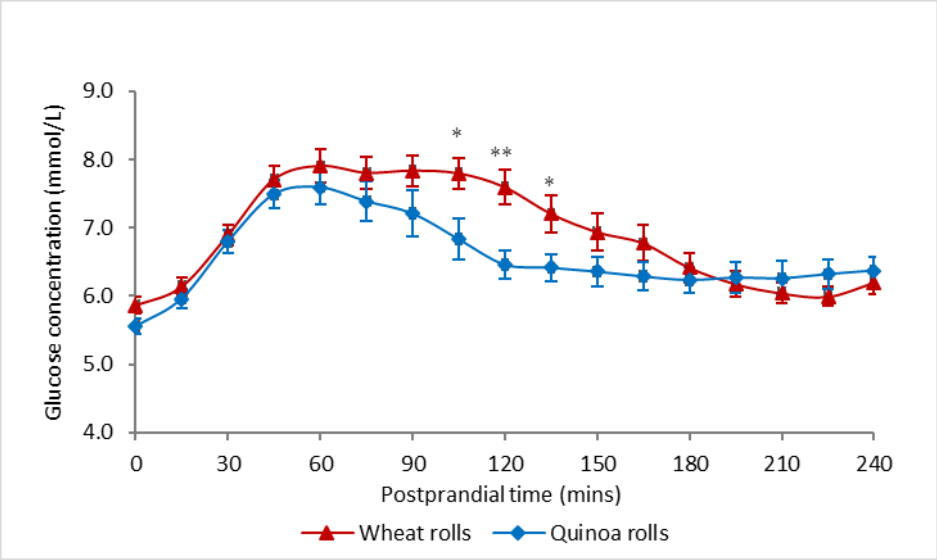


Figure 5.4. Mean concentrations of glucose responses for 240min after intake of quinoa and refined wheat breakfast meals after 4 weeks of intervention measured by FGM. Values are means with their standard errors of mean represented by vertical bars (n=24). *Mean values were significantly different between meals: *$P<0.05$, **$P<0.01$ (paired-samples *t* test with Bonferroni correction).

5.3.4 Postprandial glucose response derived from protein saver cards

The changes in postprandial capillary blood glucose response in dried blood spot samples extracted from finger-prick blood sampling on protein saver cards are shown in Figure 5.5. The ingestion order of the quinoa rolls breakfast and refined wheat rolls breakfast had no effect on the results. Values for glucose concentration are similar to those seen using the FGM system (Figure 5.4). At baseline (0 min), there were no significant differences in the concentrations of capillary blood glucose among the four test meal occasions. The peak capillary blood glucose concentrations at 60 min after all test meals were all significantly elevated compared with the respective baseline concentrations and then fell slowly thereafter. There was a suggestion that the glucose concentrations went down more quickly following quinoa rolls breakfast at week 4 but differences in capillary blood glucose concentrations between the four test meal occasions did not reach significance at any time point postprandially over 4 hours. At 120 min, following quinoa rolls consumption the glucose resulted in a significantly lower glucose concentration compared with baseline and the change in glucose response observed in the quinoa treatment at 120 min was significantly lower than that for the wheat treatment (p=0.03). By 240 min, blood glucose concentrations had not returned to baseline levels. In response to the test meal, the total IAUC for capillary blood glucose from baseline to any time points did not significantly differ between the four test meal occasions.

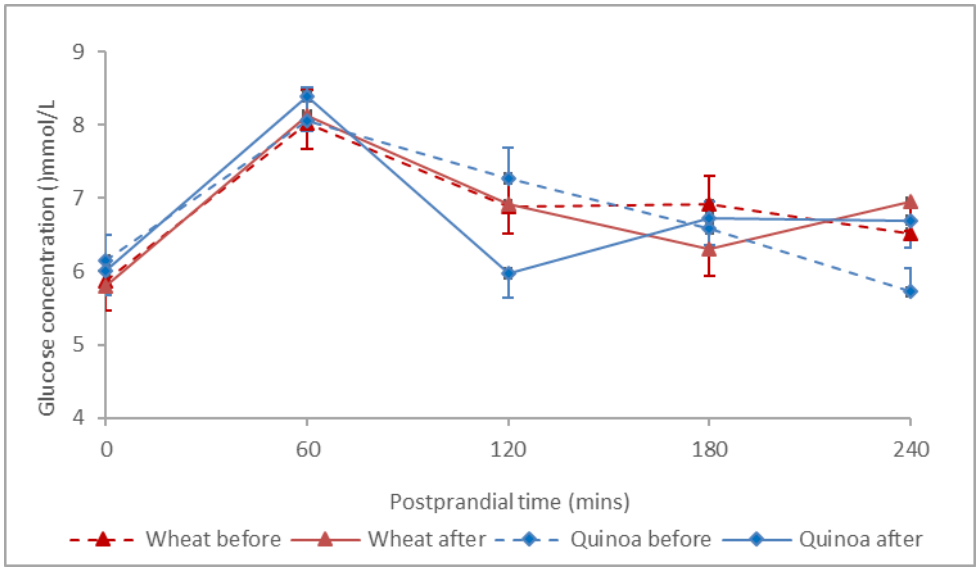


Figure 5.5. Mean concentrations of capillary blood glucose responses 240min at baseline and after 4 weeks of intervention after intake of quinoa and refined wheat breakfast meals. Values are means with their standard errors of mean represented by vertical bars (n=28).

5.3.5 Relationship between venous blood glucose, dried blood spot glucose and glucose monitor measurements

The mean values of glucose concentrations from fasting venous plasma samples and FGM appeared to be quite similar in this study, showing no differences between these two methods ($p>0.05$). DBS glucose concentrations, however, were significantly higher than venous glucose concentrations, with an overall mean difference of 0.28mmol/L; DBS glucose concentrations were not significantly differ from FGM values, however. The regression analyses of the different glucose analysis methods are presented in Figure 5.6. The correlation coefficient between venous and DBS glucose concentration values, venous and FGM glucose concentration values, DBS and FGM glucose concentration values were all significant ($p<0.01$), but the regression coefficients were relatively low, less than 0.5 ($R2=0.3973$, 0.4409 and 0.2466, respectively).

Research supports the Bland–Altman plot as an accepted statistical method of data plotting used in determining the agreement between two measures (Preiss and Fisher, 2008). As shown in Figure 5.7, Bland-Altman plots of differences in the different glucose analysis methods showed good agreement between each two methods, with few samples falling outside the 95% limits of agreement (LOA) for each comparison. However, it should be noted that the wide range of the 95% limits of agreement moderately reduced the clinical reliability, although the bias was small. As expected, Figure 5.7 (a) which shows the Bland-Altman plot of the difference between venous blood and DBS glucose concentrations for each individual observation, consistently demonstrated higher values for DBS than venous glucose concentrations.

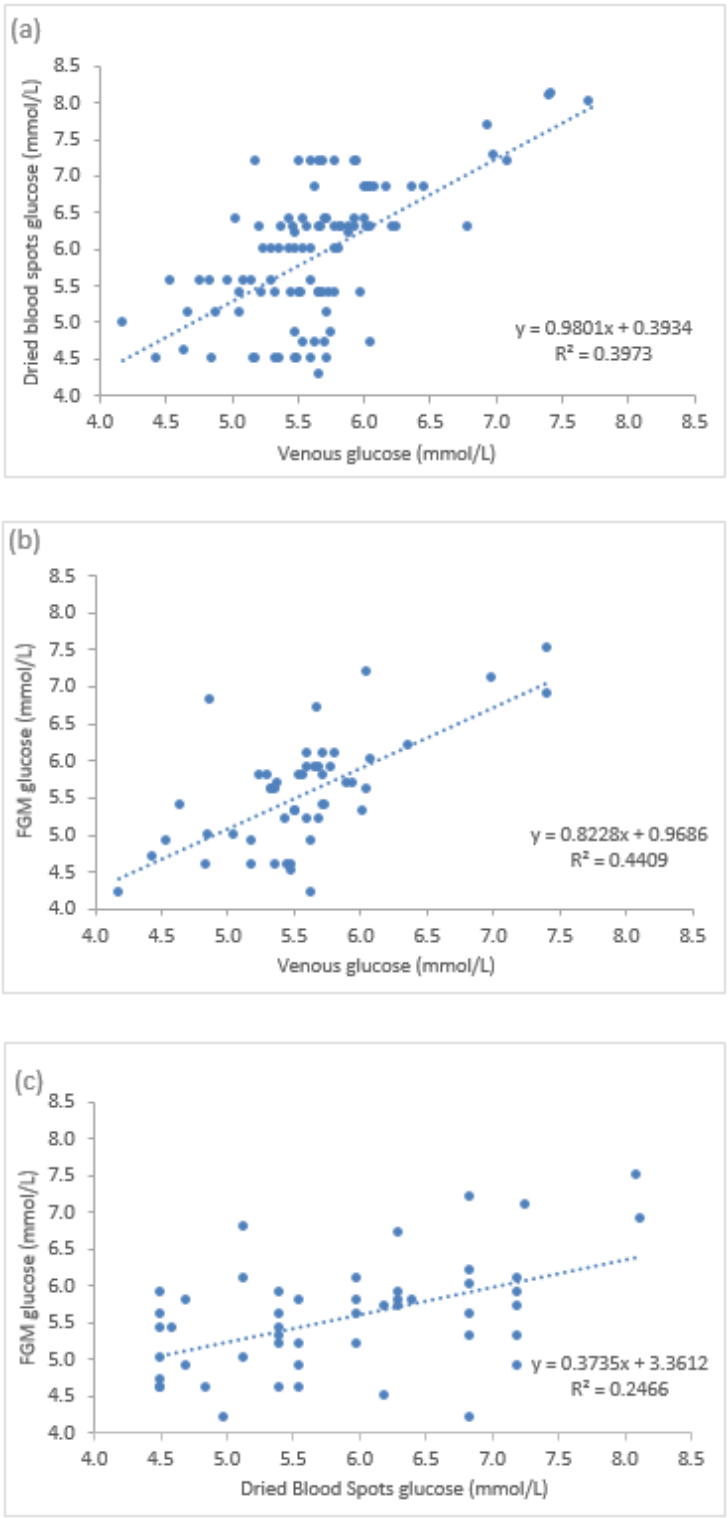


Figure 5.6. Correlation between (a) venous and DBS glucose values (n=24×4), (b) venous and FGM glucose values (n=24×2), (c) DBS and FGM glucose values (n=24×2).

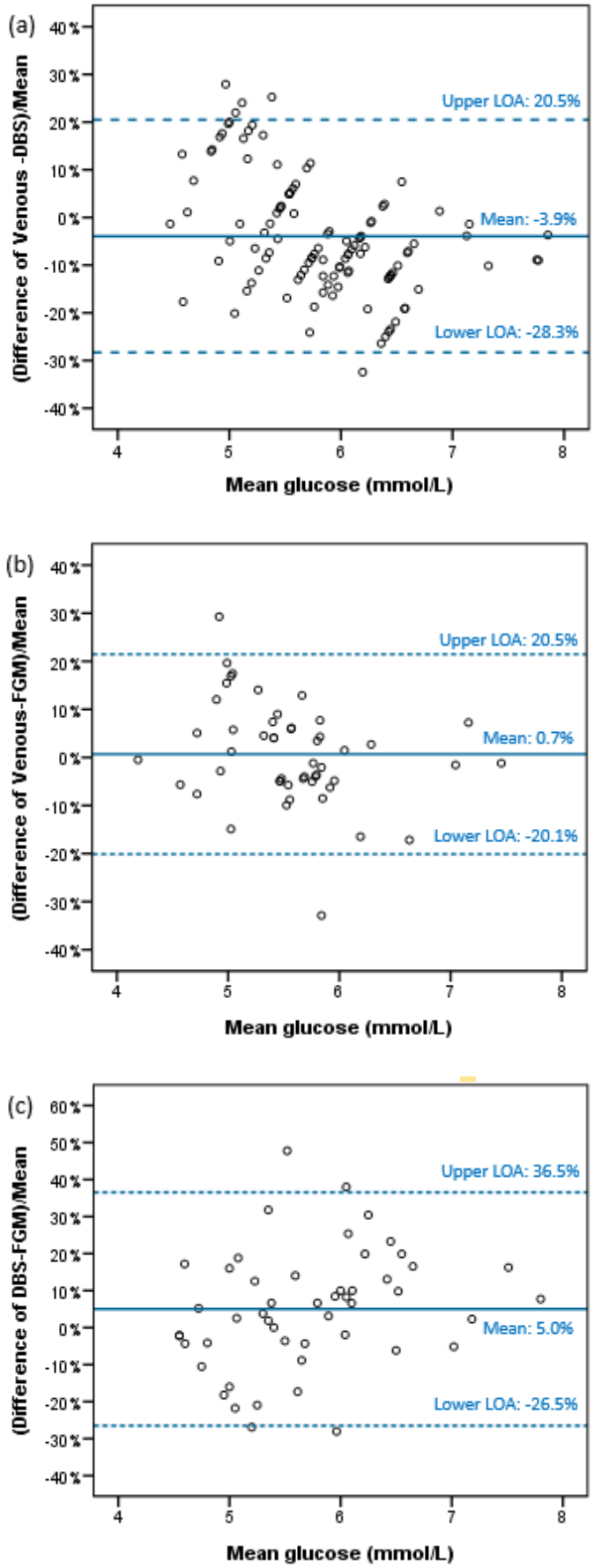


Figure 5.7. Bland-Altman plot of glucose concentrations showing relative differences between (a) venous and DBS methods (n=24×4), (b) venous and FGM methods (n=24×2), (c) DBS and FGM methods (n=24×2).

5.4 Discussion

5.4.1 CVD risk markers

Currently, whole grain cereals are generally recognized as an important part of a healthy diet, and at least 3 to 5 servings (16 g/serving) daily are specially recommended in the Dietary Guidelines for Americans 2015 (insert reference to guidelines here), while at least 6 servings are recommended for adults aged from 19 to 50 years in the 2013 Australian Dietary Guidelines (insert reference to guidelines here). Epidemiological studies consistently suggest an inverse association between the intake of whole grain food and risk of CVD, type 2 diabetes, cancer and obesity (He *et al.*, 1995; Chatenoud *et al.*, 1998; Jacobs *et al.*, 1998; Liu *et al.*, 1999b; Jacobs *et al.*, 2000; Mellen *et al.*, 2008a; O'Neil *et al.*, 2010; Ye *et al.*, 2012; Aune *et al.*, 2016; Chen *et al.*, 2016). Quinoa, as a whole grain, has attracted considerable attention recently, yet apparently little research had been done on its beneficial effects against CVD risk. To date only four quinoa intervention studies in human beings have been published, but these describe variable results (Jenkins et al., 2008; Farinazzi-Machado et al., 2012; De Carvalho et al., 2014; Zevallos et al., 2014) as discussed in Section 2.4. The present study assessed changes in CVD risk markers in response to daily intake of quinoa rolls delivering 20 g quinoa per day compared with refined wheat rolls over a relatively short period (4 weeks), using a random crossover experimental design. However, the present study cannot strongly support the hypothesis that quinoa would have beneficial effects on CVD risk markers, specifically fasting LDL cholesterol concentrations, as intake of quinoa rolls failed to produce any favourable changes in metabolic variables compared with refined wheat rolls. For example, although the reduction in LDL cholesterol (-5.7%) in the present study between baseline and after consuming quinoa was comparable to the significant reduction (-5.9%) in the study of De Carvalho et al. (2014), the absence of differences between treatments in the present study was due to an unexpected cholesterol-lowering effect after the refined wheat rolls treatment. Consequently, the difference in fasting plasma LDL cholesterol concentration was only -0.9% after 4 weeks intake of quinoa rolls compared with the control, suggesting a very modest improvement, if any, in the CVD risk profile.

The lack of improvement in fasting plasma lipids (total, LDL and HDL cholesterol, triglycerides) in the present study, is in agreement with the study of Zevallos et al. (2014) which analysed the gastrointestinal effects of 50g/d quinoa intake for 6 weeks as part of their usual diets in celiac patients, as well as the study by Jenkins et al. (2008) except for significantly increased HDL cholesterol. However, this finding appears to contrast with other two studies using similar doses and duration by Farinazzi-Machado *et al*. (2012) and De Carvalho *et al*. (2014), in which total, LDL cholesterol and triglycerides but not HDL cholesterol levels were significantly lowered after

approximately 1 month of consuming 19.5 g quinoa bars and 25 g quinoa flakes in healthy students and overweight postmenopausal women, respectively. It has been suggested that inflammation is an important contributor to the development of atherosclerosis. As a frequently used marker of inflammation, fasting plasma CRP failed to show any responses in quinoa rolls consumption in this study, which was in accordance with the result of study by Jenkins *et al.* (2008). The hepatic enzymes AST and ALT are both clinically considered to be sensitive indicators of liver damage or injury from different types of diseases or conditions, although ALT is more specific and more commonly increased than AST for the liver. In the present study, no significant changes in the levels of AST and ALT were detected after intake of quinoa rolls, although ALT concentrations were numerically decreased, in contrast to the study of Farinazzi-Machado *et al*. (2012) which reported lower values of AST, with ALT unaffected after1 month consumption of quinoa bars. In an animal study, the mean values of serum AST and ALT were significantly reduced in Male Wistar albino rats fed with a high fat diet with 60% milled quinoa. This was attributed to the high levels of quinoa polyphenols, but this has not been confirmed in human subjects to date. It is noteworthy that the results from the four studies mentioned above should be viewed or explained with caution due to inappropriate (non-refined grain) or lack of control treatments used in these studies. Difference in subject characteristics, study duration, amounts and mode of quinoa foods provided in studies may account for these apparently conflicting results.

Anthropometric and blood variables did not significantly differ between treatments at study entry. The lack of changes in anthropometric variables throughout the 8-week study period, including body weight, BMI and body fat content, suggests that subjects followed their physical activity and habitual diet routines throughout the study potentially minimizing confounding effects. Also, energy intake and overall dietary intake was not affected by the intervention. Moreover, the absence of changes in body weight or BMI between treatments was in accordance with previous studies investigating quinoa interventions, although epidemiological studies have found that intake of whole grain was related to lower BMI, and one non-peer reviewed study have shown 0.9-1.4 kg weight loss on a whole grain diet in relation to equal servings of refined grain diet over 4-6 weeks (Jacobs *et al.*, 2004; Harland and Garton, 2008; Jenkins *et al.*, 2008; McKeown *et al.*, 2009; Farinazzi-Machado *et al.*, 2012; De Carvalho *et al.*, 2014). The majority of these earlier studies, however, were based on much higher intakes of whole grain wheat, rye or oats compared with refined alternatives, and none included quinoa. Considering that subjects in this study were all overweight (BMI>25kg/m^2), the small 0.3 kg reduction in weight would indicate that the dose of quinoa used in the study was too small to cause changes in body weight.

To date, there have been no apparent indications of an improvement in glucose control after quinoa foods consumption, which may be unsurprising since none of the subjects included in the earlier studies had impaired glucose tolerance or diabetes. There has been one human intervention study which showed a significant reduction in fasting plasma glucose concentration after inclusion of two slices of quinoa bread into each individual's habitual diet for 6 months. However, this study was performed in 210 patients with type 2 diabetes (Jenkins *et al.*, 2008), and although quinoa was included in the intervention it was only part of a wider dietary change.

The presence of many 'trend' results, such as glucose, insulin and LDL cholesterol values, in the present study indicates that significant results might have been obtained with larger numbers of subjects. Besides, there were several reasons that might account for the absence of positive response. Firstly, the duration of this controlled human intervention study, representative of a very short period of dietary alteration in the context of lifelong dietary exposures, may not have been sufficiently long to detect favourable effects on markers of CVD risk. Some studies have found significant reductions in both plasma total and LDL-cholesterol after consuming diets enriched with whole grain or quinoa foods, including in healthy and normocholesterolaemic individuals at baseline with relatively short duration interventions (Behall *et al.*, 2004; Giacco *et al.*, 2010; Maki *et al.*, 2010; Farinazzi-Machado *et al.*, 2012; De Carvalho *et al.*, 2014). Secondly, the lack of intervention effects may also be a result of lower dose consumed by subjects daily. According to epidemiological studies, beneficial health effects of whole grains can be expected at a level of 3 (16g) servings per day (Anderson *et al.*, 2000). The daily amounts of quinoa provided during the present study (20g) was in the range of usual whole grain intake of about 16-25 g/d, but much lower than minimum recommended intake of 48-96 g/d (Cleveland *et al.*, 2000; Maras *et al.*, 2009). In this study, only refined wheat and wheat-quinoa rolls were provided for inclusion in the participants' usual diets. Nutritional profile analysis showed that the nutrient contents between these two rolls were similar, with the exception of a higher concentration of total dietary fibre in quinoa rolls, as expected (Section 4.3). The higher concentration of total dietary fibre in quinoa rolls might contribute to relatively higher intake of total dietary fibre higher during quinoa rolls treatment compared with refined wheat rolls treatment, according to 3-day food records shown in Table 5.1. However, it cannot deny the possibility that refined wheat and quinoa rolls have replaced healthier foods containing higher concentration of total dietary fibre, since the intake of total dietary fibre were lower by around 2 g/day at week 4 in comparison with the baseline within both two treatments. Many epidemiological studies have shown that soluble fibre was the main contributor to the cholesterol-lowering property of whole grains intake (Glore *et al.*, 1994; Truswell, 1995; Brown *et al.*, 1999). However, the higher content of insoluble dietary fibre in quinoa roll is the main cause of the

large difference in total dietary fibre between these two rolls. This may in part explain the lack of the response in plasma cholesterol in the current study. Thirdly, it may have been better to focus on an 'at risk' population with higher fasting plasma LDL cholesterol in terms of CVD risk. However, a clinically high-risk population was not chosen for the current study, because the results of this study was expected to benefit general population as a whole. Instead, the current study targeted overweight males (BMI >25 kg/m^2) which may be at elevated CVD risk, but are also representative of the general population in different countries, where overweight is likely to become normal. Fourthly, a mixture of quinoa and other grains as an experimental diet might be not a good option to deliver quinoa to humans compared with other forms of pure quinoa foods, such as bars and flakes, which were included in the studies observing significantly positive results by Farinazzi-Machado *et al*. (2012) and De Carvalho et al. (2014), respectively. For example, in the present study the unexpected reduction in LDL cholesterol concentration in the refined wheat rolls treatment made the improvement in consumption of 20 g quinoa flour insignificant. Also, the changes in quinoa treatment may be partly attributed to the 80% of refined wheat in quinoa rolls in this study, which caused some uncertainties to discuss. But it should be noted that the positive results from the above two studies may be caused by change or errors instead of successful research due to lack or inappropriate control and other reasons. Thus, taken altogether, it cannot deny the fact that there is possibility of no significant effect of quinoa consumption on CVD risk. To ensure that a study can clearly show whether an effect exists or not, a different type of study is strongly necessary. For example, short duration using much higher doses than what is feasible in a normal diet (concentrated foods without non-functional components such as starches), with a pre-determined minimal outcome, if the result is smaller than this, it can be concluded that there is no effect of quinoa consumption. However, if protective effects occurs, a concentrated food should be made by extracting the specific components such soluble fibre, saponins from quinoa and removing anything else for the future *in vivo* studies in order to explore active component that is responsible for the effects.

