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Inter-individual variability of polyphenol metabolism and colonic health

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Dedication

I would like to dedicate my thesis to my parents and my brother, Ibrahim. They are the ones who have supported me through my education abroad for the past eight years.

My parents travelled far from their home to follow me to the United States and the UK. I feel very lucky that they were able to be close to me and encourage my every step. I will be eternally grateful for their support, love and prayers.

To my brother, Ibrahim, who was always only a phone call or an email away. He has also been working hard on his education in another country, and I feel fortunate that we can experience this extraordinary time in our lives together.

I would also like to thank my grandfather, grandmother, and all of my aunts, uncles, nieces and nephews for their suppor, prayers, and well wishes.

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Abstract

It has been proposed that polyphenol-rich foods have a role in disease prevention and are associated with health benefits due to their antioxidant, anti-inflammatory, prebiotic, and antibacterial properties. However, associated health benefits depend on their intake, metabolism, and bioavailability. The metabolism and the bioavailability of polyphenols have been studied in young adults and show substantial variability. As the majority of polyphenols are metabolised in the colon, this may result in different bioactive microbial metabolites in the large intestine where they may have an impact on the risk of colorectal cancer (CRC). This variability could be due to: 1) dietary habits including intake polyphenol-rich foods; 2) ethnic-specific colonic microbiota; and 3) ageing and its effect on colonic physiology.

Little is known about the impact of ethnicity, ageing, and the risk of CRC on polyphenol metabolism. Therefore, this thesis aimed to investigate the effect of the factors that could have an impact on the colonic metabolism of dietary polyphenols in a human feeding study measuring the biomarkers of polyphenol metabolism, colonic fermentation, and gut health; and an *in-vitro* faecal fermentation study measuring the colonic metabolites of quercetin-3-O-rutinoside (rutin).

The first aim of this thesis (Chapter 3) was to examine the effect of ethnicity (Europeans versus Indians) on polyphenol metabolism. The findings of this study suggest that ethnicity could have a role on the colonic metabolism of polyphenols which could be due to the differences in disease incidence between countries such as the lowest risk of CRC in India among the world. The Indian group excreted less urinary phenolic acid after the high-polyphenol diet compared to the Europeans; however, Indians were more capable and faster in metabolizing rutin in the *in-vitro* model. This could be due to the differences in:

1. Genetics and its effect on gastrointestinal tract absorption.
2. Gut microbiota, as Indians have a significantly higher level of Bifidobacterium.
3. Gut environment, in particular the colonic pH and SCFA could have an influence as the colonic pH was lower in the Indian group.

4. Cultural daily diet between groups, as Indians significantly consumed a high amount of onions, tomatoes, chillies, spices, curry-based products, and yoghurt. These food types are high in polyphenols, fibre, and probiotics.

The second study of this thesis aimed to investigate the effect of ageing on polyphenol metabolism. The results suggest another factor, ageing, which could influence the colonic metabolism of polyphenols. The older group excreted less urinary phenolic acid and some of the acid was not detected in certain of the participants' urine compared to the younger group. However, the sum of the phenolic acid that formed after the faecal fermentation of rutin was not significantly different between the groups. This could suggest different reasons behind these variations. First, the lack of absorption of some phenolic acids by the older group as ageing was shown to decrease the colonic absorption. Secondly, the effect of ageing on gut microbiota composition and function. Thirdly, changes in dietary habits and physical activity may be influenced by ageing. Thus, this may suggest that older people can have fewer benefits of polyphenol metabolites which could be associated with an increase in risk for age-related diseases including CRC.

As the risk of CRC is different between countries and increases with age, the supportive findings of the first and second study suggest that ethnicity and ageing could have a role on the metabolism of polyphenols so this raises the questions whether a low intake of polyphenols can be one of the factors that may lead to CRC, or whether polyphenols can reduce the risk of CRC due to their colonic health benefits. Therefore, the last study examined the metabolism of polyphenols on patients who are at risk of CRC (history of polyps). No significant differences were observed between the healthy control and polypectomy groups in terms of the sum urinary phenolic acid excretion and phenolic acid formation in the faecal fluids. However, some phenolic acids were not detected in all of the urine samples of the polypectomy group as well as one acid in the faecal fermentation fluids, while some of the acids were not detected in few participants in the healthy group. No hard conclusion can be made from this study due to the small sample size. However, this study gives us an idea that there could be differences if a larger sample size were used. Therefore, more studies are needed to determine the effect of CRC risk as being one of the factors that can influence the metabolism of polyphenols.

In conclusion, the work of this thesis showed that ethnicity, ageing, and gut health are likely some of the key factors that could contribute to the variations in polyphenol metabolism which were observed previously by many *in-vivo* and *in-vitro* studies. These variations could result in bioavailability variation and consequential differences in the biological activity of polyphenol metabolites leading to differences in health and optimal health among individuals.

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Alkhaldy A, Edwards CA, Combet E. (2012) The effect of ageing on the colonic bacterial metabolism of dietary polyphenols. *Proceedings of the Nutrition Society* **71** (OCE2), E60

Alkhaldy A, Edwards CA, Combet E. (2014) Higher phenolic acid excretion in European than Indian volunteers after high polyphenol diet. *Proceedings of the Nutrition Society* **73** (OCE1), E1

Alkhaldy A, Edwards CA, Roxburgh C, Combet E. (2014) Higher phenolic acid excretion in polypectomy patients at risk of colorectal cancer. Proceedings of the Nutrition Society **73** (OCE1), E2

Abbreviations

BMI	body mass index
CBG	cytosolic β-glucosidase
COMT	catechol-O-methyltransferases
CRA	colorectal adenomas
CRC	colorectal cancer
ELISA	enzyme-linked immunoassay
EPIC	European Prospective Investigation into Cancer and Nutrition study
FAP	familial adenomatous polyposis
FFQ	food frequency questionnaires
FISH	fluorescence based in situ hybridization
FOBT	faecal occult blood test
FRAP	ferric reducing antioxidant potential
GAE	gallic acid equivalent
GC-FID	gas Chromatography – Flame Ionisation Detector
GC-MS	gas chromatography tandem mass spectrometry
HNPCC	hereditary Non-polyposis Colorectal Cancer
HPLC	high performance liquid chromatography
IQR	inter quartile range
LPH	lactate phlorizin hydrolase
MW	molecular weight
m/z	mass to charge ratio
n.d.	not detected
NEP	non-extractable polyphenols
OFN	oxygen-free nitrogen
qPCR	quantitative polymerase chain reaction
R_t	retention time
SCFA	short-chain fatty acids
SGLT	sodium-glucose-co-trasporter
UGT	uridine-5'-diphospho-glucuronosyltransferase
W.C	waist circumference
Δ	difference

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Author’s Declaration

I declare that the work in this thesis is the work of the author Areej Alkhaldy. I am responsible for the organisation, lab work, analysis and data processing, unless otherwise cited. The research for this thesis was carried out between January 2010 and May 2014.

Areej Alkhaldy

Chapter One

General Introduction

1.1 Introduction

Epidemiological and meta-analysis studies have shown that long term consumption of diets rich in plant foods, such as fruit, vegetables, and derived beverages protect against several chronic diseases including cardiovascular disease, cancer, diabetes, and neurodegenerative disease (Arts and Hollman, 2005, Graf *et al.*, 2005, Aune *et al.*, 2011). The potential protective action of these foods is due not only to amino acids, polyunsaturated fatty acids, vitamins, minerals, and dietary fibre, but also to a diversity of plant secondary metabolites, especially phenolic compounds and flavonoids (Crozier *et al.*, 2010).

Many studies have reported that higher intake of polyphenols may reduce the incidence of several chronic diseases such as: 1) cardiovascular diseases through polyphenols acting as an antioxidant, anti-inflammatory, and anti-platelet along with the polyphenols' ability to increase the level of high density lipoprotein and reduce the low density lipoprotein in plasma (Aviram *et al.*, 2000, Vita, 2005, Nardini *et al.*, 2007, Khan *et al.*, 2012); 2) cancer including stomach, colon, liver, lung, and skin cancer through the anti-estrogenic, anti-proliferation, anti-inflammatory and antioxidant properties of polyphenols, as well as their regulation of the immune system (Yang *et al.*, 2001a, Kamaraj *et al.*, 2007, Khan and Mukhtar, 2008, Pandey and Rizvi, 2009, Angst *et al.*, 2013); 3) diabetes type I and II through controlling the blood sugar by reducing and inhibiting the absorption of glucose in the gut or its uptake by peripheral tissues (Matsui *et al.*, 2002, Rizvi *et al.*, 2005, Zunino *et al.*, 2007, Dembinska-Kiec *et al.*, 2008, Jacques *et al.*, 2013); 4) neurodegenerative disease and the adverse effects of ageing through the effect of polyphenols and their antioxidant and anti-inflammatory properties (Joseph *et al.*, 2005, Shukitt-Hale *et al.*, 2008, Singh *et al.*, 2008).

In recent years, the research interest has migrated from purely examining the dietary intake of phenolic compounds to a deeper exploration their bioavailability (Crozier *et al.*, 2010). This is due to: 1) differences in polyphenol metabolism among the large variety of phenolic compounds and their classes; 2) the beneficial effect of these compounds being dependant on their bioavailability in the human body; 3) substantial variations between individuals in terms of phenolic compound metabolism.

1.2 Dietary polyphenols

Dietary polyphenols are secondary plant metabolites, classified on the basis of the number of phenol rings they contain and the structural elements that bind the rings to each other (Crozier *et al.*, 2006). There are more than 8,000 polyphenolic compounds which have been identified in different plants (Crozier *et al.*, 2006, Pandey and Rizvi, 2009). They are found in conjugated forms with one or more types of sugar (polysaccharide or monosaccharide) attached to the hydroxyl groups, though linkages between the sugar and an aromatic carbon also exist. A linkage with other compounds such as carboxylic and organic acids, amines, lipids, and phenol is also common (Kondratyuk and Pezzuto, 2004). They are classified as flavonoids and non-flavonoids (Del Rio *et al.*, 2013).

1.2.1 Flavonoids

Flavonoids are the most abundant phenolics in the plant kingdom with more than 4,000 phenolic compounds (Iwashina, 2000). They are responsible for the attractive colours of fruit and leaves (de Groot and Rauen, 1998, Hollman and Katan, 1999). They comprise 15 carbons with two aromatic rings connected by a three-carbon bridge and are based on the variation of the heterocycle type (Figure 1-1) (Vermerris and Nicholson, 2006). The majority of flavonoids naturally occur as glycosides rather than aglycones. The differences within the group arise from variation in the hydroxyl group number and arrangement and their extent of alkylation and/or glycosylation. The water solubility of flavonoids increases with the presence of sugar and hydroxyl groups, while the methyl group provides lipophilic properties (Harborne and Harborne, 1998). They are divided into flavonols, flavones, isoflavones, flavanones, anthocyanidins, and flavan-3-ols (Del Rio *et al.*, 2013).

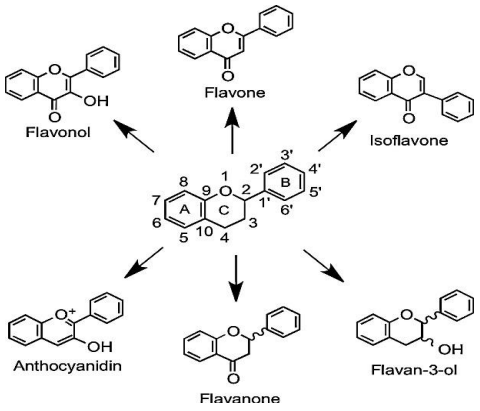


Figure 1-1: Structures of flavonoids (Del Rio et al. 2012).

1.2.1.1 Flavonols

Flavonols are the most abundant of the flavonoids in the plant kingdom with the exception of fungi and algae (Del Rio *et al.*, 2013). They are commonly found as O-glycosides such as kaempferol, rutin, quercetin, isorhamnetin, and myricetin (Figure 1-2). The conjugation position mostly occurs at position 3 of the C ring but 5, 7, 4′, 3′ and 5′ substitutions also occur (Herrmann, 1976). Kaempferol is found in many fruits and vegetables; rutin and quercetin are found in onions, tomatoes, apples, and tea; isorhamnetin can be found in onions and pears, and myricetin in berries, maize and tea (Hertog *et al.*, 1992).

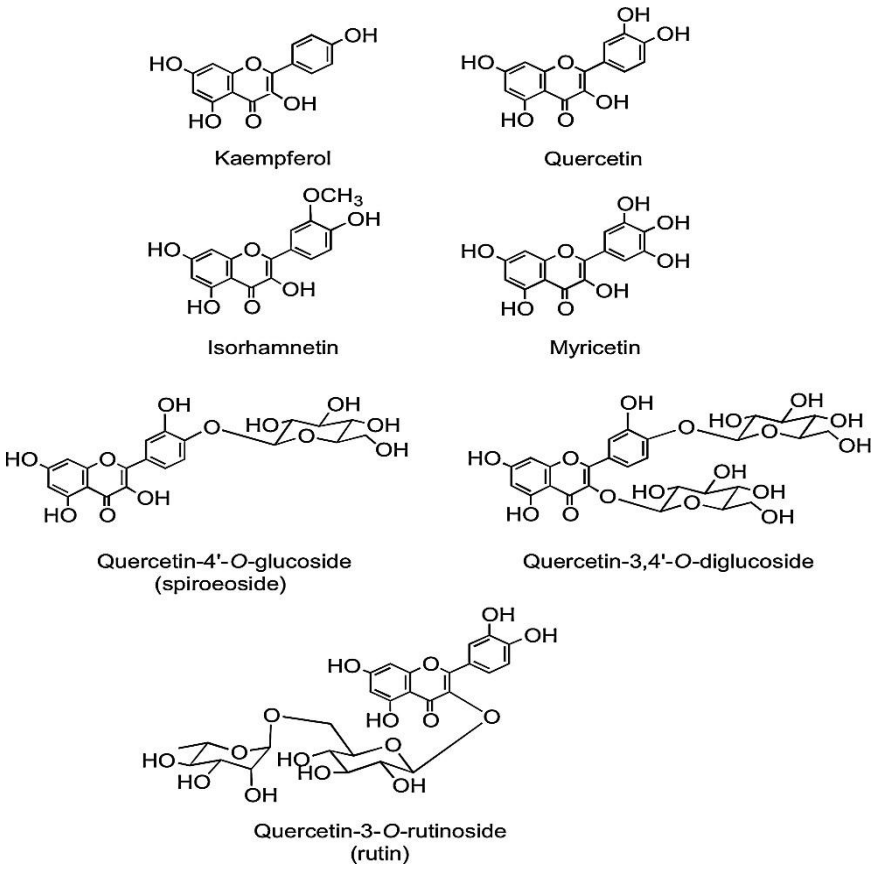


Figure 1-2: Common flavonoids structure (Del Rio et al. 2012)

1.2.1.2 Other Flavonoids

Other flavonoids can exist in different forms:

1. Flavones have the same structure as flavonols but no oxygenation at position C3. A range of substitutions is possible with flavones, including hydroxylation, methylation, O and C-glycosylation, and alkylation (Jaganath *et al.*, 2008). They have been found in celery, sweet red pepper, parsley and other herbs (Hertog *et al.*, 1992).

2. Flavanones are mainly represented as naringenin and hesperetin, which are characterized by the absence of double bond C2-C3 and the presence of a chiral centre at C-2. Flavanones occur as hydroxyl, glycosylated, and O-methylated derivatives (Del Rio *et al.*, 2013). The most common flavanone glycoside is hesperetin-7-O-rutinoside (hesperidin). They are present in high amounts in citrus fruits such as oranges in heperetin-7-rutinoside (hesperidin) and naringenin-7-rutinoside (narirutin) forms (Peterson *et al.*, 2006).

3. Anthocyanidin conjugates with sugars and organic acids to generate a multitude of anthocyanins of differing colours, ranging from orange and red to blue and purple according to the pH (Ozeki *et al.*, 2011). They can be found in red, blue or violet colour edible fruits including grapes, plums and berries (Peterson *et al.*, 2006).

4. Flavan-3-ols are naturally not conjugated to sugars. They range from the simple monomers (+)-catechin and its isomer (-)-epicatechin, to the oligomeric and polymeric procyanidins. They are typically present in various fruits and vegetables such as apples, pears, grapes and peaches as well as black tea, green tea, and red wine (Arts *et al.*, 2000, Del Rio *et al.*, 2013).

5. Isoflavones have the B ring linked at the C3 rather than C2 position (Del Rio *et al.*, 2013). Isoflavones are mostly represented by daidzein and genistein. The main dietary source is soybeans and soy products (Liggins *et al.*, 2000).

1.2.2 Non-flavonoid phenolics

1. Hydroxybenzoic acid content is generally very low with the exception of certain red fruits, black radish, and onions. Tea is also an essential source of gallic acid (Manach *et al.*, 2004).

2. Hydroxycinnamic acid is more common than hydroxybenzoic acid and consists mainly of p-coumaric, caffeic, ferulic and sinapic acids (Pandey and Rizvi, 2009). They are rarely found in free form except in processed food.

3. Stilbenes have a C6–C2–C6 structure (Langcake and Pryce, 1977). They exist in very low quantities in the human diet. The main stilbene is 3, 5, 4′-trihdroxystilbene (resveratrol). Red wines contain a range of stilbene derivatives but in very low concentrations compared to other (poly) phenolic components (Crozier *et al.*, 2010).

4. Lignans are formed by two phenylpropane units and are one of the main classes of phytoestrogens (Manach *et al.*, 2004). Linseed is the main source of lignans, which contains secoisolariciresinol and low quantities of matairesinol (Adlercreutz and Mazur, 1997). Traces of lignans are commonly found in cereal, grains, fruit and certain vegetables (Milder *et al.*, 2005).

1.3 Dietary polyphenol sources

The ubiquitous nature of polyphenols in particular flavonoids in plants ensures that they are found in large quantities in the human diet. Some polyphenols, such as quercetin, occur in all plants such as fruit, vegetables, cereal, tea, and wine, while others are found in specific foods such as flavanones in citrus fruit and isoflavones in soya (Table 1-1) (Manach *et al.*, 2004, D'Archivio *et al.*, 2007).

Table 1-1: Flavonoid dietary selected sources.

Phenolic compounds	Typical sources
Flavonols	Yellow onion
Rutin	Cherry tomato
Quercetin	Apple
Muricetin	Tomato
	Black tea
	Green tea
Flavones	Parsley
Apigenin	Celery
Luteolin	
Flavanones	Orange juice
Hesperetin	Grapefruit juice
Naringenin	
Anthocyanins	Cherries
Cyanidin	Strawberries
Pelargonidin	Plums
	Grapes
	Blackberries
	Black currants
	Red wine
Flavan-3-ols	Chocolate
Catechin	Green tea
epicatechin	Black tea
	Red wine
Isoflavones	Soy beans
Daidzein	Tofu
Genistein	Soy milk

1.4 Assessment of dietary polyphenol intake

It is important to have accurate quantitative information about polyphenol intake to establish the evidence associating the intake of dietary polyphenols with proposed health benefits. The most commonly used dietary assessment methods are diet histories, food frequency questionnaires (FFQ), and diet diaries, especially in epidemiological studies (Kristal *et al.*, 2005, Chan *et al.*, 2008, Zamora-Ros *et al.*, 2012).

Dietary assessment methods depend on using food composition databases. Previously, there was little accurate data on polyphenol content of foods but these databases have improved over the past few years. These developments include the European Food Information Resource Network (EuroFIR), internet-deployed database (EuroFIR BASIS) which combines data on food composition and biological effects for plant-based bioactive compounds and Phenol-Explorer which is an online comprehensive database of polyphenol contents in food (Costa *et al.*, 2010, Neveu *et al.*, 2010). Despite these advances, it is still challenging to measure the dietary intake of polyphenols accurately for several reasons:

1. The currently available databases have restricted information on the concentration and diversity of polyphenols in plants. For example: a) the polyphenol profiles of all varieties of apples are identical but the concentration may range from 0.1 to 5 g total polyphenols/kg fresh weight and as high as 10 g/kg in certain varieties of cider apples (Sanoner *et al.*, 1999); b) the ripeness of the plant, the time of harvest, UV exposure, condition of storage, and processing are also important factors that can affect the concentration of polyphenols (Burda *et al.*, 1990, Spanos *et al.*, 1990, Spanos and Wrolstad, 1992, Miller *et al.*, 1995, van der Sluis *et al.*, 2001); c) the distribution of polyphenols is uneven in plant tissue, with more located in the outer layers and peel (Burda *et al.*, 1990); d) cooking can also affect the content of polyphenols. For example, onions and tomatoes lose between 75 to 80% of the quercetin after boiling and 30% after micro-waving (Crozier *et al.*, 1997).

2. There is limited data on non-extractable polyphenols (NEP) in foods. Conventional extraction and detection procedures do not account for NEP. As a result, the contribution of NEP to the overall polyphenol content of foods is still underrepresented in dietary intake data and bioavailability studies (Arranz *et al.*, 2010, Perez-Jimenez *et al.*, 2013).

3. The dietary assessment methods may be affected by recall error and difficulties in estimated portion size (Rimm *et al.*, 1992, Young and Nestle, 1995, Hernandez *et al.*, 2006).

4. Relying on self-reporting of dietary habits can be affected by under-reporting of unhealthy foods such as high sugar and fat intake and over-reporting of fruit and vegetables. These methods are limited and fail to measure total intake accurately (Spencer *et al.*, 2008).

1.5 Dietary polyphenol intake

Two thirds of consumed polyphenols are flavonoids (Scalbert and Williamson, 2000). However, there is no reference for dietary intake of polyphenols. This is because polyphenols, unlike vitamins and minerals, are not essential components in the diet. However, given the potential role of flavonoids in disease prevention, it is necessary to understand the differences in flavonoids consumption patterns in relation to disease incidence. To date, there are a number of descriptive studies that have estimated the flavonoid intake in different countries (Table 1-2).

These studies have demonstrated large variations in flavonoid intake between countries, which could be due to two main reasons. The first reason is methodological, as there were differences in the analytical methods and flavonoid database used to analyse the flavonoid intake. For example, the Folin-Ciocalteu method, which was used by Kuhnau (1976), provided the highest amount of flavonoid intake because vitamin C which is present in fruit and vegetables in large quantities also reduces the Folin reagent. However, high performance liquid chromatography provided the lowest estimate of intake in Denmark, the Netherlands, and two other studies from Finland. This could be an actual underestimation because some polyphenols in the food samples may escape detection by the chromatography and be considered as unknown compounds (Santos-Buelga and Scalbert, 2000). Moreover, the differences in the presence or absence of particular polyphenol data in the flavonoid database could introduce a bias when comparing flavonoid intake between countries. For example, the oxidation products of catechins, thearubigins, are not included in the Phenol-Explorer database, while thearubigins are included in the USDA database which could explain the high amount of flavonoids in the Australian study (454 mg/day).

Secondly, there may be differences in eating habits and culture, which could affect the intake of flavonoids. For instance, Mediterranean countries are recognized for their higher consumption of fruit, vegetables, legumes, nuts, and olive oil than non-Mediterranean countries (Zamora-Ros *et al.*, 2013a).

On the other hand, the use of a single dietary recall is less likely to reflect an individual's accurate daily consumption than repeated dietary recall. Moreover, the flavonoid intake could be higher in all studies than when considering the intake of flavonoids from other sources such as spices and herbs. Lastly, data from the USDA Flavonoid & Phenol-Explorer databases are still lacking in seasonal variations between food as well as the processed and cooked foods data, which could overestimate flavonoid intake.

Many important factors should be considered when estimating the intake of flavonoids. According to Chun *et al.* (2007) the intake of flavonoids can be affected by differences between individuals in terms of their gender, age, ethnicity, health, and income level. The intake of fruit and vegetables was low in the lower socioeconomic group, which means there was a lower amount of essential nutrients, vitamins, minerals, and flavonoids, and in turn could be a variation in disease prevalence. Moreover, flavonoid intake tended to be lower in the oldest group between 65 to 74 years old (Zamora-Ros *et al.*, 2013a). In addition, the US study found that the intake of phenolic acid was lower in black men than in white men (130.6 mg/day versus 216.8 mg/day, respectively) due to differences in tea intake (Chun *et al.*, 2007). Understanding the effect of these factors could help categorise individuals depending on their flavonoid intake in relation to disease incidence and the optimum health benefit.

To date, there are some studies that have looked at the association between the intake of flavonoids and disease risk such as cardiovascular disease (McCullough *et al.*, 2012), type 2 diabetes (Knekt *et al.*, 2002, Wedick *et al.*, 2012), Parkinson's disease (Gao *et al.*, 2012), and colorectal cancer (CRC) (Zamora-Ros *et al.*, 2013b). Therefore, further studies should consider the best available dietary and analytical methods for flavonoid intake estimation while considering all previous studies' limitations.

Table 1-2: Flavonoid intake (mg/day) reported in various countries.

Country/ References	**Source of data/Subjects (n)**	**Dietary methods**	**Analytical methods**	**Flavonoid intake** (mg/day)
United States Kuhnau (1976)	Not available	Not available	FolinCiocalteau method	~1000
Denmark Justesen *et al.* (1997)	Danish Household Consumption survey, 1987. (n= not available)	Dietary history	High performance liquid chromatography	26
Netherlands Hertog *et al.* (1993)	Danish Household Consumption survey, 1987-1988. (n=4112)	48-h dietary recall	High performance liquid chromatography	23
Finland Ovaskainen *et al.* (2008)	National dietary surveys in Finland, 2003–2005. (n=2007)	48-h dietary recall	High performance liquid chromatography	33
Finland Knekt *et al.* (2002)	The Finnish Mobile Clinic Health Examination Survey. 1966–1972. (n=10054)	FFQ	High performance liquid chromatography	24.2
Australia Johannot *et al.* (2006)	National Nutrition Survey, 1995. (n=17326)	24-h dietary recall	USDA Flavonoid Database	454

*Continued overleaf

Country/ References	**Source of data/ Subjects (n)**	**Dietary methods**	**Analytical methods**	**Flavonoids intake** (mg/day)
United States Chun *et al.* (2007)	National Health and Nutrition Examination Survey, 1999-2002. (n=8809)	24-h dietary recall	USDA Flavonoid Database	189.7
Greece Dilis and Trichopoulou (2010)	*European* Prospective Investigation into Cancer and Nutrition (*EPIC*) study, 1994-1999. (n=28575)	FFQ	USDA Flavonoid Database	92
Spain Zamora-Ros *et al.* (2010)	*European* Prospective Investigation into Cancer and Nutrition (*EPIC*) study, 1992-1996. (n=40683)	Diet history questionnaire	USDA Flavonoid Database	313.3
Mediterranean /non-Mediterranean countries Zamora-Ros *et al.* (2013a)	*European* Prospective Investigation into Cancer and Nutrition (*EPIC*) study, 1992-2000 (n=35628)	24-h dietary recall software (EPIC-Soft)	USDA Flavonoid & Phenol-Explorer databases	Mediterranean Countries = 370.2 Non-Mediterranean country= 373.7

1.6 Biomarkers of dietary polyphenol intake

A nutrition biomarker can be defined as a recent or long term indicator of nutritional status, dietary intake, or an index of nutrient metabolism (Potischman and Freudenheim, 2003). Biological biomarkers such as measurements in blood and urine are essential for estimating an accurate intake of dietary polyphenols (Spencer *et al.*, 2008). There are several techniques to measure polyphenol metabolites in biological samples varying from the simple photometric to the advanced chromatography techniques which have been used as a biomarker measurement to quantify the intake of polyphenols (Robbins, 2003, van Dorsten *et al.*, 2010, Combet *et al.*, 2011, Stalmach *et al.*, 2011).

1.6.1 Simple colorimetric-based methods

The most common colorimetric method used to measure the total amount of phenolic compounds is the Folin–Ciocalteu assay, which has been used for many years to quantify phenolic compounds in plant and biological samples (Roura *et al.*, 2006, Cicco *et al.*, 2009). Medina-Remón *et al.* (2009) reported that the Folin–Ciocalteu assay is fast, environmentally friendly, and cheaper and simpler than other methods allowing measurement of a large number of samples. This assay has been validated in different study types such as prospective, randomized, crossover trials, clinical trials, and cross-sectional studies. In these studies, the total phenols in urine were correlated with the intake of polyphenols; however, this assay is not specific to phenolic compounds and other substances such as aromatic amines, sulphur dioxide, ascorbic acid, organic acids, Fe (II), and non-phenolic substances can interfere with the reading (Roura *et al.*, 2006, Medina-Remon *et al.*, 2009). The Folin-Ciocalteu method will also detect urinary (momo)-phenols (phenol, cresol, anisole) which are not all derived from polyphenols, being fermentation products of aromatic acids. Medina-Remón *et al.* (2009) showed that solid phase extraction (SPE), as a cleaning-up procedure, can improve the assay by increasing the recovery of larger numbers of polyphenols from the samples; however, using SPE does not make the Folin-Ciocalteu assay more specific for urinary phenols.

Besides quantifying phenolic compounds, some studies also measure the antioxidant power of the polyphenolic compounds in plant and biological samples using different types of assay. The ferric reducing ability of plasma (FRAP) assay is the most common because it is inexpensive, simple, and highly reproducible and offers an antioxidant index of biological fluids using technological devices that can be used in every laboratory (Benzie

and Strain, 1996, Pulido *et al.*, 2000). However, the FRAP assay was developed based on the theory that the redox reactions proceed quickly and are completed within four to six minutes. In the case of phenolic compounds, this could be a limitation as the absorbance of certain polyphenols such as caffeic acid, tannic acid, ferulic acid, and quercetin, gradually increase over several hours (Pulido *et al.*, 2000). Moreover, this assay is not specific to phenolic compounds as it can measure other antioxidant compounds such as vitamin C, vitamin E, uric acid, bilirubin, and α-tocopherol (Benzie and Strain, 1996).

1.6.2 Advanced chromatography-based methods

The simple quantitative colorimetric method is challenging due to 1) the influence of the non-phenolic components in biological or food extracts which can behave as reducing agents, and 2) the need to identify individual phenolic compounds which is not possible using the colorimetric method. In the last twenty years, chromatography techniques such as HPLC, liquid chromatography–mass spectrometry (LC-MS), and gas chromatography mass spectrometry (GC-MS) have been used to measure phenolic compounds and their metabolites in plant and animal samples (Robbins, 2003, Miniati, 2007). GC-MS has become the best identification tool to overcome the low volatility of these compounds especially phenolic acids using derivatization techniques (Robbins, 2003).

In this thesis, both simple and advanced techniques were used to measure phenolic compounds. Moreover, urine samples were used to measure the metabolites of polyphenols due to several reasons: 1) the short half-life of polyphenol metabolites in plasma, 2) lack of interferer molecules such as protein which is present in plasma, 3) the ability to detect very low quantities, and 4) greater availability to measure the outcome of diet over a longer time period (Miniati, 2007, Spencer *et al.*, 2008).

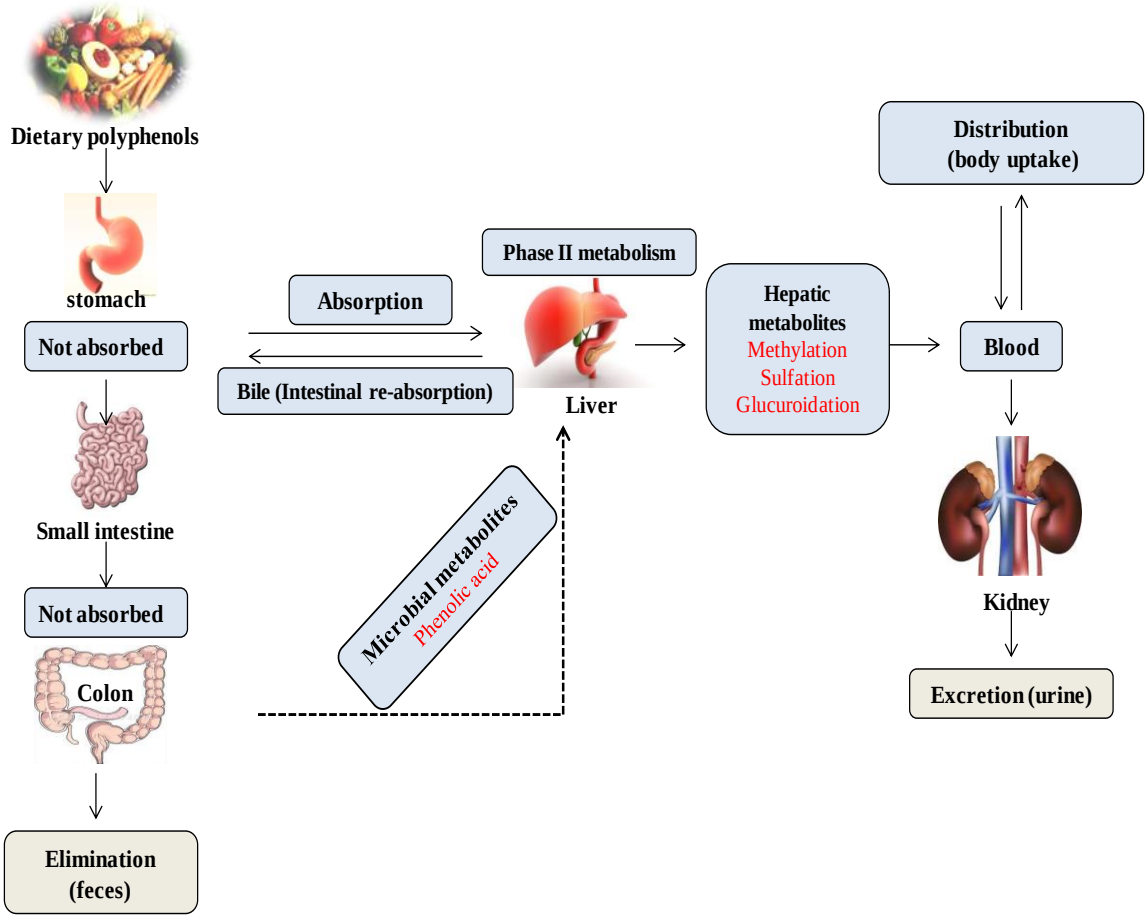
1.7 Dietary polyphenol metabolism and absorption

A high intake of flavonols has been associated with a wide range of health benefits, in particular, the possibility of reducing the risk of CRC (Jin *et al.*, 2012a, Woo and Kim, 2013b). However, large inter-individual variation in polyphenol metabolism and bioavailability in terms of flavonoid metabolites, phenolic acids, detected in plasma, urine, and faecal fluids have been reported previously (Manach *et al.*, 1998, Graefe and Veit, 1999, Moon *et al.*, 2000, Graefe *et al.*, 2001, Rechner *et al.*, 2002a, Olthof *et al.*, 2003, Jaganath *et al.*, 2006, Jaganath *et al.*, 2009, Roowi *et al.*, 2010). None of these studies

investigated the reasons behind these variations. However, these variations could be due to different factors such as differences in gut microbiota, dietary habits, and/or food matrix interactions (Manach *et al.*, 2005, Kemperman *et al.*, 2010, Bolca *et al.*, 2013) which are further dependent on ethnicity, age and colonic health. Because these factors could be the reasons behind the variations in polyphenol metabolism, bioavailability, and biological properties, and because the incidence of chronic disease, in particular CRC, is different between countries and increases with age, this raises the importance of studying the factors that can affect the metabolism of polyphenols.

Figure 1-3 show the fate of dietary polyphenolic compounds after oral consumption (Scalbert and Williamson, 2000). Most flavonoids found in food are not in free form. They are in the form of glycosides, esters, or polymers which cannot be absorbed directly (Manach *et al.,* 2004). When flavonoid compounds are consumed they are released from the matrix after mastication and interaction with saliva, which contains α-amylase for starch digestion in the mouth. Some of the flavonoids become deglycosylated in the mouth by oral epithelial cells or the microbiota (Walle *et al.*, 2005). Dietary polyphenols then pass to the stomach where the process of reducing the size of the food increases the release of the phenolic compounds from the matrix (Scalbert and Williamson, 2000, Manach *et al.*, 2005, Thilakarathna and Rupasinghe, 2013).