5.4.2 Plasma antioxidant activity

One potential mechanism for the protective properties against CVD provided by wholegrain products consumption has been attributed to their antioxidant content, which may help to reduce the oxidation of LDL cholesterol, thereby reducing cardiovascular risk. The impact of polyphenols in the diet is controversial; for example, it is not clear whether dietary polyphenols can be absorbed intact from the diet in sufficient quantity to affect 'antioxidant status'. It is possible that modified polyphenolics may instead act as signalling molecules in the body (Spencer, 2009). The effect of

wholegrain products consumption on antioxidant status has been investigated in both animal models and human subjects, but the number of studies remains small. For laboratory rats a wild rice diet fed for 8 weeks suppressed oxidative stress by enhancing antioxidant activity both in the serum and liver, even when fed a diet high in fat and cholesterol, as reported by Zhang *et al*. (2009a). In accordance with this, the antioxidant content of plasma, heart and lungs in rats was improved after 5 weeks inclusion of amaranth seeds in the diets (Paśko *et al*., 2011). Additionally, the results of the study by Khan *et al*. (2015) demonstrated that the acute intake of pasta containing 30% red whole-grain sorghum significantly improved antioxidant status and enhanced markers of oxidative stress in healthy subjects compared with baseline (0min).

In the present study the plasma antioxidant capacity analysed by FRAP and TEAC methods showed no significant difference in this study probably due to low intake of wholegrain quinoa or short duration, despite the fact that the antioxidant capacity of quinoa rolls was greater in relation to that of refined wheat rolls. In addition, the samples used in the analysis were taken after an overnight fast, therefore any acute change in plasma antioxidant content following a meal enriched with polyphenols may have subsided. A similar lack of response in plasma antioxidant capacity was seen during the whole-grain intervention study of Jones *et al*. (2004a) and Jones *et al*. (2004b), in which human subjects were provided three 23 g servings of wholegrain food per day for 8 weeks and then increased their intake of whole-grain food to six 23 g servings per day for a further 8 weeks. Thus higher doses and longer duration were not effective in changing 'antioxidant status'. Similar results were also observed in the study by Enright & Slavin, (2010), implying that addition of six or eight servings of whole-grain food per day may be insufficient to cause a significant increase in plasma antioxidant capacity. Recently, according to Price *et al*. (2012), inclusion of wholegrain wheat aleurone-rich food into habitual diets for 4 weeks failed to induce any favourable effects on antioxidant status, including superoxide dismutase activity and antioxidant activity measured by the FRAP method, although the greater amounts of cereal products consumed by subjects daily provided much higher content of total phenolics with stronger antioxidant capacity compared with the refined wheat and quinoa rolls in the present study. Together, the lack of response may, at least partly, reflect that antioxidants from whole grains were possibly not fully absorbed by the digestive tract, subsequently having no effect on the antioxidant status in animals and human subjects. Moreover, it has been suggested that the lack of response in plasma antioxidant capacity was possibly a result of homeostasis, which describes the maintenance or regulation of stable conditions, or its equilibrium. Recently, several studies have reported that plasma antioxidant capacity rapid increased after intake of antioxidants-rich foods, but then returned to baseline levels over the following hours (Serafini *et al.*, 2005; Harasym and Oledzki, 2014).

5.4.3 Postprandial glucose response: both from FGM and DBS

Postprandial hyperglycaemia has been recognised as a risk factor for CVD, resulting in elevated cardiovascular morbidity and mortality in diabetic subjects (Martín-Timón *et al*., 2014). A number of studies have clearly demonstrated that the incidence of serious diabetic complications could be decreased by strict glycaemic control via diet and its constituents (Ghannadi *et al*., 2016). Quinoa is characterized by its excellent nutritional properties, which have many potentially biochemical effects (Abugoch James, 2009; Paśko *et al*., 2010). However, research on the hypoglycaemic effects of quinoa seeds consumption in vitro, and especially in vivo, still remains scarce.

In the present study, the effects of a breakfast meal consisting of the test quinoa and wheat rolls on glycaemic responses derived from DBS and FGM (representing capillary and interstitial fluid glucose concentrations, respectively) was investigated in healthy overweight subjects. Although some important differences in glucose responses between quinoa and refined wheat rolls breakfast, such as significantly different IAUC values over 4 hours, were not observed in capillary blood samples, the glucose response curves were relatively similar; there was a more rapid fall approximately after 60 mins after the quinoa roll breakfast. These results were in agreement with those of Gabrial *et al*. (2016), showing a similar substantial drop in capillary blood glucose concentrations in healthy subjects after intake of breakfast meals delivering 80 g quinoa compared with white wheat bread as a reference breakfast meal. In this earlier study, the quinoa meal had significantly lower incremental area under curve (IAUC) values for blood glucose in diabetic subjects compared with a white wheat bread, while no significant difference was detected in healthy subjects. There were no significant differences in the peak rise of blood glucose between the two breakfast meals in both healthy and diabetic subjects. In addition, blood glucose concentrations started to decrease gradually for the quinoa but not for white wheat bread in both healthy and diabetic subjects and returned to near-fasting baseline levels by about 210 min. A test meal containing 50 g available carbohydrates as quinoa was provided to subjects in the study of Berti *et al*. (2004). The results showed that the AUC and glycaemic index (GI) for quinoa were slightly lower than those of gluten-free bread and gluten-free pasta but did not reach significance ($p<0.05$), and no significant differences in satiety, fullness and desire to eat were observed between each treatment. In an animal study, rats fed a diet with 20% quinoa exhibited lower food intake ($p<0.01$), whereas there was only 3.4% improvement in blood glucose profile in the quinoa group compared with the control group.

The postprandial glycaemic impact of foods is closely associated with the rate of carbohydrate digestion and is reliably characterized by the GI, a model which enables comparison of various starch-containing foods (Jenkins *et al.*, 2002). Predicted glycaemic indexes (pGI) can be calculated

from the AUC (g RSR/100g Total available Carbohydrates*min) of reducing sugars released (RSR). In an *in vitro* study, where starch digestibility of quinoa bread was analysed using a multi-enzyme dialysis system, the AUC and pGI for quinoa were slightly lower than of wheat bread (-5%), but there was no significant differences between these two breads (Wolter *et al.*, 2013). The lower pGI of quinoa may be explained by a significantly lower carbohydrates content of quinoa bread than that of wheat bread. In contrast to this, Berti *et al*. (2004) reported that the AUC of digested starch for quinoa was significantly lower than gluten-free bread and bread-like products, although the AUC of digested starch of quinoa and two pasta did not differ significantly. Due to the principle role played by the rate of starch digestion, measurement of this in food *in vitro* has been proposed as a cheaper and less time-consuming method to predict *in vivo* properties. However, it should be noted that some metabolic factors that affect glycaemia *in vivo*, such as glucose absorption, the rate of gastric emptying, gut hormone profiles and limited starch accessibility to α-amylase, cannot be completely mimicked through *in vivo* assays (Berti *et al.*, 2004; Fardet *et al.*, 2006).

This study is the first to show a reduction in glycaemia following regular consumption of quinoa. The AUC for glucose over the four days at the end of the quinoa treatment period was borderline significantly lower than the following four days when quinoa consumption ceased (p=0.054), and also was significantly lower than that of the four days at the end of the wheat treatment period (p=0.039) (Figure 5.3). This may have been in part due to the cumulative effect of the reduced postprandial glycaemia seen after the breakfast test meal (Figure 5.4 and Figure 5.5). The factors responsible for the positive glucose response after intake of quinoa rolls are not evident in this study. The differences in the postprandial glucose responses cannot be explained by the time taken by the ingestion of the breakfast, since the subjects were encouraged to finish the meals in a very short period of 3-5 mins. Several previous *in vitro* and *in vivo* studies have shown that polyphenols may affect carbohydrate digestion and absorption and thereby postprandial glucose responses (Kobayashi *et al.*, 2000; Hanamura *et al.*, 2006; Torronen *et al.*, 2010; Nyambe-Silavwe and Williamson, 2016). In human studies, beverages rich in polyphenols have demonstrated favourable impacts on postprandial glycaemia. For example, delayed absorption of glucose after intake of coffee and apple juice by humans has been demonstrated (Johnston *et al.*, 2002; Johnston *et al.*, 2003). As a result, a few studies have investigated the role of polyphenols in modulation of postprandial glycaemia, mainly as a component of fruits and vegetables or individual compounds, but the studies that focused on the effects of polyphenols within whole grains on postprandial glycaemia are very limited to date. In one human study, the 2-h postprandial concentrations following a meal of 30% red whole-grain sorghum pasta of plasma total polyphenols, antioxidant capacity and superoxide dismutase activity were significantly higher in healthy subjects compared

with baseline (0 min) value ($p<0.001$). Polyphenols were the major contributor to the increased antioxidant capacity, which was also associated with improved antioxidant status and enhanced markers of oxidative stress (Khan *et al.*, 2015). The study illustrates that polyphenols may be absorbed alongside glucose during the postprandial period. However, the lack of differences in fasting plasma antioxidant activity, suggests that the low dose of quinoa incorporated in the breakfast meal may only have negligible effects on the postprandial glycaemia observed.

In addition to polyphenols, dietary fibre may also have potentially influenced the postprandial glucose responses to the quinoa roll breakfast. According to Ulmius *et al*. (2009), postprandial glucose and insulin responses can be affected by fibre-rich meals, depending on the source and dose of total and soluble fibre. However, most studies exploring these beneficial effects of fibre have focused on mixtures of fibre types or only soluble fibre, but not insoluble fibre (Wilmshurst and Crawley, 1980; French and Read, 1994; Vandeven *et al.*, 1994; Tomlin, 1995; Tiwary *et al.*, 1997). The relatively similar soluble fibre content of the test rolls shown in Table 4.3, suggests that it is unlikely that the soluble fibre could solely account for the differences detected in the glucose responses. Instead, the insoluble fibre (1.33 and 2.84 g/100g breakfast refined wheat and quinoa roll, respectively) may be the most likely factor contributing to the positive effects due to more than double amounts of insoluble fibre in quinoa rolls than in wheat rolls, although the overall contribution to the meal, and the whole diet is still relatively small. One previous study have demonstrated that the intake of insoluble fibre (31.2 g/d) over 3 days was associated with improved whole-body insulin sensitivity and a reduced risk of type 2 diabetes (Weickert *et al.*, 2006). Furthermore, a high dose of insoluble fibre (a serving of 33 g) contained in a high-fibre breakfast meal supressed appetite, reduced food intake and improved glucose response to a meal after consumed 75 min later by healthy men, when compared with low-fibre cereal (1g of insoluble fibre), as reported by Samba and Anderson, (2007). Insoluble fibre consumption increases the rate of small intestine transit, thus, resulting in reduced starch hydrolysis and absorption (Jenkins *et al.*, 1978; Lewis and Heaton, 1997). Weickert *et al*. (2005) also reported that increased secretion of glucose-dependent insulintropic polypeptide, an incretin hormone, was detected in healthy women after intake of an insoluble fibre, stimulating the postprandial release of insulin. Additionally, insoluble fibres like resistant starch are additionally fermented by some bacteria in the colon, producing short-chain fatty acids, which may enter the circulation and decrease production of hepatic glucose and circulating non-esterified fatty acids (NEFA) concentration, thereby increasing glucose storage and insulin sensitivity (Brighenti *et al.*, 1995; Ostman *et al.*, 2002). Some studies have reported that starch structure of quinoa was different from that of wheat (Filho *et al*., 2017; Li and Zhu, 2018). However, up to date, no studies have showed that different starch structure of quinoa compared

with other cereals was one of the causes for reduced postprandial glucose response. Inversely, it has been demonstrated that considerable variability exists in the amylose content of quinoa (3-20%), which is regarded to be lower than in cereal as wheat (20-30%). Starch with low amylose have higher glycemic indexes. Therefore, quinoa structure of quinoa was not included in this study as one of reasons contributing to reduced postprandial glucose. However, an important limitation of this investigation was that the amounts of available carbohydrate and other macronutrients were not equalized between treatments, as well as volume and calories. Consequently, the quinoa roll was slightly lower in available carbohydrate in comparison with the wheat roll, and a lower glycemic response would perhaps be expected. Next, the "second meal effect", is an uncertain factor which may also have interfered with the postprandial glucose responses, since no standard meal was provided to subjects before the 12h fasting period before each visit (Wolever *et al.*, 1988), although participants were asked to replicate their evening meal before each visit where possible.

5.4.4 Correlation between glucose measurements from venous plasma samples, and DBS and FGM

Dried blood spots (DBS) are whole blood obtained from finger-pricks and placed onto filter paper, dried and then eluted later for further laboratory analysis. Finger-prick blood has been clinically considered as a capillary sample, but actually it is a mixture of arteriolar, capillary and venous blood (Merton *et al*., 2000). In research settings, DBS are now generally considered to be a well-established alternative to venepuncture as a method for collecting samples for analysis of biomarkers in whole blood. Currently, a GI value for various foods can be measured by both venous and finger-prick blood sampling according to guidelines from WHO and FAO (Yang *et al.*, 2012). DBS collection is simple, reliable, relatively painless, less expensive, less blood volume needed, and samples can be easily transported at ambient temperatures compared with conventional venepuncture, and needs minimal storage requirements (such as samples do not need to be immediately frozen after collection and can be stored in less space for a long time before analysis). DBS samples can be tested for a variety of blood metabolites, including glucose, cholesterol, triglycerides and haemoglobin. In the present study, the glucose concentration was slightly and significantly lower in venous blood than the results obtained from the DBS. The results in the present study are in accordance with those of Mdlalose *et al*. (2017) showing significantly higher mean glucose concentrations in DBS than venous blood observed in fish, but with a high correlation between the two methods (R2=0.898). This is most likely attributable to higher concentrations of glucose in both arterial and capillary blood than that of venous blood, since glucose absorbed in the small intestine sequentially diffuses from arteries into tissue cells through peripheral capillaries and

the de-oxygenated blood returns in the venous system (Burtis and Ashwood, 1994; Yang *et al*., 2012). However, the results in the current study contrast with the studies of Ward *et al*. (1996), which showed that the two methods did not significantly differ and the two methods were highly correlated (R2=0.96). In another study, Lacher *et al*. (2013) demonstrated that the mean of DBS glucose was significantly lower than venous blood glucose concentration with high correlation (R2=0.81). One important fact that cannot be ignored is that the method of extraction of DBS glucose from protein saver card has not been standardised. In addition, the calculated value for the plasma concentration of glucose derived from the whole blood DBS is dependent on the assumptions for 'average' haematocrit for the population (Purves *et al.*, 2004).

In contrast to earlier forms of continuous glucose monitoring systems (CGM), the newest version of FGM, does not require finger-prick calibration because the highly improved production technology makes a factory calibration possible. Even though there is a physiologic lag of around 5-15 mins between glucose concentrations due to diffusion from capillary blood into interstitial fluid, depending on the rate of change in circulating glucose concentrations, the FGM system has been regarded as a suitable device for glucose control and prevention of hypoglycaemia in patients with impaired glucose tolerance. Despite the lack of need for calibration, the accuracy is still of critical importance in delivering therapeutic value. The use of venous glucose concentrations as a reference method is the key factor and is considered the most suitable when assessing the accuracy of the FGM device. In the present study, although the correlation was lower, the mean glucose concentration values from venous blood and FGM were not significantly different similar ($p>0.05$), and there was also a good agreement between them. In the few previous studies that have focused on this field, the mean absolute relative difference (MARD) is generally used to estimate accuracy of the FGM device. The FreeStyle Libre FGM in the studies of Bailey *et al*. (2015), Bonora *et al*. (2016), Ji *et al*. (2017) and Aberer *et al*. (2017) showed a range of MARD from 10.7% to 13.2%, along with low or high correlation, when directly compared with reference venous blood glucose outcomes. However, the studies mentioned above failed to reveal the higher or lower glucose concentrations which the two methods corresponded.

5.5 Summary and conclusion

The results of the dietary intervention study indicate that consumption of 20 g quinoa per day in the form of a wheat-quinoa bread roll does not affect markers of CVD risk, although there is a suggestion that glycaemia may be improved through a reduction in postprandial glycaemic response. The primary outcome of the trial, change in LDL-cholesterol concentration was unaffected by the intervention.

To the best of our knowledge, this is the first study comparing glucose concentrations derived from DBS and FGM to date. Although the mean DBS glucose concentration was higher by 0.30 mmol/L in comparison with that of FGM, there was no significant difference between them, with a weaker correlation. The ease of capillary blood taking suggests that this should be used as the reference, instead of venous blood, to calibrate the accuracy of CGM device. The lack of need for calibration in FGM device reduces the necessity to further compare these two methods in this study.

Overall the results suggest some potential benefit of consuming quinoa on glucose response but this requires further investigation, possibly with larger doses of quinoa. The mechanisms by why quinoa may have this effect remain undetermined.

Chapter 6 Effect of quinoa consumption on the gut microbiome

6.1 Introduction

Prebiotics are defined as 'non-digestible food ingredients that confer benefits upon host well-being and health by selectively changing the composition and/or activity of one or a limited number of bacterial species already resident in the colon' (Gibson and Roberfroid, 1995). There is an increasing wealth of research to suggest the beneficial health effects of prebiotics on elevated mineral absorption, bowel habit and constipation, and recently, in decreasing the risk of some chronic diseases, such as CVD and colon cancer (Coudray *et al.*, 1997; Kleessen *et al.*, 1997). For example, it was revealed that intake of prebiotics lowered plasma total cholesterol and triglycerides concentrations in subjects especially those with hyperlipidmia (Davidson *et al.*, 1998; Causey *et al.*, 2000). Likewise, epidemiological studies strongly show that whole grain intake is protective against several chronic diseases, including CVD, cancer, type 2 diabetes and obesity (He *et al.*, 1995; Chatenoud *et al.*, 1998; Jacobs *et al.*, 1998; Liu *et al.*, 1999b; Jacobs *et al.*, 2000; Mellen *et al.*, 2008a; O'Neil *et al.*, 2010; Ye *et al.*, 2012; Aune *et al.*, 2016; Chen *et al.*, 2016). Many of these observed health benefits offered by whole grains are likely to be the result of modulation of the gut microbiota, since whole grains are rich in a variety of indigestible carbohydrates, such as β-glucan, arabinoxylan, cellulose and fructan, but most of which are lacking in refined grains owing to the removal of the bran layer of grain during refining. A number of *in vitro* fermentation and colonic studies also showed the prebiotic impacts of whole grain foods (Connolly *et al.*, 2010; Connolly *et al.*, 2012a; Connolly *et al.*, 2012b; Maccaferri *et al.*, 2012). For example, Connolly *et al.* (2010; 2012b) reported that different whole grains, including oat products, significantly stimulated both bifidobacteria and lactobacilli bacteria *in vitro*, indicating a prebiotic potential of whole grain. However, studies to support the hypothesis that intake of whole grain as prebiotics can affect the bacterial composition and activities in the gut are still limited; moreover, no information exists on the prebiotic potential of whole grain quinoa. To test this hypothesis, this study was conducted to investigate the effect of intake of bread rolls enriched with quinoa as a whole grin source on the human gut microbiota in healthy subjects compared with refined wheat rolls.

The aim was to investigate the impact of quinoa consumption on the gut microbiome. The primary objective was to examine the bacterial diversity and relative abundance upon ingestion of quinoa rolls compared with refined wheat rolls. A secondary objective was to explore possible correlation between gut microbiota and markers of CVD risk, such as body weight, glucose and lipids.

6.2 Methods

6.2.1 Experimental design and stool sample collection

The design of the dietary intervention is described in detail in Chapter 5 of this Thesis. Stool samples were collected from volunteers at the beginning and end of the treatment periods as described in Section 5.2, Methods.

6.2.2 Bacterial DNA extraction and 16S rRNA bacterial profiling

Faecal samples were collected and immediately stored at -80 °C until analysis. DNA was extracted from approximately 300 mg stool using the FastDNA^{TM} Spin Kit for Soil (MP Biomedicals) following the manufacturer's protocol. The extracted DNA was sent to for sequencing. The 16S rDNA V4 region was selected for PCR applification since it has been shown to represent the taxonomic profile of microbial communities compared with characterisation of the full length 16S gene sequences (Caporaso *et al.*, 2012). Sequencing was carried out in the Illumina MiSeq platform by using the 2x250 bp paired-end protocol yielding pair-end reads that almost completely overlap. The primers applied in amplification possessed MiSeq sequencing and single-end barcodes, which allow pooling and direct sequencing of PCR products (Caporaso *et al.*, 2012).

Phylogenetic and alignment-based approaches were incorporated into the 16S rRNA gene pipeline data to maximize data resolution. The read pairs were demultiplexed based on the unique molecular barcodes, and reads were merged using USEARCH v7.0.1090, allowing zero mismatches and a minimum overlap of 50 bases (Edgar, 2010). Merged reads were trimmed at first base with Q5. Additionally, a quality filter was used for the resulting merged reads, and reads were discarded if containing above 0.05 expected errors. 16S rRNA gene sequences were clustered into Operational Taxonomic Units (OTUs), which is a term used to categorize groups of closely related bacteria at a 97% sequence similarity level by using the UPARSE algorithm (Edgar, 2013). OTUs were mapped to an optimized version of the SILVA Database, which only contained the region of 16S V4 to determine taxonomies (Quast *et al.*, 2013). Mapping the demultiplexed reads to the UPARSE OTUs resulted in recover of abundances. A custom script constructed a rarefied OTU table from the output files generated in the previous two steps for downstream analyses of alpha-diversity, beta-diversity, and phylogenetic trends (Lozupone and Knight, 2005).

6.2.3 Calculations and statistical analyses

The data were analysed by using the SPSS 22.0 for Windows statistical program (SPSS, Inc., Chicago, IL, USA), and the results are expressed as means and standard deviations. Differences in relative

abundance of taxonomic groups that were summarised at both phylum and genus levels within and between groups were analysed with paired t-test. A p-value of less than 0·05 was considered to be significantly different. The percentage change was calculated as follows: (value at 4 week - value at baseline)/value at baseline × 100. The data of baseline and week 4 from the quinoa treatment and refined wheat treatment was combined together (28 × 4 = 112 values) to explore the Spearman's correlation between gut microbiota (bacterial diversity and relative abundance in phylum and genus levels) and markers of CVD risk. The statistical package R was applied for the analysis and visualization of microbiome communities, and the phyloseq package was used to import data and calculate alpha- and beta-diversity metrics (McMurdie and Holmes, 2013; R-Core-Team, 2014). This study rarefied each sample to 5,000 reads. Principal coordinate plots employed the Monte Carlo permutation test to estimate p-values (Dwass, 1957). All p-values were adjusted for the total number of comparisons with the use of a false-discovery (FDR) algorithm (Benjamini and Hochberg, 1995).

6.3 Results

6.3.1 Observed OTUs

Following the extraction of DNA from the faecal samples, MiSeq sequencing was used to assess the effects of quinoa rolls consumption on the profile of the faecal microbiota. Samples were sequenced using Illumina MiSeq sequencing, which is currently the most widely platform for bacterial 16S rRNA gene amplicon sequencing. This resulted in a minimum of 3,109 rarefied high-quality reads per faecal sample as shown in Figure 6.1.

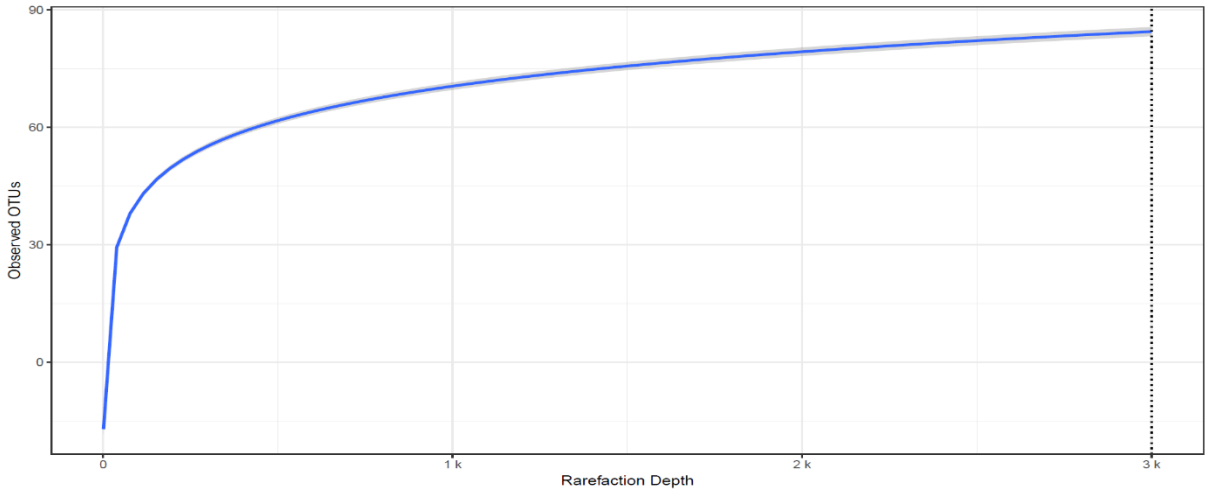


Figure 6.1. Rarefaction curves of the observed OTUs at 97% sequence identity.

Reads were clustered into operational taxonomical units (OTUs) delineated at 97% sequence identity because higher thresholds generated a dramatic increase of OTU numbers, which might represent the microdiversity at subspecies level (Caporaso *et al.*, 2010). At a threshold of 97% sequence identity, a total of 11,424 OTUs were identified in the current study (median=102 OTUs, ranging from 42 to 170 OTUs). The results, along with the calculated microbial community alpha diversity indexes, are shown in Table 6.1. Although the rarefaction curve did not plateau under the current sequencing, the number of observed OTUs of all samples had reached stable values at this sequencing depth, indicating that most of the microbial diversity had been captured in the data set despite the possibility to detect new phylotypes with additional sequencing efforts.

6.3.2 Diversity analysis

To determine if any alternations in the microbial community structure of faecal samples occurred as a result of different intervention treatments, overall association tests were performed based on alpha and beta diversity analysis. Alpha and beta diversity measures offer a holistic view of biodiversity of the gut microbiota, but they focus on different aspects. In this study, alpha diversity measures were chosen as following: the observed OTUs number (after rarefaction) as a species richness measure, and Shannon index, Simpson's index, Inverse Simpson index and Fisher's alpha parameter as an overall diversity measure including both species richness and evenness. High levels of variation were detected between subjects when the richness and diversity measures were assessed; however, none of these indices showed significant difference in stool samples within and between quinoa and refined wheat rolls treatment (Table 6.1).

Table 6.1. Estimates of alpha diversity indexes in the stool samples at baseline and week 4 after refined wheat roll or quinoa roll consumption.

	Wheat rolls (n=28)			Quinoa rolls (n=28)			P for week 4 (W vs Q)
	Baseline	Week 4	p-value	Baseline	Week 4	p-value	
Richness							
Observed OTUs	104.29	101.18	0.296	102.96	99.57	0.273	0.559
Richness and evenness							
Shannon index	3.30	3.29	0.922	3.35	3.36	0.869	0.381
Simpson's index	0.92	0.93	0.902	0.93	0.94	0.428	0.328
InvSimpson index	17.88	17.47	0.787	19.17	18.67	0.736	0.483
Fisher's alpha parameter	21.11	20.30	0.260	20.78	19.89	0.250	0.537

1 Difference between baseline and week 4 tested by paired t-test; mean values were considered significantly different if $P<0.05$.

In terms of beta diversity, which describes overall microbial community structure, the distance metrics explaining similarity between bacterial communities were obtained from the individuals using Bray-Curtis similarity and Unifrac analyses (both weighted and unweighted). Unifrac differs from Bray-Curtis similarity which only consider the abundance, in that phylogenetic distances between observed organisms was included in the computation which incorporate information on evolutionary relationships of community members. Both weighted and unweighted Unifrac, as a quantitative and qualitative measure, respectively, are commonly applied in microbial ecology, where the former focus on inter-individual differences in the relative abundance of observed organisms, whereas the latter only reflects inter-individual differences in the presence or absence of observed organisms. Overall bacterial diversity, as evaluated by Bray-Curtis similarity and Unifrac analyses, was not significantly different within and between quinoa and refined wheat rolls treatments. To further examine if samples from the same groups would cluster together, Principal Coordinate Analysis (PCoA) plot, which is used to explore and visualize the similarity or dissimilarity

between gut microbial communities of the individuals, were constructed based Unifrac distances (Figure 6.2, 6.3, 6.4). Data are shown as a 2-Dimensional plot to demonstrate the relationship, in which the different groups of individuals did not form overall visual discrete clustering in the PCoA plot across quinoa and refined wheat rolls treatment, indicating the overall microbiota was similar for both treatments.

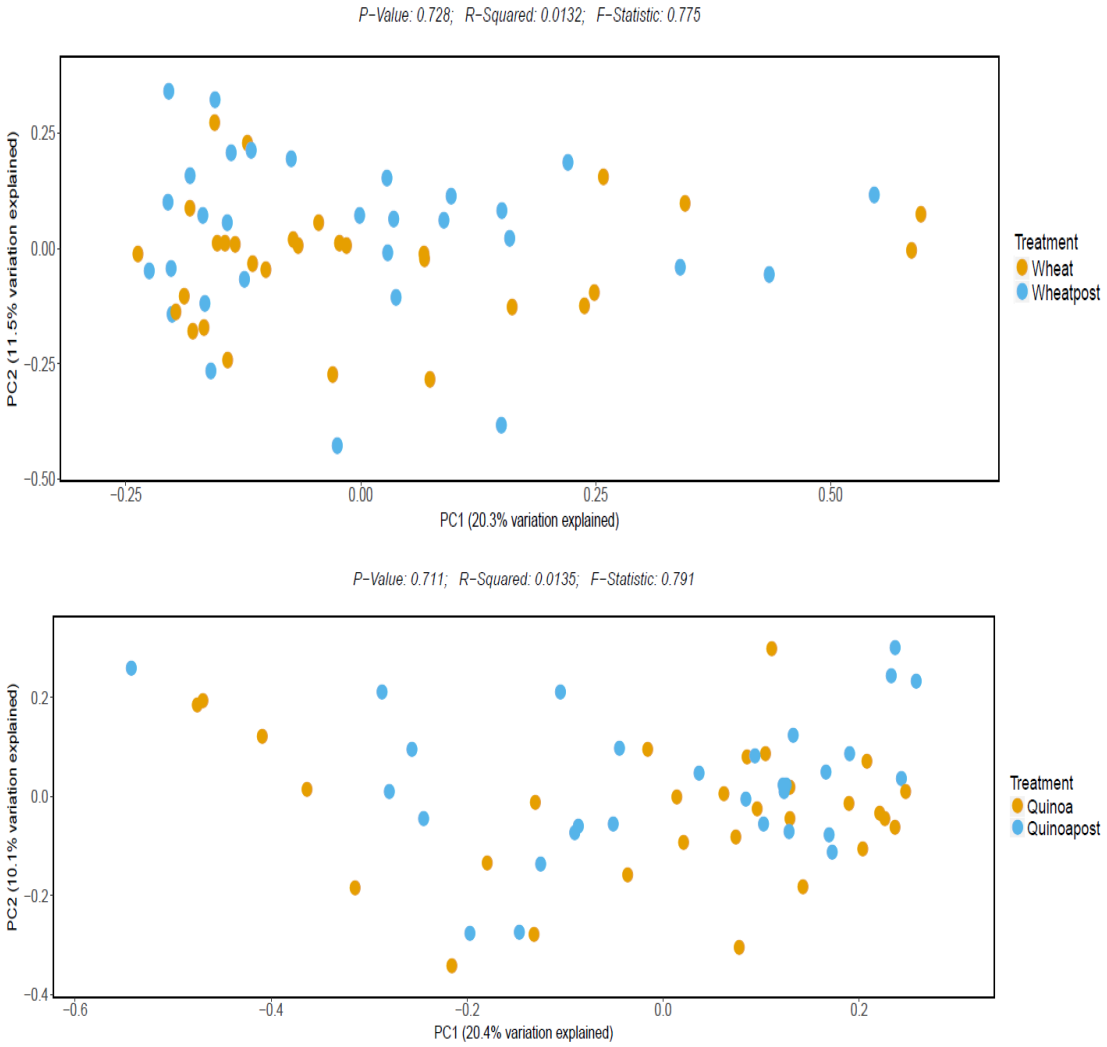


Figure 6.2. Principle coordinate analysis (PCoA) plots based on Bray-Curtis similarity. Each sample is depicted by a single symbol.

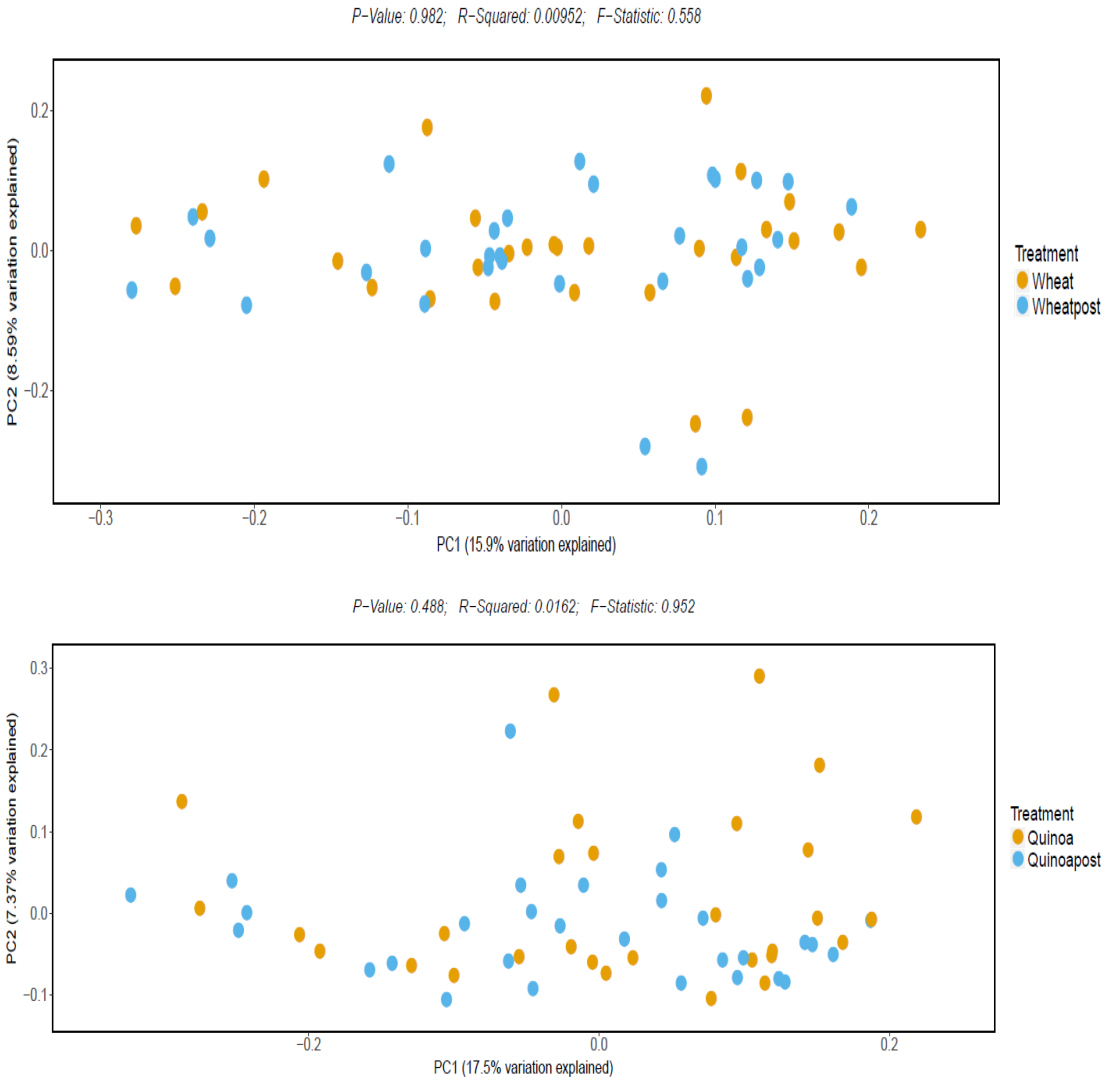
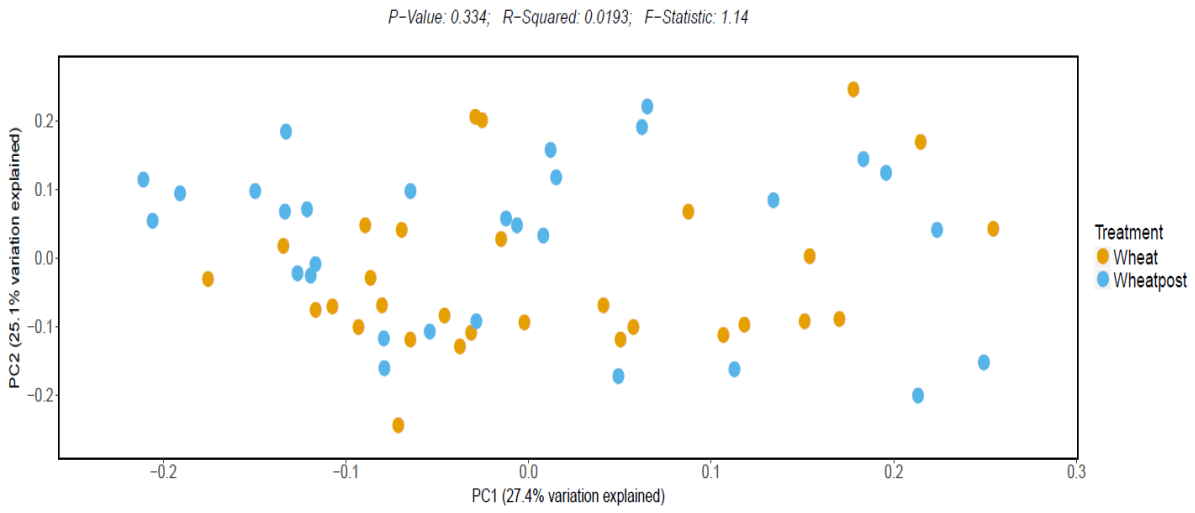


Figure 6.3. Principle coordinate analysis (PCoA) plots based on unweighted Unifrac analyses. Each sample is depicted by a single symbol.



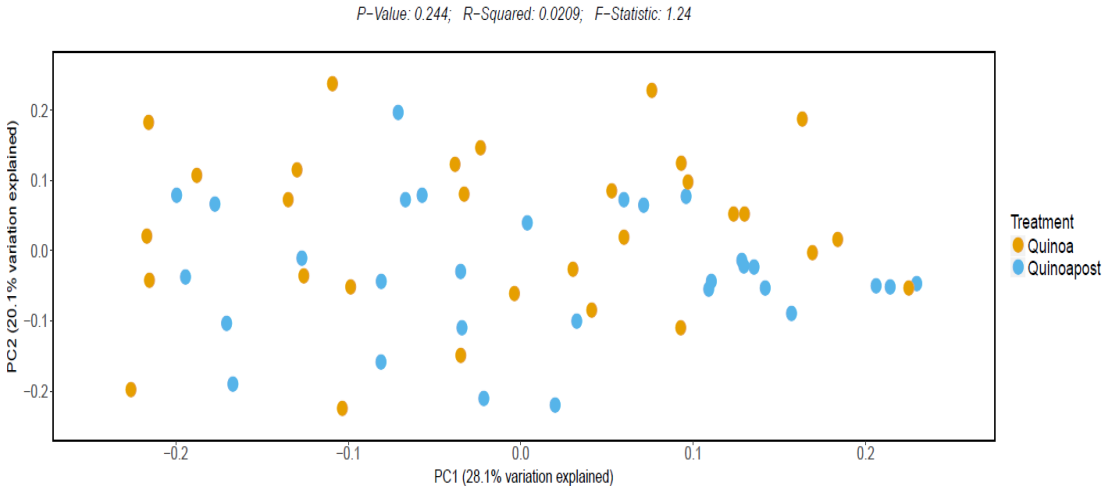


Figure 6.4. Principle coordinate analysis (PCoA) plots based on Weighted Unifrac analyses. Each sample is depicted by a single symbol.

6.3.3 Relative abundance of gut microbiota

Relative abundance of the human gut microbiota and the phylum and genus level was determined in faecal samples of twenty-eight healthy volunteers before and after each intervention arm and are presented as a percentage (%) of total microbiota. The classification of sequences from the stool samples resulted in the identification of eighteen different phyla, with the top eight phyla listed in Table 6.2. At the phylum level, no significant differences were observed at baseline between treatments, as shown in Figure 6.5. The dominant microbial groups in all samples were the Firmicutes (64.14-71.65%) and Bacteroidetes (16.56-25.01%), following by Actinobacteria, Tenericutes, Euryarchaeota, Verrucomicrobia, Proteobacteria and Cyanobacteria with much lower abundance, all of which together accounted for more than 99% of the total faecal bacteria population. Consumption of both quinoa and refined wheat rolls resulted in a significant decrease in the relative abundance of Bacteroidetes (p=0.007 for wheat and p=0.031 for quinoa), and significantly increased the abundance of Firmicutes (p=0.014 for wheat and p=0.042 for quinoa) in stool samples compared with pre-intervention. The fall in abundance of Bacteroidetes and the rise in abundance of Firmicutes were both greater after consuming the refined wheat rolls than after the quinoa rolls, but there was no significant difference between the treatments (p=0.431).

Furthermore, the ratios of Firmicutes to Bacteroidetes were higher after either consuming refined wheat rolls or quinoa rolls compared with baseline, but they were not significantly different (p=0.248 and 0.192, respectively) (Figure 6.6). None of the other phyla showed a significant change after intake of either refined wheat or quinoa rolls for 4 weeks. There were no significant differences between the two week 4 measurements of any of the phyla, indicating that quinoa had no effect on the profile of the microbiome over and above that seen by consuming the refined wheat rolls.

At the genus level, consumption of the refined wheat rolls resulted in a significant increase in the relative abundance of *Fusicatenibacter* and *Subdoligranulum*, but decreased *Bacteroides* with respect to baseline. On the other hand, consumption of quinoa rolls led to a significant increase in the relative abundance of both Anaerostipes and Dorea compared with the baseline.

There were no significant differences between treatment groups comparing the change (ΔQR vs ΔWR) in relative abundance at the phylum or genus level.