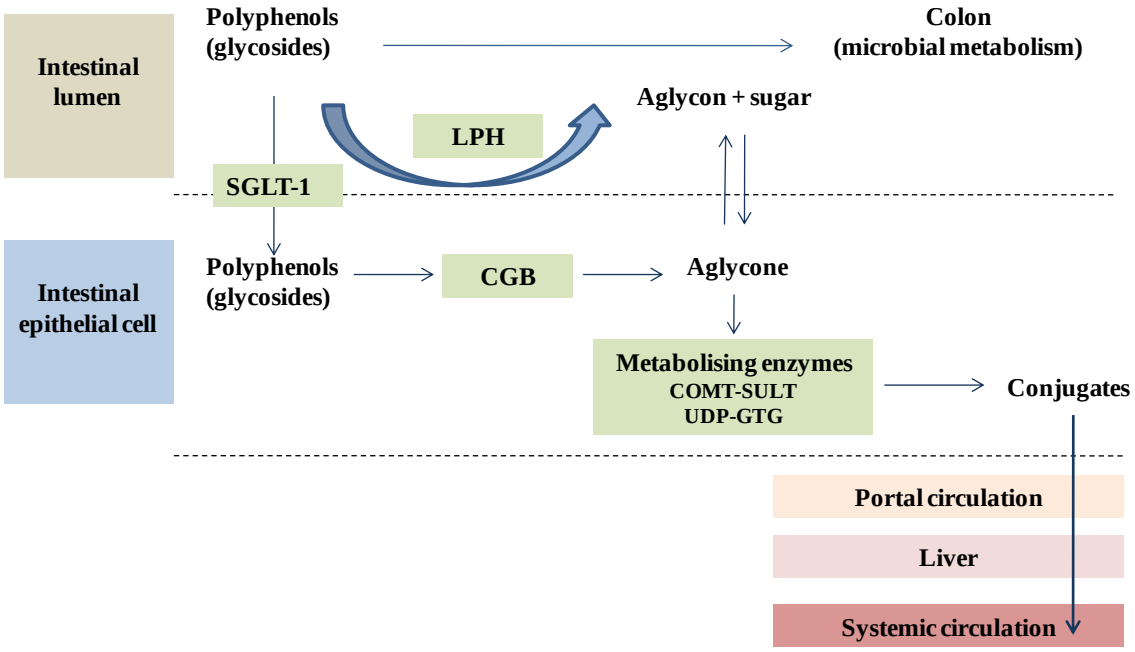
Figure 1-3: Proposed routes of polyphenols in humans (Adapted from Scalbert and Williamson 2000).



Polyphenols that reach the small intestine have two possible pathways of metabolism (Figure 1-4): First, polyphenols may form aglycones after being hydrolyzed by endogenous luminal lactase phlorizin hydrolase (LPH) before being absorbed by the intestinal mucosal brush border membrane (Hollman *et al.*, 1997, Day *et al.*, 2000). Aglycones can then enter the intestinal epithelial cell passively where they could be conjugated or cross the intestinal membrane to the portal circulation and then to the liver (Day *et al.*, 2000, Scalbert and Williamson, 2000). The second pathway of absorption may occur when the polyphenols are absorbed directly by the sodium-glucose-co-transporter (SGLT). Then, in the intestinal epithelial cell, the intact glucoside could cross the membrane directly into the portal circulation or be hydrolysed by cytosolic β-glucosidase (CBG) to form the aglycone. These two hydrolyses pathways of glucoside conjugates are recognized as "LPH/diffusion" and "transport /CBG". The released aglycones will undergo sulfation, glucuronidation and/or methylation by sulfotransferases (SULT), uridine-5'-diphospho-glucuronosyltransferase (UGT) and catechol-O-methyltransferases (COMT). The metabolites of these two pathways are absorbed into the portal vein of the blood stream

and then rapidly reach the liver for phase II metabolism. The enterohepatic recirculation may be excreted into the bile back to the small intestine (Scalbert and Williamson, 2000).

Figure 1-4: Proposed mechanisms of dietary polyphenol metabolism in the lumen and enterocytes of the digestive tract (Adapted from Nemeth et al. (2003)).



Abbreviations: SGLT, sodium-glucose-co-transporter; LPH, lactate phlorizin hydrolase; CBG, cytosolic β-glucosidase; UDP-GT, uridine-5´-diphosphate glucuronosyltransferase; COMT, catechol-O-methyltransferase; SULT, sulfotransferase

Most of the glycoside phenolic compounds that are resistant to acid hydrolysis in the stomach, and not absorbed in the small intestine or are still bound to the food matrix pass to the colon (Manach *et al.*, 2004). In the colon, phenolic compounds will be exposed to the large diverse anaerobic microbial population. These compounds are deconjugated by the colonic microbial enzymes (α-rhamnosidase, β-glucosidase, and β-glucuronidase) resulting in aglycone and phenolic acid formation (Bokkenheuser *et al.*, 1987, Aura, 2008). For example, rutin is not absorbed in the upper intestinal tract (Bokkenheuser *et al.*, 1987). The gut absorption of rhamnosides requires deglycosylation by colonic microbiota as suggested by their delayed absorption compared with glycosides.

Once the absorbed metabolites from the stomach, small intestine, and colon enter the circulatory system, they are transported to the liver via the portal vein. In the liver, phenolics can be conjugated (hepatic metabolites) to form methylated, sulphated and glucuronide metabolites during phase II metabolism (Scalbert and Williamson, 2000, Rechner *et al.*, 2001, Rechner *et al.*, 2002b, Zhao *et al.*, 2004). Circulating phenolics and

conjugated derivatives are extensively bound to albumin (Dangles *et al.*, 2001, Khan *et al.*, 2011). In the end, phenolic metabolites can be transported and distributed around the body tissues or excreted via urine as hepatic sulphate and glucuronide conjugates (Manach *et al.*, 2004, Crozier *et al.*, 2010)

1.7.1 Factors affecting polyphenol metabolism and absorption

The absorption and metabolism of polyphenols is influenced by several factors: 1) the structure of polyphenols such as molecular weight, glycosylation, and esterification (Hollman *et al.*, 1999, Olthof *et al.*, 2001, Shoji *et al.*, 2006); 2) the food matrix (Mullen *et al.*, 2008, Roowi *et al.*, 2009, Egert *et al.*, 2012); 3) changes in gut microbiota as a result of dietary habits, ageing, stress, or diseases (Gavini *et al.*, 2001, Baker *et al.*, 2009, Tiihonen *et al.*, 2010, Chen *et al.*, 2012, Flint, 2012).

1.7.1.1 Polyphenol structure

A high molecular weight (MW) can greatly affect the metabolism of polyphenols. For example, the larger molecular weight of the polymeric proanthocyanidins (MW< 578) makes it impossible to be absorbed in the small intestine. In the colon, the proanthocyanidins are catabolised by the gut microbiota and result in smaller molecules that can be absorbed in the circulation system (Donovan *et al.*, 2002). Shoji *et al.* (2006) reported that the colonic microbial metabolites of apple proanthocyanidins took one hour longer to peak in the plasma than the small molecular weight of flavan-3-ols. In addition the high molecular weight of tea theaflavins (MW568) may explain their low recovery in urine (Donovan *et al.*, 2002).

Glycosylation of most polyphenols in plants influences their absorption and thus their biological properties. In the case of flavonols, the associated sugar has an influence on the absorption. For example, quercetin glucosides from onions are absorbed five times more than quercetin-3-rutinoside (Hollman *et al.*, 1999). This is due to the enzymes in the small intestine, LPH and/or CβG, not being able to hydrolyse the rutinoside sugar. For that, rhamnoside is required which is provided later by the gut microbiota. In ileostomized volunteers, half of the quercetin glucosides in onions were absorbed in the small intestine, while the absorption of a rhamnoglucoside of quercetin was one half to one third that of quercetin glucoside (Hollman *et al.*, 1995). The maximum concentration of quercetin-3-rutinoside in the plasma was reached after nine hours of ingestion (Hollman *et al.*, 1997) suggesting colonic absorption.

Esterification can also influence intestinal absorption. For instance, in human urine the recovery of galloylated catechins after consumption of black tea was approximately 10-fold lower than the non-galloylated catechins (Warden *et al.*, 2001). Moreover, caffeic acid (95%) is better absorbed in the small intestine than its ester with quinic acid, and chlorogenic acid (33%) (Olthof *et al.*, 2001).

1.7.1.2 Food matrix

There are only a few studies on the influence of the food matrix, such as in dairy products and carbohydrates, on the absorption and excretion of polyphenols. Mullen *et al.* (2008) tested the effect of double cream on the bioavailability of pelargonidin-3-O-glucoside from strawberries in humans. Pelargonidin-O-glucuronide appeared rapidly in the blood and reached a maximum concentration of 274± 24 nmol/L after 1.1± 0.4 hours; however, when the strawberries were consumed with 100 ml of double cream the concentration was delayed by more than one hour. This could be due to the fat content in the cream (48%), which stimulated duodenal and ileal fat receptors and inhibited gastric emptying and slowed mouth to cecum transit time (Mullen *et al.*, 2008). The same group investigated the effect of yoghurt on urinary phenolic acid excretion derived from the colonic metabolism of flavanones after ingestion of orange juice. Yoghurt had an effect on the metabolism of orange juice. The excretion of phenolic acid in urine decreased after the consumption of the orange juice with yogurt from 62± 18 µmol to 9.3± 4.4 µmol. This might be due to bulk slowing of the meal to the colon (Roowi *et al.*, 2009). Moreover, Rodriguez-Mateose *et al.*(2012) reported that the intake of cocoa flavanols containing maltitol rather than sucrose significantly lowered total flavonol absorption and lowered peak metabolite concentration in the plasma. The study suggested that carbohydrates of the food matrix are important in determining the absorption of flavonol in the small intestine. Another study by Egert *et al.* (2012) examined the absorption of 130 mg of quercetin either from quercetin enriched cereal bars or quercetin capsules. The data showed that isorhamnetin (3'-O-methyl quercetin) and tamarixetin (4'-O-methyl quercetin) from the enriched cereal bars were five times higher in the plasma compared to the capsules after ingestion.

1.7.1.3 The role of colonic microbiota and its modulation by dietary habits, ethnicity and ageing

The human gut microbiota is a diverse and complex community and plays a fundamental role in human health in general and in colonic health. The gut microbiota of healthy people is thought to confer a number of health benefits such as pathogen protection, nutrition,

metabolism and immunity (Guinane and Cotter, 2013). Recently, it has been estimated that there are between 500-1000 different microbial species in the gastrointestinal tract with up to 10^{12} cells per gram in the colon (Qin *et al.*, 2010, Huttenhower *et al.*, 2012). The most common bacteria in the colon are *Bacteroides*, *Clostridium*, *fusobacterium*, *Eubacterium*, *Ruminococcus*, *Peptococcus*, *Peptostreptococcus*, and *Bifidobacterium* (Manson *et al.*, 2008).

Interaction between gut microbiota and dietary polyphenols was reported to affect the metabolism of dietary polyphenols, in particular flavonoids which are known to reach the colon (Winter *et al.*, 1989, Winter *et al.*, 1991, Manach *et al.*, 2004). So far, only a few species of gut microbiota responsible for metabolism of polyphenols have been identified. For example, *Butyrivibrio spp*. was reported to be able to cleave the C ring of rutin and quercitrin but not the C ring quercetin (Krishnam *et al.*, 1970, Krishnam.Hg *et al.*, 1970). However, quercetin is cleaved by *Eubacterium oxidoreducens* (Krumholz and Bryant, 1986). *Flavonifracror plautii* was reported to convert quercetin and taxifolin to 3,4-dihydroxyphenylacetic acid; luteolin and eriodictyol to 3-(3,4-dihydroxyphenyl) propionic acid; and apigenin, naringenin, and phloretin to 3-(4-hydroxyphenyl) propionic acid (Schneider *et al.*, 1999, Schneider and Blaut, 2000, Schoefer *et al.*, 2003). *Eubacterium ramulus* was reported to convert quercetin to 3,4-dihydroxyphenylacetic acid and luteolin to 3-(3,4-dihydroxyphenyl) propionic acid (Braune *et al.*, 2001); and Naringenin to 3-(4-hydroxyphenyl) propionic acid (Herles *et al.*, 2004).

Microbial metabolism of polyphenols plays an important role in health (anti-oxidant, anti-inflammatory, anti-microbial, anti-cancer) as the majority of polyphenols undergo microbial enzymes in the colon (Selma *et al.*, 2009). However, gut microbiota could be influenced by different factors such as dietary habits, ethnicity, ageing, as well as colonic diseases.

1.7.1.3.1 Dietary habits

Dietary habits, including the change in the amount and balance of the three main dietary macronutrients (carbohydrates, proteins and fats), are known to alter gut microbiota composition and the microbial enzymatic activities, which could impact the host's metabolic phenotype (Scott *et al.*, 2013). For example, the faecal microbiota of European adults is more similar to adults living in the USA than to adults from South America or Malawi. This may be because Europeans and Americans are high consumers of a protein-

rich diet, while people from South America and Malawi are high consumers of plant derived polysaccharides (Yatsunenko *et al.*, 2012).

The impact of diet on gut microbiota composition could be for several reasons: first, the metabolism of carbohydrates produces short-chain fatty acids (SCFA) (acetate, propionate and butyrate). These acids tend to decrease the colonic pH, which has a selective effect in the growth of bacteria species (Duncan *et al.*, 2009). Low colonic pH inhibits the *Bacteroids spp*. and increases the butyrate-producing Gram-positive bacteria (Duncan *et al.*, 2009, Etxeberria *et al.*, 2013).

Second, the change in gut microbiota depends on the source of carbohydrates in the diet. Different bacterial species have different abilities to utilise different substrates. When resistant starch, bran, and mucin were fermented, *Bifidobacterium adolescentis* was the strain that could only be recovered on starch, *Bifidobacterium bifidumonly* on mucin, while *Bifidobacterium breve* was recovered on both starch and mucin. *Eubacterum rectale* and *Bifidobacterium longum* were the only bacteria found on all three substrates. This study provides a good picture of particular roles that specific gut bacteria occupy (Leitch *et al.*, 2007).

Third, consuming a diet low in dietary fibre; for example adults in the UK are routinely not meeting the recommendation of 18 g of fibre per day, with men consuming an average of 15.2 g of fibre per day and women 12.6 g per day, which may increase the overall transit time. This increase in transit time may, in turn, change the gut microbiota (Buttriss and Stokes, 2008).

Fourth, high fat intake could cause changes in intestinal microbiota diversity and composition. Daniel *et al*. (2014) reported changes in gut microbiota composition in mice after a high fat intake for 12 weeks. *Ruminococcaceae* was decreased, which could be due to low plant polysaccharides intake; however, *Rikenellaceae* which has been recently linked with type-2 diabetes in humans (Qin *et al.*, 2012).

Lastly, high protein/low fibre intake could cause a decrease in the production of SCFA in particular butyrate and an increase in the concentration of the branched fatty acids, ammonia, and phenols. This will change the colonic bacterial activity from the saccharolytic to putrefactive metabolism and in turn affect the gut microbiota (Woodmansey, 2007).

Understanding how dietary habits affect gut microbiota should be highlighted as it can result in variations in microbial metabolites and in turn differences in the incidence of diseases associated with an imbalance in the normal gut microbiota.

1.7.1.3.2 Ethnicity

"Ethnicity refers to the social group that a person belongs to, and either identified with or is identified by others, as a result of a mix of cultural and other factors including language, diet, religion, ancestry and physical features traditionally associated with race" (Bhopal, 2004). The influence of ethnicity, from the perspective of genetics, environment and the relationship between culture and diet, could have an effect on gut microbiota and in turn on polyphenol colonic metabolism and optimum health.

Several ethnic and geographic-based studies have previously compared gut microbiota between rural African and European children (De Filippo *et al.*, 2010, Yatsunenko *et al.*, 2012); among four countries: France, Germany, Italy, and Sweden (Mueller *et al.*, 2006); among Americans, Malawians, and Amerindians (Yatsunenko *et al.*, 2012); and between Belgian and Japanese adults (Ishikawa *et al.*, 2013). These studies suggested that host ethnic origins (genetic), cultural traditions, lifestyle, and disease prevalence could contribute to the differences in gut microbiota between different ethnic groups.

The gut microbiota is more similar between twins and mother-daughter pairs compared to unrelated individuals (Dicksved *et al.*, 2008). However, considering the information of the previous section about how diet can alert the gut microbiota, it is likely that genetics has little effect on the composition of gut microbiota. The best way to distinguish between the effect of ethnicity and dietary habits on gut microbiota is to conduct observational studies of immigrants (e.g. Asian nationals living in the US or Europe). This type of study will provide a good understanding of whether effect of ethnicity on the colonic metabolism could be modulated through changing the dietary habits.

1.7.1.3.3 Ageing

Gavini *et al*. (2001) reported a decline in some of the 'beneficial' bacteria, such as *Bifidobacterium*, and an increase in the 'harmful' bacteria, such as *Enterobacteriaceae* and *Clostridium perfringens* in ageing volunteers (69-89 years old). This change could be due to different reasons: 1) a reduction in gut motility associated with ageing, leading to longer transit times (Madsen and Graff, 2004); 2) ageing-associated disease such as irritable bowel syndrome, diverticulosis, and colon cancers which are linked with changing in gut

microbiota (MADSEN, 1992, Madsen, 1992, Camilleri *et al.*, 2000, Firth and Prather, 2002); 3) reduced chewing strength, leading to different food choice and lower fibre intake (Brodeur *et al.*, 1993); 4) reduced physical activity, known to affect frequency of bowel movements (Wijhuizen *et al.*, 2007), which in turn may affect the composition / diversity of the microbiota. All of these changes can affect the dietary intake and alter nutrients metabolism, leading to an altered range of food consumed, which in turn can affect the growth and composition of gut microbiota (O'Toole and Claesson, 2010).

As the majority of polyphenols are metabolised by bacteria enzymes in the colon, this suggests the influence of ageing on the colonic metabolism of dietary polyphenols. No study has looked at the effect of ageing on the bacterial metabolism of polyphenols which raises the need to carry out such a study.

1.7.1.3.4 Summary

Not all dietary polyphenols are absorbed in the upper gastrointestinal tract; about 90-95% of them are not absorbed in the small intestine (Clifford, 2004). Dietary polyphenols in the form of esters, glycosides, or polymers reach the colon and are metabolised by colonic microflora (Aura, 2008). However, as noted above, there are several factors that could influence gut microbiota composition, which could explain the variability between individuals in bioavailability and colonic metabolism in polyphenol studies (Graefe and Veit, 1999, Rechner *et al.*, 2002a, Olthof *et al.*, 2003, Jaganath *et al.*, 2006, Gardana *et al.*, 2009, Jaganath *et al.*, 2009, Roowi *et al.*, 2010).

These factors are likely to be key contributors to the inter-individual variation seen in polyphenol metabolism. These variations are important to consider as they could impact on the implication of polyphenol intervention. However, due to the inaccessibility of the proximal colon where most of the polyphenols are fermented, it is not possible to study the colonic metabolism of polyphenols in a human feeding study without the use of complex and expensive labeling techniques. Therefore, it is necessary to carry out *in-vitro* faecal fermentations to test the effect of these factors on the colonic metabolism of dietary polyphenols.

1.7.2 *In-vitro* models to study the colonic catabolism of polyphenols

There are several *in-vitro* strategies that have been used to study the colonic metabolism of several phenolic compounds. The current available *in-vitro* models vary from the basic

faecal incubation, continuous culture system, and computerised controlled system to the multi-chamber system (Etxeberria *et al.*, 2013). The source of bacteria in these models is usually a pure bacteria strain, ileostomy fluid, or freshly voided human or animal faeces cultured in a suitable medium with substrate (Williamson and Clifford, 2010). The monitored parameters are different according to the aim of each *in-vitro* research. These parameters include examining the substrate disappearance, catabolite formation, or alteration in the flora in batch (static) system (Edwards *et al.*, 1996, Gonthier *et al.*, 2006) or semi-continuous and continuous system (dynamic) (Minekus *et al.*, 1999, Gao *et al.*, 2006, Gonthier *et al.*, 2006). None of these models reflect the natural condition in the human colon for several reasons:

1- Alteration of faecal microbiota during the sample collection process and hence may not reflect the actual gut microbiota.

2- The build-up of the degradation products (inhibitors) in the incubation vessel are not necessarily representative of the conditions that occur in the colon. These inhibitors are normally reduced by colonic absorption.

3- The concentration of any metabolite is dependent on the catabolism and absorption rate which is not the case in the *in-vitro* incubation.

4- The effect of the stationary and death phase over a short period of 24 – 48 h in batch culture system due to the depletion of nutrients, low pH, and/or the formation of an inhibitory product such as an organic acid (Jaganath *et al.*, 2009, Williamson and Clifford, 2010, Etxeberria *et al.*, 2013).

Nonetheless, the use of *in-vitro* models can provide useful information on the types of metabolite products, pathways, and the factors that could influence flavonoid degradation such as food matrix, gut microbiota, or molecular structure. Several studies have investigated the *in-vitro* metabolism of flavonoids using human faecal samples. For example, Jaganath *et al.* (2009) investigated the microbial metabolism of rutin using *in-vitro* faecal fermentation in the presence and absence of glucose in three subjects. The degradation of rutin and the release of quercetin were faster in the presence of glucose in the cultures and slower in the absence of glucose in all three donors. This suggests that fermentable fibre could speed up the colonic metabolism of polyphenols and in turn quicken the appearance of microbial metabolites. Moreover, the ability of the faecal

sample from one subject to deglycoside rutin in the absence of glucose was low compared to the other two subjects and led to 48% of rutin remaining in the fermentation vessel after 48 hours of fermentation. This suggests that a difference in gut microbiota could affect the polyphenol metabolism resulting in variations between individuals, as well as the establishing the importance of adding a source of glucose to the *in-vitro* fermentation in mimicking the *in vivo* condition.

Additionally, Nordlund *et al.* (2012) suggest that the food matrix could greatly influence the colonic fermentation of polyphenols. The study compares rye bran and aleurone, wheat bran and aleurone, and oat bran and oat cell wall concentrate in their in vitro faecal fermentation and the production of phenolic acids and SCFA, preceded by in vitro enzymatic digestion. The digested rye, wheat, and oat bran fermented more easily than the bran because of their higher water-extractable dietary fibre content and smaller particle sizes. However, wheat bran was the slowest to ferment among grains due to the high proportion of water-unextractable dietary fibre and led to the lowest SCFA production.

The effect of the molecular structure of polyphenols on their colonic metabolism was investigated by Bazzocco *et al*. (2008). Their study showed that Marie Menard apples and cider proanthocyanidins, with an average polymerization of 8.2 and 2.2 respectively, degraded faster and yielded more metabolites compared to Averolles apples with an average polymerization of 71.2. Moreover, Justesen *et al*. (2000) reported on the influence of flavonoid structure on the degradation of glycosides polyphenols (rutin, naringin, and hesperidin). The major disappearance of rutin was between 8 and 24 h with no accumulated quercetin after the fermentation, while naringin, and hesperidin were nearly degraded after 24 h of the fermentation with the appearance of naringenin and hesperetin. These findings suggest that glycosides polyphenols are not likely to have the same degradation rate or the same end microbial metabolite products in the colon, which may exhibit different biological activity and different potential health effects on the gut.

1.8 Dietary polyphenols and gut health

The absorption of polyphenolic compounds is very low in the stomach and small intestine, and the majority of these compounds pass intact to the colon where they are metabolised by the gut microflora, resulting in smaller microbial metabolites and phenolic acid (Clifford, 2004, Manach *et al.*, 2004). The majority of *in-vitro*, animal, and human studies have reported the prebiotic~~s~~ effect of the microbial metabolites of polyphenols (Sembries

et al., 2003, Sembries *et al.*, 2006, Tzounis *et al.*, 2008, Molan *et al.*, 2009, Tzounis *et al.*, 2011, Jose Pozuelo *et al.*, 2012), while other studies reported the antimicrobial properties of polyphenol metabolites (Ahn *et al.*, 1990, Ishihara *et al.*, 2001, Massot-Cladera *et al.*, 2012, Kemperman *et al.*, 2013). However, the variation in daily polyphenols consumption between individuals and the variation in gut microbiota could lead to differences in the biological activities of polyphenols metabolites among individuals and differences in the ultimate health benefits.

There are a limited number of human feeding studies (Table 1-3) investigating the effect of polyphenols on the gut microbiota. For example, a study of the intake of a high-cocoa flavonol drink (494 mg) for four weeks significantly increased the *Lactobacillus spp*. and *Bifidobacterium* while decreasing the *Clostridium histolyticum* group (a group that includes pathogen *Clostridium perfringens*) in health subjects when compared to the low-cocoa flavonol drink (Tzounis *et al.*, 2011).

Moreover, the intake of tea (0.4g/volunteer) three times a day for four weeks increased *Bifidobacterium spp.* by 16%, whereas there was a significant decrease in *Clostridium perfringens* by 26% and in *Clostridium spp* by 13% (Okubo *et al.*, 1992). Likewise, the intake of one litre of green tea per day showed a significant increase in *Bifidobacterium spp.* in healthy subject after 10 days of the intake (Jin *et al.*, 2012b).

The intake of proanthocyanidin-rich extracts from grape seeds (0.19 g/day) for two weeks significantly increased *Bifidobacterium* (Yamakoshi *et al.*, 2001). Furthermore, a wild blueberry-based drink significantly increased *Bifidobacterium spp.* and *Lactobacillus acidophilus* after six weeks of intake (Vendrame *et al.*, 2011). Another human feeding study reported that the intake of rutin or quercetin (pure or from buckwheat) for seven days increased the growth of *Eubacterium ramulus* from 0.2% to 6.9% of the total flora (Simmering *et al.*, 2002).

Some of the studies in Table 1-3 followed a crossover study design, which reduces the variation between individuals; however, the carryover treatment effect could influence the results. Because of this, the inclusion of a washout period between treatments is very important. Overall, these results provided promising evidence that the intake of polyphenols can have an effect on modulating the gut microbiota by improving the growth of beneficial bacteria such as *Lactobacillus spp*. and *Bifidobacterium* and inhibiting the growth of pathogenic bacteria such as *Clostridium histolyticum* and *Clostridium perfringens*.

The high amounts of flavonoids and their microbial metabolites in the colon may have a direct influence on the gut mucosa and protect against carcinogenic activity by inhibiting cell growth, proliferation, angiogenesis, and/or metastasis; as well as anti-inflammatory and/or antioxidant effects (Parkar *et al.*, 2008, Araujo *et al.*, 2011, Cardona *et al.*, 2013, Parkar *et al.*, 2013). More studies are needed using the most available accurate methods for gut microbiota analysis to investigate: 1) the effect of each phenolic compound on different gut microbiota growth; and 2) the effect of gut microbiota diversity and the two-way phenolic-microbiota interaction on colonic disease.

Studies on the relationship between microbial metabolism of dietary polyphenols and modulation of the gut microbiota have used different methods to identify and quantify bacteria extracted from faeces and *in-vitro* fermentation. These techniques vary from basic cultures and enumeration to microbiome shotgun sequencing (each of these methods has some advantages and disadvantages; Table 1-4). About 60 - 80 % of the bacteria are reported to be uncultureable using the culture method; therefore, the use of the modern methods such as qPCR and microbiome shotgun sequencing will give a better view of the representative bacteria in the gut (Selma *et al.*, 2009, Etxeberria *et al.*, 2013). However, the recent molecular methods are expensive and thus investigators should carefully consider the most appropriate method that will help to achieve the study's aim.

Table 1-3: Human intervention studies on the effect of polyphenols on the composition of gut microbiota.

Reference	Food/polyphenol extracts	Dose	Subjects	Duration	Methods	Anti-microbial effect	Prebiotic effect	Comment
Tzounis *et al.* (2011)	Cocoa flavanols (crossover)	High-cocoa flavanol group (494 mg cocoa flavanols/d)	12 men 10 women	4 weeks	FISH	*Clostridia histolyticum*	*Bifidobacterial Lactobacilli*	↓ plasma triacylglycerol ($p<0.05$) ↓C-reactive protein concentrations ($p<0.05$)
Okubo *et al.* (1992)	Tea polyphenols	0.4g/3 times daily	4 men 4 women	4 weeks	Bacterial cell count	*Clostridium perfringens Clostridium spp.*	*Bifidobacterium spp.*	↓ faecal pH from 6.2 to 5.8 No effect on faecal enzyme activities or ammonia.
Jin *et al.* (2012)	Green tea	1000 ml/day	4 men 6 women	10 days	qPCR		*Bifidobacterium spp.*	

*Continued overleaf

Reference	Food/polyphenol extracts	Dose	Subjects	Duration	Methods	Anti-microbial effect	Prebiotic effect	Comment
Yamakoshi *et al.* (2001)	Proanthocyanidin-rich extracts from grape seeds	0.5 g/day of 38.5% proanthocy anidin-rich extract (0.19 g/day)	5 men 19 women	2 weeks	Bacterial cell count	*Enterobacteriaceae*	*Bifidobacterium*	↓ faecal ammonia (25.6%) , ↓ faecal pH (6.6 to 6.4)
Vendrame *et al.* (2011)	Wild blueberries (crossover)	25g/day	20 men	6 weeks	RT-PCR		*Total eubacteria Bifidobacterium spp.*	No differences in Bacteroides spp., Prevotella spp., Enterococcus spp., and Clostridium coccoides
Simmering *et al.* (2002)	Flavonoids (crossover)	Quercetin (14 mg/kg body mass) Rutin (28 mg/kg body mass pure or buckwheat leaves)	9 men 19 women	7 days	FISH		*Eubacterium ramulus*	↑ 0.2% (on day 1) to 6.9% of the total flora on day 8

Table 1-4: Methods used to characterize the gut microbiota (Fraher *et al.*, 2012).

Method	Description	Advantages	Disadvantages
Bacterial Culture and enumeration	Bacteria grown on selective mediums	Inexpensive Widely available Easy to use	Slow Time consuming labour intensive Not all bacteria are culturable
DGGE/TGGE	Gel separation of DNA strands using denaturant/ Temperature	Difference in bacterial populations can be detected. Bands can be excised for additional analysis.	No identification. Bands need to be cut and sequenced. Semi-quantitative
FISH	Fluorescently labelled oligonucleotide probes designed to hybridize with specific species	Bacterial identification	Detection dependent on probe sequences and not able to identify unknown species Semi-quantitative
qPCR	Detection of individual species or genus using specific primers	Fast identification and quantitative Can detect small number of bacteria	Unable to identify unknown species
Microbiome shotgun sequencing	Enormous comparable sequencing of the whole genome	Identification Quantitative	Expensive Labour intensive

Abbreviations: DGGE, denaturing gradient gel electrophoresis; TGGE, temperature gradient gel electrophoresis; FISH, fluorescence in situ hybridization; qPCR, quantitative PCR.

1.9 Dietary polyphenols and colorectal cancer

CRC is a major cause of morbidity and mortality worldwide (WHO, 2002). It is the third most common cancer throughout the world, and the fourth most common cause of death (WCRF/AICR, 2007). The aetiology of CRC is still unclear although genetic and environmental factors are associated with an increased risk of disease. The primary risk factor for CRC is age. The majority of people who are diagnosed with CRC are older than 50 years (Haggar and Boushey, 2009, Parkin *et al.*, 2009). Moreover, people who had a polypectomy to remove colon polyps (adenomas, serrated polyps, or hyperplasic polyps) are also at high risk of recurrence (Hoensch *et al.*, 2008). Environmental factors such as diet, obesity, alcohol consumption, smoking, and low physical activity levels strongly contribute to an increased risk of CRC (Haggar and Boushey, 2009). Non-dietary factors such as a sedentary occupation and an urban lifestyle also increase the risk of CRC (Benito *et al.*, 1993). It has been shown that a diet rich in dietary polyphenols can reduce the risk of chronic disease including CRC (Bobe *et al.*, 2008, MacDonald and Wagner, 2012). For these reasons, the interest in dietary polyphenols has increased among consumers, researchers and food manufacturers during the past decade.

The biological effects of polyphenols may contribute to their role in the inhibition of carcinogenesis including anti-oxidant, anti-inflammatory and phytoestrogenic properties and an ability to inhibit cellular proliferation, invasion, angiogenesis and metastasis and promote apoptosis (Danbara *et al.*, 2005, Qu *et al.*, 2005).

The bioactivities of polyphenols can be related to the arachidonic acid-dependent pathway (Cyclo-oxygenase, lipo-oxygenase and phospholipase A2 inhibition) or arachidonic independent pathways involving nitrous oxide synthase (NOS), NF-κB and NAG (Biesalski, 2007). Despite this, the evidence for polyphenols as anti-inflammatory agents remains limited and most studies have been performed *in vitro* and have concentrated on single specific polyphenolic compound. Many dietary polyphenols have demonstrated the ability to inhibit COX-2 *in vitro* including green tea extract enriched with catechin and epigallocatechin gallate (EGCG) which inhibited COX-2 expression in mouse skin (Kundu *et al.*, 2003) and genistein, quercetin, kaempferol and resveratrol, found to down-regulate COX-2 promoter activity in human colon cancer cells (Mutoh et al., 2000). Moreover, resveratrol (Manna et al., 2000),

green tea catechins (Yang *et al.*, 2001b) and curcumin (Singh and Aggarwal, 1995) are potent inhibitors of the NF-κB signalling pathway in human and animal cell lines in vitro and the mechanisms by which they exert this effect are becoming better understood. These substances may intervene at any step in the NF-κB signalling pathway, for example, inhibition of the translocation of NF-κB to the nucleus or inhibition of any of the initial stimulatory signal transduction pathways or transcription factors (Shishodia *et al.*, 2005).

1.9.1 Epidemiological Studies

Several epidemiological studies have looked at the association between diets rich in polyphenols and the risk of CRC (Table 1-5). Some of these studies have looked at the association with total flavonoid intake, others with certain phenolics, and some have not found any association.

An Italian multi-centre hospital-based case control study by Rossi *et al.* (2006) found a reduction in the CRC risk by 36% with an increased intake of isoflavones, anthocyanidins, flavones, and flavonols. No association was found for flavan-3-ols, flavanones, or total flavonoids. Recently, a Spanish hospital-based case control study by Zamora *et al.* (2013b) reported a reduction in the CRC risk by 41% with the intake of total flavonoids, flavones, flavanols, procyanidins, and lignans but not anthocyanidines, flavanones, flavonols, flavan-3ols, or isoflavones. The Spanish study included phenolic acid in the total flavonoids which could be the reason for the significant association between total flavonoids and CRC in the Spanish study but not the Italian study. Both Italian and Spanish hospital-based case control studies used new and large flavonoid databases (USDA Flavonoid Database); however, the FFQ in both studies was not validated to estimate the flavonoid intake, which could have an effect on the studies' results. Moreover, using US flavonoid food composition is not necessarily adaptable to the Italian diet.

In addition, a Scottish case control study reported a reduction in the CRC risk by 27% with the intake of flavonols, quercetin, catechin, epicatechin, procyanidins, but not flavones, flavanones, or phytoestrogens (Theodoratou *et al.*, 2007a). On the other hand, another Scottish case control study by Kyle *et al.* (2010) did not find any association between the risk of CRC and flavonol, procyanidin, flavon-3-ol, or flavanone intakes; however, quercetin from non-tea

components may be connected with lowering the risk of developing colon cancer but not CRC ($p<0.01$). The differences between the two Scottish studies could be due to the smaller size of Kyle's study or differences in age between the subjects of the two studies. The average age for participants in Kyle's study is six years older than for Theodoratou's study.

In agreement with Kyle *et al.* (2010), recently a US study found an association between flavonols, specifically quercetin found in non-tea components, and the risk of colon cancer. The result showed a protective effect of quercetin on proximal colon cancer risk but not distal colon cancer (Djuric *et al.*, 2012). This could be due to the local bioactivities effect of the microbial metabolites of quercetin in the colon as the majority of quercetin is metabolised in the proximal colon. However, the results of this study are limited because the FFQ did not cover the onion intake, which is one of the highest sources of quercetin.

A Japanese case control study by Wang *et al*. (2013a) also suggests a decrease in CRC risk with the consumption of coffee but not with tea polyphenols. However, the subjects' number was lower in the control group (60%) compared to the case group (80%) which could have an effect on the study's result. Moreover, the study just analysed tea polyphenols, coffee polyphenols, and polyphenols other than coffee but did not consider green tea polyphenols, which could be associated with CRC risk.

Lastly, two cohort studies did not support the intake of flavonoids and the risk of CRC. The first study by Lin *et al.* (2006) showed no association between the intakes of total flavonoids, flavonols, including quercetin, myricetin, and kaempferol and the risk of CRC. However, the FFQ did not include all types of foods. Moreover, data from flavan-3-ols was not considered. The intake of apples and tea were high in this cohort, and they are the major sources of flavan-3-ols.