Table 6.2. Relative abundance of bacteria phylum and genus in stool samples of participants at baseline and week 4 after refined wheat roll or quinoa roll consumption

	Wheat rolls (n=28)				Quinoa rolls (n=28)					
	Baseline	Week 4	ΔWR	P[3]	Baseline	Week 4	ΔQR	P	ΔQR - ΔWR	P (ΔQR vs ΔWR)
Actinobacteria	4.07 ± 3.57	5.31 ± 4.20	1.24 ± 3.65	0.083	5.07 ± 4.29	5.86 ± 4.83	0.79 ± 4.24	0.330	-0.45 ± 5.41	0.665
Bifidobacterium	2.97 ± 2.90	3.96 ± 3.49	0.99 ± 2.76	0.067	3.74 ± 3.63	4.23 ± 3.86	0.49 ± 3.67	0.485	-0.50 ±4.44	0.555
Bacteroidetes	25.01 ± 13.71	16.56 ± 11.26	-8.45 ± 15.39	0.007	23.35 ± 11.70	17.92 ± 12.22	-5.43 ± 13.65	0.031	3.02 ± 20.00	0.431
Alistipes	3.45 ± 3.35	2.21 ± 2.54	-1.24 ± 3.61	0.081	3.20 ± 3.20	2.23 ± 2.40	-0.96 ± 3.69	0.180	0.28 ± 2.72	0.60
Bacteroides	15.46 ± 13.79	8.57 ± 8.58	-6.89 ± 10.76	0.002	12.45 ± 9.29	10.34 ± 10.02	-2.10 ± 8.67	0.210	4.79 ± 13.70	0.075
Cyanobacteria	0.06 ± 0.15	0.02 ± 0.06	-0.04 ± 0.13	0.112	0.08 ± 0.30	0.09 ± 0.25	0.01 ± 0.39	0.865	0.05 ± 0.41	0.491
Euryarchaeota	1.67 ± 2.83	1.32 ± 2.19	-0.36 ± 1.71	0.748	1.11 ± 1.94	1.54 ± 2.20	0.44 ± 1.28	0.084	0.79 ± 1.57	0.053
Firmicutes	64.14 ± 14.51	71.65 ± 14.29	7.51 ± 15.16	0.014	65.55 ± 11.83	69.91 ± 12.33	4.36 ± 10.82	0.042	-3.15 ± 18.43	0.374
Anaerostipes	1.96 ± 2.05	2.64 ± 2.25	0.68 ± 1.87	0.065	1.70 ± 1.62	2.36 ± 1.93	0.66 ± 1.53	0.031	-0.02 ± 2.27	0.96
Blautia	3.43 ±2.48	4.70 ± 3.57	1.27 ± 3.53	0.067	3.92 ± 4.08	4.44 ± 3.20	0.52± 2.72	0.322	-0.75 ± 5.01	0.433
Dorea	1.87 ± 1.61	2.24 ± 1.37	0.38 ± 1.54	0.204	1.90 ± 1.36	2.50 ± 1.79	0.60 ± 1.50	0.044	0.22 ± 2.19	0.602
Faecalibacterium	7.57 ± 5.07	7.68 ± 5.72	0.11 ± 5.54	0.919	7.87 ± 5.18	7.56 ± 4.44	-0.31 ± 3.88	0.672	-0.42 ± 5.72	0.7
Fusicatenibacter	1.58 ± 1.44	3.01 ± 3.09	1.42 ± 2.76	0.011	2.21 ± 1.57	2.65 ± 2.39	0.45 ± 1.89	0.221	-0.98 ± 3.24	0.122
Romboutsia	5.06 ± 8.28	4.82 ± 5.74	-0.24 ± 7.02	0.859	4.41 ± 8.21	3.81 ± 4.00	-0.59 ± 6.45	0.630	-0.36 ± 5.85	0.75
Subdoligranulum	4.07 ± 3.01	5.97 ± 4.31	1.89 ± 3.47	0.008	5.25 ± 3.12	5.28 ± 3.87	0.03 ± 4.03	0.967	-1.86 ± 5.32	0.075
Proteobacteria	1.41 ± 1.20	2.05 ± 3.38	0.65 ± 2.85	0.242	2.50 ± 5.51	2.58 ± 5.53	0.08 ± 3.46	0.903	-0.57 ± 4.28	0.491
Tenericutes	2.04 ± 5.29	1.30 ± 3.16	-0.74 ± 4.71	0.413	1.42 ± 3.68	0.89 ± 1.62	-0.52 ± 3.51	0.435	0.21 ± 6.06	0.853
Verrucomicrobia	1.51 ± 3.12	1.65 ± 2.81	0.14 ± 2.04	0.725	0.87 ± 1.70	1.18 ± 2.21	0.30 ± 1.25	0.207	0.17 ± 2.38	0.712

[1] WR, wheat rolls; QR, quinoa rolls. Mean values with their standard deviation.

[2] ΔWR or ΔQR = week 4 – baseline

[3] Difference between baseline and week 4 tested by paired t-test; mean values were considered significantly different if P<0.05

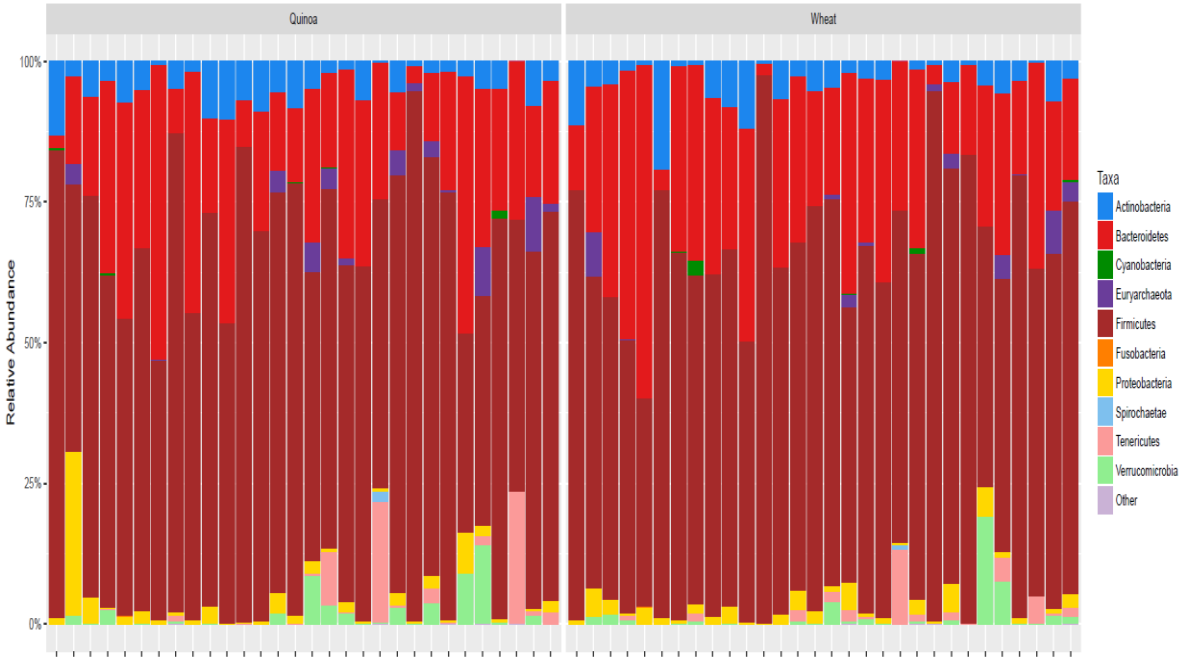


Figure 6.5. Relative abundance of gut microbiota at phylum level at baseline in quinoa and refined wheat treatment.

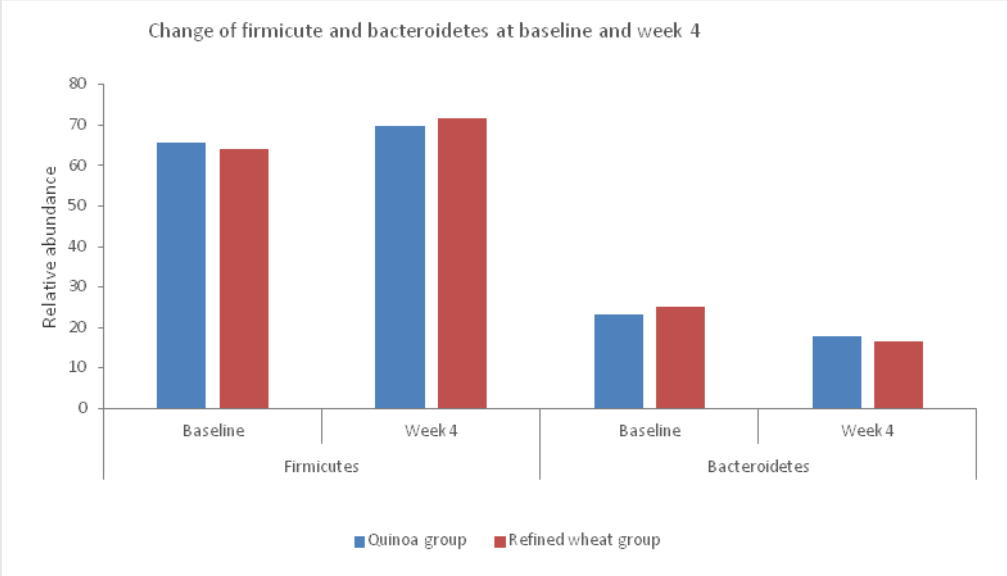


Figure 6.6. Changes of relative abundance of Firmicutes and Bacteroidetes at baseline and week 4 in quinoa and refined wheat treatment.

6.3.4 Correlation of gut microbiota and CVD risk markers

In order to explore the relationship between relative abundance of gut microbiota and markers of CVD risk and, Spearman's correlation test was carried out on the pooled data and the results were shown in Table 6.3. Very week or no correlations (Spearman's ρ between 0.01 and 0.19), week correlations (Spearman's ρ between 0.20 and 0.39) and moderate correlations (Spearman's ρ between 0.40 and 0.59) were detected between gut microbiota and markers of CVD risk.

Anthropometric variables such as age and BMI correlated positively and inversely with observed OUT as well as α diversity (Shannon, Simpson, InvSimpson index and Fisher's alpha parameter), respectively. There were consistent correlations between lipids and microbiota, in particular for Anaerostipes and Blautia, which were positively associated with total, LDL and HDL cholesterol (0.362, 0.266 and 0.335, 0.251, 0.225 and 0.196, respectively).

Table **6.3**. Correlation of markers of CVD risk and observed OUT, bacterial α diversity and the relative abundance of gut microbiota.

		α diversity				Phyla							
	Observed OTU	Shannon	Simpson	InvSimpson	Fisher	Euryarc-haeota	Actinoba-cteria	Bacteroi-detes	Cyanoba-cteria	Firmicut-es	Proteob-acteria	Teneri-cutes	Verruco-microbia
Anthropometric variables													
Age	0.464**	0.299**	0.200*	0.200*	0.464**	0.440**	-0.099	-0.070	0.181	0.038	0.234*	0.288**	0.177
Weight	-0.167	-0.187*	-0.144	-0.144	-0.167	-0.295**	-0.239*	0.232*	-0.048	-0.042	-0.185	-0.047	-0.146
BMI	-0.250**	-0.280**	-0.240*	-0.240*	-0.250**	-0.288**	-0.209*	-0.012	-0.065	0.231*	-0.420**	-0.064	-0.178
Body fat percentage	-0.064	-0.213*	-0.227*	-0.227*	-0.064	-0.124	-0.019	-0.188*	-0.010	0.344**	-0.297**	0.062	-0.016
Systolic BP	-0.156	-0.129	-0.088	-0.088	-0.156	-0.157	0.101	0.027	-0.077	0.052	-0.218*	-0.059	-0.016
Diastolic BP	-0.177	-0.115	-0.061	-0.061	-0.177	-0.142	0.261**	0.010	-0.022	0.018	-0.237*	-0.028	0.075
Blood variables													
Glucose	-0.101	-0.002	0.022	0.022	-0.101	-0.226*	-0.141	0.183	0.035	-0.037	-0.262**	-0.077	0.053
Insulin	0.355**	0.390**	0.364**	0.364**	0.355**	0.308**	0.013	-0.033	0.191*	-0.144	0.249**	0.264**	0.320**
TC	-0.092	0.017	0.051	0.051	-0.092	-0.130	-0.027	-0.036	0.102	0.018	0.002	0.016	-0.086
LDL	-0.050	0.020	0.039	0.039	-0.050	-0.050	-0.101	-0.100	0.100	0.089	0.018	0.038	-0.079
HDL	-0.007	0.064	0.135	0.135	-0.007	-0.127	0.165	-0.018	0.116	0.030	-0.199*	-0.085	-0.028
TG	-0.210*	-0.192*	-0.177	-0.177	-0.210*	-0.258**	0.121	-0.036	-0.115	0.100	-0.069	-0.049	-0.153
NEFAs	0.053	0.056	0.069	0.069	0.053	-0.039	0.156	-0.039	-0.040	0.113	0.035	-0.081	-0.112
Apo A1	-0.031	0.125	0.203*	0.203*	-0.031	-0.183	0.132	0.048	0.139	0.006	-0.100	-0.091	0.000
Apo B	-0.106	-0.010	0.013	0.013	-0.106	-0.109	-0.047	-0.066	0.075	0.070	0.007	-0.024	-0.122
Ratios													
HDL TC	0.094	0.037	0.065	0.065	0.094	0.055	0.148	0.046	0.014	-0.005	-0.225*	-0.064	0.141
ApoB/ApoA1	-0.103	-0.091	-0.121	-0.121	-0.103	-0.015	-0.161	-0.066	-0.015	0.024	0.128	0.022	-0.159
CRP	-0.061	-0.065	-0.038	-0.156	-0.064	0.088	-0.109	-0.088	-0.029	0.035	0.064	0.128	0.052
AST	-.201*	-0.090	-0.041	-0.041	-0.201*	-0.149	0.236*	-0.158	0.035	0.150	0.056	-0.216*	-0.216*
ALT	-0.447**	-0.343**	-0.249**	-0.249**	-0.447**	-0.412**	0.189*	-0.179	-0.010	0.386**	-0.311**	-0.324**	-0.271**

Continued

	Genera									
	Alistipes	*Anaerostipes*	*Bacteroides*	*Bifidobacte-rium*	*Blautia*	*Dorea*	*Faecalibact-erium*	*Fusicateni-bacter*	*Romboutsia*	*Subdoligr-anulum*
Anthropometric variables										
Age	0.101	-0.008	-0.112	-0.285**	-0.056	0.121	-0.119	-0.264**	0.022	0.064
Weight	0.011	0.090	0.036	-0.191*	0.057	-0.170	0.076	-0.227*	0.206*	0.023
BMI	-0.013	-0.008	-0.086	-0.084	0.042	-0.066	0.136	-0.027	0.170	0.120
Body fat percentage	-0.037	0.049	-.210*	-0.030	-0.020	-0.093	0.045	0.012	-0.030	0.066
Systolic BP	-0.070	0.068	-0.093	0.127	-0.037	0.036	0.101	-0.038	0.135	0.019
Diastolic BP	-0.052	0.205*	-0.051	0.269**	0.077	0.163	0.101	0.113	0.006	0.080
Blood variables										
Glucose	0.292**	0.146	0.123	-0.179	0.145	0.023	0.234*	0.016	0.099	0.092
Insulin	0.156	0.033	-0.010	-0.023	-0.038	0.125	0.068	-0.063	-0.036	0.094
TC	0.053	0.362**	0.066	-0.088	0.251**	0.319**	0.105	0.070	-0.157	0.064
LDL	0.110	0.266**	0.023	-0.171	0.225*	0.291**	0.088	0.004	-0.101	0.107
HDL	0.006	0.335**	-0.095	0.127	.196*	0.122	0.229*	0.140	0.002	-0.035
TG	-0.256**	0.115	-0.026	0.108	-0.003	0.076	-0.042	0.076	-0.125	-0.076
NEFAs	-0.078	-0.023	-0.007	0.144	0.071	0.007	0.143	0.070	-0.105	-0.003
Apo A1	0.096	0.431**	-0.009	0.071	0.254**	0.228*	0.260**	0.131	-0.056	0.030
Apo B	0.096	0.304**	0.082	-0.109	0.272**	0.341**	0.058	0.075	-0.151	0.101
Ratios										
HDL/TC	-0.002	-0.002	-0.160	0.169	-0.080	-0.192*	0.118	0.063	0.163	-0.052
ApoB/ApoA1	0.033	-0.026	0.143	-0.178	0.114	0.177	-0.132	-0.013	-0.160	0.031
CRP	0.027	-0.034	0.096	-0.122	0.086	0.186*	-0.075	0.022	-0.003	0.128
AST	-0.128	0.149	0.084	0.280**	0.063	0.091	0.111	0.168	-0.129	-0.077
ALT	-0.136	0.161	0.027	0.221*	0.135	0.122	0.259**	0.256**	-0.110	-0.071

Significant correlation: *P<0.05; **P<0.01.

6.4 Discussion

To date, this is the first study in which a bread roll enriched with quinoa as a whole grain source has examined *in vivo* the effects on bacterial diversity and composition. However, the results showed that there was no significant difference in the bacterial diversity between treatments. The lack of response was in agreement with other human intervention studies, in which intake of whole grains did not significantly alter the values of alpha and/or beta diversity (weighted and unweighted Unifrac) compared with refined grains or baseline (Langkamp-Henken *et al.*, 2012; De Angelis *et al.*, 2015; Vitaglione *et al.*, 2015; Vanegas *et al.*, 2017). In contrast, a randomised, cross-over study showed that addition of 60 g/d of whole grain barley or brown rice or an equal mixture of the two to the diets for 4 weeks significantly increased the bacterial diversity measured by Shannon's and Simpson's indices (community evenness), but not by Chao1 (total species richness) (Martínez *et al.*, 2013a). Similarly, the study of Foerster *et al*. (2014) also indicated a higher microbial diversity upon ingestion of whole grain products that delivered approximately 40 g dietary fibre per day over a short period of 3 weeks.

Despite the substantial differences in some certain bacteria within group mentioned above, the relative abundance of gut microbiota did not change significantly between groups, which was in accordance with the other intervention studies that used wheat as the main source of whole grains (Ross *et al.*, 2011; Langkamp-Henken *et al.*, 2012; Christensen *et al.*, 2013; Saa *et al.*, 2014; Ampatzoglou *et al.*, 2015; Cooper *et al.*, 2017). Similar to the comprehensive microbiota analysis of this current study, no significant microbiota changes occurred at any phylum levels after intake of whole grain, offering fibre content higher than 35g per day, compared with refined grains for 6 weeks (Vanegas *et al.*, 2017). Additionally, in another 12-wk, randomised intervention study in subjects with metabolic syndrome, inclusion of 152 g whole grain breads and fibre-rich rye bread in their diets for 12 weeks did not significantly change the microbiota composition with respect to refined white breads (Lappi *et al.*, 2013). Noteworthy, the preliminary data demonstrate that the microbiota composition of individuals with metabolic syndrome differed from that of healthy individuals (Munukka *et al.*, 2012).

In contrast, Martínez *et al*. (2013a) showed that all three treatments significantly increased and decreased the abundance of Firmicutes and Bacteroidetes, respectively, in healthy Americans. Unfortunately, the subjects' habitual diet was not described in that study. Bifidobacteria and lactobacilli, are associated with a healthy intestinal environment, and are often considered to be a positive indicator of prebiotic activity. One study that assessed the ability of daily intake of 48 g breakfast cereals, either whole grain wheat or wheat bran, to modulate faecal microbiota

populations for 3 weeks, found that populations of bifidobacteria and lactobacilli/enterococci were significantly higher upon whole grain ingestion compared with wheat bran (Costabile *et al.*, 2008). In agreement with this, significantly increased populations of bifidobacteria and lactobacilli in stool samples associated with intake of whole grain oat breakfast cereals (45 g/d) for 6 weeks, as well as total bacterial population relative to non-whole grain group have been reported, and also a bifidogenic effect of whole grain maize breakfast cereal (48 g/d) over 3 weeks has been reported (p=0.001) (Carvalho-Wells *et al.*, 2010; Connolly *et al.*, 2016).

The reasons for the inconsistent results in these human intervention studies is not completely understood, however, several factors such as differences in subject characteristics, the cereal grains used, processing conditions, not having completely controlled for other components of the diets may have been responsible for the divergent results. It is not necessarily surprising, that in the present study intake of a relatively small amount of quinoa of 20 g daily there were only modest effects on bacterial diversity and composition. It is plausible that low percentage of quinoa flour (20%) in quinoa rolls have resulted in only a marginal difference in nutritional composition between quinoa and refined wheat rolls and therefore a lack of effect on gut microbiota. For example, despite the double amounts of total dietary fibre in quinoa roll, it appeared that the relatively small difference between two rolls was insufficient to induce any change in gut microbiota.

In general, correlations between gut microbiota and markers of CVD risk markers are were weak, since the majority of correlation coefficient (ρ) were less than 0.4 in this study. A consistent correlation of Actinobacteria to body weight and BMI was observed which is in agreement with the study of Turnbaugh *et al*. (2009) who showed higher numbers of Actinobacteria in obese people.

6.5 Summary and conclusion

To the best of our knowledge, this is the first study investigating the effect of quinoa consumption on human gut microbiota. The results of the dietary intervention study show that consumption of 20 g quinoa per day for 4 weeks does not induce any changes in bacterial diversity and the relative abundance of gut microbiota between two treatments.

Chapter 7 General discussion

This thesis investigated the effect of quinoa consumption on markers of CVD risk and gut microbiota by three clear phases including; firstly, *in vitro* analysis of nutrients and phytochemicals (protein, amino acids, fibre, fat, polyphenols) in thirteen different strains of quinoa sourced from UK, Europe, South America and China, in order to select one strain of quinoa with the highest fibre content. Secondly, a bread sensory testing by feeding refined wheat bread, 20% and 30% quinoa bread to volunteers in order to select one product from two quinoa bread for use in the human intervention study. Thirdly, using 20% quinoa roll based on the sensory testing, a human intervention study was conducted to study the effect of quinoa consumption (delivering quinoa 20 g/day) for 4 weeks on markers of CVD risk and gut microbiota by measuring blood and stool samples, as well as anthropometric variables.

Based on the literature, phenolic compounds are secondary metabolites, which in whole grains may exist in three forms: free, soluble conjugated and insoluble bound forms which are attached to cell materials (Adom and Liu, 2002). In this study, free, conjugated, and bound phenolic contents in both the quinoa and buckwheat accessions tested were significantly different ($p<0.01$) from each other, with the ranking order: Free > conjugated > bound phenolic compounds. The total phenolic content of buckwheat was significantly higher than quinoa ($p<0.001$), but on average, the total phenolic contents in both quinoa and buckwheat seeds were higher than those found in other common cereals including barley, wheat, rye and millet, suggesting that quinoa and buckwheat may serve as an excellent source of phenolic compounds (Ragaee *et al.*, 2006). Even though phenolic compounds are mainly present in free form, this study also indicates that the total phenolic contents of quinoa and buckwheat could be underestimated in the previously published studies which did not include the conjugated and bound phenolic compounds in their analysis. In order to comprehensively screen and compare antioxidant activity levels among a wide accession of quinoa and buckwheat samples, three different assays of antioxidant content, FRAP, TEAC and DPPH were applied in this study. In the three antioxidant evaluation systems, antioxidant activities of free, conjugated and bound phenolic extracts accounted for less than 40% of total antioxidant activities determined by TEAC and DPPH methods, with the exception of free phenolic extracts in quinoa by FRAP. Regarding buckwheat accessions, antioxidant activities of free phenolic extracts were statistically higher than those of conjugated and bound phenolic extracts. Free phenolic extracts accounted for 64%, 67% and 46% of total FRAP, TEAC and DPPH· scavenging activities in an average among buckwheat accessions, respectively. The correlations between TPC and antioxidant activity were not always significant. Thus, it is difficult and impractical to define which phenolic component plays a dominant role in

antioxidant activity in quinoa accessions. Moreover, it is not appropriate to directly compare the results of different methods for the same samples due to differences between compounds measured by different methods. Unfortunately, since the extraction method has not been standardised so far, comparison among independent studies using different extraction procedures or solvents is often problematic. Due to the limited time available in this study, composition analysis of quinoa seeds were carried out by commercial laboratory and company. The results clearly showed that here was great variation in the proximate composition between the thirteen quinoa seed samples. In this study, the dietary fiber content, especially the soluble fiber content, was one of the determining factors for the choice of quinoa seed for the human intervention carried out in the study, based on previously published studies, which have consistently shown soluble fibre to be the most likely contributor to lower total and LDL cholesterol concentrations (Glore *et al.*, 1994; Truswell, 1995). The highest level of insoluble, soluble and total dietary fiber were all identified in the same quinoa seeds (from South America) among these included accessions; moreover, the total and insoluble dietary fiber values are much higher than other accessions.

The planned intervention study was dependent on providing the required dose of quinoa in a form which would be palatable and acceptable to (male) participants. After considering various options it was decided to base the intervention food on bread prepared from the strain of quinoa (South America). Based on the papers published previous by Milovanović *et al*. (2014), Bilgicli and Ibanoglu, (2015), by Turkut *et al*. (2016) and property of 'gluten-free', 20% and 30% quinoa bread were as the candidates involved in the bread sensory testing. From an enjoyable flavour prospective, which was the most important characteristic, the scores of refined wheat bread and 20% quinoa bread derived from all healthy volunteers and male volunteers were higher than that 30% quinoa bread, even if the scores derived from male volunteers did not reach significance level ($p>0.05$). Thus, 20% quinoa bread was selected for the further human intervention study. With the same energy content, the proximate composition of 20% quinoa rolls was slightly higher compared with refined wheat roll for ash, moisture, salt, sodium, protein, fat and soluble dietary fibre. The insoluble dietary fibre content of the quinoa rolls was more than double that of refined wheat rolls.

Using a randomized, controlled cross-over design, one human intervention study was conducted with 20% quinoa rolls. Unfortunately, anthropometric variables, blood variables and plasma antioxidant capacity did not significantly differ between two treatments. However, the presence of many 'trend' results, such as glucose, insulin and LDL cholesterol values, in the present study indicates that significant results might have been obtained with prolonged duration, higher dose and larger numbers of subjects. The duration of this controlled human intervention study, representative of a very short period of dietary alteration in the context of lifelong dietary exposures, may not have

been sufficiently long to detect favourable effects on markers of CVD risk. The lack of intervention effects may also be a result of lower dose consumed by subjects daily. According to epidemiological studies, beneficial health effects of whole grains can be expected at a level of 3 (16g) servings per day (Anderson *et al.*, 2000), although the type of whole grain consumed is not specified in this general recommendation. The daily amounts of quinoa provided during the present study (20g) was in the range of usual whole grain intake of about 16-25 g/d, but much lower than minimum recommended intake of 48-96 g/d (Cleveland *et al.*, 2000; Maras *et al.*, 2009). In addition, the similar soluble fibre content in refined wheat and 20% quinoa rolls may also explain the lack of the response in plasma cholesterol in the current study. This study is the first to show a reduction in glycaemia following regular consumption of quinoa. The AUC for glucose over the four days at the end of the quinoa treatment period was borderline significantly lower than the following four days when quinoa consumption ceased ($p=0.054$), and also was significantly lower than that of the four days at the end of the wheat treatment period ($p=0.039$). This may have been in part due to the cumulative effect of the reduced postprandial glycaemia seen after the breakfast test meal, which were derived from both DBS and FGM (representing capillary and interstitial fluid glucose concentrations, respectively). Although some important differences in glucose responses between quinoa and refined wheat rolls breakfast, such as significantly different IAUC values over 4 hours, were not observed in capillary blood samples, the glucose response curves were relatively similar; there was a more rapid fall approximately after 60 mins after the quinoa roll breakfast. The mean values of glucose concentrations from fasting venous plasma samples and FGM appeared to be quite similar in this study ($p>0.05$), but DBS glucose concentrations were significantly higher than venous glucose concentrations. This is most likely attributable to higher concentrations of glucose in both arterial and capillary blood than that of venous blood, since glucose absorbed in the small intestine sequentially diffuses from arteries into tissue cells through peripheral capillaries and the de-oxygenated blood returns in the venous system (Burtis and Ashwood, 1994; Yang *et al*., 2012). The correlation coefficient between venous and DBS glucose concentration values, venous and FGM glucose concentration values, DBS and FGM glucose concentration values were all significant ($p<0.01$), but the regression coefficients were relatively low, less than 0.5. But the Bland-Altman plots of differences in the different glucose analysis methods showed good agreement between each two methods, indicating these methods were changeable.

Through analysing the stool samples obtained from human interventions study, no significantly differences in diversity measures (alpha and beta) and relative abundance of gut microbiota were observed between two treatments. For example, although intake of both refined wheat and quinoa rolls significantly decreased the relative abundance of Bacteroidetes and significantly increased the

abundance of Firmicutes in stool samples compared with pre-intervention, the differences between these two treatment did not reach significance level (p=0.431). It is not necessarily surprising, that in the present study intake of a relatively small amount of quinoa of 20 g daily there were only modest effects on bacterial diversity and composition. It is plausible that low percentage of quinoa flour (20%) in quinoa rolls have resulted in only a marginal difference in nutritional composition between quinoa and refined wheat rolls and therefore a lack of effect on gut microbiota. For example, despite the double amounts of total dietary fibre in quinoa roll, it appeared that the relatively small difference between two rolls was insufficient to induce any change in gut microbiota. In general, correlations between gut microbiota and markers of CVD risk markers are were weak.

7.1 Strengths and limitations

The current study has both strengths and limitations that deserve to be discussed. The strength of the current study is that, to the best of our knowledge, this is the first study to demonstrate the free, conjugated and bound phenolic contents and also their corresponding antioxidant activity in quinoa and buckwheat seeds to date. Also, this is the first study to show nutritional composition of different quinoa and buckwheat accessions sourced from different regions, which can give a comprehensive view to see the variations between them, but almost previous studies only focused on one quinoa or buckwheat accession. Moreover, this is the first quinoa intervention study that examined the effect of quinoa consumption on the human gut microbiota. Additionally, strengths of the study include its randomised crossover design, and also inclusion of overweight male aged over than 35 at a potentially higher risk of CVD. The direct comparison of quinoa-enriched and refined wheat rolls adds clarity to the quinoa literature in ascertaining the differences in potential attributed to whole grains and refined grains. Also, the subjects maintained their habitual diet, with only quinoa or refined wheat roll offered during each intervention period, and they were not asked to self-select the whole grain products which may help to reduce confounding factors and improve compliance. Finally, the proximate composition of quinoa and refined wheat roll were analysed allowing this study to explore the possible mechanisms underlying beneficial effects.

As for the limitations, firstly, since no washout period was conducted before the start of the study, background diet variability acting as a confounding variable may have affected baseline levels of some CVD risk markers, such as glucose, LDL cholesterol and CRP, if subjects had previously included whole grain products in their habitual diets. Secondly, the present study may have been underpowered to observe a significant effect, because the sample size was determined assuming that no change in fasting plasma LDL cholesterol would occur from baseline in the control group. Further, the expected magnitude of decreased in LDL cholesterol of 10% between the two

intervention groups appears to have been too optimistic. Thirdly, quinoa rolls were consumed for a relatively short period of time, it remains unknown whether favourable effects on CVD risk markers would obtain with longer treatment or larger dosage.

7.2 Recommendations

1. Extraction of phenolics for individual grain should be explored, such as solvents, time for hydrolysis, in order to obtain the most accurate values.

2. For the human intervention studies, there are some improvements which would be suggested for future studies such as 1) have a washout period before the start of study to avoid background diet variability confounding the results, 2) try to recruit subjects at a potentially higher risk of CVD to show more apparent results, 3) increase the number of subjects and length to help detect small changes in markers of CVD risk, especially total and LDL cholesterol, as well as gut microbiota 4) a larger amount of quinoa consumption is essential to induce any changes, 5) other forms of pure quinoa foods rather than a mixture of quinoa and other grains as an experimental diet, such as bars and flakes, are strongly recommended to deliver quinoa to humans, since the changes in quinoa treatment may be partly attributed to the 80% of refined wheat in quinoa rolls in this study, which caused some uncertainties to discuss, 6) stool samples should be collected 24 hours before stored in the freezers.

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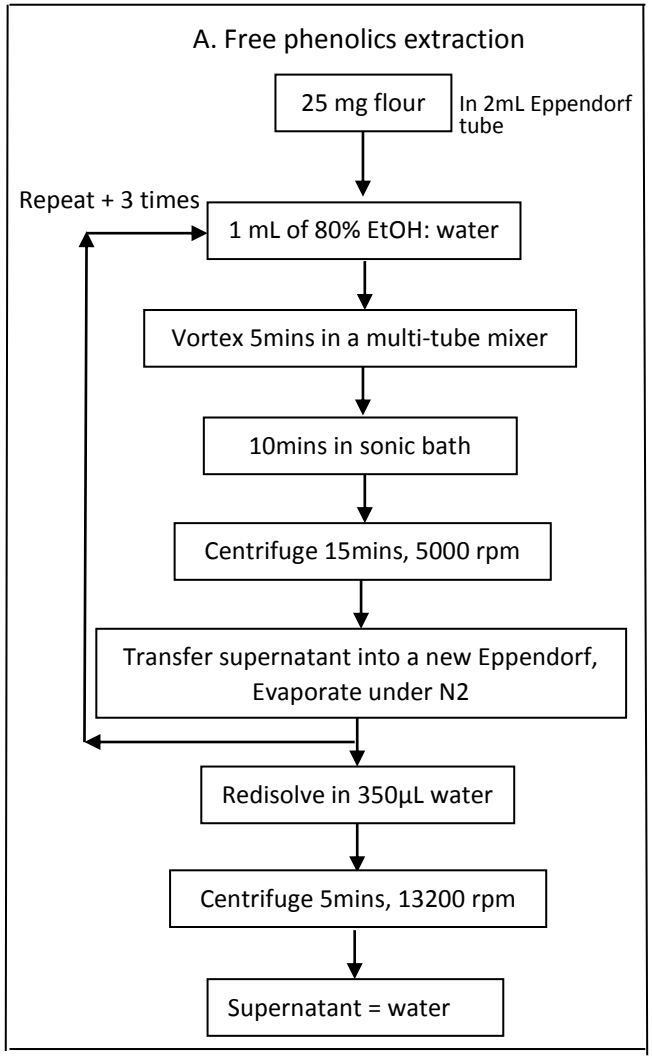
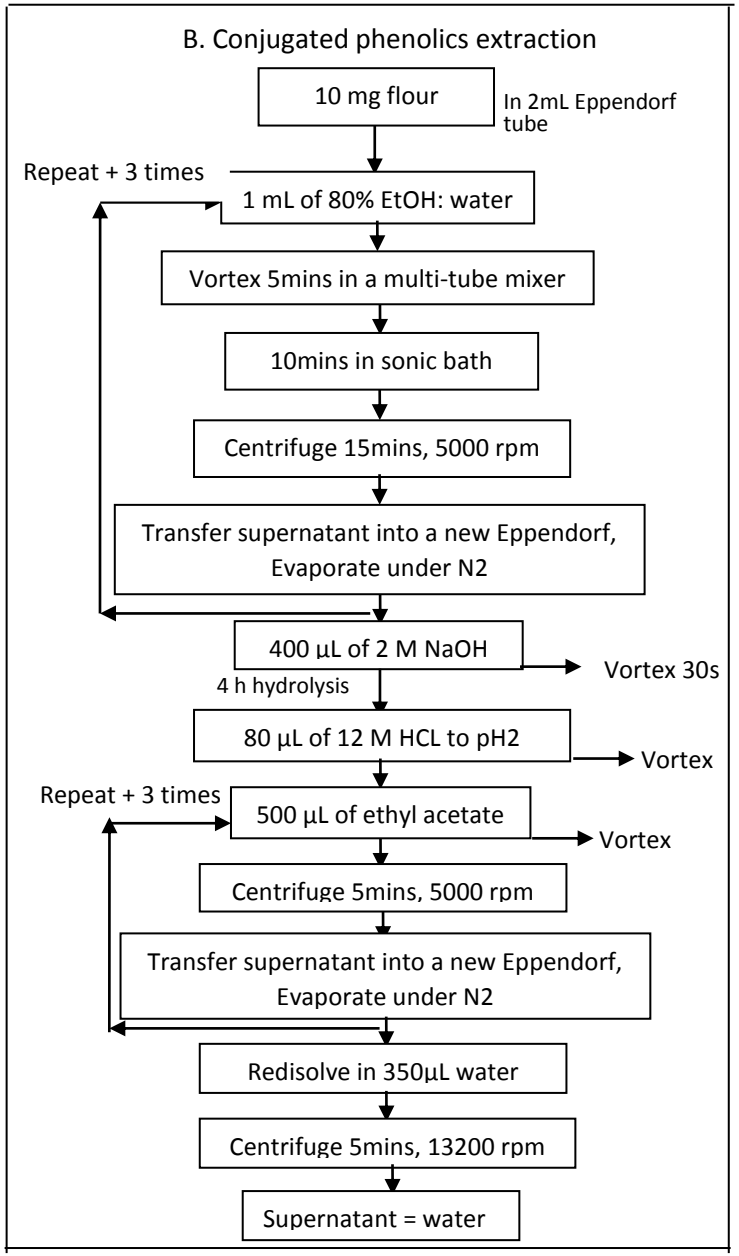
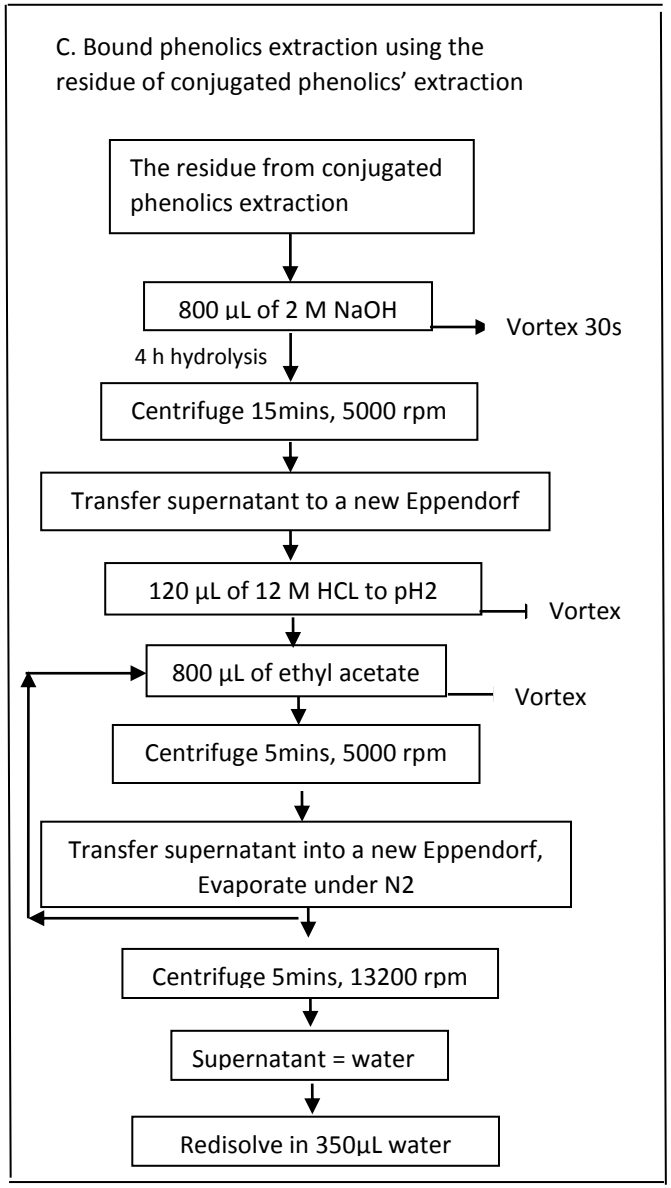
Appendix

Appendix 1. Extraction of phenolic compounds from 3 different fractions in quinoa or buckwheat seeds







Appendix 2. Summary of TPH, FRAP, TEAC and DPPH values

Table. Summary of TPH, FRAP, TEAC and DPPH values

	Phenolic content (mg GAE/g)				FRAP(μmol Fe2+/g dried weight)			
	Free	Conjugated	Bound	Total	Free	Conjugated	Bound	Total
Quinoa varieties								
Bolivia (Red)	1.32±0.04	0.41±0.01	0.80±0.01	2.54±0.07	2.97±0.12	1.27±0.10	4.58±0.49	8.82±0.71
Boilvia (White)	1.51±0.02	0.43±0.02	0.09±0.01	2.03±0.05	3.34±0.16	1.49±0.19	0.32±0.06	5.14±0.41
Ecuador (White)	1.49±0.01	0.56±0.05	0.13±0.02	2.18±0.08	1.57±0.16	1.03±0.10	0.30±0.06	2.90±0.32
Ecuador (Red)	1.38±0.03	0.40±0.02	0.63±0.02	2.42±0.06	3.92±0.29	1.43±0.02	3.63±0.39	8.97±0.70
Peru (Peru)	0.89±0.05	0.54±0.01	0.13±0.02	1.55±0.08	1.79±0.14	1.39±0.02	0.46±0.03	3.64±0.20
Peru (Tesco)	1.09±0.02	0.48±0.02	0.13±0.01	1.70±0.05	1.96±0.09	0.97±0.10	0.37±0.06	3.29±0.25
UK (Biofair)	1.06±0.08	0.43±0.02	0.62±0.04	2.12±0.13	3.59±0.22	1.57±0.08	3.74±0.26	8.89±0.57
UK (Waitrose)	1.06±0.04	0.40±0.03	0.11±0.01	1.57±0.09	1.82±0.05	0.99±0.04	0.42±0.04	3.23±0.13
USA	2.12±0.06	0.38±0.03	0.06±0.01	2.56±0.11	5.79±0.21	1.75±0.11	0.26±0.07	7.80±0.40
Netherlands	1.61±0.01	0.35±0.03	0.06±0.01	2.01±0.06	4.27±0.27	1.65±0.21	0.28±0.01	6.20±0.50
South America	1.61±0.08	0.76±0.06	0.32±0.04	2.69±0.18	4.45±0.25	1.23±0.12	0.66±0.05	6.34±0.42
China, Tibet	1.50±0.03	0.37±0.05	0.10±0.02	1.97±0.10	3.43±0.28	1.70±0.23	0.30±0.04	5.43±0.55
China, Shanxi	2.13±0.02	0.67±0.01	0.25±0.04	3.05±0.07	6.44±0.08	3.51±0.17	0.94±0.24	10.89±0.49
Average	1.44±0.38	0.48±0.12	0.26±0.25	2.18±0.45	3.49±1.52	1.54±0.65	1.25±1.58	6.27±2.62
Buckwheat varieties								
YunNan (T)	1.36±0.01	0.72±0.12	0.14±0.03	2.22±0.16	4.38±0.10	4.00±0.17	3.01±0.07	11.39±0.34
YunNan (C)	3.03±0.15	0.75±0.09	0.08±0.01	3.87±0.25	7.54±0.04	1.39±0.19	1.83±0.06	10.76±0.28
SiChuan (T)	2.06±0.08	1.16±0.05	0.64±0.07	3.86±0.21	10.53±1.22	5.29±0.39	3.08±0.13	18.90±1.74
GuiZhou (C)	2.38±0.03	1.23±0.19	0.08±0.01	3.69±0.23	10.27±0.16	1.94±0.12	2.12±0.10	14.33±0.38
ZhangJiaKou (C)	2.26±0.03	1.26±0.02	0.10±0.01	3.62±0.07	8.90±0.52	1.45±0.03	2.02±0.10	12.37±0.65
NeiMengGu (C)	1.99±0.08	1.10±0.02	0.09±0.01	3.18±0.11	9.75±0.22	1.49±0.20	1.84±0.03	13.09±0.45
FuJian (C)	2.22±0.13	1.12±0.03	0.10±0.01	3.44±0.17	8.31±0.39	3.10±0.20	2.11±0.04	13.52±0.63
Netherlands (C)	2.01±0.24	1.00±0.03	0.10±0.01	3.11±0.28	6.55±0.24	2.41±0.11	1.20±0.01	10.16±0.36
USA (C)	2.28±0.06	1.53±0.11	0.14±0.01	3.96±0.17	10.96±0.19	2.97±0.21	1.30±0.01	15.23±0.41
Average	2.18±0.44	1.10±0.25	0.16±0.18	3.44±0.54	8.58±2.14	2.67±1.32	2.06±0.65	13.30±2.67