The second cohort study conducted by Simons *et al.* (2009) used a case control approach. The cohort showed no association between total flavonols, flavones, and total catachin and the risk of CRC after 13.3 years. However, in overweight men there was a significant inverse association of intake of catechin and rectal cancer. Contrary to men, a significant inverse association between the intake of catechin and women with a body mass index (BMI) less than $25kg/m^2$ was found. According to the study, BMI may have an effect on the association

between the flavonol intake and colorectal cancer due to the effect of insulin like growth factor 1 (IGF-1) and estrogens inhibiting apoptosis and promoting cell cycle progression with EGCG lowering IGF-1 levels. For that, it might be that flavonoids only significantly reduced risk when IGF-1 levels are fairly high, in which adipose tissue secretes IGF-1, might be the reason for the association between the flavonol intake with women and overweight men but not in normal weight men. Despite the large sample size and long follow-up of this cohort, the single measurement of dietary intake at the baseline may not be reflecting the intake for the 13.3 years.

The varied outcomes of these nine studies might be due to various reasons:

1- Variations in study design and data analysis, which include differences in FFQ, food composition table, and flavonoid databases used.

2- Inconsistency in inclusion of cofounders such as levels of activity, smoking habits, and alcohol intake.

3- A small number of subjects, with fewer cases to compare to the controls (Kyle *et al.*, 2010, Rossi *et al.*, 2010)

4- The effect of seasons as the intake of fruit and vegetables is usually higher in summer compared to winter.

5- An insufficient intake of food rich in polyphenols. Slattery *et al.* (2004) reported that a minimum of five servings of vegetables per day must be consumed in order to reduce the risk of rectal cancer.

It is still not feasible to conclude an inverse association between the intake of flavonoids and the risk of CRC as it is difficult to distinguish between the effect of fruit and vegetables versus total flavonoid intake on the reduction of the CRC. The reduction of the CRC could be due to other nutrients in the fruit and vegetables such as fibre, vitamins, and/or salicylates. Moreover, dietary habits could also be affected by a recent cancer diagnosis.

Table 1-5: Epidemiological studies on the dietary polyphenols and the risk of CRC.

References	Countries	Study type /duration	Aims	Subjects	Dietary assessment and analysis	Conclusions
Rossi *et al*. (2006)	Italy	Case-control 1992-1996	Investigated the association between total flavonoids without phenolic acid and individual flavonoid subgroups (anthocyanidines, flavones, flavonols, flavan-3-ols, flavanones, and isoflavones) and CRC.	Cases= 1953 Control= 4154	FFQ/ USDA Flavonoid Database	An association was found between isoflavones (p_{trend}=0.001), anthocyanidines(ptrend<0.001), flavones ($p_{trend=}$0.004) and flavonols (p_{trend}<0.001) but not flavan-3ols, flavanones, or total flavonoids.
Zamora-Ros *et al.* (2013)	Spain	Case-control 1996-1998	Assessed the association between total flavonoids with phenolic acid and individual flavonoid subgroups (anthocyanidines, flavones, flavonols, flavan-3-ols, flavanones, proanthocyanidins, isoflavones, and lignans) and CRC.	Cases=424 Control=401	FFQ/ USDA Flavonoid Database	An association were found between total flavonoids (p_{trend}=0.04), flavones (p_{trend}=0.04), flavanols (p_{trend}=0.04), procyanidins ($p_{trend=}$0.02), and lignans ($p_{trend=}$0.03) but not anthocyanidines, flavanones, flavonols, flavan-3ols, andisoflavones.
Theodoratou *et al.* (2007)	Scotland	Case-control 1999- 2003	Examined the association between 6 main classes of flavonoids (Flavones, flavonols, flavan-3-ols, catechins, procyanidins, flavanones, and isoflavones) and the risk of CRC.	Cases= 1456 Control= 1456	Scottish collaborative groups FFQ /database for flavonoids from Kyle and Duthie(Kyle and Duthie, 2006)	An association was found between flavonols (p_{trend}=0.01),quercetin (p_{trend}=0.001), catechin (p_{trend}=0.0005), epicatechin(ptrend<0.01), and procyanidins($p_{trend=}$0.03) but not flavones, flavanones, or phytoestrogens.

*Continued overleaf

References	Countries	Study type /duration	Aims	Subjects	Dietary assessment and analysis	Conclusions
Kyle *et al.* (2010)	Scotland	Case-control 1998 - 2000	Examined the association of total dietary and non-tea consumption of four flavonoid subclasses and the incidence of developing CRC in people with a high intake of tea and its relationship to the high occurrence of CRC.	Cases= 264 Control= 408	Scottish collaborative groups FFQ /database for flavonoids from Kyle and Duthie (2006)(Kyle and Duthie, 2006)	No association between the risk of CRC and flavonol, procyanidin, flavon-3-ol, or flavanone intakes, however, quercetin from non-tea components may be connected with lowering the risk of developing colon cancer but not CRC ($p<0.01$).
Djuric *et al.* (2012)	United states	Case-control 2002-2005	Evaluated dietary quercetin intake and risk of CRC	Cases= 1163 Control= 1501	FFQ (Block Dietary Data Systems, Berkeley, CA (Bowman *et al.*, 1998).	Quercetin had protective effects only on proximal, not distal colon ($p=0.003$)
Wang *et al.* (2013)	Japan	Case-control 2000-2003	Investigated the associations between polyphenols intake and CRC.	Cases=816 Control=815	FFQ/Phenolic-Explorer database & USDA Flavonoid Database	Decreased risk of CRC associated with coffee consumption ($p_{trend}=0.01$)
Lin *et al.* (2006)	United states	Cohort 1990-2000	Looked at the association between intakes of flavonoids and flavonoid subgroups and risk of CRC in men and women from two prospective cohort studies, the Nurses' Health Study (NHS) and the Health Professionals Follow-up Study (HPFS).	498 women 380 men	FFQ/ USDA Flavonoid Database	Unable to validate inverse associations between flavonoid intake and risk of CRC.

*Continued overleaf

References	Countries	Study type /duration	Aims	Subjects	Dietary assessment and analysis	Conclusions
Simons *et al.* (2009)	Netherlands	Cohort 13.3 years	Investigated the association between the intake of dietary flavonol, flavone and catechin and CRC endpoints within the Netherlands Cohort Study on diet and cancer (NLCS).	Case= 1,444 male 1,041 female Control= 2,191 male 2,247 female	FFQ/ flavonol and flavones were gathered from the Netherlands Hertog et al. (1997)	Did not support an association between dietary flavonol, flavone and catechin intake and the CRC. The intake of dietary catechin may be associated with reducing the rectal cancer risk in overweight men(p_{trend}=0.04) The intake of dietary flavonol and catechin may be associated with reducing colon risk in normal weight women(p_{trend}=0.04)

1.9.2 Clinical trials and colorectal cancer

Human intervention trials with polyphenol rich supplements are limited (Table 1-6). Only three trials used polyphenols such as anthocyanin, apigenin, epigallocatechin gallate, and mirtocyan as a treatment to prevent or reduce the risk of recurrence (LS *et al.*, 2007a, Hoensch *et al.*, 2008, Thomasset *et al.*, 2009); though, one trial used dietary advice of increasing the fruit and vegetables as an approach to increase the polyphenol intake (Bobe *et al.*, 2008).

Considering the results together, no hard conclusions can be made:

First, only three studies have been carried out, testing the hypothesis that polyphenol-based interventions may impact on CRC recurrence. These studies had small sample sizes, between 51 and 87 subjects. No sample size calculation was supplied.

Second, the trial duration varies from seven days to four years, using different interventions and analytical methods. The trial by Bobe *et al.* (2008) used the US Department of Agriculture flavonoid database to examine the association between the flavonoids intake from the fruit and vegetables and the polyp recurrence, while other studies measured the effect of polyphenols on health markers in blood, colonic tissue, or cancer recurrence (LS *et al.*, 2007b, Hoensch *et al.*, 2008, Thomasset *et al.*, 2009).

Third, different types of polyphenols were used as interventions with different doses varying from 0.5 to 60 g/day. This is challenging to speculate whether the association between the prevention and the reduction in the CRC recurrence is due to the effect of one phenolic or a combination of phenolics or even the effective dose to reduce the risk of CRC.

In general, the most appropriate approach for the clinical trial was to measure the effect of dietary intervention on health biomarkers in blood, urine, or tissue which allowed measuring the effect of polyphenols directly on health biomarkers. However, using the US flavonoid database to analyse, and the FFQ is not accurate as it could be affected by under/or over-reporting and memory. Moreover, Bobe *et al*. (2008) asserted that flavonoid is associated with low risk of CRC; however, this association may be cofounded by other bioactivities in fruit and vegetables (fibre, vitamins, salicylates) which were not taken into consideration in their

analysis. More clinical trials (phase I) are needed to test the effective dose of the polyphenols and how much can be given safely to reduce the risk of CRC, especially for phenolic compounds that are known to reach the colon.

It is very difficult to ascribe health benefits to a particular polyphenol as there could be interactions between polyphenols and selected food components, such as proteins, carbohydrates, fiber, and fat. In well-controlled clinical trials, the use of purified polyphenols (powder or extract) could help to understand the effect of a particular polyphenol on the gut microbiota or colonic health; however, the translation of this type of study to the everyday life is limited as food intake could result in a different outcome due to the effect of other food components. From a nutritionist's point of view, a recommendation of a diet that is high in fruit and vegetables will provide a high amount of polyphenols, helping to maintain a healthy colon and reducing the risk of CRC. However, when looking at polpyhenols from a pharmacologic point of view as a pure compound to treat or to reduce the recurrence of CRC, more factors need to be investigated. There is a need to test the effects of a food matrix and if polyphenols need to be taken before, after, or with the meal, dosing, and duration. For example, there is a recommendation to avoid tea, coffee, and eggs 2 hours before and after taking iron supplementation. The same concept could be applied to polyphenols if they are proven to prevent and reduce the risk of CRC to decrease the components that are found in the meal which could affect polyphenols.

Table 1-6: Clinical trial studies on the dietary polyphenols and the risk of CRC

Reference	Patients	Aims	Intervention	Duration	Conclusions
Bobe *et al.* (2008)	Cases= 958 Control= 947	Examined the association between flavonoids with the risk of any or advanced adenoma recurrence in the Poly Prevention.	Dietary advice to increase the fruit and vegetables and reduce fat intake.	4-years	High intake of flavonols was associated with decreased risk of adenoma recurrence (p=0.0006).
Hoensch *et al.* (2008)	36 patients with resected colon cancer and 51 after polypectomy were divided into two groups (treated and untreated).	Examined the biological prevention of flavonoids on the recurrence of neoplasia in resected colon cancer and polypectomy patients.	20 mg/day of apigenin& 20mg/day epigallocathecin-gallat.	3-4 years	Among resected colon cancer treated patients, 14 had no cancer recurrence but one developed adenoma, however, cancer recurrence rate of the 15 matched untreated controls was (3 of 15) and adenomas (4 of 15)
Thomasset *et al.* (2009)	25 CRC patients scheduled resection of primary tumour or liver metastases	Pre-surgical model to evaluate the effect of the oral administration of mirtocyan on the proliferation of colorectal tissue and the circulating levels of IGF-I and IGFBP-3	Mirtocyan 1.4, 2.8, or 5.6 grams (containing 0.5-2.0 grams anthocyanins) daily.	7 days Before surgery	• Proliferation was reduced by 7% • Not significant reduction in circulating *of*(IGF-I and IGFBP-3) • <0.5 gram bilberry anthocyanins are required to find whether they may be suitable for CRC chemopreventive agents.
Wang *et al.* (2007)	25 colon cancer patients	Pre-surgical model looked at the effect of anthocyanin rich black raspberry as antiangiogenic in patients diagnosed with colon cancer.	60 g/day of anthocyanin rich black raspberry power	2-4 weeks	↓ Proliferation and ↑ apoptosis in the colon tumour but not in normal crypts.

The studies in this chapter speculate that the colonic metabolism of dietary polyphenols could be affected by several factors such as dietary habits, gut microbiota, and/or food matrix interactions and their further dependency on ethnicity, age and colonic health. These factors could be the reasons behind the large inter-individual variations in polyphenol metabolism that have been reported previously by several *in-vitro* and *in-vivo* studies. Inter-individual variations in polyphenol metabolism could lead to differences in polyphenol bioavailability and bioactivities between individuals and in turn contribute to differences in disease incidence. Therefore, it is important to investigate the possible factors that could cause the variations in polyphenol metabolism to be able to manage their influence especially polyphenols that are known to be metabolised by gut microbiota. This is justified by the promising data linking the polyphenol microbial metabolites with colonic health benefits such as inhibiting cell growth, proliferation, angiogenesis, and/or metastasis; besides the anti-inflammatory and/or antioxidant properties.

1.10 Hypothesis to be tested in this thesis

In order to clarify the health benefits associated with dietary polyphenols intake, we must study the factors affecting their colonic metabolism. In this thesis, the effect of ethnicity, ageing, and the risk of CRC (polypectomy patient), as factors that could influence the metabolism of dietary polyphenols, were examined. As the majority of polyphenols are metabolised in the colon, the interplay between dietary habits, ethnic food, genetics, ageing, gut health and gut microbiota could have a major influence on colonic polyphenol metabolism and the (derived) bioactivities.

The following hypotheses are to be tested:

1. Colonic metabolism of polyphenols differs between Europeans and Indians (Study1).
2. Colonic metabolism of polyphenols differs between younger and older adults (Study 2).
3. Colonic metabolism of polyphenols differs between polypectomy patients (at increased risk of CRC) and healthy older adults (Study 3).

To test these hypotheses, two study designs will be used for each study:

1. A human intervention (*in-vivo*), to study the colonic metabolism of dietary polyphenols after low-high polyphenol diets between two groups (Europeans versus Indians; younger versus older adults; and polypectomy patients versus healthy older adults). Outcome measures include biomarkers of polyphenol metabolism (urinary phenolic acid, urinary total phenols, and urinary antioxidant power), colonic fermentation (faecal SCFA, faecal pH, faecal ammonia), and gut health (faecal calprotectin).

2. *In-vitro* fermentations, using faecal samples collected during the dietary intervention, to study the metabolic capacity of the samples to ferment the polyphenol rutin. Outcome measures include formation of phenolic acids, production of SCFA, changes in fermented faecal fluid, and gas production.

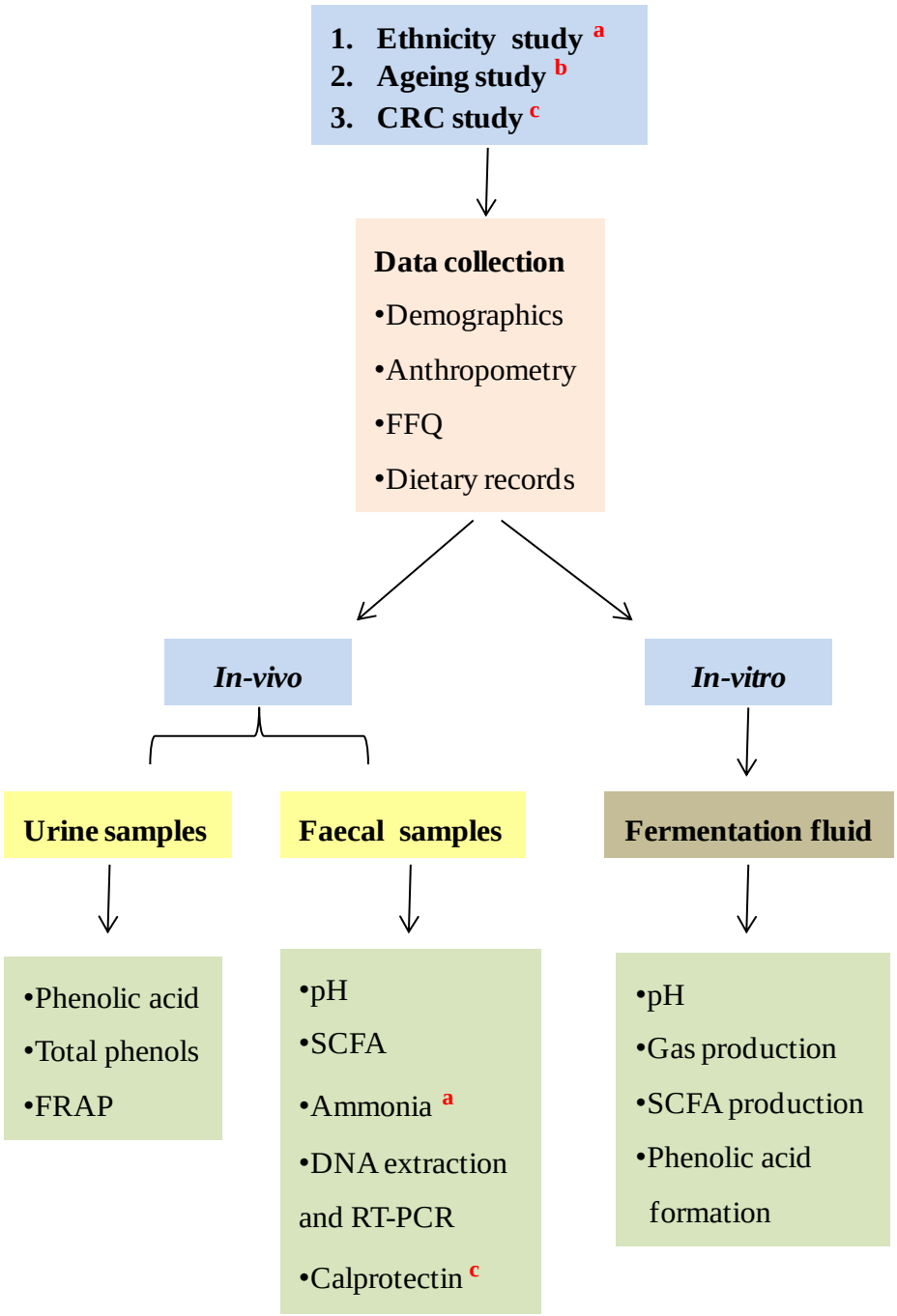
Chapter Two

General Materials and Methods

2.1 Introduction

This chapter describes the general materials and methods used throughout this thesis. All analyses presented in this thesis were performed either at the Human Nutrition or the Biochemistry and Molecular Biology laboratories at the University of Glasgow, UK. Figure 2-1 shows the three studies which were carried out during this PhD and their relevant analyses.

Figure 2-1: Types of studies and relevant analyses. Analyses applied to only one study are indicated by the relevant letters a, b or c.



2.2 Collection of data and samples

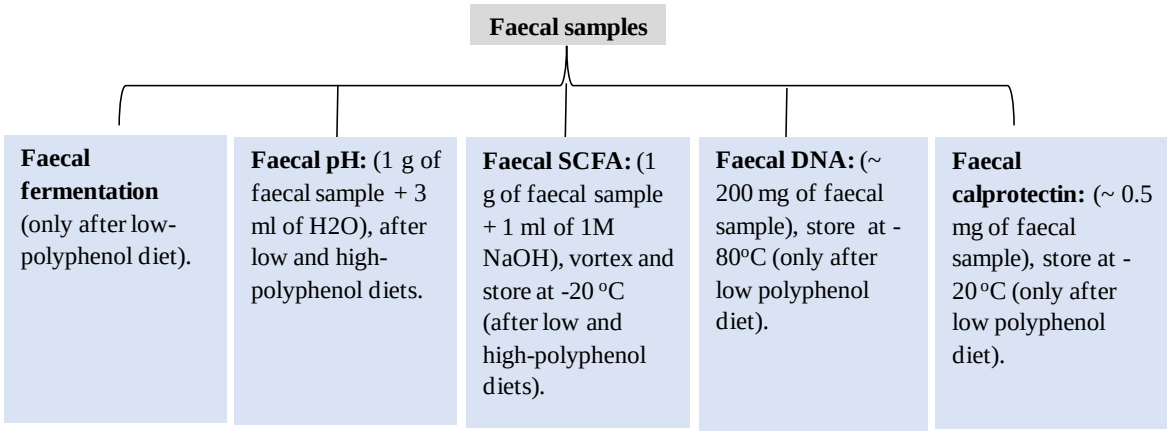
2.2.1 Demographics and anthropometry

Demographics information such as name, date of birth, gender, and ethnicity were collected. The anthropometric measurements (height, weight, waist circumference, and body mass index) and blood pressure were also collected at the baseline. Anthropometric measurements were conducted according to standard procedures. Weight was measured in kilograms using a calibrated digital scale (Seca, UK) and subjects were weighed in light clothing, without shoes. Height was measured using a wall-mounted Stadio meter (Seca, UK) according to the Frankfurt plane position. BMI was computed as the fraction of weight to the squared height. The waist circumference was taken at the level of the narrowest point between the lowest costal border and the iliac crest.

2.2.2 Collection of faecal samples

Participants were asked to pass an entire bowel movement using the kit provided (plastic bag inside a pot, using the support of a paper bedpan on the toilet seat). The pot was sealed in a bag with an anaerobic gas kit (Anaerocult® A Merck KGaA 62471 Darmstadt, Germany), to induce anaerobic conditions. An indicator strip (Anaerotest® A Merck KGaA 1151120001 Darmstadt, Germany) was included to ensure anaerobic conditions (Appendix 1). The stool sample was kept at 0^{o}C (in an insulated bag with frozen ice blocks) to slow ongoing bacterial metabolism, and was transferred to the laboratory within two hours of passage. Fresh faecal samples were used for faecal fermentation as well as the measurements of colonic fermentation and inflammatory biomarkers (Figure 2-2).

Figure 2-2: Faecal sample processing chart.



2.2.3 Collection of urine samples:

Urine samples were collected in an opaque container (to prevent UV degradation of phenolic compounds), stored in an insulated bag with frozen ice blocks after each diet (Appendix 1). The urine volume was recorded and urine aliquoted (2 ml) and immediately stored at – 80^{o}C.

2.3 *In vitro* fermentation (faecal incubation)

2.3.1 Preparation of fermentation medium

All chemicals and reagents used in the fermentation preparation including medium, buffers, macromineral, micromineral, and reducing solutions were supplied by Fisher Scientific (Leicestershire, UK), Sigma-Aldrich Company Ltd. (Dorset, UK), and BDH AnalaR Laboratory (Dorset, UK). Rutin (2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-[[(2R,3R,4R,5R,6S)-3,4,5-trihydroxy-6methyloxan-2-yl]oxymethyl]oxan-2-yl]oxychrome) was purchased from Sigma-Aldrich Company Ltd. (Dorset, UK) and phenolic acids from Fisher Scientific (Leicestershire, UK), Sigma-Aldrich Company Ltd. (Dorset, UK), and AASC Ltd (Southampton, UK). The chemicals for the SCFAs extraction (orthophosphoric acid, sodium hydroxide, diethyl ether, and external & internal standards) were obtained from Fisher Scientific (Leicestershire, UK), Sigma-Aldrich Company Ltd (Dorset, UK).

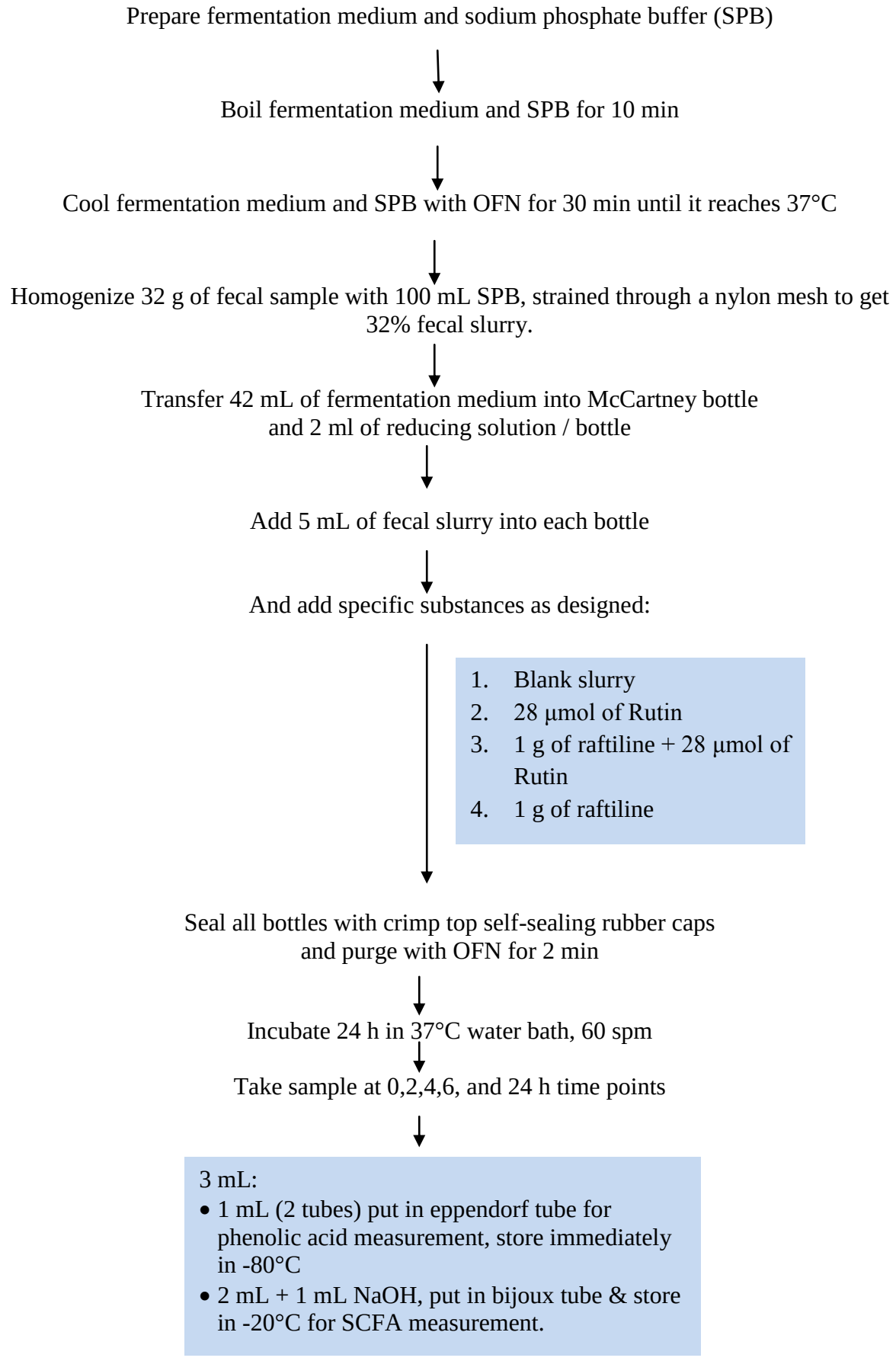
The basal medium was prepared according to Jaganath *et al.* (2009) by mixing 2.25g of tryptone in 450ml of distilled water with 112.5µl micromineral solution (13.2g $CaCl_2.2H_2O$, 10g $MnCl_2.4H_2O$, 1g $CoCl_2.6H_2O$, 8g $FeCl_3.6H_2O$, and distilled water to 100ml). After vortexing, 225ml buffer solution (2g NH_4HCO_3, 17.5g $NaHCO_3$ in 500 ml distilled water), 225 ml macromineral solution (2.85g $Na_2HPO_4.H2O$, 3.1g$KH_2PO_4.H_2O$, 0.3g $MgSO_4.7H_2O$ in 500 ml distilled water) and1.125 ml of 0.1% resazurin solution as a redox indicator were added. The medium was boiled for five minutes to remove oxygen and to sterilize it. The pH of the medium was adjusted to 7.0 using a hand-held pH meter (Hanna pH20instruments, USA) using HCl. The medium was then purged with oxygen-free nitrogen (OFN) to create an anaerobic condition, indicated by a colour change from indigo to colourless. The reducing solution (312.5mg of cysteine hydrochloride, 2 ml of 1M NaOH, 312.5 mg Na_2S, and 50 ml of distilled water) was added at 2ml per 50ml of the medium before adding the faecal slurry. The substrates used were as follows a) control (no substrate), b) 28 µM rutin, c) 28 µM rutin with 1 g raftiline, and d) 1 g raftiline. The

concentration of rutin used in the faecal fermentation was based on the rutin levels collected in ileal fluid 0-24 h after the ingestion of tomato juice supplemented with rutin in the human feeding study (Jaganath *et al.*, 2006). The amount of fibre was used according to Parker *et al.* (2013) as 1 g of fibre (20 mg/ml of medium) which is approximately equal to 8g/day of resistant starch intake (Cummings and Macfarlane, 1991).

2.3.2 Preparation of fermentation (faecal incubation)

Faecal samples were collected from study participants of all groups, and brought to the laboratory. The entire procedure is summarised in Figure 2-3. Freshly voided human faecal samples were homogenised with a sodium phosphate buffer (0.1 M, pH 7.0) in a blender (Braun™) to make a 32% faecal slurry (16g faecal sample with 50ml sodium phosphate buffer). Five millilitres of the faecal slurry was added to 44 ml of the fermentation medium (42 ml of pre-reduced basal media and 2 ml of reducing solution) in 100 ml fermentation glass sterilised bottles. The substrate (1 ml of rutin) was added to the faecal slurry in the presence and absence of 1 g of a highly fermentable fibre (raftiline) as a source of glucose to mimic the *in-vivo* environment. Faecal fluids containing no substrate were incubated at the same time as controls. Fermentation bottles were sealed and purged with OFN. Bottles were kept upright in a shaking water bath and incubated at 37^{o}C, 60 stokes/min for 24 hr to simulate the colonic lumen conditions. Two samples (3ml) of fermented fluids were collected after 0,2,4,6 and 24 hr. One sample for each time point was immediately stored at -80^{o}C for phenolic acid analysis. The other was mixed with 1M NaOH (1 ml) for stabilization and to prevent oxidation and stored at -20^{o}C for SCFAs production measurements.

Figure 2-3: Feacal fermentation flow chart.



2.4 Measurements of colonic fermentation markers

2.4.1 Determination of pH

The pH was measured directly in fresh and fermentation fluid. The fresh faecal slurry was prepared by a suspension ~1 g of homogenized faecal sample with three-fold volume of distilled water. The slurry was vortexed at full power until completely mixed. An auto calibrated portable digital pH meter model (Hanna pH20instruments, USA) was used. The fermented faecal of the fermented medium was determined at different time points (0, 2, 4, 6, and 24 hr) using universal pH indicator paper from 1 to 14 (Fisher Brand, UK). The pH meter was not used for this measurement as the volume at each time point was too small (1ml). According to Mansourian (2014), there was no major difference in the measurement when pH paper was used instead of a pH meter.

2.4.2 Determination of faecal ammonia

An automated photometric ammonia analyser (high range; Hanna 93828, USA) was used to measure faecal ammonia by taking an aliquot (100µl) of the diluted samples (used to measure faecal pH) and diluting it to a final volume of 40 ml with distilled water in a conical polypropylene centrifuge tube. The slurry was mixed and filtered using 0.22 µm Millipore filters before measuring the ammonia. The measurement process is carried out in two phases: first the meter is zeroed and then the actual measurement is performed.

2.4.3 Determination of water content

Water content (%) was measured in all faecal samples to standardize dry material weight and avoid confounding by faecal water levels. One gram of faecal sample was transferred into a 5 ml bijou tube with an equal volume of 1M NaOH for stabilization. The pre-weighted NaOH-stabilized faecal samples were freeze-dried in an Edwards apparatus (Freezer Dryer Micro Modulyo) for 24 hr. The water content was calculated using the following equation:

$$\text{Water content (\%)} = \frac{\text{(Weight of tube with wet sample- weight of tube with dry sample)}}{\text{Weight of sample}} \times 100$$

2.4.4 Determination of short chain fatty acids

The residual short chain fatty acids after absorption were measured in dry faecal samples according to Laurentin & Edwards (2004) as a proxy for SCFA production and in fermented faecal fluid samples as a marker for fermentation. The internal standard solution (86.1 mM 2-ethylbutyric acid, 100µl) was added to 800µl (50 mg of pre-stabilized dry faecal sample with 1M NaOH and 800 µl distilled water) or 800µl fermented faecal slurry previously stabilized with 1 ml of 1M NaOH (1:3), along with orthophosphoric acid (100µl). The mixture was mixed for 15 sec and was extracted three times with 3ml of diethyl ether (mixing 1 min each time). The upper phase was pooled and combined in 15 ml tubes.

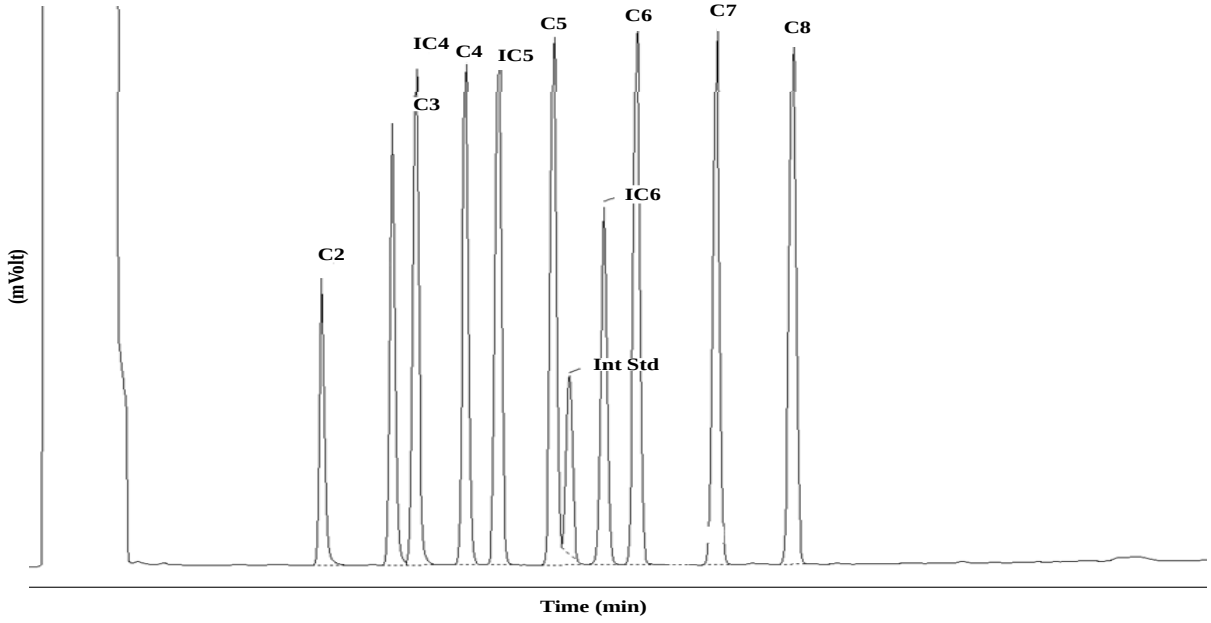
2.4.4.1 Analysis of SCFA by GC-FID

The short chain fatty acids were measured by GC-FID using a TRACE™ 2000 gas chromatograph (Thermo Quest Ltd, Manchester, UK) equipped with a flame ionisation detector (250°C) and a Zebron ZB-Wax capillary column (15 m x 0.53 mm id x 1 µm film thickness)(catalogue No.7EK-G007 22, Phenomenex, Cheshire, UK). The carrier gas was nitrogen (30 ml/min). Samples (1 ul) were automatically injected (230°C, splitless) onto the column. The column temperature was held at 80°C for 1 min, increased at 15°C/min until 210°C and held for 1min. Analyses of GC-FID data were performed on Chrom-Card 32-bit software version 1.07β5 (2000) Thermo Quest (Milan, Italy). Identification of SCFAs was achieved by comparison with retention times of authentic standards. Quantification was based on the averaged area ratio of each external standard (166.5mM acetic, 135.0mM propionic, 113.5mM isobutyric, 113.5 mM n-butyric, 97.9mM isovaleric, 97.9mM n-valeric, 86.1mM n-hexanoic, 76.8mM heptanoic and 69.3mM n-octanoic acid solution (concentrations based on expected values in normal/healthy faecal samples)) batch-wise, with grouped analysis of samples from the same individuals to reduce inter-assay error. A set of five calibrated standards was extracted and analysed before and after the samples. A signal standard as a quality control calibrated was run every 16 samples. Samples were extracted in duplicate and their results were averaged. Standards were extracted once and injected twice. Table 2-1 shows the SCFA measured in the dry faecal and fermented faecal fluids samples in all studies. The typical GC-FID trace is illustrated in Figure 2-4.