Continue

	TEAC(μmol TE/g dried wieght)				DPPH (μmol TE/g dried wieght)			
	Free	Conjugated	Bound	Total	Free	Conjugated	Bound	Total
Quinoa varieties								
Bolivia (Red)	6.63±0.16	3.02±0.22	11.16±0.48	20.08±0.86	6.70±0.45	7.95±0.74	5.81±0.34	20.45±1.53
Boilvia (White)	8.33±0.32	4.24±0.05	1.16±0.22	13.72±0.59	5.19±0.23	4.03±0.50	1.83±0.09	11.05±0.82
Ecuador (White)	7.41±0.24	5.50±0.19	1.15±0.18	14.06±0.61	1.81±0.12	8.11±0.50	6.25±0.67	16.17±1.29
Ecuador (Red)	8.06±0.56	3.42±0.19	9.85±0.47	21.33±1.21	7.23±0.60	10.73±0.24	9.12±0.53	27.07±1.36
Peru (Peru)	6.96±0.09	5.45±0.18	2.01±0.19	14.42±0.46	4.96±0.64	8.29±0.09	1.76±0.11	15.01±0.84
Peru (Tesco)	7.61±0.33	5.06±0.38	2.68±0.26	15.34±0.97	2.27±0.35	4.67±0.31	8.40±0.42	15.34±1.08
UK (Biofair)	10.02±0.63	4.31±0.21	11.63±0.13	25.96±0.98	6.67±0.18	7.27±0.37	7.62±0.39	21.57±0.94
UK (Waitrose)	8.70±0.61	5.64±0.15	2.16±0.28	16.50±1.04	2.62±0.30	6.05±0.43	6.82±0.51	15.49±1.24
USA	12.87±0.15	5.47±0.08	1.34±0.05	19.68±0.28	6.43±0.53	10.77±0.52	4.02±0.20	21.22±1.25
Netherlands	9.59±0.59	4.97±0.33	1.27±0.08	15.83±1.01	5.40±0.07	11.15±1.10	3.78±0.39	20.33±1.56
South America	7.54±0.17	4.57±0.32	3.91±0.19	16.02±0.68	3.02±0.15	3.09±0.20	1.92±0.29	8.03±0.64
China, Tibet	8.33±0.25	5.24±0.24	1.52±0.11	15.09±0.60	5.81±0.26	2.07±0.44	6.37±0.26	14.25±0.96
China, Shanxi	9.83±0.37	8.41±0.39	3.25±0.27	21.49±1.03	9.72±0.38	3.79±0.38	2.00±0.44	15.50±1.20
Average	8.61±1.67	5.02±1.30	4.08±3.98	17.71±3.75	5.22±2.28	6.77±3.08	5.05±2.65	17.04±4.99
Buckwheat varieties								
YunNan (T)	12.02±0.25	11.68±0.30	4.24±0.18	27.94±0.74	7.64±0.70	8.26±0.31	9.29±0.08	25.19±1.09
YunNan (C)	32.09±1.07	11.74±0.64	1.16±0.30	45.80±2.01	12.72±0.93	4.76±0.14	10.37±1.22	27.86±2.29
SiChuan (T)	21.51±1.30	16.95±0.10	6.85±0.40	45.31±1.79	17.84±0.12	11.30±1.02	11.41±0.46	40.54±1.60
GuiZhou (C)	37.73±1.07	12.28±0.25	1.07±0.17	51.08±1.50	17.29±0.20	11.79±0.28	9.75±0.71	38.82±1.20
ZhangJiaKou (C)	31.36±0.48	11.43±0.29	0.99±0.11	43.78±0.87	14.59±0.46	4.59±0.33	6.76±0.53	25.94±1.31
NeiMengGu (C)	32.48±1.33	11.99±0.40	0.89±0.08	45.37±1.81	15.28±0.73	4.43±0.25	7.19±0.52	26.90±1.51
FuJian (C)	32.67±1.62	11.44±0.54	0.86±0.07	44.97±2.23	14.45±0.93	9.23±0.23	9.25±0.78	32.93±1.94
Netherlands (C)	23.65±0.93	9.26±0.65	1.39±0.09	34.30±1.67	10.56±0.19	7.02±0.26	8.83±0.25	26.41±0.71
USA (C)	31.72±1.02	10.54±0.17	1.35±0.05	43.62±1.23	14.63±0.51	4.40±0.21	10.10±0.80	29.14±1.53
Average	28.45±7.91	11.93±2.09	2.09±2.07	42.26±6.97	13.89±3.20	7.31±2.98	9.22±1.48	31.41±5.74

Appendix 3. Information sheet of bread sensory testing

Consumer Evaluation of Quinoa Bread

What is involved?

- Three different types of bread have been prepared:
  - Refined wheat bread
  - 20% quinoa bread
  - 30% quinoa bread
- Volunteers will test each bread product and compare the flavour/characteristics of the 3 different kinds of bread.

Information sheet for volunteers

Newcastle University
NU-Food
School of Agriculture, Food and Rural Development
Agriculture Building
Newcastle upon Tyne
NE1 7RU

Study Contact: NU-Food
Telephone: 0191 208 3592
Email: NU.Food@ncl.ac.uk

You are invited to take part in a research study. Before you start, it is important you understand what it will involve. Please take time to read the following information carefully. Please ask if there is anything that is not clear or if you would like more information.

What is the purpose of this study?

The aim of this study is to see if there is a difference in taste between bread made with different amounts of quinoa (0%, 20% or 30% of the bread).

More information about the quinoa bread

Quinoa has been cultivated and consumed by humans for thousands of years in the Andean region of South America. It has been described as ''golden grain'' due to its high nutritional value, especially the high quality protein, fiber and antioxidants. We want to see if bread can be used as a way of adding quinoa into our diet so we have worked with a baker to make bread with differing amounts of quinoa we want to use in a new intervention study.

Do I have to take part?

After reading this information sheet, if you are interested in volunteering, you will be asked to sign a consent form to show that you agree to take part. You can withdraw from the study at any time without giving a reason. If you enjoy eating bread and have no known allergies or intolerance to bread, then you are an ideal candidate!

What will volunteering involve?

The taste testing will take place in the NU-Food sensory facility, which contains a well-equipped kitchen located in the basement of Newcastle University's Agriculture Building. The tasting session will take about 5 minutes. You will be asked to taste 3 different kinds of bread, sipping water between each sample and scoring them on an anonymous score sheet.

What are the possible disadvantages of taking part?

There are no known disadvantages. However, if you allergy to gluten or other ingredients in the test foods, you will not be allowed to participate. The researcher has a Level 2 Food Handler certificate and will take great care during bread sample preparation.

What are the possible benefits of taking part?

The study will not provide any benefit to you personally in the short term. However, the knowledge gained from this study will support the future development and production of better tasting quinoa bread, so your volunteering is greatly appreciated!

What happens if I decide I want to quit during the study?

If you wish to withdraw from the study then please inform NU-food staff about it. You have the right to withdraw from the study without providing a reason. If you decide to leave before completing the taste testing, then your data will not be used.

What happens if something goes wrong?

Any complaints you have about this study should be made to Professor Chris Seal, Newcastle University (chris.seal@ncl.ac.uk or 0191-2087650) and will be fully investigated.

Will my taking part in this study be kept confidential?

Yes. You do not need to give us any contact details or other information to keep, in order to take part in the study, other than your name. For safety reasons, while you are in the building we need to have a sheet with your contact details, but this sheet will be destroyed as soon as the tasting session is completed and you have left the building.

If you agreed to be contacted for further research, we will keep the contact details, but any information you provided will still be kept strictly confidential.

What will happen to the study results?

The results will be included in a PhD thesis and published in a scientific journal. You will not be personally identified in any publications. If you wish, we will let you know where you can obtain a copy of any published results once it is available, and we will be happy to discuss the results with you then.

Who is organising the study?

This study is being organised by Newcastle University

Finally, thank you for having taken the time to read this information sheet and for your interest in the study.

Appendix 4. Consent form of bread sensory testing

nu-food
Food & Consumer Research Facility

Newcastle University

Consumer Evaluation of Quinoa Bread

CONSENT BY VOLUNTEER TO PARTICIPATE IN A FOOD TASTING STUDY

Study contact: NU-Food **phone:** 0191-208-3592 **e-mail:** nu.food@ncl.ac.uk

I, the undersigned, confirm that (please initial each box as appropriate):

1.	I have read and understood the information about the project, as provided in the Information Sheet dated 28/06/16.	
2.	I have been given the opportunity to ask questions about the project and my participation.	
3.	I understand that I can stop at any time without having to give any reason and that I will not be penalised for stopping.	
4.	I understand that the researcher is a qualified food handler.	
5.	I do not have any known allergy or intolerance to quinoa, wheat or bread (gluten)	
6.	The use of the data in research, publications and marketing material has been explained to me and I agree that the data can be used in this way.	
7.	I understand that the questionnaire is anonymous and cannot be traced back to me.	
8.	I agree to participate in the project	

_____	_____	_____
Name of Participant	Signature	Date
_____	_____	_____
Name of researcher	Signature	Date

For safety reasons, please provide a set of contact details:

The contact details are only required for safety reasons, so we, for example, can ensure you get the appropriate help in case of an accident.
This information will not be linked to your completed questionnaire.
Your contact details will be destroyed as soon as the test is completed, unless you explicitly allow us to keep them for longer, by selecting 'Yes' in the option below

Name_____

Address_____

_____postcode_____

Phone number _____

Email address_____

Would you like us to e-mail you with the results of the trial, once the results are published?

Yes ☐ No ☐

Appendix 5. Basin information of volunteers involved in bread sensory testing

Newcastle University

Number:

nu-food
Food & Consumer Research Facility

Before you start, please complete the following brief questions about you:

Male ☐ **Female** ☐

Age ≤20 ☐ 21-35 ☐ 35-55 ☐ ≥56 ☐

Are you a current smoker? **Yes** **No**

Have you smoked in the past? **Yes** **No**

If yes, when did you stop smoking?_____

Do you currently have a cold/feel congested? **Yes** **No**

Do you suffer from hay fever? **Yes** **No**

How often do you eat bread? (Tick only one)

Never ☐

Once or twice a week ☐

Almost once every day ☐

More than once every day ☐

What type of bread do you eat? (Tick all that apply

None ☐

White bread ☐

Wholemeal bread ☐

Granary bread ☐

Rye bread ☐

Other speciality bread (e.g. ciabatta, Nan bread) ☐

Which of the above bread do you eat most? _____

Appendix 6. Questions for bread sensory testing

Newcastle University

nu-food
Food & Consumer Research Facility

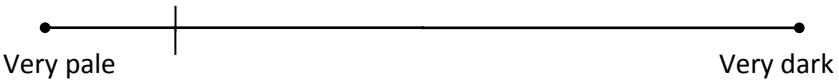
Sensory testing of bread

Three bread samples will be presented to you on three separated plates, each sample will be presented with its corresponding symbol. Please use the scales to assess the appearance and taste of the bread.

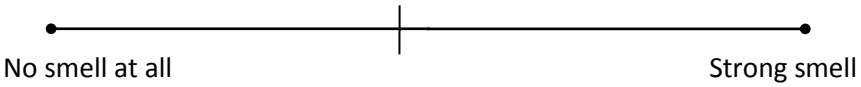
SAMPLE:

Please mark each horizontal line with your response as shown below:

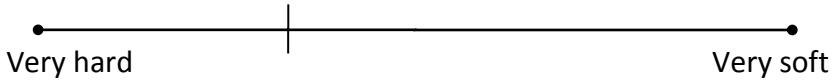
1. How would describe the colour of the bread?



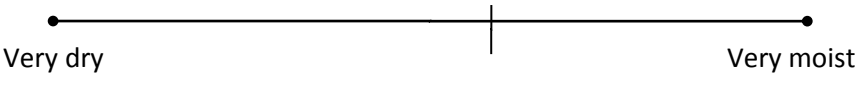
2. How would describe the aroma/smell of the flavour of bread?



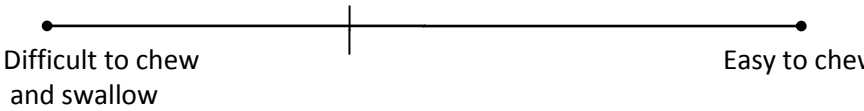
3. How soft does the bread feel?



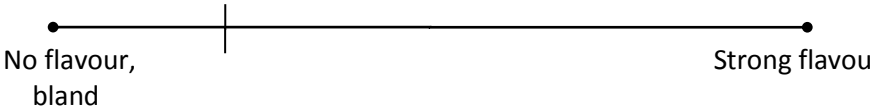
4. How would describe the moistness of the bread when you eat it?



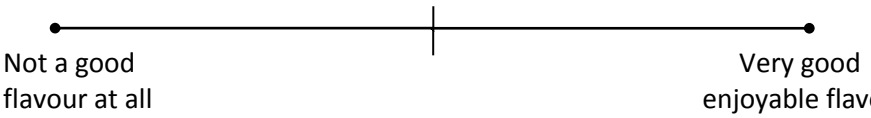
5. How chewy would you describe the bread?



6. How much flavour does the bread have?



7. How would you describe the flavour of the bread?



Newcastle University

nu-food
Food & Consumer Research Facility

Bread Ø

1. How would describe the colour of the bread?

Very pale — Very dark

2. How would describe the aroma/smell of the flavour of bread?

No smell at all — Strong smell

3. How soft does the bread feel?

Very hard — Very soft

4. How would describe the moistness of the bread when you eat it?

Very dry — Very moist

5. How chewy would you describe the bread?

Difficult to chew and swallow — Easy to chew

6. How much flavour does the bread have?

No flavour, bland — Strong flavour

7. How would you describe the flavour of the bread?

Not a good flavour at all — Very good enjoyable flavour

Please add any other comments you would like to make about two samples of bread:

Bread Δ

1. How would describe the colour of the bread?

Very pale — Very dark

2. How would describe the aroma/smell of the flavour of bread?

No smell at all — Strong smell

3. How soft does the bread feel?

Very hard — Very soft

4. How would describe the moistness of the bread when you eat it?

Very dry — Very moist

5. How chewy would you describe the bread?

Difficult to chew and swallow — Easy to chew

6. How much flavour does the bread have?

No flavour, bland — Strong flavour

7. How would you describe the flavour of the bread?

Not a good flavour at all — Very good enjoyable flavour

Please add any other comments you would like to make about two samples of bread:

Newcastle University

nu-food
Food & Consumer Research Facility

Bread ¶

1. How would describe the colour of the bread?

Very pale — Very dark

2. How would describe the aroma/smell of the flavour of bread?

No smell at all — Strong smell

3. How soft does the bread feel?

Very hard — Very soft

4. How would describe the moistness of the bread when you eat it?

Very dry — Very moist

5. How chewy would you describe the bread?

Difficult to chew and swallow — Easy to chew

6. How much flavour does the bread have?

No flavour, bland — Strong flavour

7. How would you describe the flavour of the bread?

Not a good flavour at all — Very good enjoyable flavour

Please add any other comments you would like to make about two samples of bread:

Appendix 7: Poster for recruiting volunteers

Appendix 8: Pre-screening questionnaire for human intervention study

Effect of quinoa consumption on cardiovascular disease risk and gastrointestinal health

Pre-Screening Questionnaire

Please explain more about the study – assure confidentiality of personal information at the start of the telephone interview.

	YES	NO
Date of birth --/--/----		
Male aged 35-70 years? *(exclude if outside this range)*	☐	☐
Do you smoke? *(exclude if yes)*	☐	☐
Are you allergic to wheat or gluten? *(exclude if yes)*	☐	☐
Do you have any other food allergies or intolerances?	☐	☐

If yes, please give details.

……

	YES	NO
Do you have any dietary restrictions? *(such as being on 'detox' or other slimming diets, exclude if yes)*	☐	☐
Are you currently suffering from any illness *(prompt volunteers with – heart disease, diabetes, cancer, celiac disease, high blood pressure (requiring treatment), anaemia) (exclude if any major illness)*	☐	☐
Do you eat breakfast cereal?	☐	☐
Do you eat bread?	☐	☐
Do you eat wholegrain foods?	☐	☐
Would you be willing to stop eating whole grain foods for three months during the study?	☐	☐

Are you currently taking any prescribed medication? *If yes give details.* ……	**YES** ☐	**NO** ☐
Are you currently taking any other medication that can be purchased over the counter? *If yes give details.* ……	**YES** ☐	**NO** ☐
Are you currently taking any dietary supplements (vitamins/minerals etc.)? **If taking dietary supplements, would you be prepared to stop taking them for 12 weeks during the study period?**	**YES** ☐ **YES** ☐	**NO** ☐ **NO** ☐
Do you take drugs for recreational use?	**YES** ☐	**NO** ☐
Do you drink alcohol?	**YES** ☐	**NO** ☐
If yes, do you drink more than the recommended amount of alcohol per week? *[prompt – 3-4 units/ day recommended for men and 2-3 units/day recommended for women. One unit = ½ pint beer or 1 spirit (25 ml) or 1 small glass of wine]* **Exclude if yes**	**YES** ☐	**NO** ☐

Diet and BMI			
Estimate of current weight	St/lb kg	**Estimate of current height**	Ft/in m

Estimated BMI: _____

Exclude if estimated BMI < 25 kg/m ^{2}

Have you had a weight change of more than 3 kg (7lbs) in the past 2 months?	**YES** ☐	**NO** ☐
Are you planning to lose/gain weight in the next 3-4 months? **If yes, Exclude**	☐	☐

	YES	NO
2. Are you a vegetarian/vegan?	☐	☐

Availability Information

	YES	NO
Would you have any anticipated difficulties with your availability or transport arrangements to attend each of the study visits?	☐	☐

	YES	NO
Are you planning to go away on a holiday in the next 12 weeks?	☐	☐

Please state dates

…….

Suitability

	YES	NO
Suitable?	☐	☐

If yes, book Induction Visit

Date ……. Time …….

ID code | I | H | | | |

	YES	NO
If no, ask volunteers if they wish to take part in any future human nutrition studies and have their details retained within our confidential database	☐	☐

Participant details
Name ……. **M** ☐ **F** ☐ **Address** …… …..…… …… **Post Code** …… **E-mail address** ……..…… **Phone Number:** Day……..…….. Evening ……..…… Mobile…… **Best time to phone** …….. **Preferred Telephone Number (circle)** Day/Evening/Mobile
General Practitioner Name…… **Of (medical practice)**……..……..…….. …… **GP telephone number**……

Please mention to interested volunteers that they will be sent:

(i) an official Invitation Letter, to attend the Screening Visit
(ii) the Study Information Sheet, containing more information about the study

1. **File this form in 'Pending Induction Visit' Folder** ☐
2. **On obtaining consent, file form in individual folder** ☐
3. **If no consent, shred this form using a shredder** ☐

Signature ……..… **Date** ……

Newcastle University

Appendix 9: Information sheet

Effect of quinoa consumption on markers of cardiovascular risk and gastrointestinal health

Information Sheet for Participants

Chief Investigator: Pro. Chris.Seal
Coordinator: Liangkui Li

Newcastle University
School of Agriculture, Food & Rural Development
Agriculture Building
Newcastle upon Tyne
NE1 7RU

For further information please contact:
Email: l.li11@newcastle.ac.uk or NU.Food@newcastle.ac.uk

Telephone: 07706273008 or 0191 208 3592

http:www.ncl.ac.uk/hnrc

You are being invited to take part in a research study. Before you decide it is important you understand why the research is being done, and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Please ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. Thank you for reading this.

What is the purpose of this study?

Quinoa is possible alternative to wheat, corn and rice due to its high nutritional value and possible properties against cardiovascular diseases and for improving gut health. There are some animal studies to suggest that some important class of compounds like fiber, antioxidants and protein, found naturally in quinoa, have the beneficial effects against markers of cardiovascular diseases.

However, the effects of quinoa on humans have rarely been investigated. This study will investigate the effects of quinoa on markers of cardiovascular risk, including blood cholesterol and resting blood pressure, and on gut health.

Why have I been chosen?

We are looking for men who are non-smokers and over the age of 35 years to take part in this study. We will be recruiting 25 volunteers in total from the Newcastle Upon Tyne area.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be asked to sign a consent form on your 'Screening Visit'; you will be given a copy of this to keep.

What will happen to me if I take part?

If you decide to take part and you are a suitable volunteer for the study, we will ask you to sign a full consent form. However, you will be free to withdraw from the study without giving a reason anytime up to the

end of your final visit. Shortly after this, all data will be fully annonymised, and therefore, from this point forward it will not be possible to withdraw any data from the study.

What will happen to me if I take part?

If you decide to take part, we will then ask you some questions about your medical history to check whether you can participate. You will not be included in the study if you have any medical conditions or if you are taking medications that will affect the measurements in the study. If you are a suitable volunteer for the study, you will be randomly allocated into either a quinoa bread or refined wheat bread diet. For each of the two diets we will provide you bread and ask you to substitute these for some of the bread you normally eat in your diet every day. For one period the bread will be made with quinoa, in the other period the bread will be made only from refined wheat. We will ask you to consume two and half slices of these breads for 4 weeks. Following the first intervention phase of the study above, a 4-week 'wash-out' period will then begin (weeks 5-8) where you must avoid some foods on a 'Food to Avoid' list but no other supplementary food will be provided. After this 'washout period', we will ask you to consume either quinoa or refined wheat bread for the final 4 weeks, in a cross-over design. For example, if you eat quinoa bread during the first intervention phase, after the wash period, you will then consume the refined wheat bread. We will provide you with the study breads regularly (every 2 weeks) and will also advise you on the amounts of the bread we would like you to eat.

What else do I have to do?

If you agree to take part, we will ask you to visit **the NU Food,** Newcastle university, on four occasions. If you are suitable, the first visit is to assess your blood pressure and a fasted blood sample will be collected for cholesterol and glucose analysis, and there will be 3 further visits at four-weekly intervals during the intervention periods. On the evening before each visit, you will need to fast from 8 pm; this means that you should not eat or drink anything except water until you complete your visit the following morning.

At each visit, we will take a blood sample (20ml/4 teaspoons of blood) from your arm and will also measure your height, weight, waist and hip circumference, body fat and blood pressure using non-invasive procedures and will ask you to complete a digestive health questionnaire. We will also ask you to collect your urine for 24 hours the day before and collect a small sample of your stool, and bring them to this visit (appropriate containers and full instructions on how to do this will be provided). Each visit will last approximately 45 minutes.

You will then be offered a test breakfast of one of the test breads. After finishing the breakfast, we will ask you to collect 5 finger prick blood samples in the following 5 hours (1 time per hour). These processes can by carried out at NU-Food by staff or if you

prefer you can do this at home yourself using a 'home test kit' we will provde for you to use.

Finally, we will ask you to record what you eat for 3 days (2 week days, 1 weekend day) in a 3-day food diary during each four-week period and complete a food-frequency questionnaire (FFQ) on-line during the visit to NU-Food.

What will happen to the samples I provide?

Blood and urine samples provided will be tested for substances present in quinoa or refined wheat foods (e.g. antioxidants or their metabolites). lipid profile (e.g. cholesterol), glucose and insulin. The stool sample will be analysed for the number and type of bacteria present, and for some metabolites produced by the bacteria when they break down the fibre in the quinoa. A small amount of the blood taken at study visits will be stored (for up to 10 years) for future tests to confirm results. This may include genetic tests on DNA if necessary as part of this study. All stored plasma and DNA samples will be coded so that no one can be identified from these samples.

What are the possible disadvantages and risks of taking part?

Taking blood samples may cause minor discomfort and there is a small chance of minor bruising afterwards. If a new diagnosis of high blood pressure is made, this could affect your future insurance status (e.g. for life insurance or private medical insurance).

What are the possible benefits of taking part?

If we discover any abnormalities of significance in your lipid profile, blood glucose or blood pressure, we will inform you and your GP. Although you will derive no further individual benefit, the knowledge gained from this study will help our research into identifying the effects of quinoa bread on health.

What will happen if anything goes wrong?

Any complaints you have about this study should be made to Prof. Chris Seal, Newcastle University (chris.seal@ncl.ac.uk or 0191-2087650) and will be fully investigated.

Will my taking part in this study be kept confidential?

Any information which is collected about you during the course of the research will be kept strictly confidential. Your GP will be notified that you are participating in this study. He/ She will be notified if any abnormal results of significance to your health are found.

What will happen to the study results?

We will publish the results of the study in a scientific journal and on the project website. You will not be personally identified in any publications. We will be happy to discuss the overall results with you

when the study is completed, and will let you know where you can obtain a copy of the published results if you wish.

Will I be reimbursed for my time?

Any travel expenses will be paid. In recognition of your time commitment, you will be paid an honorarium of £120 in the form of Eldon Square vouchers at the completion of the study.

Contact for further information

If you would like any further information about this study, please do not hesitate to contact **Liangkui Li or NU-Food**

Telephone: 07706273008 or 0191 208 3592
Email:l.li11@newcastle.ac.uk
NU.Food@newcastle.ac.uk

And finally…

Thank you for having taken the time to read this information sheet and for your interest in the study

Appendix 10: Volunteer guidance

Volunteer ID: ……
Visit No. ……

Welcome to

The 'Effects of quinoa consumption on markers of cardiovascular risk and gastrointestinal health' study

Thank you for agreeing to take part in this study, conducted by the Human Nutrition Research Centre, Newcastle University.

You will find all the information you need for the study inside this information pack.

Please read the information carefully before you begin.

Guidelines:

- Sample schedule
- Food and drink
- Urine collection guidance
- Stool sample collection guidance
- Home cholesterol test kit
- Test food consumption record
- Reminder of key days

If there is anything you are not sure about, please don't hesitate to ask.

We hope you enjoy taking part!

Liangkui Li
Tel: 07706273008
Email: l.li11@newcastle.ac.uk

Sample Schedule

Screening	Run in	First 4-wk intervention period				4-wk wash out period (5-8 week)		Second 4-wk intervention period			
	Week: 0	1	2	3	4	5-7	8	9	10	11	12
At NU Food: Questions about health and lifestyles, assessment of eligibility.	FFQ, 3 day food dairy, 24 hour urine samples	At NU Food: Fasting blood, Finger prick			At NU Food: 3 day food dairy, fasting blood and 24 urine samples, Finger prick		At NU Food: Fasting blood, 24 hour urine samples Finger prick				At NU Food: 3 day food dairy, fasting blood and 24 urine samples, Finger prick
		Avoiding all the foods on the list provided									

Food (Breads)

Intervention period (weeks 1-4, and weeks 9-12):

- The test breads will be provided fresh every two weeks during the intervention phases. You will need to freeze the bread when you get home and then defrost it as you need it.
- Consume two and half slices of the test breads (provided by the researcher) per day. You can eat the bread in any way you wish (toasted, as a sandwich etc.).
- Avoid all foods on the 'Foods to Avoid' list (provided in these instructions) but otherwise you can eat freely as you normally would. Please try not to change your 'normal' diet during the study apart from avoiding these foods.

'Wash out' period (week 5-8):

You will not have to consume any supplementary foods during the 'wash-out' period and are free to eat as you normally would EXCEPT avoiding those foods in the 'Foods to Avoid' list.

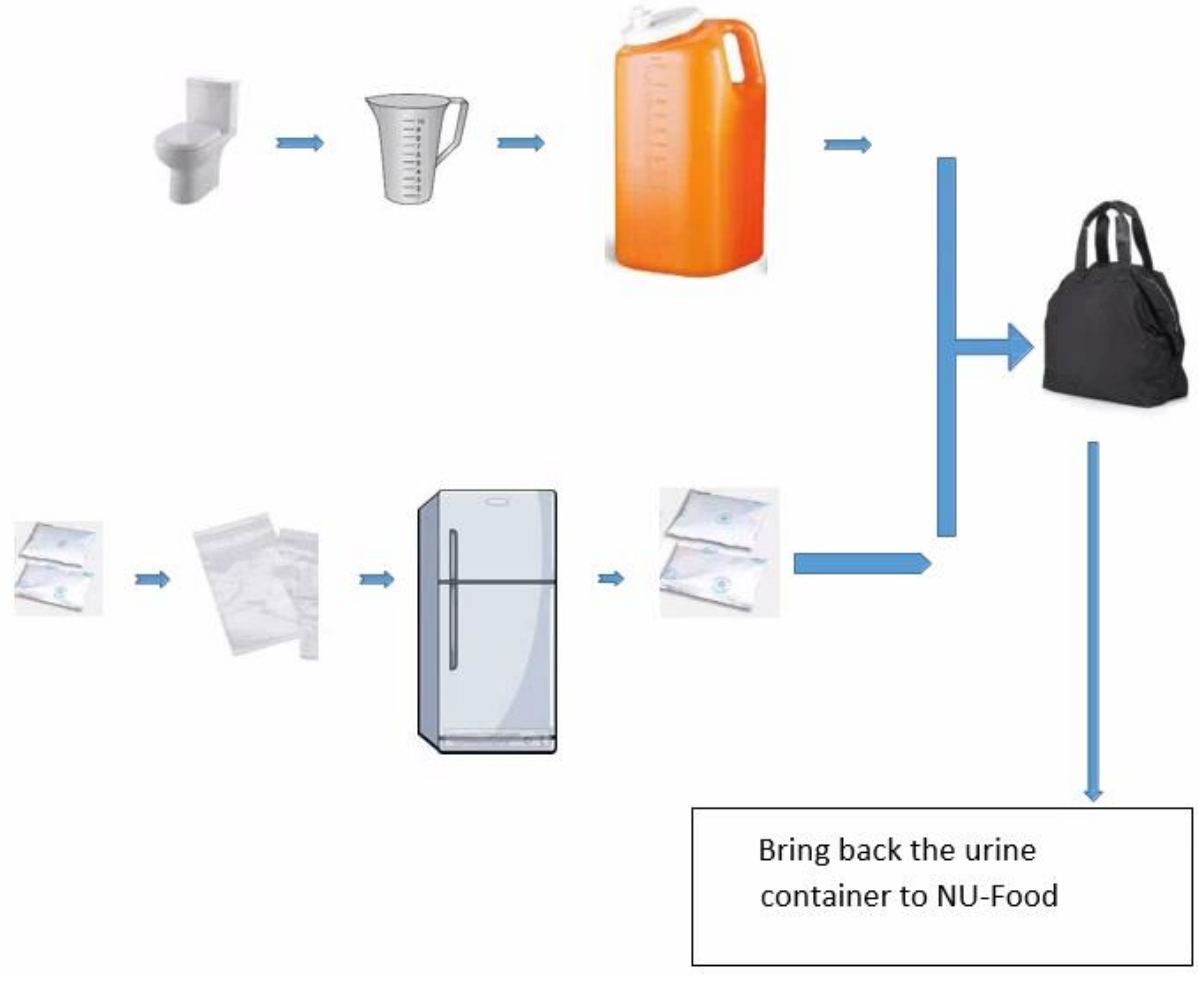
Once the final blood samples are taken and the urine and stool samples collected, at the end of the full 12 week period, you may eat freely again.

Urine Collection Guidance

Collect a urine sample **every time** you visit the toilet from waking on the 24 hours **before** the sample collection day.

How you collect urine samples at home:

Collect urine in the plastic jug provided, and then pour all sample into the urine container in the 24 hours. Please bring all urine samples that you have collected when you attend the sample collection day.



If you are at work or out during the day please remember to take your sample collection kit and cool bag with you and transfer them to your fridge when you return in the evening.

How to collect the stool sample

Numbers correspond to numbers on the attached pictures below:

1. Use the complete kit that you received by mail (see kit's picture below).
   It contains:

   i) A stool catcher to place over the toilet

   ii) Sticky labels for plastic tube, transparent bag and opaque (silver) bag

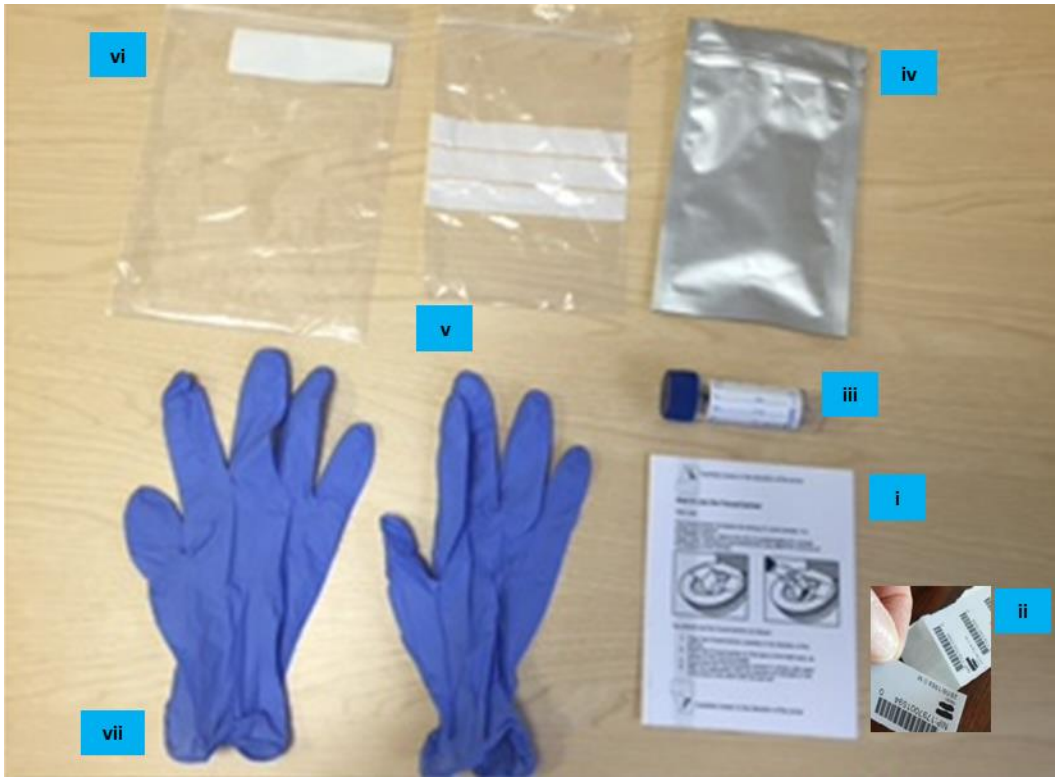
   iii) Plastic tube with a spoon attached to the lid

   iv) A small (silver) opaque plastic bag

   v) A small transparent plastic bag

   vi) A large transparent plastic bag

   vii) 1 pair of rubber gloves for stool collection



2. Label the tube (iii), the small transparent plastic bag (iv) and the opaque (silver) bag (v) with the labels provided (ii). Alternatively, place your initials the plastic tube prior to stool collection.

3) If possible try to urinate (pass water) before stool collection as this may affect the sample.

THEN PLEASE WASH HANDS YOUR HANDS

Read the instructions on the stool catcher. Place the stool catcher on the toilet seat of the toilet, ensuring that the position is correct to catch the stool sample. Then follow the instructions on the stool catcher.

THEN PLEASE WASH HANDS YOUR HANDS

4) After opening your bowels, put on the gloves provided (ensure your hands are dry or these will be difficult to put on).

If you are able to do this in clinic, one of the nurses or a member of the research team will collect the stool for you once you have finished. If you are at home please continue to the next step.

5) Using the plastic tube with the spoon attached (as seen in the below image), and place stool sample into the tube.

Attempt to fill the tube at least a quarter.

This will be around 3-4 spoons.

Then place the spoon into the tube and secure the lid.

After stool collection the remaining stool sample and stool catcher can then be released from the toilet (as described in the instructions) and flushed away. The rubber gloves should be placed in your bin as normal rubbish.

6) Place the tube with stool sample into the small transparent plastic bag. Then seal the bag tightly.

7) Place the small transparent bag inside the opaque (silver) bag and seal the silver bag tightly. Then place the opaque (silver) bag into the large transparent bag and seal tightly.

Then do one of the following:

- If you are attending clinic on that day bring the sample with you.
- If you collect the sample at home then place the sample in the bags with the ice pack into the cooler bag and into your freezer until your next clinic appointment, or a member of the research team will collect the sample as soon as possible.

8) Ideally the stool sample should be returned to us as soon as possible (within a few days). Please keep the sample frozen in the bags provided.

Blood glucose meter

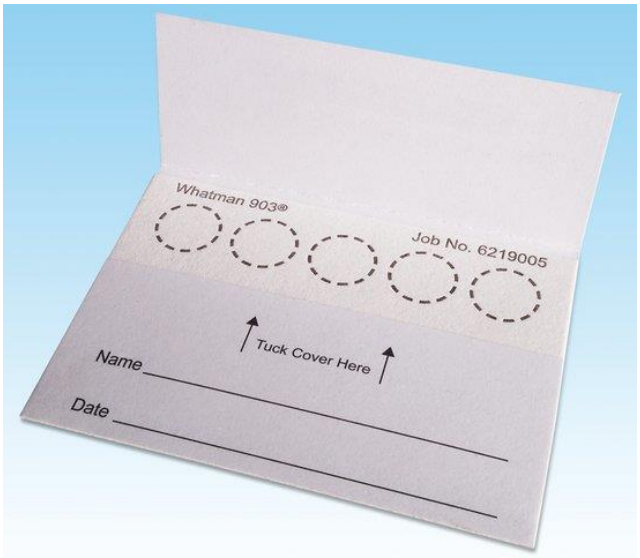
1. The trained staff will apply the FreeStyle Libre sensor on to the back of your upper arm with a simple, disposable device called an applicator. When the sensor is applied, a thin, flexible and sterile fiber is inserted just under the skin. It is held in place with a small adhesive pad.
2. Perform a quick, painless 1-second scan of the reader over the sensor in order to obtain a glucose reading.
3. You should wear the sensor 14 days, which will automatically measure glucose, day and night. The sensor must be scanned at least once every 8 hours. Each scan of the reader over the sensor gives a current glucose reading, the last 8-hours of glucose history, and a trend arrow showing if glucose is going up, down, or changing slowly.

Notes: sensor is water-resistant in up to 1 metre (3 feet) of water for a maximum of 30 minutes, so you can wash or have a shower freely.

Finger pricks on 903 protein saver card

To use the protein saver card

Wash your hands thoroughly, then prick your finger with the small lancet provided. Put a drop of blood within the half-inch circle on the 903 protein saver card from left to right in the following 5 hours (one finger prick per hour). You also should remain that the cover is folded over the sample when the blood sample become dry after each finger prick.



Test Food Consumption Record

Instructions

Please record:

- date
- time
- whether you consumed the portion
- if you left any uneaten
- any another comments

Please do this each time you consume any of the bread provided. If you miss a portion, please record this on this recording sheet.

Date	Time	Portion consumed	Any left overs?	Other comments
E.g. 08/05/2016	*8.00am*	*Yes*	*No*	*No*

Reminder of key dates:

Study Day 1:

- Eat as you normally would and during the week leading up to your visit record everything you ate on 2 week days and 1 weekend day in the 3-day food diary.
- On the day before coming to the blood collection session **Date**..... : collect all urine passed and collect a stool sample. Eat the standard meal provided no later than 8pm, and drink the water provided and remain fasted (no food or drink except water) for at least 12 hours before test (**from……pm**). Fill in the Food Frequency Questionnaire (FFQ).

- Test session- **Date..... :** Come to NU Food **fasted,** bring urine and stool samples with you and the completed 3-day food diary and FFQ. You will have your blood taken. You will then be given a test breakfast of wheat bread, and after the breakfast, you will give 5 finger prick blood samples in the following 5 hours (1 time per hour).

Study Day 2:

- Eat as you normally would EXCEPT avoiding foods on the Foods to Avoid List **and** eating a portion of the test food. During the week leading up to your visit record everything you ate on 2 week days and 1weekend day in the 3-day food diary.
- On the day before coming to NU food for the blood collection session, in the 4th week, **Date**.....**:** collect all urine passed, eat the standard meal provided no later than 8pm, and drink the water provided and remain fasted (no food or drink except water) for at least 12 hours before test (**from……pm**). Fill in the FFQ.

- Test session- **Date..... :** Come to NU Food **fasted,** bring urine and stool samples with you and the completed 3-day food diary and FFQ. You will have your blood taken. You will then be given a test breakfast of either wheat or quinoa bread, and after the breakfast, you will give 5 finger prick blood samples in the following 5 hours (1 time per hour).

Study Day 3:

- Eat as you normally would EXCEPT avoiding foods on the Foods to Avoid List. During the week leading up to your visit record everything you ate on 2 week days and 1 weekend day in the 3-day food diary.
- On the day before coming to NU food for the blood collection session, in the 8th week, **Date**..... : collect all urine passed and collect a stool sample, eat the standard meal provided no later than 8pm, and drink the water provided and remain fasted (no food or drink except water) for at least 12 hours before test (**from……pm**). Fill in the FFQ.

- Test session- **Date**……..: Come to NU Food fasted, bring urine and stool samples with you. You will have your blood taken. You will then be given a test breakfast of wheat bread, and after the breakfast, you will give 5 finger prick blood samples in the following 5 hours (1 time per hour).

Study Day 4:

- Eat as you normally would **EXCEPT** avoiding foods on the Foods to Avoid List **and** eating a portion of the test food. During the week leading up to your visit record everything you ate on 2 week days and 1weekend day in the 3-day food diary.
- On the day before coming to NU food for the blood collection session, in the 12th week, **Date**..... : collect all urine passed and collect a stool sample, eat the standard meal provided no later than 8pm, and drink the water provided and remain fasted (no food or drink except water) for at least 12 hours before test (**from……pm**).

- Test session- Date……..: Come to NU Food fasted, bring urine and stool samples with you. You will have your blood taken. You will then be given a test breakfast of either wheat or quinoa bread, and after the breakfast, you will give 5 finger prick blood samples in the following 5 hours (1 time per hour).

You will receive reminders of these dates as the study progress.

Appendix 11: Food frequency questionnaire

Volunteer ID	I	H			
Visit no		Week commencing (date)			

Effect of quinoa consumption on cardiovascular disease risk and gastrointestinal health

Food Frequency Questionnaire

- Please read the instructions on pages 2 and 3 before completing the questionnaire.
- All information collected will be kept completely confidential.
- Thank you for taking time to complete this questionnaire.

If you have any queries please contact:

Wendy Bal Tel: 0191 208 6619 or 07510567098

or email ingrained.health@ncl.ac.uk

How to answer the questions

There are several types of question in this booklet. Most of them can be answered by ticking one box (ONLY) beside each food types.

For example:

FOODS & AMOUNTS	AVERAGE USE IN THE LAST WEEK							
FISH *(medium serving)*	None	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Fried fish in batter, as in fish and chips		✓						

Please put ONE tick in the appropriate box (✓) on each line to indicate how often, **on average**, you have eaten each food **during the past week.**

- Answer every question by putting ONE tick (✓) on every line

- Do not leave **ANY** lines blank.

Another example of questions requiring boxes to be ticked:

Q. Do you usually add salt to food while cooking?

Yes……❒

No……❒

Some of these questions have several boxes and you may be asked to tick ONE only.

For example:

What kind of fat did you most often use for frying, roasting, grilling etc?

Select one only

Butter……❒	Olive oil……☑
Lard/dripping……❒	Walnut Oil……❒
Solid vegetable fat……❒	Soya Oil……❒
Margarine……❒	None……❒
Vegetable Oil……❒	Other……❒

Some of these questions have several boxes and you may be asked to tick all the boxes you think apply to you.

For example:

14. What kind of fat did you use for cooking?

Please tick <u>all</u> that apply

Butter……☑

Lard/dripping……❒

Solid vegetable fat……❒

Margarine……❒

Vegetable oil……☑

Olive oil……❒

Walnut Oil……❒

Soya Oil……❒

None……❒

Other……❒

If "other" selected in question 14, please state……

What do I do if I make a mistake?

Cross out the incorrect answer, and put a tick where you think the right answer should be. We'll verify your answers at your next appointment visit.

If you have any problems filling in this dietary questionnaire, we will discuss them at your next visit. If you have any questions do not hesitate to contact Mrs Wendy Bal, contact details are provided on the front cover of this questionnaire.