Table 2-1: Retention times present in chromatograms spectra in standard solution and faecal samples.

No.	SCFAs	t_R (min)
1	Acetic acid (C2)	2.6
2	Propionic acid (C3)	3.2
3	Isobutyrate acid (IC4)	3.4
4	Butyric acid (C4)	3.9
5	Isovalerate acid (IC5)	4.2
6	Valeric acid (C5)	4.7
7	Isocaproic acids (IC6)	5.1
8	Caproic acid (C6)	5.4
9	Enanthic acid (C7)	6.1
10	Caprylic acid (C8)	7.8
11	2-ethylbutyric acid (IS)	4.8

Figure 2-4: Chromatogram of a standard sample containing SCFA.



2.4.5 Production of gas

A 50 ml disposable syringe and a three way tap were used to measure the gas production in each fermentation bottle at different time points (0, 2, 4, 6, 24 hr).

2.5 Determination of faecal bacterial diversity and composition

Eubacterium ramulus and *Flavonifractor plautii* were selected to be quantified in faecal samples because they contribute to the colonic metabolism of flavonoids. Also, *Bifidobacterium*, *Bacteroides,* and total bacteria were measured since they are abundant in the human colon. Real-time PCR with species-specific probes was used to provide a sensitive and accurate method to quantify individual species, bacterial populations, and total bacteria. Real-time PCR is more reliable than other methods such as single-strand conformation polymorphism analysis, temperature gradient gel electrophoresis, and fluorescence *in situ* hybridization (Corless *et al.*, 2000, Guiver *et al.*, 2000).

2.5.1 Extraction of DNA

Total bacterial DNA was extracted from human faecal samples using the chaotropic method, modified by Godon *et al.* (1997). Extractions were carried out over a period of two days. During the first day of extraction, approximately 200 mg of homogenized faecal material were transferred to a 2 ml Eppendorf screw cap tube using sterile wooden sticks and stored at -80°C. Samples were thawed at room temperature before use.

Guanidine thiocyanate (250 µl, 4 M/Tris-Cl 0.1 M, pH 7.5) and 10%N-lauroylsarcosine (40 µl) were added to each sample and vortexed briefly. Samples were centrifuged for 3 sec at 15000*g* and incubated at room temperate for 10 min. N-lauroylsarcosine (5%, 500 µl) in phosphate buffer 0.1 M (pH 8.0) was added followed by vortexing and centrifuging for 3 sec. The tubes were then incubated for 60 min at 70°C in a Dri-Block® heater (Techne, UK) with vortexing every 20 min. After this, approximately 750 mg of sterile 0.1 mm zirconia glass beads (Biospec Products, USA) were added and the tube was vortexed for 30 sec and vigorously agitated in a FastPrep®-24 bead beater (MP Biomedicals, USA) three times for 60 sec at 4.5 m/s followed by a 5min rest on ice. The bead beating process was repeated once more with 5 min of rest on ice. The tubes were centrifuged for 3 sec, 15 mg of polyvinylpyrrolidone (PVPP) powder added and the tube homogenised by vortexing upside down to dissolve the pellet followed by shaking for 5 min and centrifugation (3 min, 15000*g*, 4°C). The supernatant was then transferred to a sterile 2 ml safe-lock tube.

The remaining pellet was washed twice with 500 µltris-EDTA-sodium chloride buffer (TENP), vortexed, shaken for 3 min and centrifuged again (3 min, 15000 *g*, 4°C). Supernatants were recovered after centrifugation and added to the 2 ml safe-lock tube. The

pooled supernatant (approx. 2 ml) was centrifuged for 10 min at 20000 *g* and 4°C and was split into three safe-lock tubes. Supernatant (750 µl) was pipetted into two 2 ml tubes and the rest (~ 500 µl) transferred to a 1.5 ml tube. An equal volume of isopropanol was added to each volume of supernatant. Samples were mixed by inverting and then incubated for 10 min at room temperature. Samples were centrifuged for 5 min at 15000 *g* and 4°C and the supernatant was discarded. Two hundred and twenty-five microlitres of phosphate buffer 0.1 M (pH 8) and 25µl 5M of potassium acetate were added to each tube followed by vortexing, shaking for 5 min, and then they were stored at -4°C overnight.

The next day, the tubes were shaken for 10 min and centrifuged for 30 min at 15000 *g* and 4°C. The supernatants were combined into a new 2 ml eppendorf tube, and 5µl RNAse (10 mg/ml) was added, vortexed, centrifuged for 3 sec, and incubated for 45 min at 37°C, with a vortex step after every 15 min. Samples were then centrifuged for 3 sec and 50 µl3 M sodium acetate and 1 ml ice-cold absolute ethanol was added. Tubes were inverted and stored at -20°C for 1 hr.

After that, samples were shaken for 10 min and centrifuged for 10 min at 15000 *g* and 4°C. The supernatant was discarded and 800 µl ice-cold ethanol (70%) was added to the pellet. The sample was then shaken at a medium speed for 5 min and the pellet was dissolved by pipetting up and down, followed by repeated shaking and centrifugation (10 min, 15000 *g*, 4°C). The supernatant was discarded and the washing was repeated two more times. Finally the pellet was dried under a laminar-flow hood for 45 min and re-suspended in 300 µlRNase-free water. Five aliquots of 60 µl were prepared and stored at -20°C for further use (Godon *et al.*, 1997).

2.5.2 DNA concentration and purity assessment

Total DNA concentration and purity were determined by measuring 1.5 µlof undiluted DNA extract with a NanoDrop ND-1000 (software version 3.7.4; Fisher Scientific, UK). A260/A280 ratio was measured to assess the purity of the DNA. An absorbance ratio of greater than 1.8 is considered to be of high purity and anything lower than that indicates the presence of contaminants such as protein. Also, the absorbance ratio at 230/260nm was checked to assess the guanidium salt carried over from extraction which should be greater than 1.5 (a good level of purity). Furthermore, 2µl of the DNA template was added to 3µl of loading buffer (Bromophenol x6; B0126, Sigma) and 2µl of distilled water and separated by gel electrophoresis on a 1.5 % (w/v) agarose gel containing 0.01% ethidium

bromide. Electrophoresis was performed in 1x TBE (Tris Borate EDTA) for 30 min at 100V alongside a 100bp DNA ladder (G210A, 100 bp DNA Ladder, Promega). Genomic DNA should appear as a compact, high-molecular-weight band with no low-molecular-weight smears.

2.5.3 Reverse transcription polymerase chain reaction (RT-PCR)

2.5.3.1 Preparation of bacterial standards and standard curve

DNA standards for *Eubacterium ramulus* and *Flavonifractor plautii* were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). Standard DNA for *Bifidobacterium* (*Bifidobacterium longum*) *Bacteroides* (*Bacteroides vulgatus*), and total bacteria (*Bacteroides vulgatus*) were available in-house (Gerasimidis, 2009). A set of seven bacterial standard references was made for each target. The serial dilution to measure the *Eubacterium ramulus* and *Flavonifractor plautii* was 1:5, and 1:10 to measure *Bifidobacterium, Bacteroides*, and total bacteria. A new standard curve was run on each plate and the dilutions of the standard curve were prepared freshly each time. Table 2-2 shows the stock concentration of each standard.

Table 2-2: Bacterial groups and corresponding standards used for quantitative analysis using qPCR.

Target Group	Standard species used	Stock concentration
Eubacterium ramulus	*Eubacterium ramulus* DSM 15684	3.15 ng/µl
Flavonifractor plautii	*Flavonifractor plautii* DSM 4000	6.3 ng/µl
Bifidobacterium spp.	*Bifidobacterium longum subsp. longum* DSM 20219T	9.8ng/µl
Bacteroides	*Bacteroide svulgatus* DSM 1447T	27.2 ng/µl
Total bacteria	*Bacteroidesvulgatus* DSM 1447T	27.2 ng/µl

2.5.3.2 Design of the oligonucleotide (probes and primers) sequences

Fluorescently labeled probes and primers were designed to detect *Eubacterium ramulus* and *Flavonifractor plautii* using the PrimerQuest tool (Integrated DNA Technology, http://www.idtdna.com/Primerquest/Home/Index, accessed as of February, 2013). The parameters of the tool were customized to fall within the guidelines for designing probes and primers for RT-qPCR assay: the temperature of a hydrolysis probe was customized to be 5–10°C higher than that of the primers; the length was selected to be less than 30 nucleotides with no G at its 5' end (because this could quench the fluorescence signal even after hydrolysis), and with a GC content of 30–80%.

The parameters for primers were set as follows: the amplicon size was set between 50-150 bp, the primers to probe distance were adjusted not to exceed 100 bp, the GC content was kept between 30-80%, and the primers' temperatures were designed to be close to 60°C. In addition, we considered the region of validated probe to design the *Eubacterium ramulus* probe (Simmering *et al.*, 1999) and a validated PCR probe region to design the *Flavonifractor plautii* probe (Schoefer *et al.*, 2003).

We chose the set of primers and probes for *Eubacterium ramulus* that met all the required parameters and covered the same validated FISH probe by Simmering *et al.* as detailed in Figure 2-5. The same parameters were applied to *Flavonifractor plautii* and also covered the validated PCR probe by Schoefer et al (2003) (Figure 2-6).

Figure 2-5: Design of qPCR assay for *Eubacterium ramulus* at a specific location with PrimerQuest.

				Start	Stop	Length	Tm	GC%
Forward	GGTGGAGCATGTGGTTTAATTC (Sense)			934	956	22	62	45.5
Probe	TGACATCCCGGTGACAGAACATGT (Sense)			985	1009	24	68	50
Reverse	GACACGAGCTGACGACAA (AntiSense)	Hairpin	Blast	1052	1070	18	62	55.6

Base	Sequence
1	NNNTTTATGAGAGTTTGATCCTGGCTCAGGATGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGAAGCACTTTACCTGATTTCCTTCGGGGATG
101	ATTGTTCTGTGACTGAGTGGCGGACGGGTGAGTAACGCGTGGATAACCTGCCTCACACAGGGGGATAACAGTTGGAAACGRCTGCTAATACCGCATAAGC
201	GCACAGCACCGCATGGTGCAGTGTGAAAAACTCCGGTGGTGTGAGATGGATCCGCGTCTGATTAGCTTGTTGGCGGGGTAACGGCCCACCAAGGCGACGA
301	TCAGTAGCCGGCCTGAGAGGGCGACCGGCCACATTGGGACTGAGACACGGCCCNGNCTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAA
401	ACCCTGATGCAGCGACGCCGCGTGAGCGATGAAGTATTTCGGTATGTAAAGCTCTATCAGCAGGGNAGATAATGACGGTACCTGACTAAGAAGCTCCGGC
501	TAAATACGTGCCAGCAGCCGCGGTAATACGTATGGAGCNAGCGTTATCCGGATTTACTGGGTGTAAAGGGAGCGTAGGCGGTCCTGCAAGTCTGATGTGA
601	AAGGCCGGNGCTCAACCCCNNNACTGCATTGGAAACTGTAGGNCTAGAGTGTCGGAGGGGTAAGTGGAATTCCTAGTGTAGCGGTGAAATGCGTAGATAT
701	TAGGAGGAACACCAGTGGCGAAGGCGGCTTACTGGACGACTACTGACGCTGAGGCTCNNAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCA
801	CGCCGTAAACGATGAATACTAGGTGTTGGCAGGTAAGNCCTGTCNGTGCCGCAGCAAACGCAATAAGTATTCCACCTGGGGAGTACGTTCGCAAGAATGA
901	AACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGANNNAACGCGAAGAACCTTACCAAGTCTTGACATCCCGGTGACA
1001	GAACATGTAATGTGTTTTCCCTTCGGGNCACCGGNGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGC
1101	GCAACCCTTATCCCCAGTAGCCAGNATTTCGGATGGGCNCTCTGTGGAGACTGCCAGGGACAACCTGGAGGAAGGTGGGGATGACGTNNAATCATCATGC
1201	CCCTTATGACTTGGGCTACACACGTGCTACAATGGCGTAAACANAGGGAAGCGAGACCGCGAGGTGGAGCAAATCCCNAAAATAACGTCCCAGTTCGGAT
1301	TGTAGTCTGCAACTCGACTACATGAAGCTGGTATCGCTAGTAATCGCAGATCAGAATGCTGCGGTGAATACGTTCCCNNGTCTTGTACACACNGNNCGTC
1401	ACACCATGGGAGTCGGAAATGCCCGAAGCCGGTGGCCTAACCGCAAGGAAGGAGCCGTCGAAGGCAGGTCTGATAAC

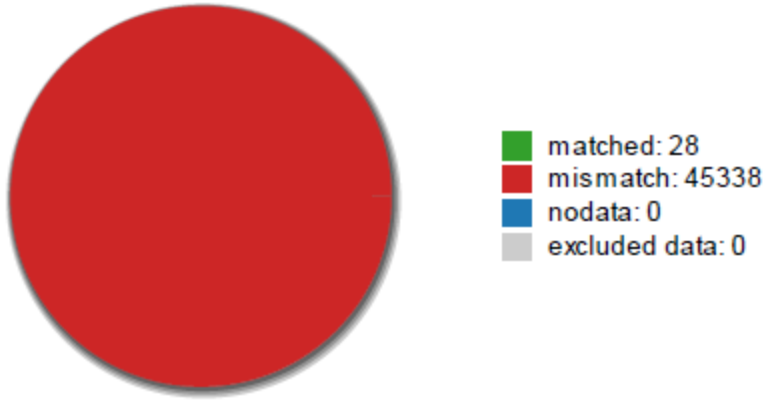
Figure 2-6: Design of qPCR assay for *Flavonifractor plautii* at a specific location with PrimerQuest.

		Start	Stop	Length	Tm	GC%
Forward	GGGAATAACACTCCGAAAGGA (Sense)	124	145	21	62	47.6
Probe	CATGCGACCCAACTGCTTCATGC (AntiSense)	156	179	23	68	57
Reverse	CATCTCAGAGCGATAAATCTTTGG (AntiSense)	189	213	24	61	41.7

Base	Sequence
1	CGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGGGGTGCTCATGACGGAGGATTCGTCCAATGGATTGAGTTACCTAGTGGCGGACGGGTGAGTAACG
101	CGTGAGGAACCTGCCTTGGAGAGGGGGAATAACACTCCGAAAGGAGTGCTAATACCGCATGAAGCAGTTGGGTCGCATGGCTCTGACTGCCAAAGATTTAT
201	CGCTCTGAGATGGCCTCGCGTCTGATTAGCTAGTAGGCGGGGTAACGGCCCACCTAGGCGACGATCAGTAGCCGGACTGAGAGGTTGACCGGCCACATTG
301	GGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGGCAATGGGCGCAAGCCTGACCCAGCAACGCCGCGTGAAGGAAGAAGGC
401	TTTCGGGTTGTAAACTTCTTTTGTCGGGGACGAAACAAATGACGGTACCCGACGAATAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAG
501	GTGGCAAGCGTTATCCGGATTTACTGGGTGTAAAGGGCGTGTAGGCGGGATTGCAAGTCAGATGTGAAAACTGGGGGCTCAACCTCCAGCCTGCATTTGA
601	AACTGTAGTTCTTGAGTGCTGGAGAGGCAATCGGAATTCCGTGTGTAGCGGTGAAATGCGTAGATATACGGAGGAACACCAGTGGCGAAGGCGGATTGCT
701	GGACAGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGGATACTAGGTGTGGGGGGT
801	CTGACCCCCTCCGTGCCGCAGTTAACACAATAAGTATCCCACCTGGGGAGTACGATCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGC
901	GGTGGAGTATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGGCTTGACATCCCACTAACGAGGCAGAGATGCGTTAGGTGCCCTTCGGGGAAA
1001	GTGGAGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTGTTAGTTGCTACGCAAGA
1101	GCACTCTAGCGAGACTGCCGTTGACAAAACGGAGGAAGGTGGGGACGACGTCAAATCATCATGCCCCTTATGTCCTGGGCCACACACGTACTACAATGGT
1201	GGTTAACAGAGGGAGGCAATACCGCGAGGTGGAGCAAATCCCTAAAAGCCATCCCAGTTCGGATTGCAGGCTGAAACCCGCCTGTATGAAGTTGGAATCG
1301	CTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTCGGGAACACCCGAAGTCCGTAGC
1401	CTAACCGCAAGGAGGGCGCGGCCGAAGGTGGGTTCGATAATTGGGGTGAAGTCGTAACAAGGTAG

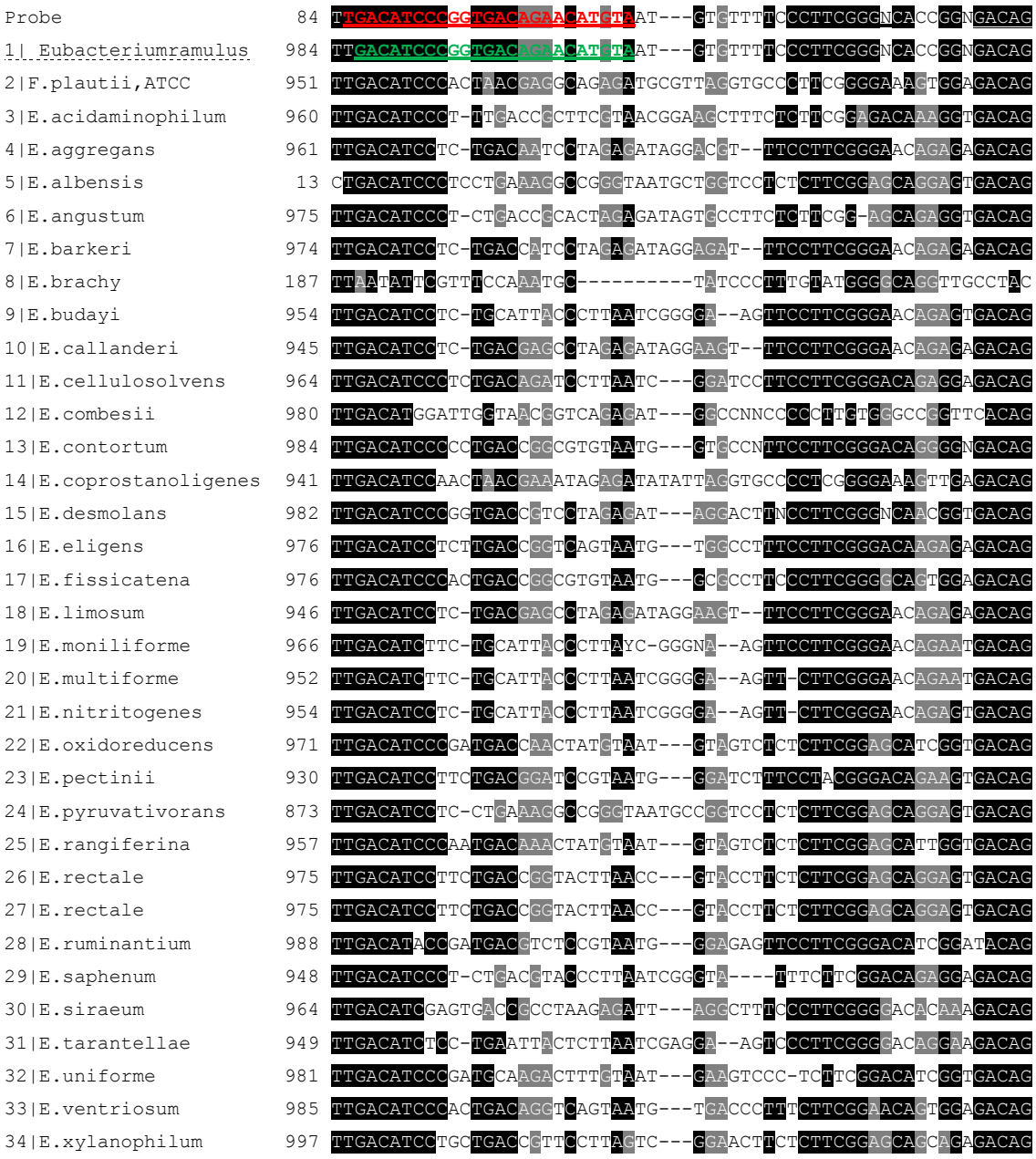
The probes were checked for specificity using the SILVA Probe Match and Evaluation Tool (TestProbe 3.0), http://www.arb-silva.de/search/testprobe/, which is a comprehensive on-line resource for quality check and aligned ribosomal RNA sequence data and can detect and display all occurrences of a given probe or primer sequence in the SILVA datasets. The designed probe for *Eubacterium ramulus* matched 28 uncultured bacteria (AF371611, AM693454, AY992786, AY992790, AY992794, AY993033, DQ796036, DQ796331, DQ799885, DQ801873, DQ801919, DQ802672, DQ806278, DQ806278, DQ809017, DQ809219, DQ809811, EF402928, EU462315, EU462328, EU530190, EU765639, EU765653, EU778157, FJ368719, FJ507401; Figure 2-7).

Figure 2-7: Chart for the matched and mismatch sequences within the selected taxonomic group.



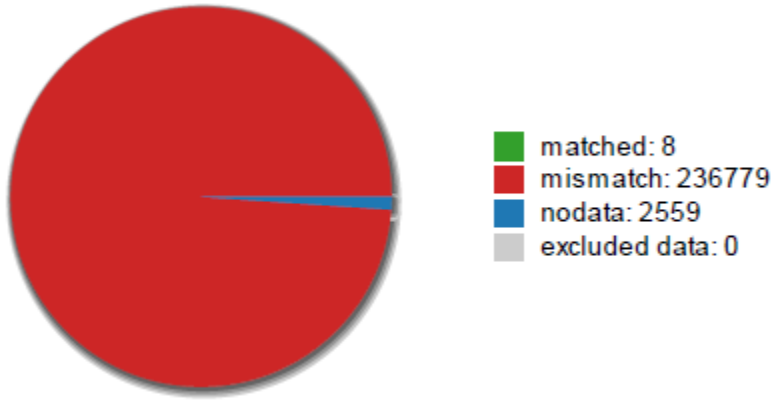
The probe was aligned with 34 selected 16S sequences of closely related DNA sequences to ensure specificity. We used the Multiple Sequence Alignment (Clustal omega) online tool, http://www.ebi.ac.uk/Tools/msa/clustalo/, which can align three or more biological sequences of similar length and BoxShade for shading multiple aligned sequence files. Figure 2-8 shows the alignment of *Eubacterium ramulus* probe with 34 related organisms.

Figure 2-8: Alignment of *Eubacterium ramulus* probe with 34 related organisms using Clustal omega online tool.



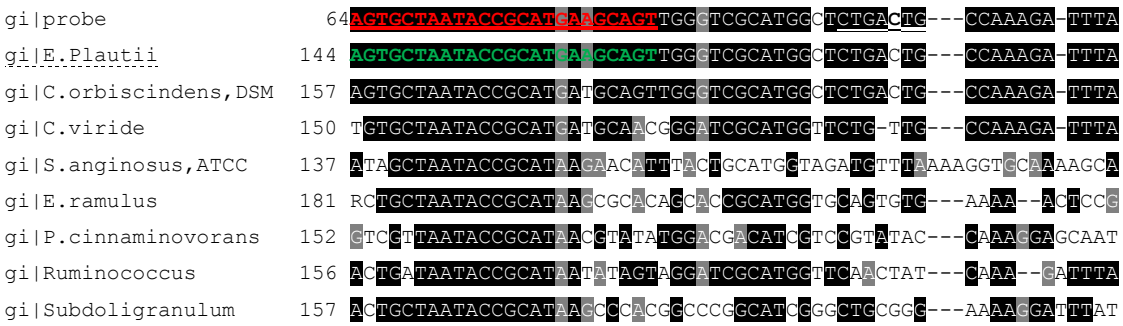
The designed probe for *Flavonifractor plautii* matched eight sequences. One of them was AY724678 (*Flavonifractor plautii*) and the rest were uncultured bacteria (DQ803890, DQ904982, EF434371, EF644509, EU777135, GU303208, HM008848; Figure 2-9).

Figure 2-9: Chart for the matched and mismatch sequences within the selected taxonomic group.



The *Flavonifractor plautii* probe was aligned with seven other related organisms as shown in Figure 2-10.

Figure 2-10: Alignment of *Flavonifractor plautii* probe with 34 related organisms using Clustal omega online tool.



2.5.3.3 Preparation of the qPCR assay and running conditions

The qPCR assays were prepared under sterile conditions in a SterilGARD® safety cabinet (Class II Type A/B3, The Baker Company, USA) in triplicate using a 7500 Real-Time PCR System (Applied Biosystems, UK). A 25 µl reaction was prepared in MicroAmp™ optical 96-well reaction plates which were sealed with MicroAmp® 96-& 384-well optical adhesive film (both Applied Biosystem, UK). The TaqMan® assay reaction mixes were made up as in Table 2-3.

Table 2-3: qPCR reagents used for TaqMan assay.

TaqMan®	**Single species** (*Eubacterium ramulus & Flavonifractor plautii*)	**Group species** (*Bifidobacterium, Bacteroides, & total bacteria*)
TaqMan® gene expression master mix	12.5µl	12.5µl
2.5 uMTaqMan probe	2.5µl	2.5µl
10 uM Forward primer	2.25µl	2.25µl
10 uM Reverse primer	2.25µl	2.25µl
Dilution DNA sample	2µl (50 ng/ul)	1.5µl (5 ng/ul)
Nuclease free water	3.5µl	4µl
Total	25µl	25µl

Template free controls containing 1.5µl of water in place of the sample were run on each plate as a negative control. All oligonucleotides were manufactured by Applied Biosystems (UK), and sequences, concentrations as well as cycling conditions are listed in Table 2-4.

Table 2-4: Bacterial Species/Groups showing TaqMan probes, primers, and cycling conditions.

Target organism	Probes and primers concentrations	Sequence 5' – 3'	Cycling conditions	Sources of reference
Eubacterium ramulus	TaqMan probe: 250 nM	TGACATCCCGGTGACAGAACATGT	95°C for 10 min; 45 cycles of 95°C for 30 sec and 60 °C for 1 min	this PhD (2014)
	Forward primer: 900 nM	GGTGGAGCATGTGGTTTAATTC		
	Reverse primer: 900 nM	GACACGAGCTGACGACAA		
Flavonifractor plautii	TaqMan probe: 250 nM	CATGCGACCCAACTGCTTCATGC	95°C for 10 min; 45 cycles of 95°C for 30 sec and 60°C for 1 min	this PhD (2014)
	Forward primer: 900 nM	GGGAATAACACTCCGAAAGGA		
	Reverse primer: 900 nM	CATCTCAGAGCGATAAATCTTTGG		
Bifidobacterium	TaqMan probe: 250 nM	CTCCTG GAAACGGGTG	95°C for 10 min; 45 cycles of 95°C for 30 sec and 60 °C for 1 min	Furet*et al.* (2009)
	Forward primer: 900 nM	CGGGTGAGTAATGCGTGA CC		
	Reverse primer: 900 nM	TGATAGGAGCGACCCCA		
Bacteroides	TaqMan probe: 250 nM	AAGGTCCCCCACATT G	95°C for 10 min; 45 cycles of 95°C for 30 sec and 60 °C for 1 min	Furet*et al.* (2009)
	Forward primer: 900 nM	CCTWCGATGGATAGGGGT T		
	Reverse primer: 900 nM	CACGCTACTTGGCTGGTTCAG		
Total bacteria	TaqMan probe: 250 nM	CTTGTACACACC GCCCGT C	95°C for 10 min; 45 cycles of 95°C for 30 sec and 60°C for 1 min	Furet*et al.* (2009)
	Forward primer: 900 nM	CGGTGAATACGTTCCCGG		
	Reverse primer: 900 nM	TACGGCTACCTTGTTACGACTT		

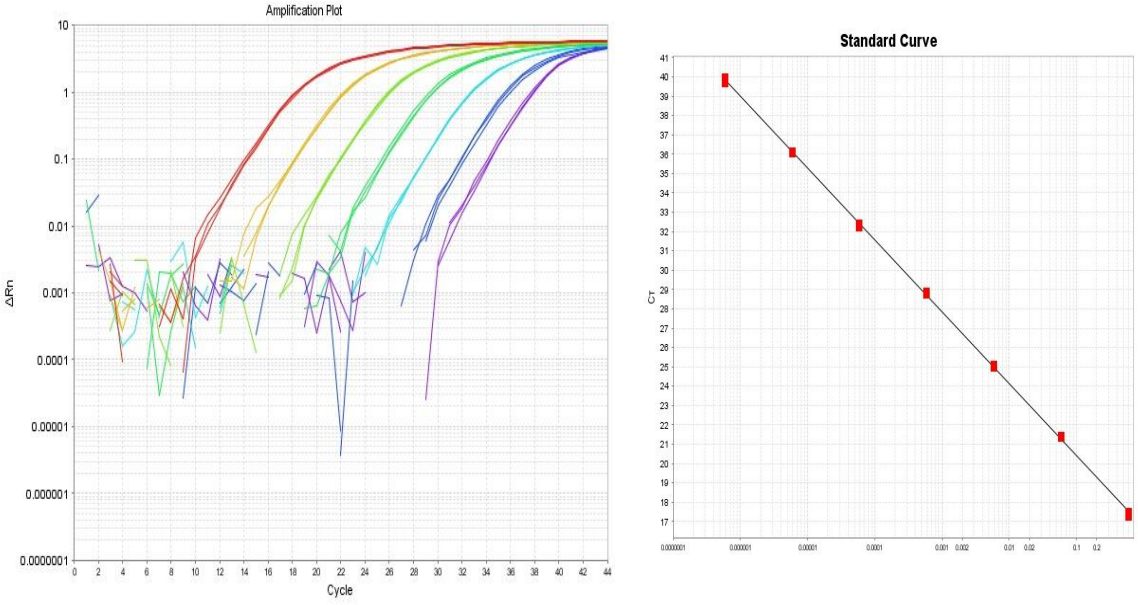
2.5.3.4 Analysis of qPCR data:

All qPCR experiments were analysed using the Applied Biosystem 7500 RT-PCR system software (version 2.0.5). After each run, the amplification efficiency, slope, and coefficient of determination (R^2) were calculated by the software and Microsoft Excel using the serially diluted DNA curve (Figure 2-11). A slope of -3.32 cycles (=3.32 cycles) between each serial 1:5 or 1:10 means that the assay was well-optimized, and it was mathematically 3.32 which gives a reaction efficiency of 100%. Generally, an amplification efficiency between 90% and 105% is acceptable. The following formula was used to calculate the efficiency:

Amplification efficiency (%) = $(10^{(-1/slope)}-1) \times 100$

The automatic baseline and threshold feature of the SDS software (Auto Ct) were adjusted manually and the same value was used for all runs of one species/group bacteria to make sure that the conditions of the analyses were identical for all runs.

Figure 2-11: Example of amplification plot for the qPCR and the standard curve for total bacteria.



To quantify the absolute bacterial DNA in all unknown samples in the qPCR reaction, we used a known concentration of an external standard and followed the equation below:

$$\text{DNA (ng)perqPCR reaction} = \frac{\text{Average DNA concentration (ng/ul)}}{\text{Dilution after DNA extraction}} \times \frac{\text{DNA volume used in qPCR reaction}}{\text{qPCR reaction volume}}$$

A further calculation was used to determine the total gene copy numbers using the following equation:

$$\text{Gene copy number per wet faeces} = \frac{\text{Amount of DNA detected (ng) x sample dilution factor in qPCR x resuspension volume after DNA extraction}}{\text{Weight of faecal sample (g) x mean genome weight (ng)}}$$

To calculate the average genome weight, we averaged the sizes of all fully sequenced genomes of species that are known to be detected by the same respective primer and probe. The genome size was obtained from The National Centre for Biotechnology Information (NCBI) (http:www.ncbi.nlm.nih.gov/genome). The molecular weight of one genome was calculated by assuming that 1 ug of 1000 bp DNA was equal to $9.1x10^{11}$molecules (New England BioLabs, Nucleic Acid Data), which was calculated using the following equation:

1) $\frac{1\mu g}{1000\ bp} = 9.1 \times 1011\ genomes \quad : \quad \frac{x}{2.25 \times 10^6\ bp} = 1\ genome$

2) $\frac{1\mu g}{1000\ bp \times (9.1 \times 10^{11})} = 1\ genome \quad : \quad \frac{x}{2.25 \times 10^6\ bp} = 1\ genome$

3) $\frac{1\mu g}{1000\ bp \times (9.1 \times 10^{11})} \qquad \frac{x}{2.25 \times 10^6\ bp}$

4) $X\ (\text{weight of 1 genome}) = \frac{1\mu g \times 2.25 \times 10^6\ bp}{1000\ bp \times (9.1 \times 10^{11})}$

5) weight of 1 genome = 2.47 fg

The gene copy numbers in dry faecal samples material was calculated using the following formula:

$$\frac{\text{Gene copy numbers}}{\text{(g dry faeces)}} = \frac{\text{bacterial gene copy numbers per g wet faeces}}{\text{1-(\% of water content/100)}}$$

2.6 Measurement of faecal calprotectin

Calprotectin is a calcium and zinc binding protein found in the cytosol of human neutrophils and macrophages. It is released extracellularly from stressed or damaged cells and can be detected in faecal samples, and thus can be used as a sensitive marker for investigation and diagnosis in patients with lower gastrointestinal symptoms (Smith and Gaya, 2012). In this study faecal calprotectin was measured as a non-invasive marker of intestinal inflammation in healthy older people and patients at risk of CRC.

A commercially analytically validated quantitative enzyme-linked immunoassay (CALPROLAB™ Calprotectin ELISA test, ALP, Germany) with ready-to-use reagents was used according to the manufacturer's instructions. The CALPROLAB™ Calprotectin ELISA (ALP) is based upon the preparation of an extract of faeces using the Faecal Extraction Buffer. The level of calprotectin was determined by testing the extract in an enzyme-linked immunoassay (ELISA) specific for calprotectin. In the ELISA, samples and standards were incubated in separate microtitre wells coated with monoclonal antibodies which bind the calprotectin. After incubation and washing of the wells, bound calprotectin was allowed to react with enzyme-labelled, immunoaffinity-purified calprotectin-specific antibodies. After this reaction, the amount of enzyme bound in the microtitre wells was proportional to the amount of calprotectin in the sample or standard, by incubation with a substrate for the enzyme giving a coloured product. The colour intensity is determined by absorbance using an ELISA plate reader.

Briefly, approximately 100 mg of thawed faecal extract was suspended with the extraction buffer to a weight: volume ratio of 1:50 and mixed for 30 sec using a vortex mixer and for 30 min on a shaker (IKA ® Vibrax VXR basic) at 1000 rpm then allowed to settle on the bench for 5 min. Five hundred microlitres of the homogenised extract was transferred to new Eppendorf tubes using a pipette. All tubes were stored in the freezer at -20°C to be analysed by ELISA. The faecal extract was diluted 1:100 and mixed by vortexing. One

hundred microlitres of each standard, control, and diluted sample was added in duplicate in the wells. The plate was covered with a sealing foil and incubated at room temperature for 40 min on a horizontal plate shaker. At the end of the incubation time, the liquids were removed and washed by adding 300 µl of washing solution to each well three times. One hundred microlitres of the enzyme was conjugated to each well using a multichannel pipette. The plate was covered again and incubated for another 40 min and the same washing step was applied three times. One hundred microlitres of enzyme substrate solution was added to each well. The plate was incubated in the dark at room temperature for 25 min. In the last step, 100 µl of 1M NaOH was added to stop the reaction. The optical density values were measured at 405 nm using Thermo Fisher, Multiskan Spectrum Spectrophotometer (1MSPT013, Finland). The concentration of faecal calprotectin in the samples was calculated using the standard curve as a reference and values were expressed as mg/kg of wet material. According to the manufacturer's protocol, values above 50mg/kg were considered as a positive calprotectin.

2.7 Polyphenol intake and antioxidant activity

2.7.1 Determination of phenolic acid:

Phenolic acid extraction and derivatisation was carried out as described by Combet *et al.* (2011). Thirty microlitres (1mg/ml) of internal standard (2, 4, 5-trimethoxycinnamic acid) was added to samples containing either 500 µl of faecal slurry, urine, or standards in dH_2O prior to vortexing. Samples were acidified to precipitate the phenolic acids by adding 60µl of 1M HCl, prior to mixing for 30 sec and stored at 4°C for 10 min. Anhydrous ethyl acetate (1.5 ml) was added to the samples, which were mixed for 30 sec and centrifuged at 4000*g* for 10 min. The upper organic layers were transferred to amber glass vials, which were placed in an aluminium block held at 37^{o}C. Samples were dried under a gentle flow of nitrogen. The extraction was repeated once more and the upper organic layers were pooled for each sample. Two hundred microlitres of dichloromethane was added to rinse the walls of the vials and dried under nitrogen at 37°C. N, O-Bis (trimethylsilyl)trifluoroacetamide (BSTFA) + 10%trimethylchlorosilane (TMCS,) was added to the vials (50 µl), and the headspace was flushed with nitrogen prior to sealing. Samples were derivatized at 80^{o}C for 4 hr with mixing every 30 min. Anhydrous hexane (99%, 350 µl) was added to each sample, prior to analysis by gas chromatography-mass spectrometry (GC-MS). A set of standard calibration solutions, containing phenolic acids ranging from 1.5- 15µg/ml, was extracted and analysed before and after the samples.

2.7.1.1 Phenolic acid analysis by GC-MS:

Derivatized phenolic acids were analysed on a Trace GC interfaced to a DSQ mass spectrometer equipped with a split/splitless injector and an AI3000 autosampler (Thermo Fisher, Hemel Hempstead, UK). Each sample was injected in split mode, with a 1:25 split ratio. The inlet temperature was maintained at 220°C. The oven was programmed from 40°C (held for 0.1 min) to 160°C at 20°C/min, to 200°C at 1.5°C/min, to 250°C at 10 °C/min to a final temperature of 300°C at 40°C/min, held for 5 min. The transfer line was maintained at 310°C. The carrier gas flow (helium) was constant at 1.2 ml/min on a fused silica capillary column (30 m × 0.25 mm i.d.) coated with cross-linked 5% phenylmethylsiloxane (film thickness 0.25 μm) (Phenomenex, Macclesfield, Cheshire, UK). Acquisition was performed in positive EI (electron ionization) mode in full scan (*m*/*z* 50–550) with an ionization energy of 70 eV, from 6 to 35 min. Acquisition and analysis of GC-MS data were performed on Xcalibur version 2. Identification of phenolic acids was achieved by comparison with retention times and mass spectra of authentic standards. Quantification was based on 2.5 to 15 μg calibration curves of derivatized and analysed phenolic acid standards and the area ratio of each standard was averaged and the coefficient of variance calculated (R^2>0.98). All samples were analysed batch-wise, with grouped analysis of samples from the same individuals to reduce inter-assay error. Samples and standards were extracted in duplicate and their results were averaged.

2.7.2 Determination of urinary total phenol using FolinCiocalteu

The Folin–Ciocalteu assay which also called the Gallic Acid Equivalence method (GAE) has been used for many years to measure the total phenolics in natural products; nonetheless, this assay is affected by a number of interfering substances, for instance sugars, aromatic amines, sulphur dioxide, ascorbic acid, organic acids, Fe (II), and non phenolic substances that react with the Folin reagent (Roura *et al.*, 2006). The urinary total phenol Folin-Ciocalteu assay method by Singleton and Rossi (1965) was modified to the 96-well plate. Briefly 20ul of urine samples or standards (triplicate) were added to the 96-well plate followed by (1:10,100ul) Folin reagent (Sigma-Aldrich, UK) and dH_2O (70 ul). The reaction was incubated for 5 min prior to the addition of sodium carbonate (1:10, 70 ul). The reaction was incubated for two hours at room temperature. The absorbance was read on a Thermo Fisher, Multiskan Spectrum Spectrophotometer (1MSPT013, Finland) at 765 nm wavelength.

2.7.3 Determination of antioxidant activity

Total antioxidant activity was measured by ferric reducing antioxidant power (FRAP) assay. The method was modified from Benzie and Strain (1996) for a 96-well plate. The antioxidant power was measured in the 24hr urine samples after low and high polyphenol diets. FRAP regent was prepared by mixing 8.33% 10 mM TPTZ, 8.33% 20 mM $Fe^{+}2$ solution, 20 mM Ferric Chloride ($FeCl3.6H_2O$) and 83.3% Acetate Buffer. The standard $Fe^{+}2$ (1mM ferrous sulphate) was used for the calibration curve from 0.1 to 1 mM. Twenty-five microlitres of each standard and sample was added to the well. Two hundred and twenty five microlitres of FRAP reagent was added to each well in less than 30 sec and the plates were incubated for 4 min at room temperature. The absorbance was measured using a Thermo Fisher Multiskan Spectrum Spectrophotometer (1MSPT013, Finland) at 593 nm wavelength.

2.8 Dietary assessment methods

2.8.1 Dietary records

Dietary records were used during the study after low and high diet-polyphenol diets were completed (three days each) to estimate the dietary intake of each participant and to measure the participants' compliance (Appendix 2). Participants were asked to record their entire dietary intake, including food types, portion sizes, and cooking methods for each day (breakfast, lunch, dinner, and three snacks between meals). They were also asked to include the names of commercially prepared foods consumed to allow more accurate nutritional assessments. Participants were provided with an electronic scale to measure the portion sizes.

2.8.1.1 Estimation of macro and micronutrients intake

These dietary records were analysed using WinDiets Nutritional Analysis Software (Commercialisation, Research, and European Development Office, The Robert Gordon University, Aberdeen, UK) (Wise, 2008) which is based on McCance & Widdowson's The Composition of Foods (Food Standards Agency, 2002) to calculate the amount of macro and micronutrients. The WinDiets software allows analysis of up to seven days' food intake including breakfast, morning snack, lunch, afternoon snack, evening meal and evening snack, which is sufficient for the purposes of this study. Moreover, WinDiets enables analyses of the recipes by entering individual ingredients to create a recipe that can

then be used in the same way as other foods in the database. This procedure was used for cooked dishes and a wide variety of sandwiches, wraps and filled rolls.

2.8.1.2 Estimation of flavonoid intake

An advanced search was carried out using an online comprehensive database on polyphenol content in foods, the Phenol-Explorer database (www.phenol-explorer.eu/contents, Neveu et al., 2010) to retrieve mean content values of flavonoids contained in the dietary records food. Flavonoid content in foods that are low in polyphenols such as pasta, bread, biscuits, cakes and pastry were estimated from their wheat-flour content using the data from Chlopicka *et al.* (2012).

2.8.2 Food frequency questionnaires

A modified food frequency questionnaire based on the European Prospective Investigation into Cancer and Nutrition study (EPIC) questionnaire was used. A total of 112 food items and food groups with an addition of a spices intake section were added to EPIC FFQ. The questionnaire took approximately 15 min to complete (Appendix 3). For each food item or food group, subjects were asked how frequently (never or almost never, 1–3 times per month, once per week, 2–4 times per week, 5–6 times per week, once per day, 2–3 times per day, 4–6 times per day, and more than six times per day) they consumed the food or food groups. Food intake was grouped into food groups (beverages, fruits and vegetables, prepared meals, eggs, fish and meats, potatoes, pasta, rice, breads, sweets and treats, dairy and fats, and seasonings and sauces). The analysis was performed using Microsoft Office Excel to calculate the servings of each item per week. After that, food items within the same group were combined. For example, green tea, black tea, coffee, hot chocolate and herbal infusions were all combined under the hot beverages section. Likewise, marrow, sweet pepper, sweetcorn, avocado, apple, pear, orange, grapefruit, banana, grapes, melon, apricot, and berries were combined under the fresh fruit intake. Vegetable, cereal, meat, milk, and sweets were calculated the same way.

2.9 Statistical analysis

The statistical analysis for all data was carried out using Minitab 16. Normality test was run for all data using the Anderson-Darling test to determine if a data set is well-modelled by a normal distribution and to compute how likely it is for a random variable underlying the data set to be normally distributed. Descriptive statistics are presented as mean and standard deviation, or medians and inter quartile range (IQR). The ranges were also used for continuous data. Comparisons between groups were done using Mann-Whitney test for non-normally distributed data and paired test and 2 sample t-tests for the normally distributed data. The correlation coefficients were calculated for the excretion of the urinary phenolic acid with total phenol and FRAP. Moreover, the general linear model (GLM) with Bonferroni post-hoc test was used to analyse the *in-vitro* data to understand the effect of the 1) ethnicity, 2) time, 3) and compounds on the metabolism of rutin. Lastly, a post-hoc test was used using the G-power software to obtain the sample size and effect size to determine what the power was in the study.

Chapter Three

The Effect of Ethnicity on the Colonic Metabolism of Dietary Polyphenols

3.1 Introduction

The ubiquitous nature of dietary polyphenols, in particular the flavonoids, ensures that they are found in large quantities in the human diet (Manach *et al.*, 2005, Jaganath *et al.*, 2006). Dietary polyphenols are mostly found in fruits, vegetables, chocolate, and beverages, such as tea, coffee, or wine (Scalbert and Williamson, 2000, Manach et al., 2005). The total daily intake of dietary polyphenols is estimated to be between 150 and 1000 mg (Scalbert and Williamson, 2000).

These compounds have been associated with a large number of biological activities which include anticarcinogenic (Pereira *et al.*, 1996, Coates *et al.*, 2007, Kamaraj *et al.*, 2007, Khan and Mukhtar, 2008) and antioxidant properties (Jacob *et al.*, 2008, Giftson *et al.*, 2010). A high level of polyphenol metabolites in plasma has been associated with a lower risk of colorectal adenomas (Kuijsten *et al.*, 2006). Furthermore, epidemiological evidence links diets rich in polyphenols with a decreased risk of developing gastrointestinal diseases, including CRC (Theodoratou *et al.*, 2007b, Jedrychowski *et al.*, 2009, Kyle *et al.*, 2010).

The aetiology of CRC indicates the role of a strong environmental component connected to dietary habits (Cappellani *et al.*, 2013). Likewise, the World Cancer Research Fund emphasized the role of nutrition and physical activity in CRC incidence; with a high calorie intake, reduced fruit and vegetable intake, increased fat consumption, excess body weight, and reduced physical activity all considered major risk factors for disease (Stewart and Kleihues, 2003).

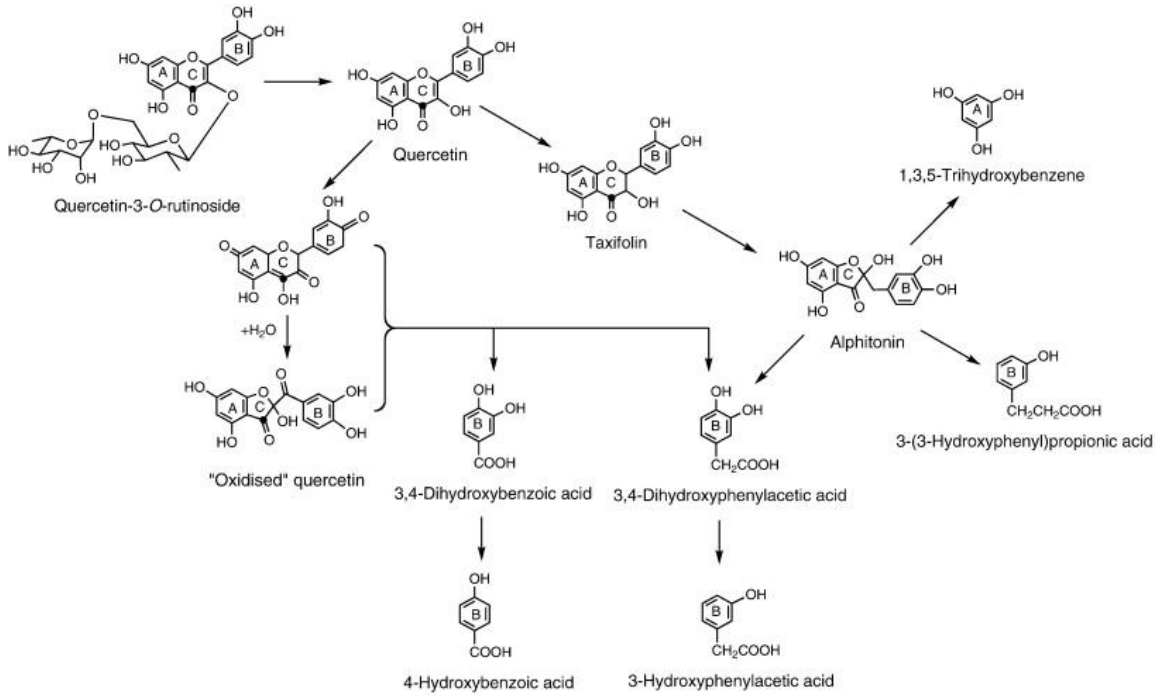
The incidence of CRC varies from country to country, with at least a 25-fold difference in incidence worldwide (Wild *et al.*, 2006). The highest incident rates are found in North America, Australia/New Zealand, Western and Eastern Europe (Wild *et al.*, 2006). In contrast, the reported CRC incidence in India during the past three decades has remained consistently low (4.3 per 100,000 for males, 3.4 per 100,000 for females) compared to the United Kingdom (36.2 per 100,000 for males, 23.5 per 100,000 for females) (Mohandas, 2011). This is thought to be related to a number of environmental and lifestyle factors, including: a lower intake of sugars, calories, meat, and fat-rich food; a higher consumption of vegetables, fruits, grains, and spices, which are rich in polyphenolic compounds; and adequate rates of physical activity and a low rate of overweight and obesity compared to

other countries. Yet India has an increasing problem with obesity-related type II diabetes (Tanaka *et al.*, 2008, Pathy *et al.*, 2012).

Flavonoids in practical flavonols such as rutin have shown significant activity in reducing azoxymethanol-induced hyperproliferation of colonic epithelial cells, as well as focal areas of dysplasia incidence in female mice (Deschner *et al.*, 1991). This suggested the ability of rutin to suppress tumour development. However, the beneficial systemic health effects of polyphenols are dependent on their capacity to be metabolised and absorbed by the human body and locally via the exposure of the gastrointestinal tract to their metabolites (Del Rio *et al.*, 2013).

Rutin is usually found in vegetables, particularly tomatoes and onions, in a glycosylated form (Mullen *et al.*, 2006, Crozier *et al.*, 2010). The daily intakes of rutin and its aglycone and quercetin are estimated to be between 50 and 500 mg/day (Lipkin *et al.*, 1999). The glycosylation of rutin with rhamnose-glucose disaccharide influences its absorption (Aura *et al.*, 2002, Jaganath *et al.*, 2006). The glycosides resist the acid hydrolysis in the stomach and thus pass intact to the large intestine (Gee *et al.*, 1998). The gut absorption of rhamnosides requires deglycosylation by colonic microbial enzymes (α- rhamnosidase, β-glucosidase, and β-glucuronidase). Rutin is not deglycosylated within the endogenous human small intestine by LPH and/or the CBG enzymes. The deglycosylation by human enzymes is for the flavonols that are attached to glucose, arabinose, or xylose (Jaganath *et al.*, 2006). Thus, rutin is hydrolyzed and degraded in the colon by the microbiota to low molecular weight, phenolic acids (Figure 3-1); (Erlund *et al.*, 2000, Manach *et al.*, 2005, Jaganath *et al.*, 2006, Jaganath *et al.*, 2009). The phenolic microbial metabolites are then subjected to the phase II liver metabolism, resulting in their sulphated and glucuronidated derivatives. In the end, phenolic metabolites are excreted via urine as hepatic sulphate and glucuronide conjugates (Manach *et al.*, 2004, Crozier *et al.*, 2010).

Figure 3-1: Proposed pathways for the human colonic degradation of rutin (Jaganath *et al.*, 2009).



Using urinary phenolic acids as biomarkers of polyphenol-rich food intake has some advantages over the plasma concentration, largely because it provides a better index of intake of the small and large intestinal metabolites. Moreover, it allows for total polyphenol absorption to be more accurately measured (Mennen *et al.*, 2006, Spencer *et al.*, 2008). The urinary excretion of polyphenol metabolites varies, depending on the polyphenol, and ranges from 0.3% to 43% (Manach *et al.*, 2005). According to Sawai et al.(1987), the total phenolic metabolites excreted in the urine accounted for 50% of the ingested dose of 75 mg rutin among volunteers. However, studies focusing on the bioavailability and the colonic metabolism of polyphenols have found a large inter-individual variability between participants (Gardana *et al.*, 2009, Jaganath *et al.*, 2009, Gill *et al.*, 2010, Gross *et al.*, 2010).

A study by Gill *et al.* (2010) noted a large inter-individual variation in the phenolic acid profiles of the faecal water of 10 participants after the intake of raspberry puree (200g/day) for four days. A number of the phenolic acids were significantly increased across the group of participants, including phenylacetic acid, 3,4-dihydroxyphenylacetic acid, and 3-hydroxyphenylacetic acid; however, it was noted that 4-hydroxyphenylacetic acid was only increased in a single subject. Furthermore, the concentration of 3-hydroxyphenylacetic acid and 4-hydroxybenzoic acid in faecal water samples varied two to three folds between subjects. Moreover, Gross *et al.* (2010) studied *in-vitro* colonic metabolism fermentation of

black tea and red wine/grape juice using faecal samples collected from ten participants and reported varying metabolite pathways among participants.

No clear mechanism has yet been established to account for this inter-individual variability; however, some of the observed variability is suggested to be a consequence of genetics, dietary habits and gut microbiota diversity (Scalbert and Williamson, 2000).

Studies have reported that genetics could influence the gut microbiota. A study by Yatsunenko *et al.* (2012) reported that both children and adults from the United States have a different gut microbiota from people in Malawi and the Amazonas state in Venezuela. Moreover, Turnbaugh *et al.* (2009) suggested more similarities in the gut microbiota structure between identical twins than adult dizygotic twins. Observable patterns in the composition of individuals' gut microbiota may also be detectable at a population level. For example, a study by Mueller *et al.* (2006) suggested an influence of geography in the differences in gut microbiota composition.

Cultural factors, particularly diet, have been shown to be essential in determining gut microbiota. Differences in dietary habits could have an influence on the colonic fermentation and production of short-chain fatty acids which may lead to changes in the colonic pH, which has a selective effect on the growth of bacteria species (Walker *et al.*, 2011, Claesson *et al.*, 2012). Hayashi *et al.* (2002) also illustrated differences in gut microbiota between vegetarians and individuals who follow a Western diet (which may be linked to fibre intake, level of fat consumption, lifestyle etc.). Moreover, the effect of the food matrix on the colonic metabolism of polyphenols has been reported previously by Manach *et al.* (2004). Direct interactions between polyphenols and other components in foods such as polysaccharides, proteins, or dietary fibre (Manach *et al.*, 2004), milk (Serafini *et al.*, 2009) or yogurt (Mullen *et al.*, 2008, Roowi *et al.*, 2009) have been shown to influence the colonic metabolism of dietary polyphenols.

There are a number of human faecal bacteria species that have been identified to degrade the C-ring system of flavonoids, such as, *Clostridium scindens, Flavonifractor plautii*, *Eubacterium desmolans*, and *Eubacterium ramulus* (Schneider *et al.*, 1999, Simmering *et al.*, 1999, Braune *et al.*, 2001, Schoefer *et al.*, 2003). Yet, there remains a lack of information on the type and occurrence of the flavonoid-degrading bacteria in the human intestinal tract, the effect of ethnicity and dietary habits on the quantity of these bacteria in

the human gut, and the variability of phenolic microbial metabolite excretion in relation to health and disease.

Therefore, the ethnic origin of subjects, which may impact on dietary habits, gut function and gut microbiota colonisation, should be considered when evaluating the bioavailability, metabolism, and possible health effects of dietary polyphenols. These factors might influence the metabolism of dietary polyphenols (to phenolic acids), which have been proposed to be more bioactive in the colon than their parent compounds (Olthof *et al.*, 2003, Parkar *et al.*, 2013).

This chapter consequently focuses on the impact of ethnicity on the colonic metabolism of dietary polyphenols, particularly flavonols, known to accumulate in the colon. Two ethnic groups were studied: adults from European countries, and adults from India (having resided less than 5 years in the UK, while maintaining the dietary habits of their home country). We know that CRC prevalence is lowest in India, and that diet is an important predictor of CRC risk. Since flavonol intake is different between the two ethnic groups, we hypothesized that the colonic metabolism of polyphenols would differ between Europeans and Indians, a possible reason behind the differences in CRC risk between the two groups. To test this hypothesis, two study designs were used.

1. A human cross-over dietary intervention (*in-vivo*) to study the colonic metabolism of dietary polyphenols (after low and high polyphenol diets) between the two groups, focusing on urinary phenolic acid excretion in the light of gut bacterial diversity (especially polyphenol-degrading bacteria).

2. *In-vitro* fermentations, using faecal samples collected during the dietary intervention, to study the metabolic capacity of the samples when specific flavonols were fermented.

3.2 Subjects and study design

3.2.1 Subjects and recruitment

Participants were recruited using local advertisements, printed poster displays, and online social networking sites. Exclusion criteria included alcohol consumption (>4 units/day), obesity (BMI>30kg/m^2), taking dietary supplements, pregnancy or at risk of pregnancy,

smoking, taking any medication, having any conditions known to affect bowel function, or Indians who had been in the UK more than 5 years and did not follow their native diet There was no consideration whether the individual followed a vegetarian diet. A full ethical application was prepared for this study and submitted to the University of Glasgow, College of Medical; Veterinary & Life Sciences (approval in Appendix 6). All participants gave informed written consent.

3.2.2 Sample size and power calculation

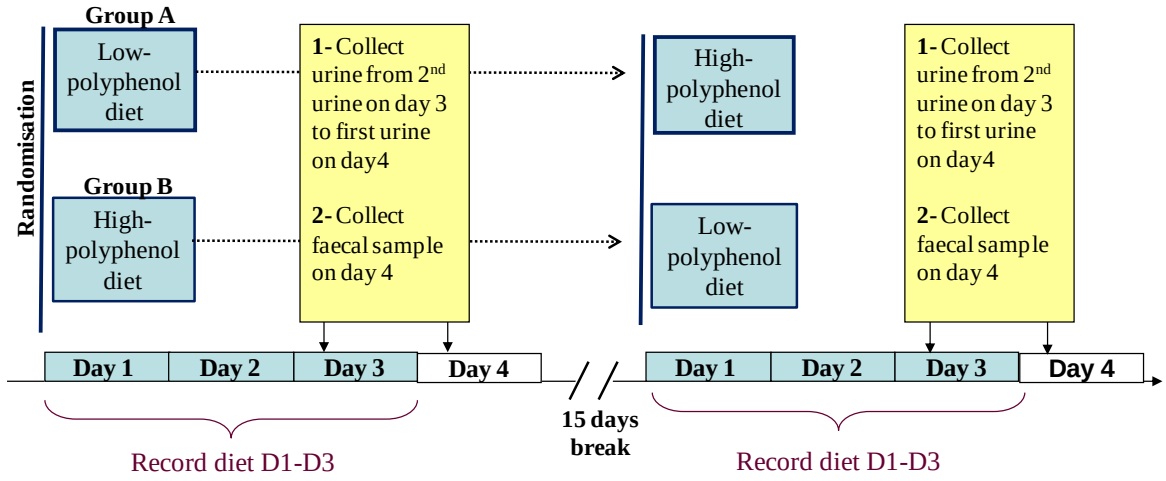
The required sample size was estimated based on a previous study (Combet *et al.*, 2011), using the excretion of the sum of phenolic acids relevant to flavonoid metabolism, as a primary outcome. The difference in urinary phenolic acid excretion after low-high diet was 60.45± 36.03µmol/day in the European groups and 1.91± 10.18 µmol/day in the Indian group. Based on this, it was calculated that a total sample size of n=16, allowing for the recruitment of n=8 participants of European background and n=8 participants of Asian Indian background, would detect a difference of one standard deviation between the two populations at a power of 90% $p<0.05$, allowing for a 20% drop-out rate.

3.2.3 Study design and sample collection

A crossover design with randomised allocation was carried out. Participants picked the diet allocation in sealed envelopes from a bag. Each low and high-polyphenol diet lasted three days, with a washout period of 15 days in-between. Three days for each diet was enough for polyphenol rich foods to be supplied to the colons and fermented over the course of several meals. Moreover, a 15 day washout period was used to separate the effects of each diet and to give the participant a break to resume normal eating patterns and prepare for the second part of the diet. During the low-polyphenol diet, participants were asked to avoid all fruits, vegetables, onions, coffee, tea, chocolate, vanilla and similar flavourings, whole meal products, alcohol, spices, and all dietary supplements (vitamins, minerals, and herbal products). During the high-polyphenol diet, participants were asked to follow a specific diet including polyphenol-rich foods, which were provided along with cooking guidance and recipe sheets. Examples of foods to be included during the low polyphenol diet, and a detailed menu of the high-polyphenol diet are given in Appendix 4 and 5. Urine and faecal samples were collected after the low-polyphenol diet (day 4) and high-polyphenol diet (day 4) for the human feeding study measurements (Figure 3-2). Stool samples collected after the low-polyphenol diet (day 4) were used in *in-vitro* faecal fermentations.

Sociodemographic and anthropometric measurements (height, weight, BMI, and waist circumference), and blood pressure for each participant were also collected (details described in Chapter 2, page 44).

Figure 3-2: Overall study design and samples collection.



3.3 Dietary assessment

A food frequency questionnaire was used to assess the participants' habitual diet over the past year, as described in Chapter 2, page 68).

3.4 Dietary records

Participants kept a 3-day weighed dietary record for the duration of the low and high-polyphenol diets. Diaries were used to estimate the intake of macronutrients, micronutrients, and flavonoids of each participant during the study and to measure the participants' compliance by reviewing the food types and portion size according to the provided instruction (details described in Chapter 2, page 67).

3.5 Outcome measures for the human feeding and *in-vitro* faecal fermentation studies

Phenolic acid (GC-MS), total phenols (Folin–Ciocalteu), and ferric reducing antioxidant power (FRAP) were measured in urine samples. The pH, short chain fatty acids (GC-FID), ammonia, and bacterial composition (Taqman real-time quantitative PCR) were measured in the faecal samples.

Phenolic acid, pH, short chain fatty acids, and gas production were measured in the fermentation supernatants (details described in Chapter 2).

3.6 Results

3.6.1 Subjects characteristics

Sixteen participants were recruited aged between 18 – 43 years old, with 8 Europeans and 8 Asian Indians (who had lived in the UK for less than 5 years; two of whom were vegetarians). The baseline data for both groups (Europeans and Indians) are presented in Table 3-1. There were no significant differences between groups in terms of their age or anthropometric characteristics. The male to female ratio was different between groups, with 2 males and 6 females in the European group and 7 males and 1 female in the Indian group.

Table 3-1: Baseline data in Europeans (n=8) and Indians (n=8) participants.

	All		European (n=8)		Indian (n=8)		p value
	Median	**IQR**	**Median**	**IQR**	**Median**	**IQR**	
Age (years)	24.0	5.8	23.0	6.5	25.0	4.3	0.4
Height (cm)	174.0	15.5	163.0	19.0	175.5	8.5	0.1
Weight (kg)	70.2	20.6	63.0	13.3	74.8	10.8	0.2
BMI (kg/m^2)[a]	22.7	4.3	22.0	5.8	23.0	3.7	0.7
W.C (cm)[b]	82.5	14.3	80.0	3.0	92.0	11.4	0.1
Systolic BP	113.5	15.0	122.0	19.0	1 13.0	4.0	0.7
Diastolic BP	71.5	10.3	69.0	13.8	73.0	5.5	0.3
			n	**%**	**n**	**%**	
Normal weight			5	62.5	5	62.5	
Overweight			3	37.5	2	25	
Obese			0	0	1	12.5	

[a]BMI cut-off points (European=25, Indian = 23; Snehalatha *et al*., 2003)
[b]WC cut-off points (European women=80 cm, man=94; Indian women=80, man=90; Misra *et al*., 2006)

Bowel movements (self-reported on the questionnaire as twice daily or more, daily, every 2-3 days or less than twice a week) were significantly different between Europeans and Indians using chi-square test (p=0.02; Table 3-2). The Indian group reported daily bowel movements, while the European varied from twice daily to every two to three days.

Table 3-2: Bowel movement in Europeans (n=8) and Indians (n=8) participants.

	Twice daily or more	Daily	Every 2-3 days	Less than twice a week
Europeans (n=8)	37.5%	37.5%	25%	0%
Indians (n=8)	0%	100%	0%	0%

*Data presented as percentage of frequency

3.6.2 Dietary assessment

3.6.2.1 Estimation of habitual dietary habits

The Indian group consumed more servings of onion, tomato, chilli, or curry- based products, yoghurt, and spices & chilli than the European group (p=0.008, 0.04, 0.0009; respectively). The European group consumed more seafood (p=0.01) and cheese (p=0.002) than the Indian group. In terms of meat intake, two male vegetarians were in the Indian group; however, there was no significant difference in median meat intake between the two groups (Table 3-3).

Table 3-3: Estimation of dietary habits (serving/week) using the FFQ European (n=8) and Indian (n=8) participants.

Food item	European		Indian		p value
	Median	IQR	Median	IQR	
Hot Beverages (Green tea, black tea, coffee, hot chocolate, herbal infusion)	15.4	24.4	8.8	7.9	0.2
Milk drinks	3.0	15.2	2.0	6.5	0.6
Milk substitutes(soya, rice, and oat milk)	0.5	0.0	3.8	9.5	0.5
Fruit juices	1.5	6.5	2.5	5.9	0.6
Soft drink	0.4	0.4	6.3	9.1	0.1
Alcohol	1.6	0.7	3.6	4.6	0.8
Wholemeal products (pasta, rice, and brown rice)	7.6	7.3	1.3	0.7	0.2
Refined products (pasta, rice, and crisps and crackers)	4.9	4.2	13.1	10.3	0.1
Breakfast cereals	3.8	5.1	0.9	2.1	0.09
Fresh fruit	16.0	11.2	7.5	13.0	0.1
Dried fruits	0.5	0.8	0.3	0.3	0.1
Vegetables	10.1	9.1	19.4	13.2	0.2
Potatoes	1.8	2.0	2.8	4.3	1.0
Legumes	2.0	0.6	5.0	8.4	0.9
Onion, tomato, chili, or curry- based products (inc. fresh tomatoes, ketchup, and soup)	11.9	7.9	29.1	18.6	0.008
Meat (beef, chicken, lamb, pork, bacon, and sausages)	2.0	4.3	3.0	1.6	0.7
Sea food (White fish, oil-rich fish, and shellfish)	2.9	1.6	0.6	1.4	0.01
Biscuits, cakes, and sweet	11.0	12.9	17.4	10.3	0.4
Yoghurt	1.8	2.5	5.9	3.6	0.04
Dairy dessert	0.4	0.3	0.3	0.5	0.3
Cheeses	2.4	3.4	0.0	0.6	0.002
Eggs	2.0	3.0	3.0	3.0	0.5
Ice cream	0.5	0.8	0.0	0.3	0.3
Fats & oils	6.1	9.6	3.4	6.6	0.1
Seasonings	6.8	19.6	8.8	1.9	0.9
Herbs	6.0	5.9	5.0	7.1	0.4
Spices & Chili	2.5	3.3	14.0	0.0	0.0009

3.6.2.2 Macronutrients intake during low and high polyphenol diets

There was no significant difference between the two groups in terms of energy, fat, protein, carbohydrate, total sugars, starch, alcohol, or dietary fibre consumption during the low-polyphenol diet. However, Europeans consumed significantly more total sugar ($p=0.01$) during the high-polyphenol diet than during the low-polyphenol diet (Table 3-4). When considering the difference in macronutrient intake (Δ low-high diet), there was no significant difference between the groups.

3.6.2.3 Micronutrient intake during low and high polyphenol diets

There was no significant difference between groups for the vitamin or dietary mineral intake, with the exception of vitamin B12 intake during the low-polyphenol diet ($p=0.03$), which had a notably higher intake in the European group (Table 3-5).

Table 3-4: Energy intake and macronutrients from the dietary records after low and high-polyphenol diets in European (n=8) and Indian (n=8) participants.

Group	Low-polyphenol diet					High-polyphenol diet				
Diet	**European**		**Indian**		**p value**	**European**		**Indian**		**p value**
Nutrient	**Median**	**IQR**	**Median**	**IQR**		**Median**	**IQR**	**Median**	**IQR**	
Energy (KJ)	8016	2466	9663	7430	0.6	8227	2442	7549	1033	0.7
kcal (Kc)	1910	585	2292	1775	0.6	1949	586	1782	257	0.7
Fat (g)	95.8	27.8	78.1	45.3	0.4	74.3	25.8	68.2	8.0	0.4
Protein (g)	80.4	7.2	85.8	33.5	0.7	54.9	15.6	58.3	21.4	0.6
Carbohydrate (g)	195.0	83.3	301.9	326.7	0.4	247.1	109.5	244.4	78.7	0.9
Total sugars (g)	36.1	26.4	31.7	28.5	0.4	115.9	50.0	90.4	40.5	0.01
Starch (g)	140.5	62.8	237.0	308.3	0.4	70.6	44.2	115.2	86.4	0.4
Alcohol (g)	0.0	0.0	0.0	0.0	-	0.0	0.0	0.0	0.0	-
Dietary fibre*(g)	9.1	2.0	9.0	11.2	0.8	27.5	3.2	30.5	8.7	0.4

* Non-starch polysaccharide

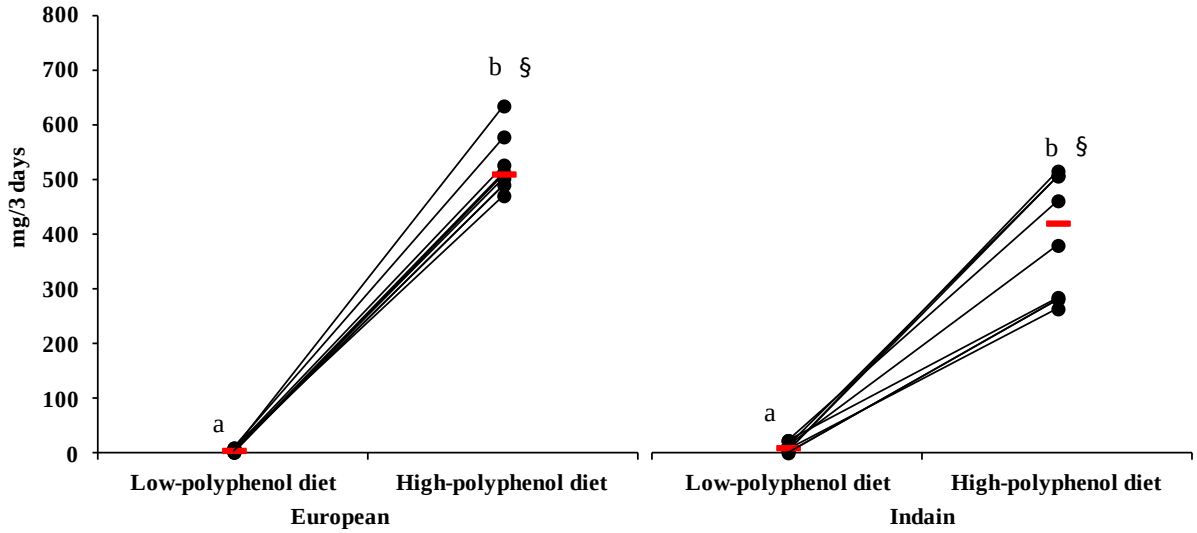
Table 3-5: Estimated micronutrients intake from the dietary records after low and high-polyphenol diets in European (n=8) and Indian (n=8) participants.