For **Questions 1-12**, please put **ONE tick** in the appropriate box (✓) on each line to indicate how often, **on average**, you have eaten each food **during the past week.** Please **DO NOT leave any lines blank.**

8.1.1.1.1.1.1.1.1 FOODS & AMOUNTS	**Average Use In LAST WEEK (Tick ONE per line)**							
1. MEAT ***(medium serving)***	None	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Beef: e.g. roast, steak, mince, stew, casserole, curry, Bolognese								
Beefburgers *(single burger)*								
Corned beef, Spam, luncheon meats *(2 slices – a sandwich's-worth)*								
Lamb: e.g. roast, chops, stew, curry								
Chicken, turkey or other poultry: e.g. casserole, sliced, curry								
Breaded or fried poultry products: e.g. chicken nuggets, deep fried chicken pieces *(1 breaded chicken portion or c.6 nuggets)*								
Pork: e.g. roast, chops, stew, curry								
Bacon and ham *(2 rashers/slices – a sandwich's-worth)*								
Sausages *(one)*								
Savoury pies, e.g. meat pie, pork pie, pasties, steak & kidney pie, sausage rolls, scotch egg *(single pie/savoury)*								
Game and Wild-fowl: e.g. duck, rabbit, grouse								
Kidneys or liver; including liver pate, liver sausage								
FOODS & AMOUNTS	**Average Use In LAST WEEK (Tick ONE per line)**							
2. FISH and SEAFOOD	None	Once	2-4	5-6	Once	2-3	4-5	6+ per

(medium serving)		a week	per week	per week	a day	per day	per day	day
White fish - not coated e.g. cod, halibut, haddock, whiting, plaice, sole, etc *(per portion)*								
White fish- in batter or crumbs e.g. cod, haddock, plaice, etc *(per portion)*								
Oily fish e.g. herring, mackerel, salmon- not tinned, trout, kippers etc *(per portion)*								
Tinned fish e.g. Sardines, Pichards, Tuna, Salmon etc *(per can, or portion)*								
Prawns, shellfish and other fish *(within dish or one sandwich's-worth)*								
Fish cakes, Fish fingers *(one)*								
Fish based dishes e.g. fish pie, fish curry, kedgeree								
Roe and roe products including taramasalata, caviar								

FOODS & AMOUNTS	**Average Use In LAST WEEK (Tick ONE per line)**							
3. BREAD & SAVOURY BISCUITS ***(one slice or biscuit)***	None	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
White bread and rolls, white pitta bread (per slice/roll)								
Scones, teacakes, crumpets, muffins or croissants (each)								
Brown bread and rolls (per slice/roll)								
Wholemeal pitta bread (each)								
Wholemeal bread/rolls (per slice/roll)								
Granary bread (per slice/roll)								
Rye bread (per slice/roll)								
Naan bread, chapatti (each)								
Garlic bread (per serving)								
Cream crackers, cheese biscuits (each)								

Wholemeal crackers (per cracker)								
Crispbreads e.g. Ryvita, Ryvita currant crunch (one)								
Oatcakes (one)								
Other speciality breads (each) (please state and tick for frequency) 1. 2.								

FOODS & AMOUNTS	**Average Use In LAST WEEK (Tick ONE per line)**							
4. CEREALS ***(one bowl)***	None	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Porridge, Readybrek								
Sugar coated cereals e.g. Sugar Puffs, Cocoa Pops, Frosties								
Non-sugar coated cereals e.g. Cornflakes, Rice Crispies								
Muesli								
Bran containing cereals e.g. All Bran								
Cheerios								
Branflakes								
Weetabix								
Shredded Wheat, Shreddies								
Wholegrain cereals with fruit e.g. Sultana Bran, Fruit n Fibre								

FOODS & AMOUNTS	**Average Use In LAST WEEK (Tick ONE per line)**							
5. POTATOES, RICE & PASTA *(medium serving)*	None	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Boiled, mashed, instant or jacket potatoes *(about 1/3 of a plate)*								
Chips, potato waffles *(side order with meal – chip-shop portions count as 2)*								

Roast potatoes *(3 – 5 potatoes)*								
Yorkshire pudding, pancakes, dumpling *(each medium)*								
Potato salad *(per small tub, c. 2 tablespoons)*								
White rice *(1/2 plateful, or in a dish e.g. rice salad, risotto etc)*								
Brown rice *(1/2 plateful, or in a dish e.g. rice salad, risotto etc)*								
White or green pasta, e.g. spaghetti, macaroni, noodles, *(1/2 plate)*								
Tinned pasta, e.g. spaghetti, ravioli, macaroni *(1/2 standard tin)*								
Super noodles, pot noodles, pot savouries *(per pot)*								
Wholemeal pasta/spaghetti *(1/2 plate)*								
Pasta dishes e.g. Lasagne, moussaka, cannelloni *(as individual ready-meal)*								
Pizza *(10" = 1, 12" = 2, 12"+ = 3-4)*								
Wholegrain dishes not mentioned (Please state and tick for frequency) 1. 2. 3.								
FOODS & AMOUNTS	**Average Use In LAST WEEK (Tick ONE per line)**							
6. (a) DAIRY & EGG PRODUCTS	None	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Single or sour cream *(tablespoon)*								
Double or clotted cream *(tablespoon)*								
Low fat yoghurt, fromage frais *(125g carton)*								
Full fat or Greek yoghurt *(125g carton)*								

Dairy desserts *(125g carton)*, e.g. mousse								
Cheese, e.g. Cheddar, Brie, Edam *(medium serving)*								
Cottage cheese, low fat soft cheese *(medium serving)*								
Eggs as boiled, fried, scrambled, omelette etc. *(one)*								
Quiche *(medium serving = 1/6 of pie)*								

FOODS & AMOUNTS	**Average Use In LAST WEEK (Tick ONE per line)**							
6.(b) DAIRY PRODUCTS & FATS used on bread *(teaspoon/curl)*	None	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Butter (e.g. Anchor, Country Life, Lurpak, St Helens Farm Goat Butter, Yeo Valley, Own Brand Butter)								
Vegetable oil based spreads (e.g. Flora Original, I Can't Believe It's Not Butter! Original, Pure Soya Dairy Free, Pure Sunflower Dairy Free, Vitalite, Own Brand Sunflower Spread)								
Olive oil based spreads (e.g. Benecol Olive, Bertolli, Pure Olive Dairy Free, Own Brand Olive Spread)								
Margarine (e.g. Stork, Own Brand Margarine)								
Margarine and Butter Blend Spreads (e.g. Anchor Spreadable, Benecol Buttery, Clover, Country Life Spreadable, Flora Buttery, Lactofree spreadable, Lurpak Spreadable, Utterly Butterly, Yeo Valley Spreadable, Own Brand Marge and Butter Blend Spread)								
Reduced/Low Fat Vegetable Oil Based Spreads (e.g. Benecol Light, Flora Light, I Can't Believe It's Not Butter! Light, Own Brand Reduced/Low Fat Sunflower Spread)								

Reduced/Low Fat Olive Oil Based Spreads (e.g. Betolli Light, Own Brand Reduced/ Low fat Olive Oil Based Spread)								
Reduced/Low Fat Margarine								
Reduced/Low Fat Margarine and Butter Blend Spreads (e.g. Anchor Lighter Spreadable, Clover Lighter, Country Life Lighter, Flora Spread Buttery Light, Lurpak Lighter, Lurpak Lighter with Olive Oil, Lurpak Lightest, Own Brand Reduced/Low Fat Margarine and Butter Blend Spreads)								

FOODS & AMOUNTS	**Average Use In LAST WEEK (Tick ONE per line)**							
6.(c) DAIRY PRODUCTS & FATS used on vegetables *(teaspoon/curl)*	None	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Butter (e.g. Anchor, Country Life, Lurpak, St Helens Farm Goat Butter, Yeo Valley, Own Brand Butter)								
Vegetable oil based spreads (e.g. Flora Original, I Can't Believe It's Not Butter! Original, Pure Soya Dairy Free, Pure Sunflower Dairy Free, Vitalite, Own Brand Sunflower Spread)								
Olive oil based spreads (e.g. Benecol Olive, Bertolli, Pure Olive Dairy Free, Own Brand Olive Spread)								
Margarine (e.g. Stork, Own Brand Margarine)								
Margarine and Butter Blend Spreads (e.g. Anchor Spreadable, Benecol Buttery, Clover, Country Life Spreadable, Flora Buttery, Lactofree spreadable, Lurpak Spreadable, Utterly Butterly, Yeo Valley Spreadable, Own Brand Marge and Butter Blend Spread)								

Reduced/Low Fat Vegetable Oil Based Spreads (e.g. Benecol Light, Flora Light, I Can't Believe It's Not Butter! Light, Own Brand Reduced/Low Fat Sunflower Spread)								
Reduced/Low Fat Olive Oil Based Spreads (e.g. Betolli Light, Own Brand Reduced/ Low fat Olive Oil Based Spread)								
Reduced/Low Fat Margarine								
Reduced/Low Fat Margarine and Butter Blend Spreads (e.g. Anchor Lighter Spreadable, Clover Lighter, Country Life Lighter, Flora Spread Buttery Light, Lurpak Lighter, Lurpak Lighter with Olive Oil, Lurpak Lightest, Own Brand Reduced/Low Fat Margarine and Butter Blend Spreads)								

FOODS & AMOUNTS	**Average Use In LAST WEEK (Tick ONE per line)**							
7. SWEETS & SNACKS *(medium serving)*	None	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Chocolate coated sweet biscuits, e.g. Penguin, kit-kat, chocolate digestive *(one)*								
Sweet biscuits, plain, e.g. Nice, ginger *(one)*								
Cakes e.g. fruit, sponge, sponge pudding *(medium slice)*								
Sweet buns & pastries e.g. doughnuts, Danish pastries, cream cakes *(each)*								
Flapjacks (*each)*								
Fruit pies, tarts, crumbles (per individual pie/medium serving)								
Milk puddings, e.g. rice, custard, trifle *(medium serving)*								
Ice cream, choc ices *(one)*								
Chocolates,, toffee, sweets and other confectionary *(medium bar of*								

chocolate, one snack bar, one packet)								
Sugar added to tea, coffee, cereal *(teaspoon)*								
Crisps or other packet snacks e.g. Wotsits *(one packet)*								
Peanuts *(one packet)*								
Walnuts (medium serving)								
Other nuts (medium serving)								
FOODS & AMOUNTS	**Average Use In LAST WEEK (Tick ONE per line)**							
8. SOUPS, SAUCES AND SPREADS	None	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Vegetable soups *(medium bowl)*								
Meat soups *(medium bowl)*								
Sauces, e.g. white sauce, cheese sauce, gravy *(1/3 of plate or in dish)*								
Tomato based sauces e.g. pasta sauces *(1/3 of plate or in dish)*								
Tomato ketchup, brown sauce *(per tablespoon)*								
Relishes e.g. pickles, chutney, mustard *(per tablespoon)*								
Marmite, Bovril *(per teaspoon/slices of bread)*								
Jam, marmalade, honey, syrup *(per teaspoon/slices of bread)*								
Peanut butter *(per teaspoon/slices of bread)*								
Chocolate spread, chocolate nut spread *(per teaspoon/slices of bread)*								
FOODS & AMOUNTS	**Average Use In LAST WEEK (Tick ONE per line)**							
8. SOUPS, SAUCES AND SPREADS (continued)	None	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Dips e.g. houmous, cheese and chive *(per tablespoon/slices of bread)*								

Salad cream, mayonnaise (per tablespoon)								
Other salad dressing (oil, vinegar or cheese based per tablespoon)								
Reduced/ Low fat salad cream, mayonnaise (per tablespoon)								
Reduced/ Low fat other salad dressing (oil, vinegar or cheese based per tablespoon)								
FOODS & AMOUNTS	**Average Use In LAST WEEK (Tick ONE per line)**							
9. DRINKS	None	Once A Week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Tea *(cup)*								
Coffee, instant or ground *(cup)*								
Coffee whitener, e.g. Coffee-mate *(teaspoon)*								
Cocoa, hot chocolate *(cup)*								
Horlicks, Ovaltine *(cup)*								
Wine *(glass)*								
Beer, lager or cider *(half pint)*								
Port, sherry, vermouth, liqueurs *(glass)*								
Spirits, e.g. gin, brandy, whisky, vodka *(single)*								
Low calorie or diet fizzy soft drinks *(glass)*								
Fizzy soft drinks, e.g. Coca cola, lemonade *(glass)*								
Pure fruit juice (100%) e.g. orange, apple juice *(glass)*								
Fruit squash or cordial *(glass)*								

FOODS & AMOUNTS	**Average Use In LAST WEEK (Tick ONE per line)**							
10. FRUIT *(1 fruit or medium serving)*	None	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Apples *(each)*								

Pears *(each)*								
Oranges *(1x)*, satsumas, mandarins, tangerines, clementines *(all 2x)*								
Grapefruit *(1/2 a fruit)*								
Bananas *(each)*								
Grapes *(per small handful)*								
Melon *(1 medium slice)*								
Peaches *(1x)*, plums, apricots, nectarines *(2 – 3x)*								
Strawberries, raspberries *(per small handful)*, kiwi fruit *(each)*								
Tinned fruit *(1/2 tin)*								
Dried fruit, e.g. raisins, prunes, figs *(per small handful)*								
All Other Fruit (medium serving)								

FOODS & AMOUNTS	**Average Use In LAST WEEK (Tick ONE per line)**							
11. VEGETABLES Fresh, frozen or tinned *(medium serving)*	None	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Carrots *(2-3 table spoonfuls)*								
Cooked Spinach *(major ingredient in dish (e.g. curry) or per 2 – 3 tablespoonfuls)*								
Broccoli *(per 4 – 5 florets)*								
Brussels sprouts *(2-3 tablespoonfuls)*								
Cabbage *(2-3 tablespoonfuls)*								
Peas *(2-3 tablespoonfuls)*								
Green beans, broad beans, runner beans *(2-3 tablespoonfuls)*								
Marrow, courgettes *(major ingredient in dish or 2-3 tablespoonfuls)*								
Cauliflower *(major ingredient in dish (e.g. curry) or 2-3 tablespoonfuls)*								

Parsnips, turnips, Swedes *(2-3 tablespoonfuls)*								

FOODS & AMOUNTS	**Average Use In LAST WEEK (Tick ONE per line)**							
11. VEGETABLES Fresh, frozen or tinned ***(medium serving)***	None	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Leeks *(2-3 tablespoonfuls)*								
Onions *(per onion)*								
Garlic *(2 cloves)*								
Mushrooms *(handful of uncooked mushrooms, or 2-3 tablespoonfuls)*								
Sweet peppers *(per ½ pepper)*								
Beansprouts *(major ingredient in dish or (2-3 tablespoonfuls)*								
Mixed salad leaves, lettuce, rocket *(side-salad or per 1/3 plate)*								
Cucumber *(per ¼ cucumber)*								
Mixed vegetables (frozen or tinned) *(2-3 tablespoonfuls)*								
Watercress *(per bunch, or as a major ingredient in salad)*								
Red tomatoes *(2 medium tomatoes, ½ can of tomatoes)*								
Sweetcorn *(2-3 tablespoonfuls)*								
Beetroot *(1 medium)*								
Radishes *(3-4 pieces)*								
Coleslaw *(2-3 tablespoonfuls)*								
Avocado *(per ½ fruit)*								
Baked Beans *(per ½ tin)*								
Dried lentils, beans, peas *(2-3 tablespoonfuls, or major ingredient)*								
Tofu, soya meat, TVP, (*in dish e.g. curry),* Vegeburger *(each)*								

Other vegetables (medium serving)								
Yellow tomatoes *(2 medium tomatoes, ½ can of tomatoes)*								

YOUR DIET IN THE LAST WEEK, *continued*

12. What type of milk did you most often use?

Select one only

Full cream……❒

Channel Islands……❒

Dried milk……❒

Semi-skimmed……❒

Skimmed……❒

Soya……❒

Other……❒

None……❒

13. Approximately, how much milk did you drink each day, including milk with tea, coffee, cereals etc?

None……❒

Quarter of a pint (roughly 125mls)……❒

Half a pint (roughly 250mls)……❒

Three quarters of a pint (roughly 375mls)……❒

One pint (roughly 500mls)……❒

More than one pint (more than 500mls)……❒

14. What kind of fat did you use for cooking?

Please tick <u>all</u> that apply

Butter……❒

Lard/dripping……❒

Solid vegetable fat……❒

Margarine……❒

Vegetable oil……❒

Olive oil……❒

Walnut Oil……❒

Soya Oil……❒

None……❒

Other……❒

If "other" selected in question 14, please state……

15. Do you usually add salt to food while cooking?

Yes……❒

No……❒

16. Do you usually add salt to any food at the table?

Yes……❒

No……❒

17. Do you usually eat the fat on cooked meats?

Yes……❒

No……❒

18. Do you usually eat the skin on cooked meats?

Yes……❒

No……❒

19. Do you usually add sugar to drinks i.e. tea/coffee?

Yes……❒

No……❒

20. On average, in the past week, how many portions of fruit and vegetables did you eat per DAY?

Please estimate:……

21. On average, in the past week, how many servings of wholegrain foods did you eat per DAY?

Please estimate:……

22. Have you taken any of the following during the past week?

	None	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day
Vitamins (e.g. multivitamins, vitamin B, vitamin C, folic acid)						
Minerals (e.g. iron, calcium, zinc, magnesium)						
Fish oils (e.g. cod liver oil, omega-3)						
Other food supplements (e.g. oil of evening primrose, starflower oil, royal jelly, ginseng)						

- **Did you use any other food supplements? Please state below:**

1 _____
2 _____
3 _____

Thank you for taking the time to complete this questionnaire!!

Appendix 12: Values of phenolic in quinoa and buckwheat extracted by water, ethanol and methanol

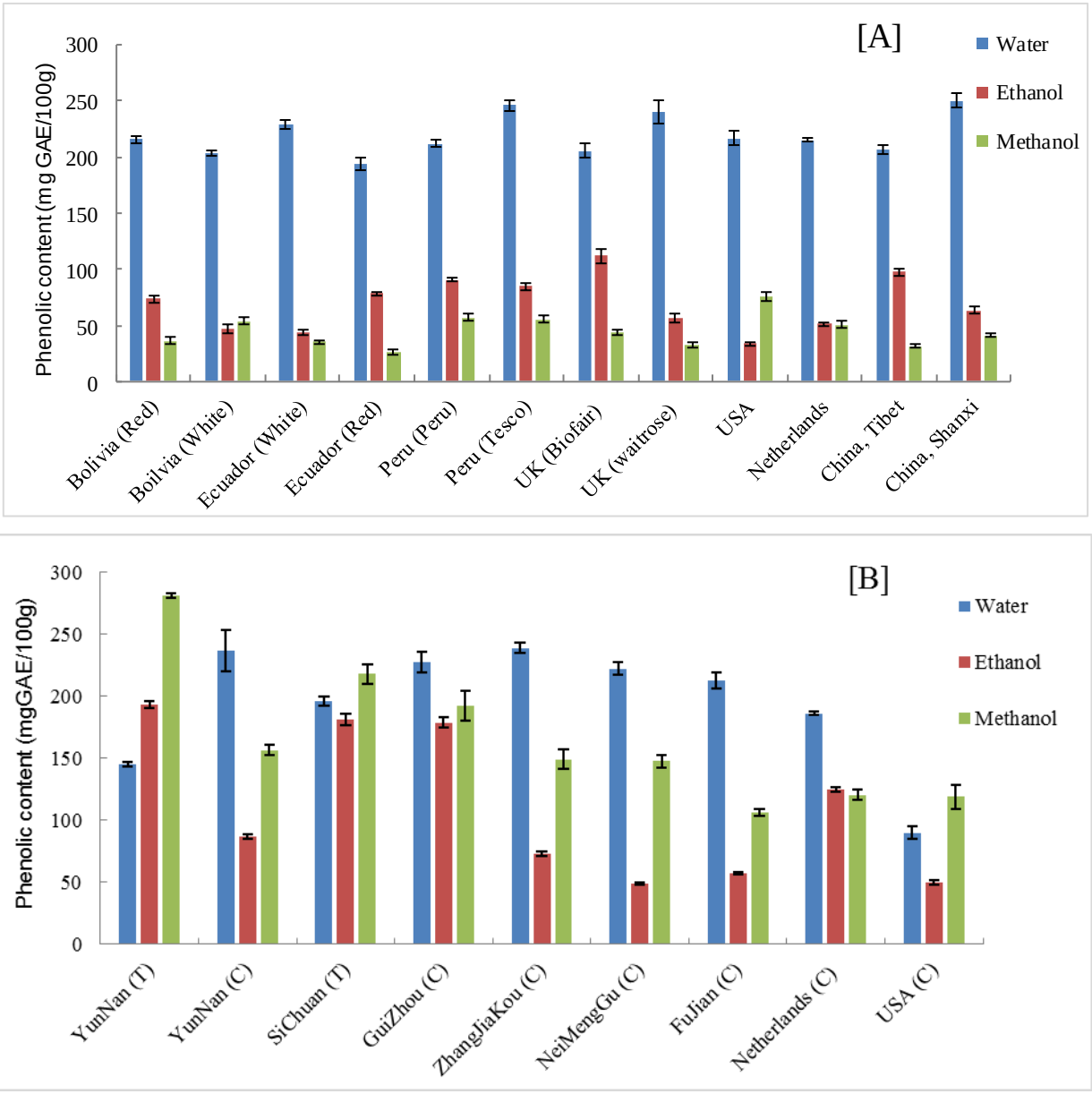


Figure. Total phenolic content (Gallic acid equivalent/100g) of water, ethanol and methanol extracts of different varieties and sources of quinoa[A] and buckwheat [B] T, Tartary; C, common.

Table. Value of phenolics in quinoa and buckwheat extracted by water, ethanol and methanol

	Phenolic content (mg GAE/g)		
Solvent	water	ethanol	methanol
Quinoa accessions			
Bolivia (Red)	216±3	73±3	36±3
Boilvia (White)	203±3	46±4	54±3
Ecuador (White)	229±4	44±3	35±1
Ecuador (Red)	193±6	78±2	27±2
Peru (Peru)	212±3	91±2	57±3
Peru (Tesco)	246±5	84±4	55±3
UK (Biofair)	206±7	112±6	43±2
UK (Brand Waitrose)	239±10	56±4	32±3
USA	217±7	33±2	76±3
Netherlands	215±2	51±2	51±3
China, Tibet	207±4	97±3	31±2
China, Shanxi	250±6	64±4	41±1
Buckwheat accessions			
YunNan (T)		193±3	281±2
YunNan (C)	236±16	87±2	156±4
SiChuan (T)	196±4	181±5	218±8
GuiZhou (C)	227±8	179±4	192±12
ZhangJiaKou (C)	238±4	73±2	149±8
NeiMengGu (C)	222±5	49±1	148±5
FuJian (C)	212±6	57±1	106±3
Netherlands (C)	186±1	124±2	120±4
USA (C)	90±5	50±2	119±10