Diet	Low-polyphenol diet				p value	High-polyphenol diet				p value
Group	European		Indian			European		Indian		
Nutrient	Median	IQR	Median	IQR		Median	IQR	Median	IQR	
Vitamin A (µg)	632.0	201.6	530.5	485.3	0.3	362.2	98.1	284.0	169.1	0.7
Thiamine (mg)	1.2	0.3	0.9	0.8	0.2	0.6	0.3	0.7	0.4	0.2
Riboflavin (mg)	1.7	0.5	1.5	1.6	0.9	0.6	0.4	0.5	0.2	0.5
Niacin (mg)	28.6	4.9	29.4	22.9	0.9	19.5	14.7	13.5	9.1	0.6
Vitamin B6 (mg)	1.5	0.2	1.5	1.2	0.9	0.7	0.5	0.8	0.5	0.8
Vitamin B12 (µg)	5.2	1.3	2.7	2.2	0.03	1.6	2.1	0.6	1.6	0.6
Folic acid (µg)	155.2	70.5	170.7	150.2	0.7	95.5	33.3	108.8	49.7	0.3
Pantothenic acid (mg)	4.6	1.4	3.8	2.0	0.5	2.2	0.5	1.9	0.9	0.7
Biotin (µg)	26.0	5.9	23.2	11.9	0.5	18.5	6.4	15.7	4.4	0.4
Vitamin C (mg)	17.2	8.3	16.2	16.7	0.6	62.4	11.0	49.1	38.4	0.3
Vitamin D (µg)	2.0	1.3	1.4	1.3	0.2	1.2	1.1	0.4	0.7	0.2
Vitamin E (mg)	5.8	3.8	6.9	2.1	0.7	11.7	5.0	8.9	1.6	0.3
Calcium (mg)	1302.3	539.5	955.3	1033.4	0.6	358.8	69.7	346.0	101.8	0.7
Magnesium (mg)	182.7	65.8	142.3	118.3	0.7	146.5	55.1	168.3	78.4	0.9
Sodium (mg)	3009.5	1340.0	3324.0	1971.8	0.5	2698.5	713.2	2320.7	971.0	0.9
Potassium (mg)	1934.2	686.1	1725.0	1441.3	1.0	1987.7	680.3	1931.5	915.1	1.0
Chlorine (mg)	4394.5	1702.3	5101.8	2889.2	0.6	2612.2	721.5	2563.3	1338.8	0.8
Phosphorus (mg)	1486.7	227.8	1354.7	816.8	0.6	568.2	329.1	686.0	257.2	0.4
Iron (mg)	8.6	2.5	10.2	9.3	0.6	6.2	2.4	6.9	4.2	0.3
Zinc (mg)	8.5	1.6	6.5	3.3	0.2	4.1	1.5	5.2	3.2	0.1
Copper (mg)	0.7	0.3	0.6	0.3	0.6	1.1	0.2	1.1	0.4	0.5
Manganese (mg)	1.3	1.0	1.2	0.8	0.8	2.5	0.5	2.7	1.0	0.7
Selenium (µg)	41.0	9.8	36.5	15.1	0.2	22.5	15.8	16.5	16.5	0.4
Iodine (µg)	198.7	127.9	175.8	152.0	0.6	58.7	57.0	63.3	34.7	0.5

3.6.2.4 Flavonoid intake during low and high polyphenol diets

Dietary assessment of average flavonoid intake estimated that Europeans consumed significantly more (p=0.03) flavonoids over the 3 days high-polyphenol diet (510.4 mg/day, IQR 40.1) than Indians (420.8 mg/day, IQR 222.5; Figure 3-3).

Figure 3-3: Median flavonoid intake per day over 3 days after low and high-polyphenol diets in European (n=8) and Indian (n=8) participants.



Each circle indicates the estimated average daily flavonoid intake for each participant after low and high-polyphenol diets. Median flavonoid intake for each group is indicated by a red horizontal line.
a,b symbols indicate differences within group (low to high-polyphenol diet).
§ symbol indicates differences between groups (high vs. high).

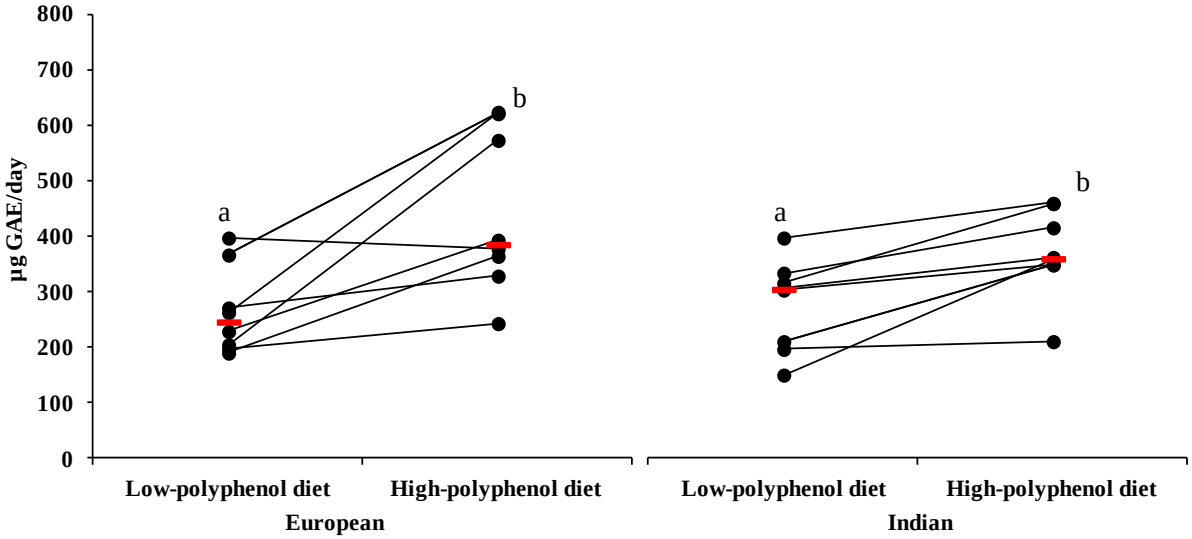
As the urinary phenolic acid excretion has been shown to be markedly increased between 8 to 24 h following ingestion (Roowi *et al.*, 2010), urinary phenolic acid excretion was corrected for flavonoid intake on day 3 of the diet, given that the 24 h urine collection was carried out from the second urine of day 3, and including the first urine of day 4. A similar result was obtained when the flavonoid intake of day 3 only was considered, with significantly higher levels (p=0.02) in the European group (553.1 mg, IQR 76.0) compared to the Indian group (420.9 mg, IQR 158.7).

3.6.3 Measurements performed on urine samples

3.6.3.1 Urinary total phenols using Folin-Ciocalteu assay

Urinary total phenols were significantly higher after the high-polyphenol diet in both European (p=0.02) and Indian (p=0.03) participants. The urinary total phenol increased from 245.9 µg GAE/24h (IQR 91.9) to 385.4 µg GAE/24h (IQR 229.7) in the European group and from 304.7 µg GAE/24h (IQR 112.9) to 359.9 µg GAE/24h (IQR 77.1) in the Indian group. However, there was no significant difference in urinary total phenols excretion (Δ low-high diet) between groups (Figure 3-4).

Figure 3-4: 24-hour urinary total phenols (µg GAE/24h) after low and high-polyphenol diets in European (n=8) and Indian (n=8) participants.

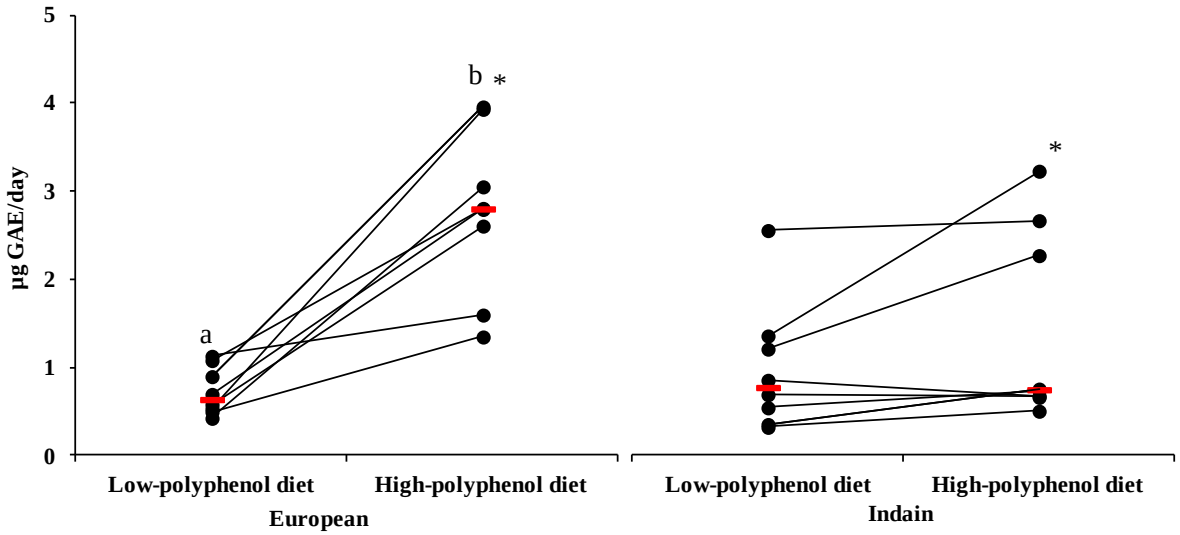


Each circle indicates the measurement of urinary total phenols for each participant after low and high-polyphenol diets. Median urinary total phenols for each group are indicated by a red horizontal line.
a,b symbols indicate differences within group (low to high-polyphenol diet).

3.6.3.2 Total antioxidant activity (FRAP) of urine samples after low and high polyphenol diets

The urinary FRAP value was increased significantly in European participants after the high-polyphenol diet compared to the low-polyphenol diet, from 0.6 mM Fe+2/day (IQR 0.4) to 2.8 mM Fe+2/day (IQR 0.9; p=0.0009), indicating a rise in total antioxidant activity in this group. However, urinary FRAP did not change in Indian participants, with similar values recorded after both low (0.8 mM Fe+2/day, IQR 0.8) and high polyphenol diets (0.7 mM Fe+2/day, IQR 1.7). The difference (Δ low-high diet) in FRAP value was higher in the European group (p=0.003; Figure 3-5).

Figure 3-5: 24-hour urinary antioxidant activity (mM F^{+2}/day) after low and high-polyphenol diets in European (n=8) and Indian (n=8) participants.

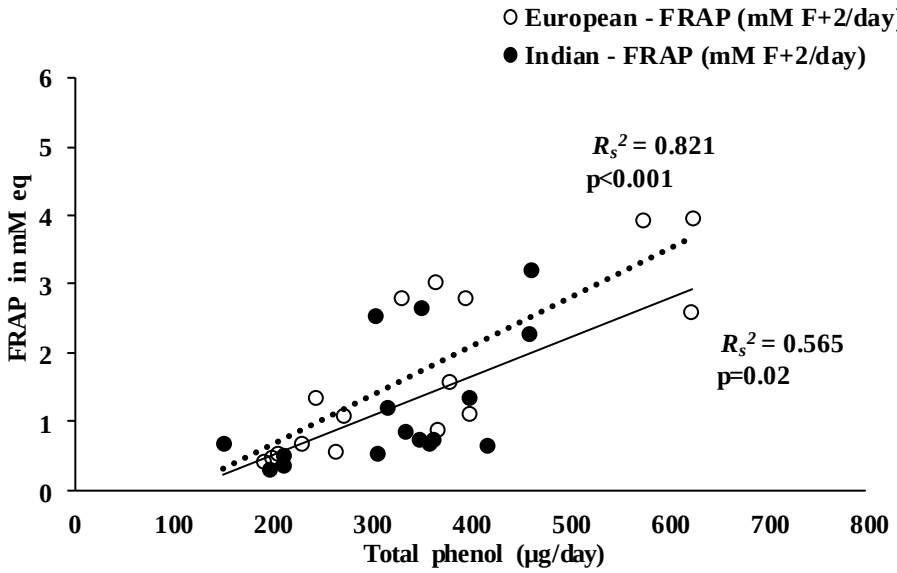


Each circle indicates the measurement of urinary FRAP for each participant after low and high-polyphenol diets. Median urinary FRAP for each group is indicated by a red horizontal line.
a,b symbols indicate differences within group (low to high-polyphenol diet).
*symbols indicate differences in changes (Δ) between groups.

3.6.3.2.1 Correlation between urinary total phenols (measured with Folin-Ciocalteu) and urinary FRAP

The relationship between urinary total phenols (measured with Folin-Ciocalteu) and urinary FRAP was analysed by linear regression (Spearman rank correlation). There was a very good relationship (R_s^2=0.8; p<0.001) between FRAP and total phenols in the European group and a moderate relationship between the variables (R_s^2=0.6; p=0.02) in the Indian group (Figure 3-6).

Figure 3-6: Correlation between urinary FRAP and the urinary total phenols (Folin-Ciocalteu) in European (n=8) and Indian (n=8) participants.



Dotted line indicates Europeans; solid line indicates Indians.

3.6.3.3 GC-MS measurement of urinary phenolic acid excretion after low and high-polyphenol diets

Eighteen phenolic acids were identified and quantified in the 24 hr urine of participants using GC-MS (A typical GC-MS trace of standards and sample are illustrated in Figure 3-7 and for sample in Figure 3-8, respectively.

Table 3-6). The identification of phenolic acids was based on retention time (t_R) and target ions (Stalmach *et al.*, 2013). A typical GC-MS trace of standards and sample are illustrated in Figure 3-7 and for sample in Figure 3-8, respectively.

Table 3-6: Retention times and target ions present identified in chromatograms mass spectra of standard solution and urine samples.

No.	Phenolic acids	t_R (min)	Target ion (m/z)
1	Benzoic acid (BA)	6.95	105
2	Phenylacetic acid (PAA)	7.49	164
3	Mandelic acid (MA)	10.06	179
4	3-Hydroxybenzoic acid (3-OHBA)	12.19	267
5	3-Hydroxyphenylacetic acid (3-OHPAA)	13.35	164
6	4-Hydroxybenzoic acid (4-OHBA)	13.95	267
7	4-Hydroxyphenylacetic acid (4-OHPAA)	14.3	179
8	4-Hydroxyphenylpropionic acid (4-OHPPA)	18.62	179
9	Vanillic acid (VA)	18.72	297
10	Homovanillic acid (HVA)	18.97	209
11	4-Hydroxymandelic acid (4-OHMA)	19.28	267
12	3,4-Dihydroxybenzoic acid (3,4diOHBA)	20.99	193
13	3,4-Dihydroxyphenylacetic acid (3,4diOHPAA)	21.33	179
14	Hippuric acid (HA)	22.06	206
15	3-(3,4-Dihydroxyphenyl) propionic acid (Dihydrocaffeic acid)	24.39	340
16	4-Hydroxy-3-methoxy-phenylpropionic acid (3,4diOHPPA)	26.76	179
17	Gallic acid (GA)	27.46	281
18	3-Hydroxyhippuric acid (3-OHhippA)	37.09	294

Figure 3-7: GC-MS total ion chromatogram profile of phenolic acids in the standards.

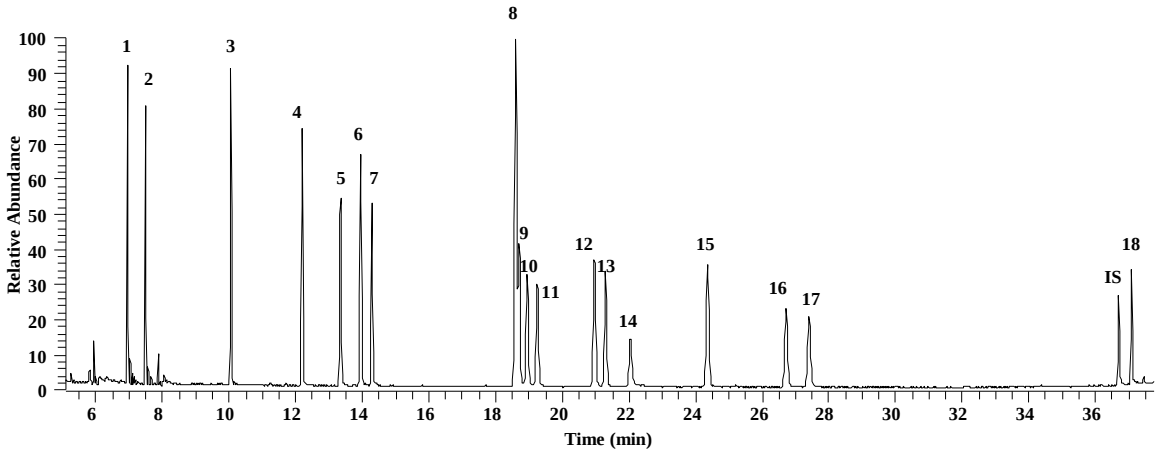
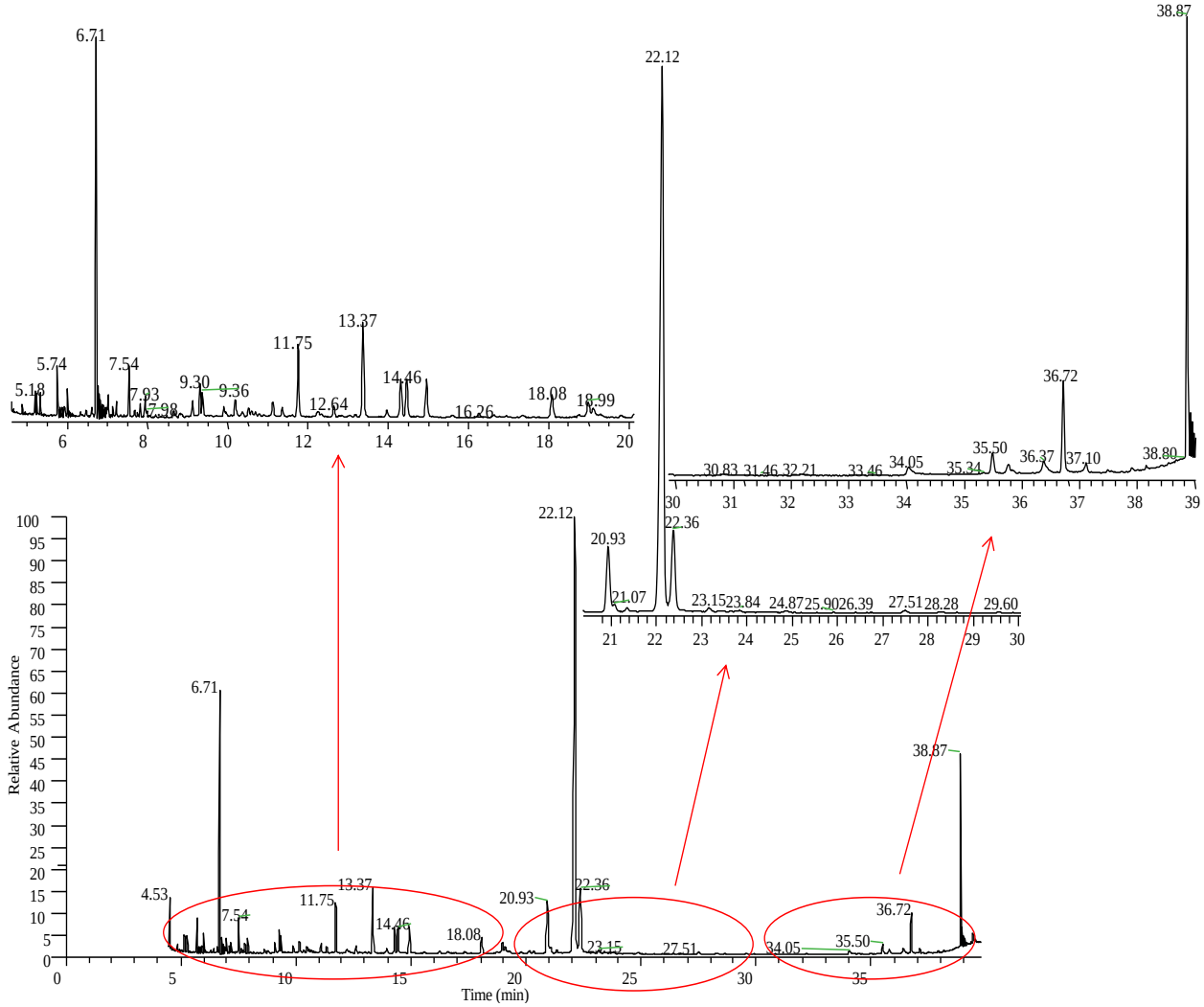


Figure 3-8: Profiling of phenolic acids in urine samples after the high-polyphenol diet.

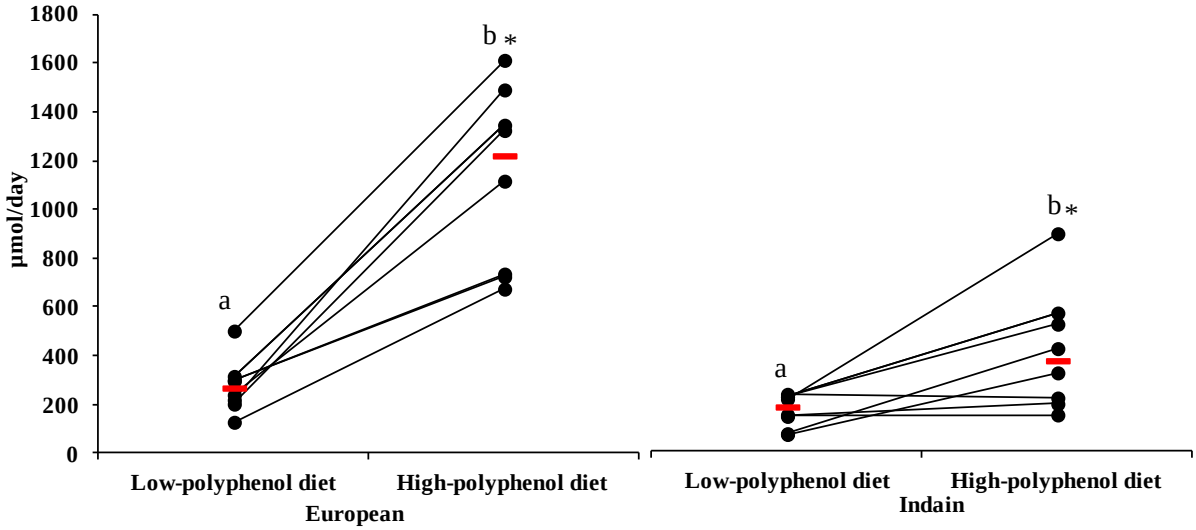


All urinary phenolic acid concentrations increased in both groups after the high-polyphenol diet intake with the exception of 4-OHPAA. Therefore, 4-OHPAA was excluded from the sum of urinary phenolic acid excreted for between-group comparisons.

As expected, the sum of urinary phenolic acids increased after the high-polyphenol diet, with an increase from 268.2 µmol/day (IQR 89.3) to 1220.4 µmol/day (IQR 649.5) in the European group ($p<0.001$) and from 183.4 µmol/day (IQR 97.3) to 372.9 µmol/day (IQR 320.2) in the Indian group ($p=0.04$). The difference in urinary excretion (Δ low-high diet) was significantly higher ($p=0.001$) in the European group (Δ953.5 µmol/day, IQR 593.9) compared to the Indian group (Δ274.7 µmol/day, IQR 307.5; Figure 3-9).

After correcting for flavonoid intake, the Indian group still excreted significantly ($p<0.001$) less phenolic acid (633.3 µmol/mg of flavonoids, IQR 860) than the European group (1629.4 µmol/mg of flavonoids, IQR 1185.3). As the differences between groups before and after the correction were the same, the actual (non-corrected) values for urinary phenolic acid were used.

Figure 3-9: 24-hour urinary phenolic acids profile excretion (µmol/day) after low and high-polyphenol diets in European (n=8) and Indian (n=8) participants.



Each circle indicates the measurement of urinary phenolic acids profile for each participant after low and high-polyphenol diets. Median urinary phenolic acids profile for each group is indicated by a red horizontal line.
a,b symbols indicate differences within group (low to high-polyphenol diet).
*symbol indicates differences in changes (Δ) between groups.

There were several differences in the excretion of the individual phenolic acids (Δ low-high diet). The increase in benzoic acid ($p=0.04$), 3-hydroxybenzoic acid ($p=0.02$), 3, 4-dihydroxyphenylacetic acid ($p=0.05$), and hippuric acid ($p=0.001$) urinary excretion was significantly higher in Europeans than Indians. In addition, the concentration of urinary3-OHPAA was higher in Europeans after both the low-polyphenol diet ($p<0.001$) and high-polyphenol diet ($p=0.04$) when compared to the Indian participants. Likewise, the homovanillic acid concentration was higher in the urine of Europeans after low-polyphenol diet ($p=0.05$) and high-polyphenol diet ($p=0.05$) compared to Indian participants (Table 3-7).

Table 3-7: Amount of phenolic acid in 24 h urine (µmol/day) after low and high-polyphenol diets in European (n=8) and Indian (n=8) participants.

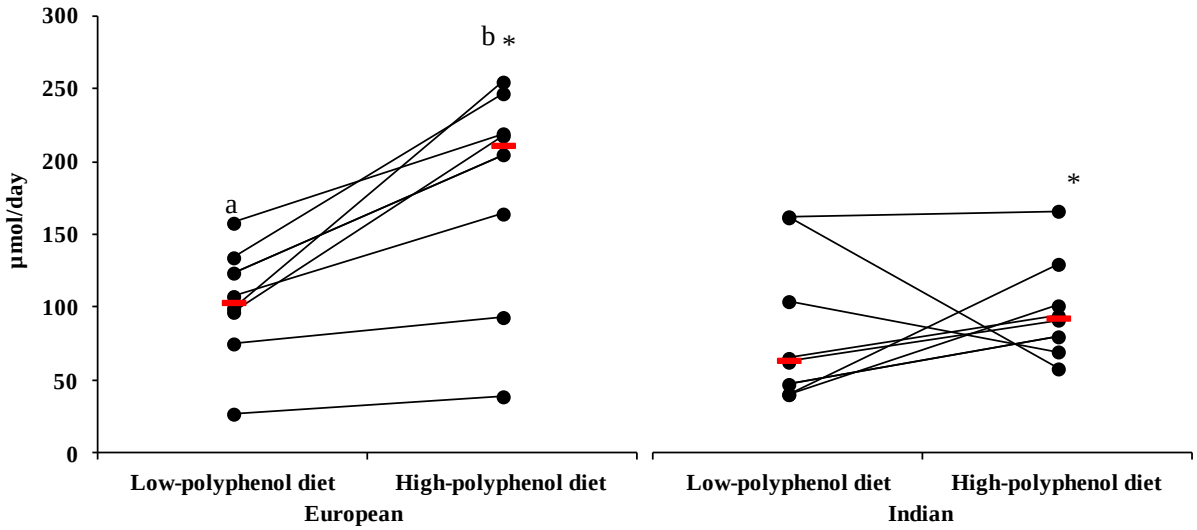
Group	Europeans					Indians					
Diet	**low-polyphenol diet**		**High- polyphenol diet**			**low-polyphenol diet**		**High- polyphenol diet**			
Acid	**Median**	**IQR**	**Median**	**IQR**	Δ	**Median**	**IQR**	**Median**	**IQR**	Δ	**p value**
BA	16.6	14.3	31.1	32.9	**14.5**	15.1	22.6	11.4	17.6	**-3.7**	0.04
PAA	3.2§	2.2	3.2	4.8	**0.0**	0.5§	1.6	1.7	2.1	**1.1**	0.9
MA	0.7§	0.5	1.1	1.2	**0.4**	0.2**§	0.0	0.4**	0.2	**0.2**	0.3
3-OHBA	0.6*§	0.5	0.9*†	1.2	**0.3**	< 0.1§	0.1	0.1†	0.1	**0.0**	0.02
3-OHPAA	2.3*§	1.3	19.2*†	23.3	**16.9**	0.9§	0.5	2.4†	10.3	**1.5**	0.08
4-OHBA	2.1§	1.0	3.8	1.9	**1.7**	1.1§	0.4	1.6	0.7	**0.5**	0.3
4-OHPAA	38.0	21.4	42.9†	15.0	**4.9**	17.5	32.3	17.7†	9.1	**0.2**	0.4
4-OHPPA	0.4§	0.4	0.5	0.9	**0.2**	< 0.1§	0.0	0.1	0.1	**0.0**	0.2
VA	0.7*	0.4	2.8*	2.7	**2.1**	0.3**	0.2	1.8**	1.2	**1.5**	0.6
HVA	0.8*§	4.4	19.8*†	12.7	**9.0**	4.4§	5.7	8.9†	5.6	**4.5**	0.2
4-OHMA	4.9	1.5	3.9	1.6	**-1.0**	2.8	1.9	3.1	0.8	**0.3**	0.2
3,4diOHBA	1.0*§	0.2	2.1*†	1.1	**1.1**	0.3**§	0.2	0.8**†	0.3	**0.5**	0.05
3,4diOHPAA	1.5*§	0.4	2.9*†	5.3	**1.5**	0.6§	0.3	1.3**†	2.4	**0.6**	0.3
HA	155.7*§	95.7	969.8*†	540.2	**814.1**	68.7**§	55.9	257.5**†	372.3	**188.8**	0.001
Dihydrocaffeic acid	1.3*	0.9	2.3*	2.2	**0.9**	0.6	0.8	1.1	0.8	**0.4**	0.3
3,4diOHPPA	0.5*§	0.4	1.6*	1.2	**1.1**	0.1**§	0.2	0.6**	0.4	**0.5**	0.08
GA	0.6§	0.7	0.8†	1.4	**0.2**	< 0.1§	0.1	0.2†§	0.4	**0.2**	0.05
3-OHhippA	21.0*§	10.2	68.6*	41.4	**47.6**	13.3**§	9.2	33.9**	25.2	**20.6**	0.2

Δ Difference in urinary excretion (high diet minus low diet).
p value is the difference in urinary phenolic acid excretion (Δ low-high diet) between groups.
* Significant increase in Europeans after the high-polyphenol diet $p \leq 0.01$
**Significant increase in Indians after the high-polyphenol diet $p \leq 0.01$
§Significant difference between groups after the low-polyphenol diet $p \leq 0.05$
†Significant difference between groups after the high-polyphenol diet $p \leq 0.05$

Hippuric acid (HA) was the most abundant acid in urine samples after the high-polyphenol diet in both groups (89.2 and 83.3% of the total of all phenolic acids for Europeans and Indians, respectively). The sum of the phenolic acids minus hippuric acid was considered as it is most likely to be formed in the liver by conjugation of benzoic acid and glycine. Dietary sources of benzoic acid and precursors (quinic acid, aromatic amino acid tryptophan, tyrosine, and phenylalanine) should be considered (Self *et al.*, 1960, Grumer, 1961). Other sources of benzoic acid are benzoates (E numbers 210-219) which are commonly used in food, medications, and mouthwashes.

The sum of phenolic acids minus hippuric acid was significantly increased in the European group after following the high-polyphenol diet, from 103.6 µmol/day (IQR 34.9) to 211.2 µmol/day(IQR 79.6; $p<0.01$). The sum of phenolic acids minus hippuric acid changed from 63.9 µmol/day (IQR 72.9) to 92.8 µmol/day (IQR 30.9) in the Indian group. The difference in urinary excretion (Δ low-high diet) was significantly higher ($p=0.03$) in the European group (Δ 71.2 µmol/day, IQR 67.6) compared to the Indian group (Δ 29.0 µmol/day, IQR 45.4; Figure 3-10).

Figure 3-10: 24-hour urinary phenolic acids profile excretion without hippuric acid (µmol/day) after low and high-polyphenol diets in European (n=8) and Indian (n=8) participants.

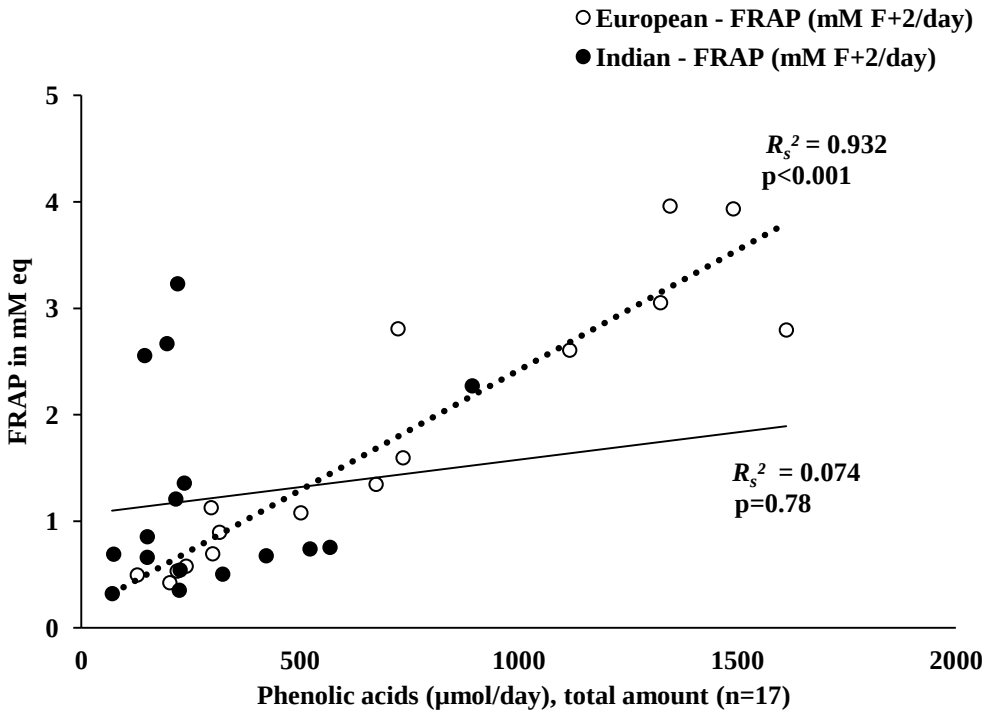


Each circle indicates the measurement of urinary phenolic acids profile without hippuric acid for each participant after low and high-polyphenol diets. Median urinary phenolic acids profile without hippuric acid for each group is indicated by a red horizontal line.
a,b symbols indicate differences within group (low to high-polyphenol diet).
*symbol indicates differences in changes (Δ) between groups.

3.6.3.3.1 Correlation between urinary phenolic acid (measured with GC-MS) and urinary FRAP

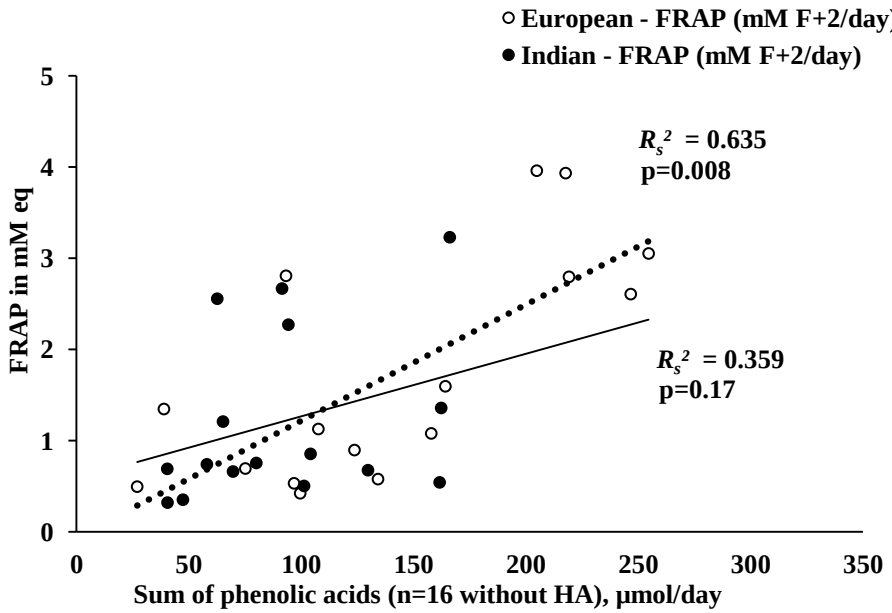
The relationship between urinary phenolic acid (measured with GC-MS) and urinary FRAP was analysed by linear regression (Spearman rank correlation). There was a strong correlation between FRAP and the sum of the phenolic acids in the European group (R_s^2=0.9; p=0.001); however, there was no correlation between FRAP and total phenolic acids in the Indian group (Figure 3-11). The data without the hippuric acid showed no difference in the direction of the association (Figure 3-12).

Figure 3-11: Correlation between urinary FRAP and the phenolic acid (GC-MS) in European (n=8) and Indian (n=8) participants.



Dotted line indicates Europeans; solid line indicates Indians.

Figure 3-12: Correlation between urinary FRAP and the phenolic acid without hippuric acid (GC-MS) in European (n=8) and Indian (n=8) participants.



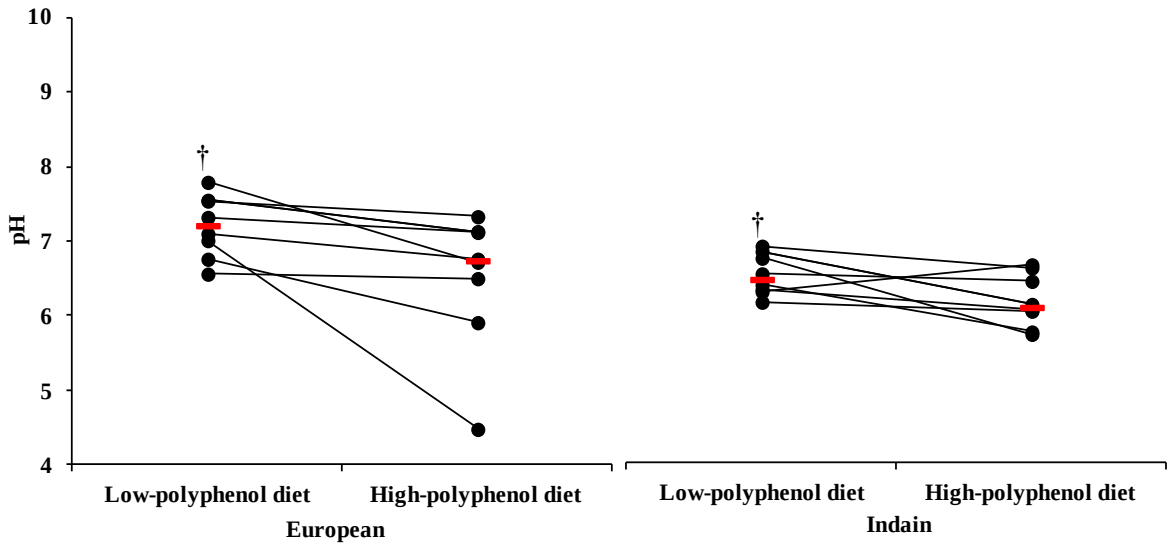
Dotted line indicates Europeans; solid line indicates Indians.

3.6.4 Measurements performed in faecal samples

3.6.4.1 Faecal pH after low and high-polyphenol diets

The faecal pH did not change in either group from low to high-polyphenol diet (Europeans from 7.2, IQR 0.6 to 6.7, IQR 0.8; Indians from 6.5, IQR 0.5 to 6.1, IQR 0.5). The faecal pH was lower in the Indian group after the low-polyphenol diet than the European group (p=0.003; Figure 3-13).

Figure 3-13: Change in faecal pH after low and high-polyphenol diets in European (n=8) and Indian (n=8) participants.

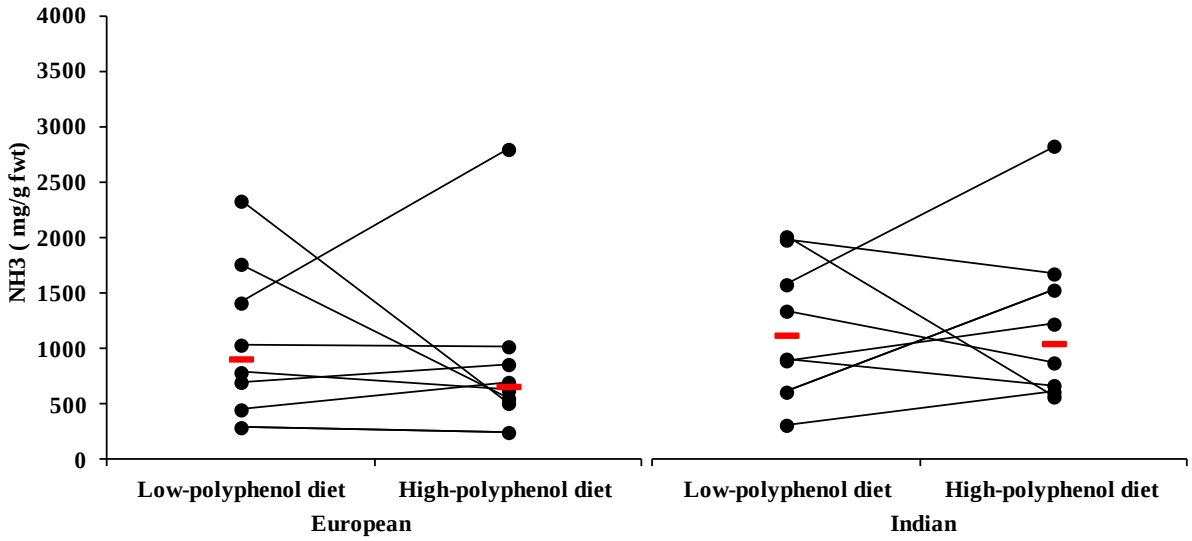


Each circle indicates the measurement of faecal pH for each participant after low and high-polyphenol diets. Median faecal pH for each group is indicated by a red horizontal line.
† symbol indicates differences between groups (low vs. low).

3.6.4.2 Faecal ammonia after low and high-polyphenol diets

The change in faecal ammonia was not significant in either group from low to high-polyphenol diet (in Europeans, from 910.5 mg/g wet weight, IQR 863.2, to 662.8 mg/g wet weight, IQR 356.9; in Indians, from 1124.3 mg/g wet weight, IQR 856.3 to 1048.9 mg/g wet weight, IQR 910.5; Figure 3-14).

Figure 3-14: Change in faecal ammonia after low and high-polyphenol diets in European (n=8) and Indian (n=8) participants.

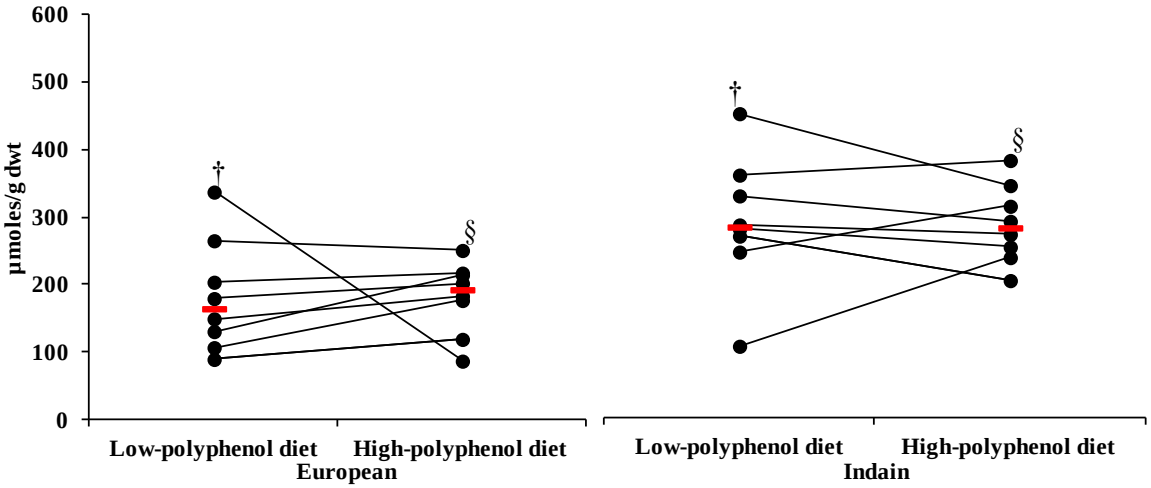


Data presented as mg/g of wet weight. Each circle indicates the measurement of faecal ammonia for each participant after low and high-polyphenol diets. Median faecal ammonia for each group is indicated by a red horizontal line.

3.6.4.3 Faecal SCFA after low and high-polyphenol diets

Total faecal SCFA concentration (sum of all SCFA) did not significantly increase from a low to high-polyphenol diet in either group. Values of 164.3 µmoles/g dwt (IQR 94.5) to 192.4 µmoles/g dwt (IQR 52.8) were recorded in the European group, and from 288.7 µmoles/g dwt (IQR 75.0) to 287.4 µmoles/g dwt (IQR 73.8) in the Indian group. There were no significant differences (Δ low-high diet) in either group; however, the SCFA concentration was higher for the Indian group after both low (p=0.02) and high-polyphenol (p=0.002) diets, compared to the European group (Figure 3-15).

Figure 3-15: Faecal SCFA after low and high-polyphenol diets in in European (n=8) and Indian (n=8) participants.



Each circle indicates the measurement of faecal SCFA for each participant after low and high-polyphenol diets. Median faecal SCFA for each group is indicated by a red horizontal line.
† symbol indicates differences between groups (low vs. low).
§ symbol indicates differences between groups (high vs. high).

There was no difference between groups for change (Δ low-high diet) in levels of individuals SCFA (Table 3-8); however, the concentration of the acetic acid was significantly higher after both low (p=0.007) and high (p=0.002) diets in Indians compared to the Europeans. Moreover, the level of propionic acid was significantly higher in the Indian group after the high-polyphenol diet (p=0.004), and valeric acid was significantly higher (p=0.05) in the Indian group after the low-polyphenol diet.

Table 3-8: Faecal concentration of SCFA (µmol/g dwt) in European (n=8) and Indian (n=8) participants after low and high-polyphenol diets.

Group	Europeans					Indians					
Diet	**Low-polyphenol diet**		**High-polyphenol diet**		Δ	**Low-polyphenol diet**		**High-polyphenol diet**		Δ	**p value**
	Median	**IQR**	**Median**	**IQR**		**Median**	**IQR**	**Median**	**IQR**		
Acetic acid	97.8§	35.8	130.2†	25.4	**32.4**	168.3§	30.4	168.6†	16.8	**0.3**	0.95
Propionic acid	25.6	25.7	25.9†	11.4	**0.3**	75.4	46.4	53.5†	25.4	**-21.9**	0.23
Isobutyric acid	3.2	2.9	2.5	0.6	**-0.7**	3.9	3.2	2.7	2.4	**-1.3**	1.00
Butyric acid	19.4	23.0	17.5	8.5	**-1.9**	38.4	25.4	32.7	28.2	**-5.8**	0.96
Isovaleric acid	5.7	4.6	3.6	2.2	**-2.1**	6.7	5.0	4.1	3.8	**-2.6**	0.94
Valeric acid	4.9§	3.7	3.3	1.5	**-1.6**	8.9§	10.1	6.6	6.8	**-2.2**	0.45
Isocaproic acid	0.0	0.5	0.0	0.0	**0.0**	0.0	0.0	0.1	1.0	**0.1**	0.13
Caproic acid	1.7	2.4	1.1	4.0	**-0.6**	1.0	1.6	2.7	3.4	**1.7**	0.19
Enanthic acid	1.2	2.3	1.8	4.2	**0.6**	0.0	0.0	0.7	2.1	**0.7**	0.56
Caprylic acid	1.7	1.6	2.3	3.7	**0.6**	0.0	1.4	1.6	1.9	**1.6**	0.67
Total SCFA	***161.2***	***102.4***	***188.3***	***61.5***	***27.1***	***302.7***	***123.4***	***273.3***	***91.7***	***-29.4***	***0.96***
Proportional ratio	59.4	16.2	66.2	8.0	**6.8**	54.5	9.3	60.8	2.9	**6.3**	
%Acetic acid	15.6	8.1	13.0	3.5	**-2.6**	23.1	9.1	17.8	8.5	**-5.2**	
%Propionic acid	2.0	0.7	1.3	0.7	**-0.7**	1.6	0.9	0.9	0.8	**-0.7**	
% Isobutyric acid	10.7	6.0	9.7	2.3	**-1.0**	13.3	4.5	11.6	9.5	**-1.7**	
%Butyric acid	3.9	1.4	2.2	0.7	**-1.7**	2.8	1.6	1.4	1.1	**-1.4**	
% Isovaleric acid	2.5	1.6	1.9	1.0	**-0.6**	3.0	2.7	2.7	1.8	**-0.4**	
%Valeric acid	0.0	0.3	0.0	0.0	**0.0**	0.0	0.0	0.0	0.3	**0.0**	
%Isocaproic acids	1.2	1.5	0.5	1.9	**-0.7**	0.3	0.4	0.9	1.1	**0.6**	
%Caproic acid	0.9	1.0	1.0	2.2	**0.0**	0.0	0.0	0.2	0.8	**0.2**	
%Enanthic acid	0.9	0.7	1.2	0.4	**0.3**	0.0	0.4	0.6	0.8	**0.6**	

Δ difference in faecal concentration of SCFA (high diet minus low diet).

p values are the difference in urinary excretion (Δ low-high diet) of each acid between groups.

§Significant difference between groups after the low-polyphenol diet $p \leq 0.05$

†Significant difference between groups after the high-polyphenol diet $p \leq 0.05$

3.6.4.4 Concentration of bacterial DNA isolated from faecal samples after low-polyphenol diet

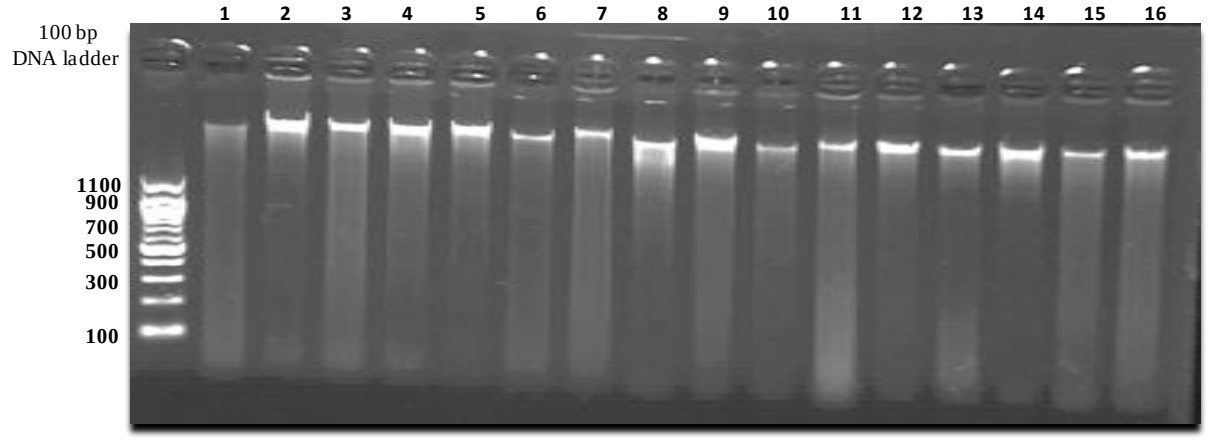
High quality and high yield DNA was obtained from all samples. The purity and yield of the extracted DNA as measured by Nano-Drop was very high and similar between groups (Table 3-9).

Table 3-9: The DNA concentration and purity measured using Nano-Drop in European (n=8) and Indian (n=8) participants.

Group	European			Indian		
Measurements	**Median**	**IQR**	**Range**	**Median**	**IQR**	**Range**
DNA concentration (ng/µl)	519.5	98.6	298.0-570.5	520.1	296.3	421.6 - 1031.4
DNA purity 280/260 nm	1.81	0.06	1.7 - 1.9	1.84	0.10	1.7- 2.0
DNA purity 260/230 nm	1.40	0.41	1.1 - 1.8	1.46	0.36	1.0-1.8

The faecal DNAs were intact and appear compact as a high-molecular-weight band in 1.5% agarose gel (Figure 3-16).

Figure 3-16: Agarose gel showing the purity of DNA extracted for bacterial qPCR analysis in European (1-8) and Indian (9-16) participants.



3.6.4.5 Validation of the assay for the qPCR run:

Probes and primers for *Eubacterium ramulus* and *Flavonifractor plautii* were validated using pure DNA for each of them. *Eubacterium ramulus* (DSM 15684) was used to validate the *Eubacterium ramulus* (Figure 3-17) and *Flavonifractor plautii* (DSM 4000) was used to validate *Flavonifractor plautii* (Figure 3-18).

Figure 3-17: TaqManqPCR amplification plot showing the serial dilution of standard curve used to validate *Eubacterium ramulus*.

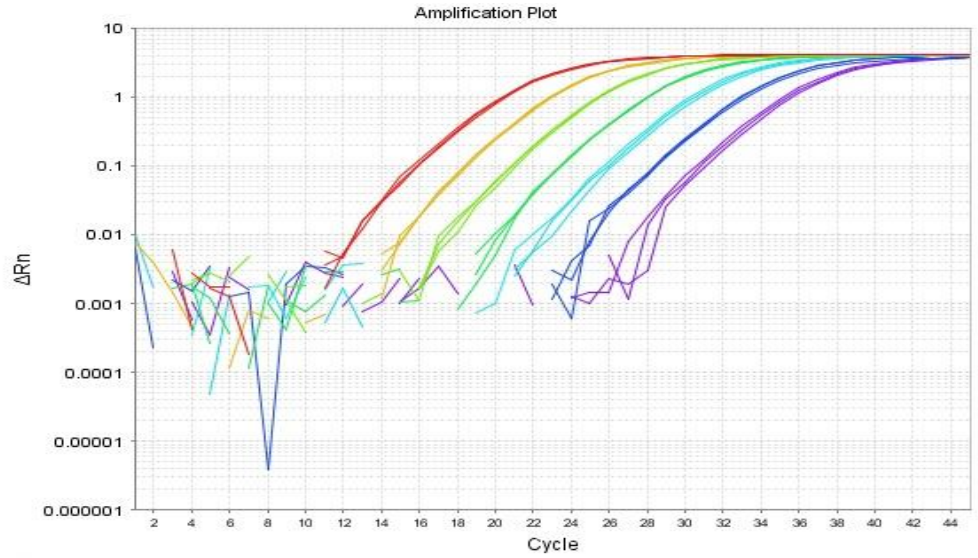
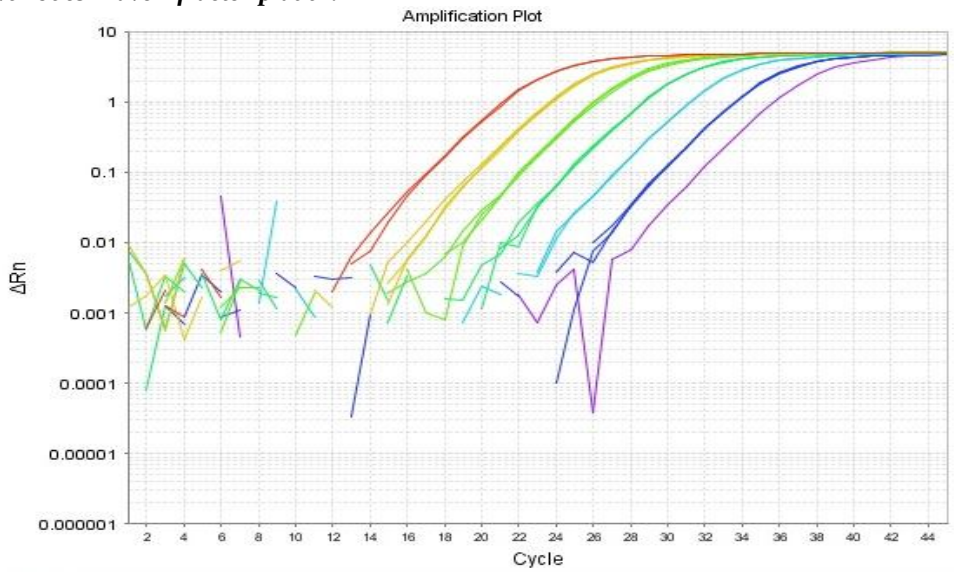


Figure 3-18*:* TaqManqPCR amplification plot showing the serial dilution of standard curve used to validate *Flavonifractor plautii*.



The cross-specificity was also checked for each target by running the designed qPCR set (probe & primers) with the known positive DNA and nine other different DNAs

(*Akkermansia, Bacteroides vulgatus, Blautiacoccoides, Bifidobacterium longum subsp., Desulfovibrio piger, Enterococcus faecalis, Escherichia coli, Faecalibacterium prausnitzii*, and *Lactobacillus plantarum subsp*.). Both Figure 3-19 and Figure 3-20 show that the designed probes and primers were successfully specific for each target.

Figure 3-19: *Eubacterium ramulus* cross-specificity test using the qPCR.

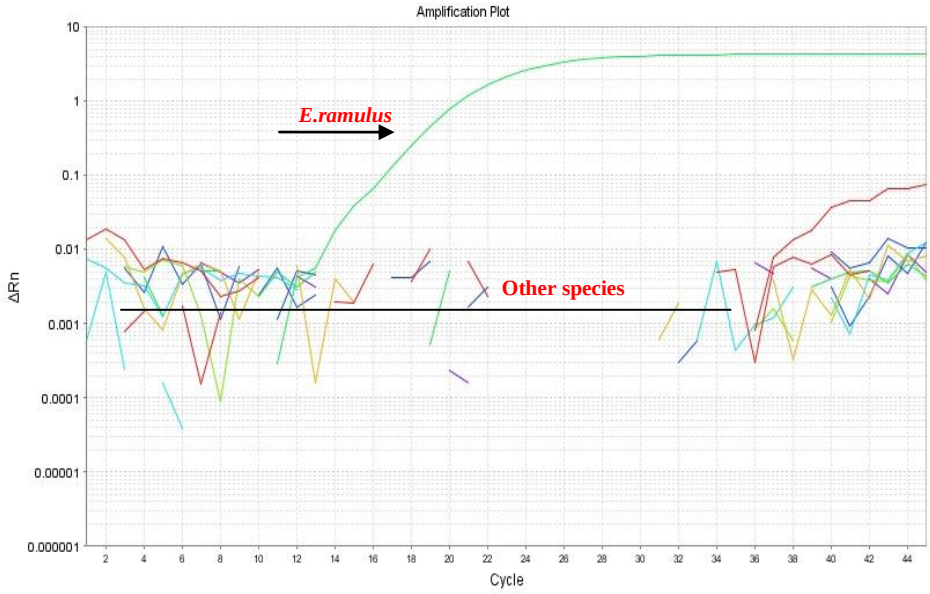
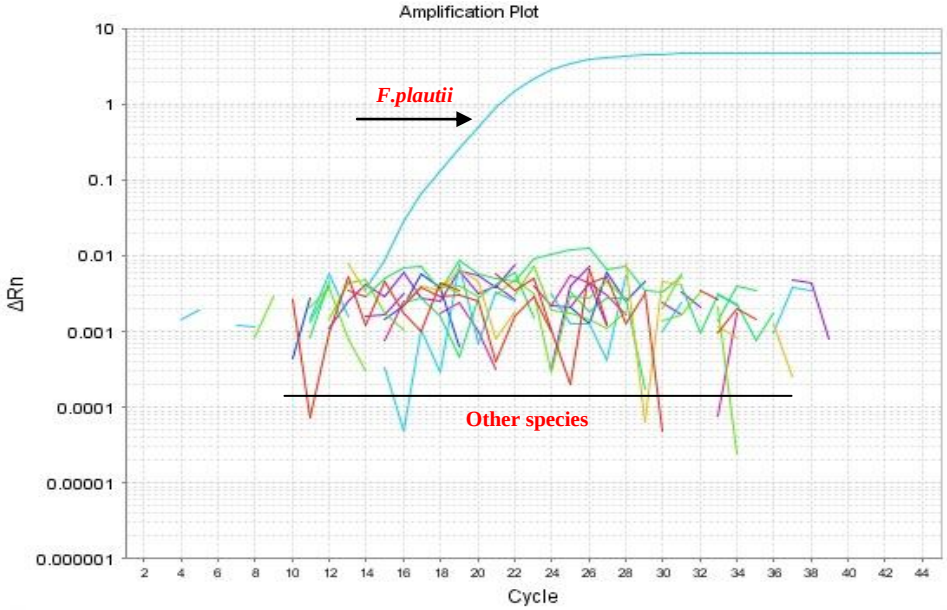


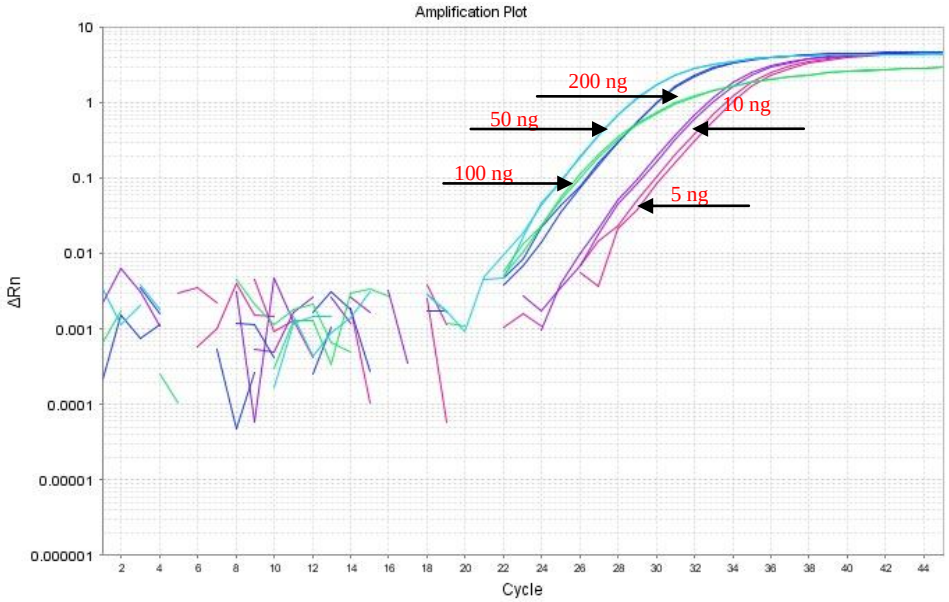
Figure 3-20: *Flavonifractor plautii* cross-specificity test using the qPCR.



Lastly, to define the right concentration of the faecal DNA template for the qPCR reaction, diluted faecal DNA of five different concentrations (200, 100, 50, 10, and 5 ng/µl per reaction)

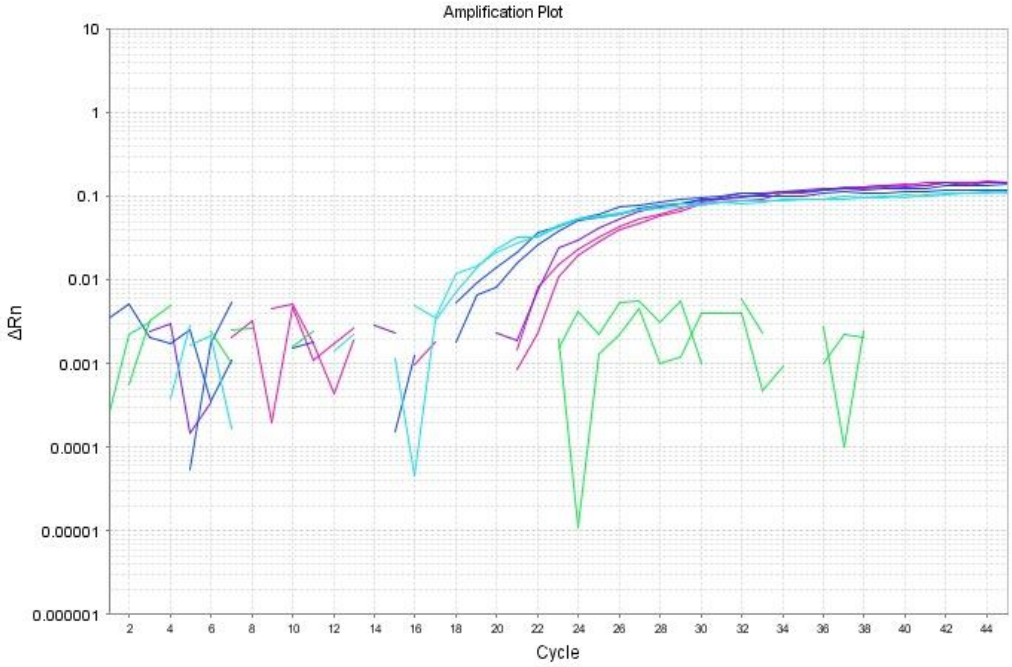
were run. The 50 ng/µl concentration shows the best dynamic plateau to measure *Flavonifractor plautii* (Figure 3-21).

Figure 3-21: Run of five different concentrations of *Flavonifractor plautii* faecal DNA template in the qPCR.



However, the qPCR amplification curve for the five concentrations for the *Eubacterium ramulus* did not reach a plateau (Figure 3-22).

Figure 3-22: Run of five different concentrations of *Eubacterium ramulus* faecal DNA template in the qPCR.



In order to solve this problem, some exploratory analyses were performed. Two issues were thought to be behind this low amplification. The first could be due to inhibitors in the faecal samples inhibiting the qPCR reaction. For that, pure standard of *Eubacterium ramulus* DNA was run alongside four random faecal DNA samples spiked with the pure *Eubacterium ramulus* DNA (standard DNA) to see if the faecal DNA would inhibit the amplification reaction of the pure DNA.

The result (Figure 3-23) suggested that the low curve was due to the inhibitor products in the faecal DNA. Therefore, the faecal DNA sample was further purified to remove the inhibitor. First, the Qiagen kit was used; however, this treatment was not successful. Next, bovine serum albumin (BSA) was used to inactivate the inhibitors in the qPCR mixture (Garland *et al.*, 2010). Again, adding BSA did not improve the reaction (Figure 3-24).

Figure 3-23: Inhibition experiment to test the DNA faecal sample for measuring *Eubacterium ramulus* using the qPCR.

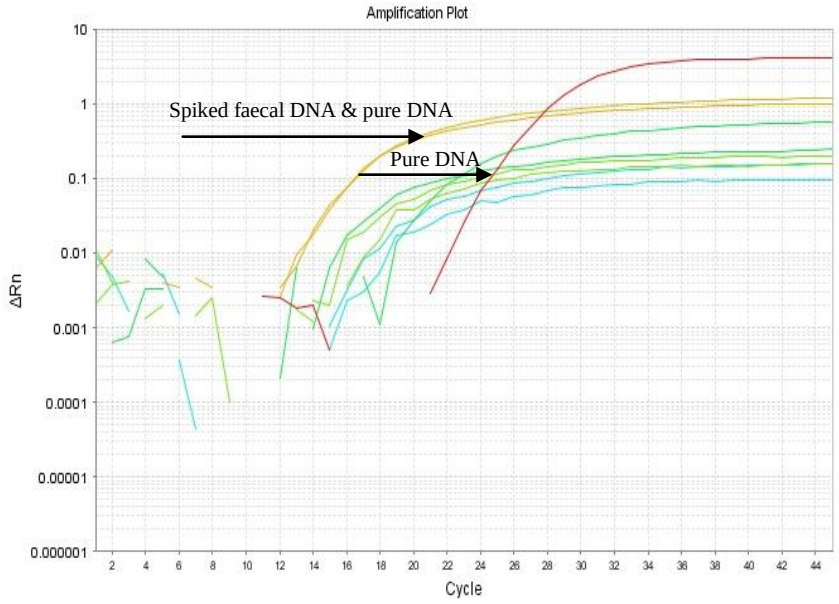
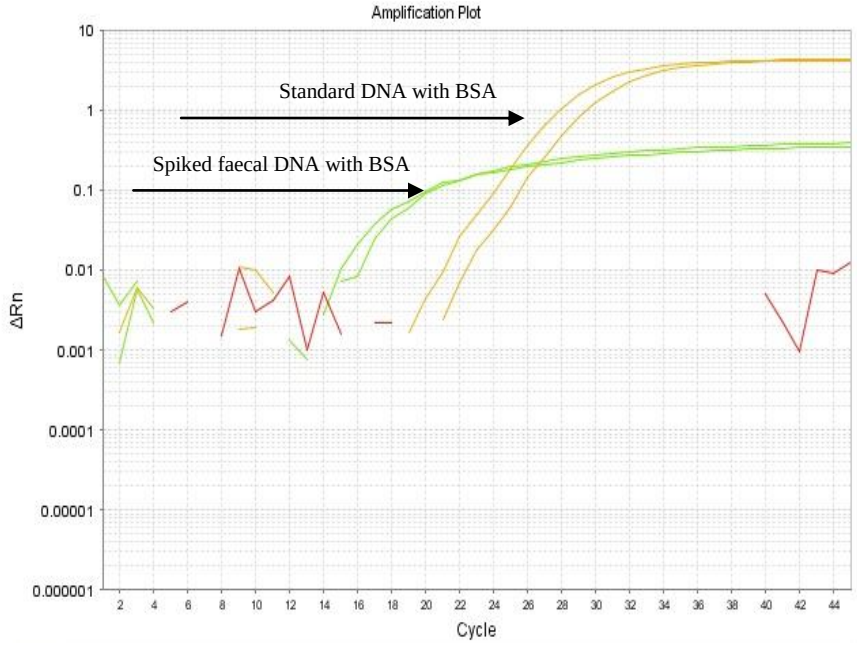


Figure 3-24: Effect of BSA additions on DNA faecal sample inhibitors for measuring *Eubacterium ramulus* using the qPCR.



In summary, these experiments for the DNA faecal samples show that the low values were due to some inhibitor in the faecal DNA not because of the low target concentration in the faecal samples. In the present study the *Eubacterium ramulus* was not able to be measured due to the low amplification.

3.6.4.6 Characteristics of the qPCR run condition

The characteristics of the qPCR runs of each bacterial group and species including the slope, amplification efficiency, and the coefficient of determination (R^2) for both groups are described in Table 3-10. The amplification efficiency of *Bacteroides – Prevotella*, *Bifidbacterium,* and *Eubacterium ramulus* were just below the ideal range (90% - 105%); however, as efficiency was similar and both groups were run together on the same plate to minimise confounding variables, it was considered acceptable for this experiment.

Table 3-10: Characteristics of qPCR runs for each bacteria species/groups in European (n=8) and Indian (n=8) participants.

Bacteria species/groups	Slope	Amplification efficiency (%)	R^2
Total Bacteria	-3.444	95.1	0.995
Bacteroides – Prevotella	-3.629	89.1	0.997
Bifidobacteria	-3.72	86.6	0.999
Flavonifractor plautii	-3.581	90.2	0.998

3.6.4.7 Absolute concentration of bacterial species and groups using qPCR

Absolute levels of total bacteria, *Bacteroides*, and *Flavonifractor plautii* did not differ between European and Indian groups; however, the absolute level of *Bifidobacteria* was significantly higher ($p \leq 0.05$) in Indians (Table 3-11).

Table 3-11: Absolute (log^{10}/g) faecal concentrations and relative abundance of bacterial groups/species in European (n=8) and Indian (n = 8) participants.

Target	Wet weight sample				Dry weight sample*			
	European (n=8)		Indian (n=8)		European (n=8)		Indian (n=8)	
Log^{10}	**Median**	**IQR**	**Median**	**IQR**	**Median**	**IQR**	**Median**	**IQR**
Total bacteria	11.5	0.2	11.4	0.3	11.9	0.3	11.9	0.5
Bifidobacterium spp.	9.7[a]	0.5	10.1[b]	0.5	10.1[†]	0.8	10.6[§]	0.3
Bacteroides+Prevotella	10.5	0.8	10.1	0.6	11.0	1.0	10.6	0.5
Flavonifractor plautii	8.4	0.7	8.7	0.6	8.9	0.8	9.2	0.4
(%)								
Bifidobacterium spp.	1.6		5.0		1.6		5.0	
Bacteroides+Prevotella	10.0		5.0		12.6		5.0	
Flavonifractor plautii	0.08		0.20		0.10		0.20	

a, b Difference between ethnic groups p value 0.04

†, §Difference between ethnic groups p value 0.05

* Calculated using the weight (g) and the moisture percentage (%) of the wet faecal samples.

3.6.5 *In-vitro* fermentation of rutin

Fourteen sets of fermentations were performed using the stool samples of 6 Europeans (1 male and 5 females) and 8 Indians (7 males and 1 female), collected after the low-polyphenol diet. The data of two Europeans were excluded as the initial pH of the fermentation was very high at nine due to an experimental error adjusting the pH of the medium. The flavonols rutin was fermented for 24 h with and without raftiline to test the metabolic capacity of the gut faecal contents, including the microbiota, in the context of ethnicity.

3.6.5.1 Rutin fermentation and pH of faecal fluids

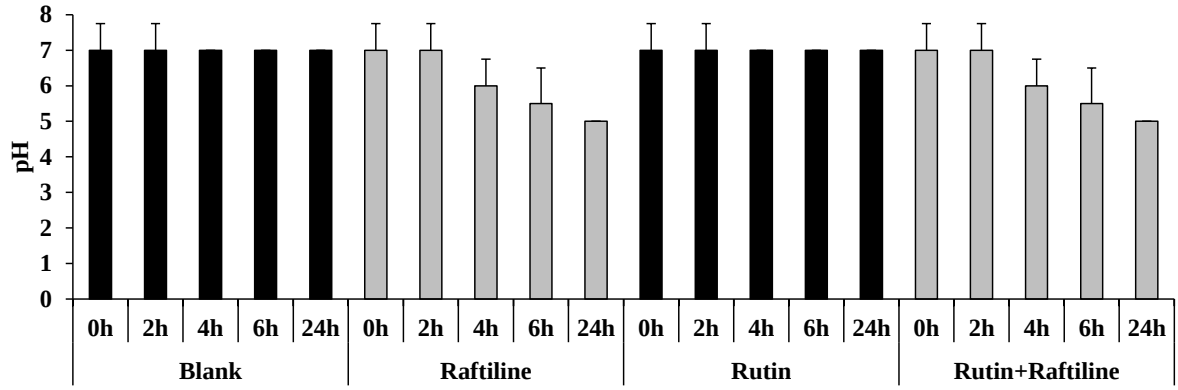
There was no change in the pH of the fermented faecal fluids containing rutin alone, over time, in either group (Figure 3-25). However, the combination of rutin with raftiline significantly ($p<0.01$) reduced the pH level at the end of the fermentation in both groups. In addition, the pH values of the fermented faecal fluids of raftiline and the combination of rutin with raftiline were significantly lower in the Indian samples than in the European samples after 24 h of fermentation ($p<0.01$).

3.6.5.2 Rutin fermentation and gas production

In both European and Indian groups, the combination of rutin with raftiline increased the gas production more than rutin alone ($p<0.05$). Moreover, the gas production was higher in the Indian fermented faecal fluids at 2 h of fermentation ($p<0.05$) than European. Indians fermented faecal fluids of rutin or combination of rutin with raftiline produced more gas after 2 h of fermentation; however, the gas production was higher in the European faecal fluids at 4h of fermentation. There was no difference in total gas production between groups after 24 hours of fermentation (Figure 3-26). However, there was a higher variability in gas production within the Indians fermented faecal fluids of rutin (SD 11.9 versus 5.5) or combination of rutin with raftiline (SD 30.3 versus 10.6) compared to the European group.

Figure 3-25: Change in pH of fermented faecal fluids over 24 h in A) European (n=6) and B) Indian (n=8) participants. Data presented as median and IQR.

(A)



(B)

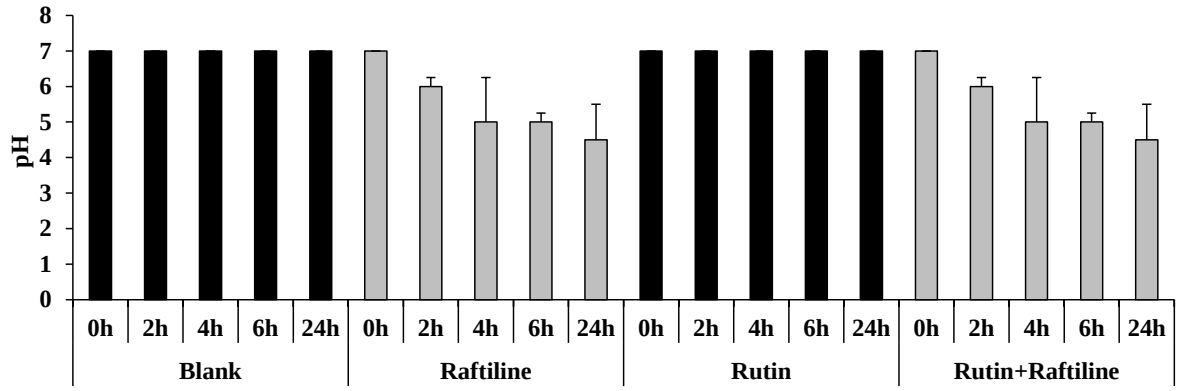
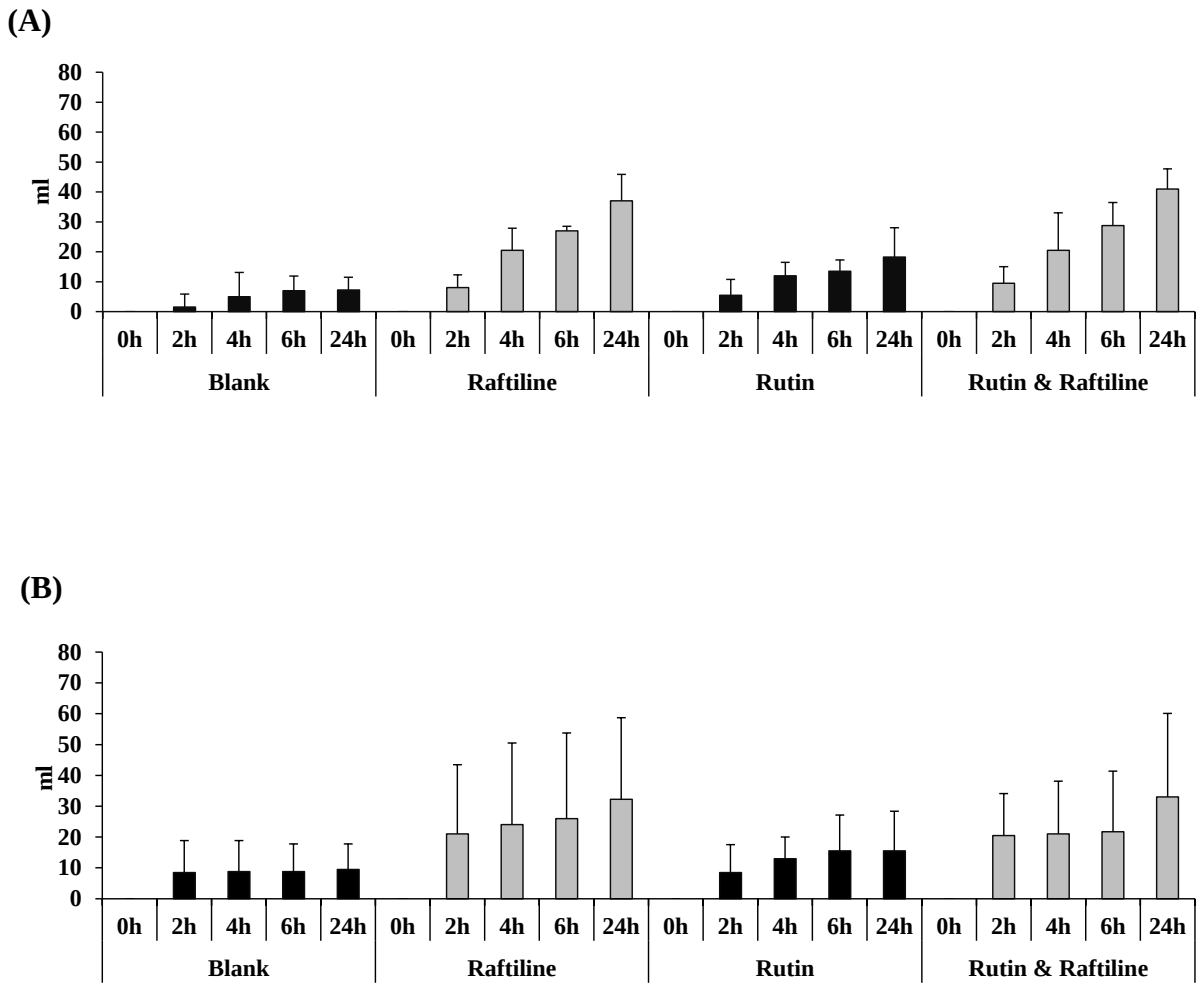


Figure 3-26: Cumulated gas production from fermented faecal fluids over 24 h of incubation in A) European (n=6) and B) Indian (n=8) participants. Data presented as median and IQR.



3.6.5.3 Metabolism of rutin in faecal fluids and phenolic acids formation

Only seven metabolites were found and identified using GC-MS after the fermentation of rutin with or without raftiline in the fermented faecal fluids of European and Indian groups at 0, 6, and 24 hr (Table 3-12). PAA, 3-OHPAA, 4-OHBA, 3-OHPPA, 4-OHPPA, and 3,4diOHPPA were detected in both faeces only fluids and fermented faecal fluids of rutin with or without raftiline. 3,4diOHPAA was only detected in the fermented faecal fluids of rutin with or without raftiline.

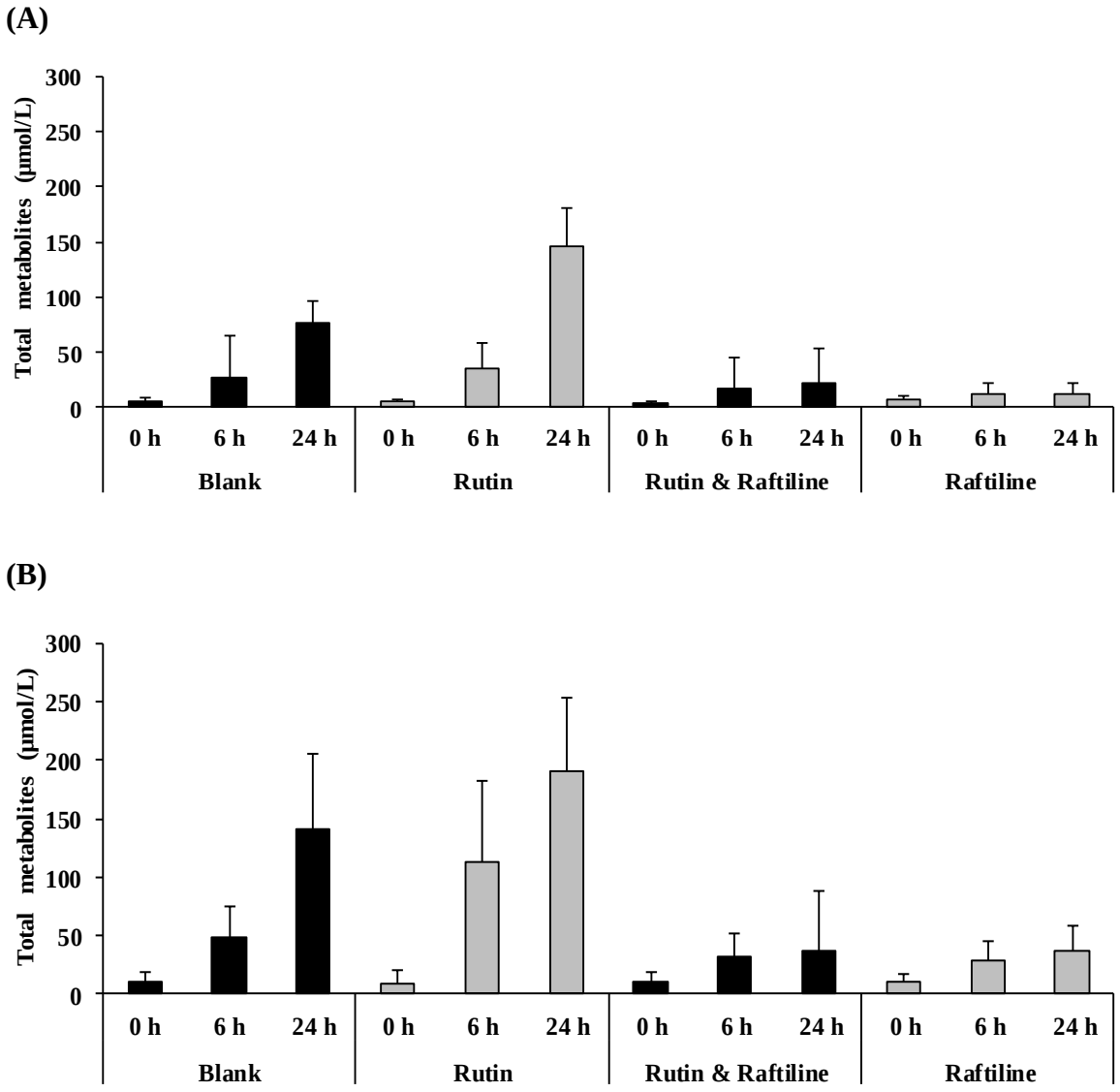
Table 3-12: Phenolic acids detected by GC-MS following the *in vitro* fermentation of rutin ± raftiline for 24 h.

No.	Phenolic acids	t_R (min)	Target ion (m/z)
1	Phenylacetic acid (PAA)	7.46	164
2	3-Hydroxyphenylacetic acid (3-OHPAA)	13.26	164
3	4-Hydroxybenzoic acid (4-OHBA)	13.87	267
4	3-hydroxyphenylpropionic acid (3-OHPPA)	17.24	205
5	4-Hydroxyphenylpropionic acid (4-OHPPA)	18.48	179
6	3,4-Dihydroxyphenylacetic acid (3,4diOHPAA)	21.18	179
7	3,4-Dihydroxyphenylpropionic acid (3,4diOHPPA)	26.58	179

3.6.5.3.1 The sum of seven phenolic acid metabolites formed during fermentation of rutin

The sum of the seven metabolites significantly increased over time in the fermented faecal fluids of rutin in both the European ($p<0.01$) and Indian ($p<0.001$) groups. The addition of raftiline significantly reduced the formation of phenolic acids in Europeans ($p=0.02$) and Indians ($p<0.001$), with no significant differences between groups. Fermentation of rutin alone led to higher concentration of phenolic acids in the Indian group ($p=0.001$) compared to the European group (Figure 3-27).

Figure 3-27: The sum of seven phenolic acids (µmol/L) in A) Europeans (n=6) and B) Indians (n=8) fermented faecal fluids over 24 h of fermentation. Data presented as median and IQR.



3.6.5.3.2 Individual phenolic acid formed after the fermentation of rutin

PAA increased significantly over time in the fermented faecal fluids of rutin and the combination of rutin with raftiline in Europeans ($p<0.001$; $p=0.03$) and Indians ($p<0.001$; $p<0.001$), respectively. 3-OHPPA significantly increased just in the fermented faecal fluids of rutin in the Europeans ($p=0.02$) and Indians ($p=0.009$). 3-OHPAA was only increased in the fermented faecal fluids of rutin in the Indian group ($p=0.007$; Table 3-13).

3.6.5.3.3 The impact of raftiline on the fermentation of rutin

The addition of raftiline reduced the level of PAA by 89.1% in the Europeans ($p=0.005$) and by 85.4 % in the Indians ($p<0.001$) when compared to fermented faecal fluids of rutin alone. Moreover, the level of 3-OHPAA was significantly reduced in the Indian group ($p=0.004$).

3.6.5.3.4 The impact of ethnicity on the fermentation of rutin

The Indian group produced significantly more PAA ($p=0.002$), 3-OHPAA ($p=0.001$), and 4-OHBA ($p=0.003$) compared to Europeans in the fermented faecal fluids of rutin. The highest amounts of 3-OHPPA, 4-OHPPA, and 3,4diOHPAA were detected at 6 hr in the Indian group and 24 hr in the European group.

Table 3-13: Accumulation of seven phenolic acids (µmol/L) produced after 0, 6, and 24 h of fermentation in faecal fluids from European (n=6) and Indian (n=8) participants. Data presented as median and IQR.

Metabolite	Group	Substrates	0h		6h		24h	
			Median	IQR	Median	IQR	Median	IQR
PAA	Europeans	Blank	3.7	1.0	15.2	22.1	66.1	25.3
		Raftiline	2.7	0.8	8.6	4.5	6.6	6.2
		Rutin	3.3	1.6	6.0	7.8	81.9	51.1
		Rutin+raftiline	4.3	2.3	8.3	1.5	8.9	3.6
	Indians	Blank	4.8	2.0	28.9	30.6	116.8	70.6
		Raftiline	3.9	3.4	15.7	11.9	25.6	19.0
		Rutin	3.8	1.0	29.1	56.0	127.4	39.2
		Rutin+raftiline	4.7	2.4	11.8	7.9	18.5	16.8
3-OHPAA	Europeans	Blank	0.2	<0.01	0.2	<0.01	0.5	0.2
		Raftiline	0.3	<0.01	0.2	<0.01	0.1	<0.01
		Rutin	0.2	<0.01	1.5	0.9	4.4	23.3
		Rutin+raftiline	0.1	<0.01	1.3	<0.01	1.3	0.8
	Indians	Blank	nd	nd	0.1	0.1	0.3	<0.01
		Raftiline	0.1	<0.01	0.1	<0.01	0.1	<0.01
		Rutin	0.3	<0.01	1.0	0.4	30.6	7.6
		Rutin+raftiline	0.1	<0.01	0.3	0.1	0.2	0.1
4-OHBA	Europeans	Blank	nd	nd	nd	nd	nd	nd
		Raftiline	nd	nd	0.1	<0.01	0.1	<0.01
		Rutin	0.1	<0.01	0.1	<0.01	0.1	<0.01
		Rutin+raftiline	nd	nd	0.1	<0.01	0.1	<0.01
	Indians	Blank	0.4	0.2	0.4	0.2	0.4	0.2
		Raftiline	0.5	0.1	0.4	0.4	0.4	0.4
		Rutin	0.5	0.3	0.4	0.1	0.3	0.1
		Rutin+raftiline	0.3	<0.01	0.4	0.3	0.4	0.1

*Continued overleaf

Metabolite	Group	Substrates	0h		6h		24h	
			Median	IQR	Median	IQR	Median	IQR
3-OHPPA	Europeans	Blank	1.6	0.8	2.8	1.3	2.9	1.6
		Raftiline	0.5	1.3	0.9	0.5	1.3	1.4
		Rutin	0.4	0.8	1.8	0.8	4.8	8.0
		Rutin+raftiline	0.4	0.9	1.6	4.5	1.2	2.6
	Indians	Blank	0.7	1.2	2.1	1.2	2.5	1.3
		Raftiline	0.9	2.9	1.1	1.7	1.0	1.1
		Rutin	0.6	1.1	4.1	6.9	1.8	4.4
		Rutin+raftiline	0.6	1.0	1.6	1.5	1.6	1.5
4-OHPPA	Europeans	Blank	0.4	0.3	6.5	6.7	1.2	2.6
		Raftiline	0.6	0.4	3.8	1.2	3.9	1.0
		Rutin	0.5	0.3	1.1	0.8	4.9	6.1
		Rutin+raftiline	0.5	<0.01	1.0	1.3	1.2	1.0
	Indians	Blank	0.9	1.1	2.7	7.8	0.6	0.9
		Raftiline	0.6	1.2	2.4	4.6	2.1	3.0
		Rutin	0.6	1.2	3.7	4.8	0.6	1.2
		Rutin+raftiline	0.7	1.2	2.1	1.5	2.1	1.4
3,4diOHPAA	Europeans	Blank	nd	nd	nd	nd	nd	nd
		Raftiline	nd	nd	nd	nd	nd	nd
		Rutin	nd	nd	16.1	27.2	32.9	56.8
		Rutin+raftiline	0.2	<0.01	5.0	30.4	7.1	30.0
	Indians	Blank	nd	nd	nd	nd	nd	nd
		Raftiline	nd	nd	nd	nd	nd	nd
		Rutin	nd	nd	39.2	32.8	15.1	60.0
		Rutin+raftiline	<0.01	<0.01	3.0	22.2	3.2	19.6
3,4diOHPPA	Europeans	Blank	nd	nd	nd	nd	nd	nd
		Raftiline	nd	nd	nd	nd	nd	nd
		Rutin	nd	nd	0.2	<0.01	0.4	0.1
		Rutin+raftiline	nd	nd	nd	nd	nd	nd
	Indians	Blank	2.5	1.8	1.2	0.4	0.7	0.6
		Raftiline	0.7	<0.01	0.7	<0.01	0.8	<0.01
		Rutin	0.7	1.8	2.8	15.7	5.1	23.6
		Rutin+raftiline	0.7	<0.01	7.5	6.1	8.3	6.5

Not detected (nd)

3.6.5.4 Metabolism of rutin in faecal fluids and SCFA production

Ten SCFA were identified and quantified in the fermented faecal fluids after the fermentation of rutin in the presence or absence of raftiline in younger and older groups at 0, 2, 4, 6 and 24 h (details of the method described in Chapter 2, page 49). Isocaproic, caproic, enanthic, and caprylic acid were either detected in limited amounts or not detected at all.

The SCFA concentration significantly increased over time in the fermented faecal fluids of rutin with or without raftiline in both Europeans and Indians ($p<0.05$). With raftiline, significantly higher levels of SCFA were detected in the fluids compared to rutin alone ($p=0.03$ for the European group; $p=0.02$ for the Indian group). The SCFA concentration in the fermented faecal fluids of rutin was not different between groups; however, the addition of raftiline significantly increased the SCFA concentration in Indians compared to Europeans ($p<0.01$; Figure 3-28).

Acetic, propionic, and butyric acid significantly increased over time in the fermented faecal fluids of rutin and the combination of rutin with raftiline in Europeans and Indians ($p<0.05$); however, Indians produced more acetic ($p=0.008$), propionic ($p=0.03$), and butyric acid ($p=0.002$) compared to Europeans (Table 3-14).

Figure 3-28: SCFA production (µmol/ml) in A) Europeans (n=6) and B) Indians (n=8) fermented faecal fluids over 24 h of fermentation. Data presented as median and IQR.

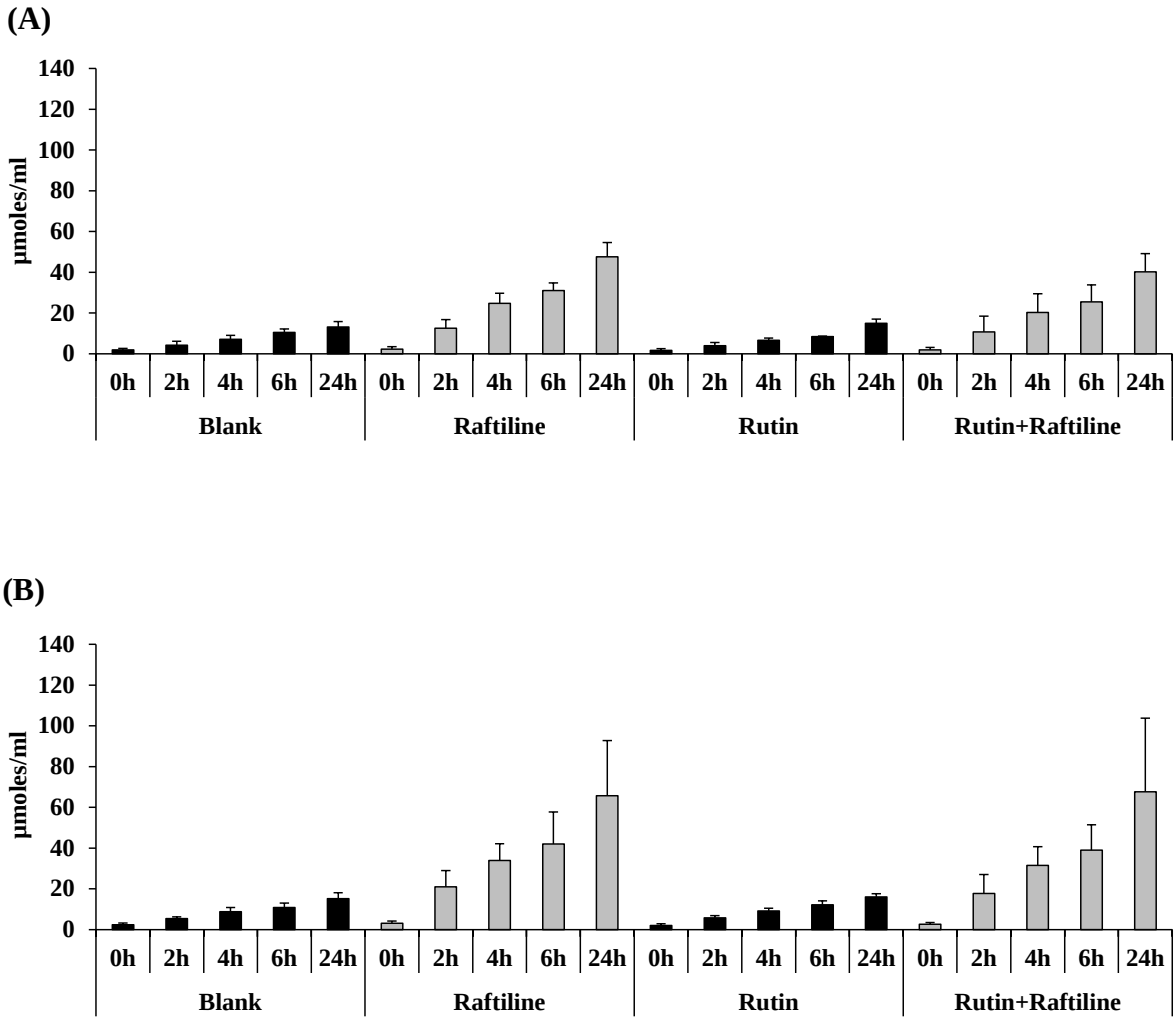


Table 3-14: SCFA concentration (µmole/ml) after 0,2,4,6 and 24 h. of fermentation from European (n=6) and Indian (n=8) participants. Data presented as median and IQR.

SCFA	Group	Substrates	0h		2h		4h		6h		24h	
			Median	IQR	Median	IQR	Median	IQR	Median	IQR	Median	IQR
C2	Europeans	Blank	1.2	0.4	2.2	0.7	4.1	0.7	6.0	1.3	7.8	2.4
		Raftiline	1.5	0.4	8.4	2.6	18.1	5.0	23.1	4.1	30.7	7.2
		Rutin	1.0	0.4	2.3	0.6	3.7	0.5	5.2	0.8	9.3	2.0
		Rutin+Raftiline	1.2	0.9	6.8	5.0	14.4	5.6	17.5	8.1	32.1	14.4
	Indians	Blank	1.3	0.5	3.1	0.7	5.1	0.5	5.9	0.5	7.5	1.5
		Raftiline	2.0	1.0	12.6	2.8	20.2	7.9	26.7	11.5	37.4	14.0
		Rutin	1.3	0.3	3.0	0.7	5.0	0.9	6.3	0.6	7.8	1.1
		Rutin+Raftiline	1.7	0.4	11.3	4.2	18.5	8.9	24.9	11.9	33.5	20.2
C3	Europeans	Blank	0.4	0.1	1.1	0.4	1.7	0.7	2.2	0.7	1.8	0.6
		Raftiline	0.4	0.2	2.4	1.6	4.2	0.9	5.3	2.1	5.7	4.8
		Rutin	0.3	0.2	1.2	0.8	1.7	1.0	2.0	1.0	2.4	1.0
		Rutin+Raftiline	0.4	0.3	2.5	2.1	4.0	3.0	5.3	2.3	6.8	3.5
	Indians	Blank	0.5	0.2	1.2	0.3	1.9	0.5	2.2	0.4	2.6	0.8
		Raftiline	0.6	0.2	3.9	2.9	6.0	7.0	9.7	10.4	11.4	12.5
		Rutin	0.5	0.1	1.2	0.4	1.9	0.3	2.3	0.3	2.9	0.5
		Rutin+Raftiline	0.5	0.2	3.7	2.8	5.8	6.1	9.3	10.1	12.1	12.2
iC4	Europeans	Blank	0.1	<0.01	0.1	<0.01	0.1	<0.01	0.1	0.1	0.3	0.5
		Raftiline	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
		Rutin	0.1	<0.01	0.2	<0.01	0.1	<0.01	0.1	<0.01	0.6	0.3
		Rutin+Raftiline	nd	nd	0.2	<0.01	<0.01	<0.01	0.1	<0.01	0.1	<0.01
	Indians	Blank	nd	nd	0.1	<0.01	0.2	<0.01	0.3	0.3	0.7	0.1
		Raftiline	nd	nd	0.1	<0.01	0.1	0.1	0.2	0.1	0.2	0.2
		Rutin	nd	nd	0.1	<0.01	0.2	0.1	0.3	0.3	0.7	0.1
		Rutin+Raftiline	nd	nd	0.1	<0.01	0.1	0.1	0.1	0.1	0.1	0.2

*Continued overleaf

SCFA	Group	Substrates	0h		2h		4h		6h		24h	
			Median	IQR	Median	IQR	Median	IQR	Median	IQR	Median	IQR
C4	Europeans	Blank	0.3	0.1	0.5	0.3	0.9	0.5	1.3	0.9	1.2	0.7
		Raftiline	0.2	0.1	1.0	0.4	1.8	1.6	2.5	1.4	6.8	5.3
		Rutin	0.2	0.2	0.5	0.2	0.8	0.3	1.0	0.2	1.5	0.5
		Rutin+Raftiline	0.2	0.1	0.8	0.7	1.0	1.7	1.6	1.6	3.9	3.3
	Indians	Blank	0.2	0.2	0.6	0.2	1.1	0.5	1.6	0.4	2.1	0.7
		Raftiline	0.3	0.1	1.3	1.6	2.0	4.3	2.4	6.3	7.8	19.0
		Rutin	0.2	0.1	0.6	0.2	1.1	0.4	1.8	0.5	2.3	0.5
		Rutin+Raftiline	0.3	0.1	1.2	1.4	1.8	4.4	2.5	5.9	7.8	19.8
iC5	Europeans	Blank	0.1	0.4	0.1	0.1	0.2	0.1	0.2	0.2	0.8	0.6
		Raftiline	0.4	5.1	0.1	0.2	0.1	0.2	0.1	0.1	0.1	<0.01
		Rutin	0.5	0.4	0.1	<0.01	0.2	0.1	0.2	0.1	1.3	0.4
		Rutin+Raftiline	0.1	0.1	0.1	0.2	0.1	0.1	0.1	0.4	0.1	<0.01
	Indians	Blank	0.1	0.1	0.2	0.2	0.5	0.6	0.9	0.8	1.4	0.2
		Raftiline	0.1	<0.01	0.1	<0.01	0.2	0.1	0.2	<0.01	0.4	0.4
		Rutin	0.1	<0.01	0.2	0.1	0.4	0.5	0.8	0.8	1.4	0.1
		Rutin+Raftiline	0.1	<0.01	0.1	<0.01	0.2	0.1	0.2	<0.01	0.4	0.3
C5	Europeans	Blank	0.1	0.1	0.1	0.1	0.2	0.1	0.4	0.3	0.5	0.4
		Raftiline	<0.01	<0.01	0.1	<0.01	0.2	0.1	0.2	0.1	0.2	0.3
		Rutin	0.1	<0.01	0.1	0.1	0.1	<0.01	0.2	0.1	0.9	0.3
		Rutin+Raftiline	<0.01	<0.01	0.1	0.1	0.1	<0.01	0.1	0.1	0.1	0.1
	Indians	Blank	0.1	<0.01	0.3	0.1	0.4	0.2	0.7	0.4	1.1	0.5
		Raftiline	0.1	<0.01	0.3	0.2	0.6	0.3	0.6	0.5	2.8	4.8
		Rutin	0.1	<0.01	0.2	0.1	0.4	0.2	0.7	0.4	1.0	0.7
		Rutin+Raftiline	0.1	<0.01	0.3	0.2	0.5	0.3	0.5	0.7	2.6	6.7

Not detected (nd), acetic acid (C2), propionic acid (C3), isobutyric acid (iC4), butyric acid (C4), isovaleric acid (iC5), valeric acid (C5).

3.7 Discussion

This study was carried out to test the hypothesis that ethnicity may affect the colonic metabolism of dietary polyphenols. To our knowledge, this is the first dietary semi-controlled study investigating the colonic metabolism of dietary polyphenols in different ethnic groups using human feeding and *in-vitro* faecal fermentation designs.

The present study demonstrated a clear difference between ethnic groups in terms of the urinary excretion of phenolic acid after a low-high polyphenol diet as well as the phenolic acid formation after the fermentation of rutin with faecal slurry. Possible proposed mechanisms for this variation include different colonic microbiota which might be affected by ethnic genetics and dietary habits.

The low level of urinary phenolic acid excreted by Indians could not be due to their lack of compliance with the high-polyphenol diet, as the corrected urinary phenolic acid for flavonoid intake indicated that Indians still excreted significantly less phenolic acid than the Europeans. There is no clear indication as to why Indians excreted less phenolic acid than Europeans. However, a number of possible mechanisms can be hypothesised based on the evidence presented here and previously published related studies:

First, it could be connected to genetics and its impact on gut microbiota composition. Yatsunenko *et al.* (2012) reported both children and adults from the United States have a different gut microbiota from people in Malawi and the Amazonas state in Venezuela.

Second, it may be related to the ability or/and the composition of colonic microbiota on the metabolism of polyphenols, which could affect the urinary excretion of phenolic acid. The faecal *in-vitro* fermentation of rutin showed that Indians formed significantly more phenolic acids, in particular PAA and 3-OHPAA. These have been reported previously as metabolites of rutin, formed in the colon (Olthof *et al.*, 2003). This suggested that Indians may have a greater capacity to metabolise rutin than Europeans.

Third, it could be due to differences in intestinal environment. Indians could have higher small intestinal absorption and tissue uptake compared to Europeans due to the variation in β-glucosidase activity that is involved in the absorption and metabolism of dietary polyphenols. Previous studies have showed considerable variation in β-glucosidase activity, suggesting the presence of diverse CBG populations with diffrent genetic

identities (Cheetham *et al.*, 1978, Nemeth *et al.*, 2003). For example, Namath et al. (2003) reported about 35-fold variation in β-glucosidase activity measured from samples of human cells from 10 healthy individuals. Moreover, the deficiency of LPH (known genetic polymorphisms) which causes lactose intolerance demonstrated the link between ethnicity and intestinal enzymes activity as it is a common disorder apart from Northern European adults (Wang *et al.*, 1995).

Fourth, it could be due to their cultural daily diet and its impact on gut microbiota. The significant high level of *Bifidobacterium* in Indians may be a result of their intake of yoghurt (3-fold), onion, tomato, chilli, and curry- based products (1.5-fold) , and spices (5.5-fold) than in Europeans. These food types are high in prebiotics (polyphenols and fibre) and probiotics (yogurt). A study by Parkar *et al.* (2013) investigated the biotransformation of four polyphenols; rutin, quercetin, chlorogenic acid, and caffeic acid, using an *in-vitro* culture model of human gut microbiota. Their data indicated that the biotransformation of these four polyphenols increased the growth of *Bifidobacterium* in culture, while the intact plant polyphenols had no effect on gut microbiota. The study concluded that the habitual intake of a high polyphenol diet which is associated with a higher amount of phenolic acid in the colon may result in promoting optimum gut health (Parkar *et al.*, 2013).

Another possibility is that colonic phenolic acids are metabolized to support the growth of the gut microbiota or/and are absorbed by body tissues resulting in a low amount excreted by Indians. This was supported by the faecal fermentation of rutin and raftiline. The reduction of the phenolic acid with the addition of raftiline to the faecal fluids could be from the effect of fiber increasing the bacterial growth and in turn their need to use the phenolic compounds as a source of carbon (Jaganath *et al.*, 2009).

Finally, the frequent daily bowel movements in the Indian group compared to the European group may be accountable for lower urinary phenolic acid excretion in Indians. As the majority of flavonoids are metabolised in the colon, the influence of the transit time on the colonic pH and SCFA driven changes in the microbiota may impact on polpyhenol metabolism (ElOufir *et al.*, 1996). Since the study groups are not matched in gender, whether gender has an impact on the transit time or not is still controversial. There are some studies that have suggested a longer transit time in women compared to men (Degen and Phillips, 1996, Sadik *et al.*, 2003), whereas other studies have reported no significant differences between men and women (Hinds *et al.*, 1989, Soffer *et al.*, 2000). Moreover,

the link between gender and dietary habits might have an effect on the colonic metabolism of polyphenols as men tend to consume more meat, while women tend to consume a larger proportion of vegetables, fruit, fish and dairy products (Jensen and Holm, 1999). However, despite these facts, a study by Cerda *et al.* (2005) indicated that the variability in the metabolism of ellagitannins was not related to gender as the amount of ellagitannins consumed from different foods (strawberries (250 g), red raspberries (225 g), walnuts (35 g), and oak-aged red wine (300 mL)) was not proportionally related to the amount of urinary metabolites excreted. The differences in the metabolites were more dependent on the type of food consumed. The lowest excretion was observed in the red wine group, while the highest excretion was observed in the volunteers who consumed walnuts. This could suggest the role of a food matrix and gut microbiota on categorising individuals to "high and low metabolite excreters."

In addition, the high production of SCFA, especially acetic and propionic acid in Indians may be related to the increase in growth of *Bifidobacterium. Bifidobacteria* may use phenolic acids as a source of energy (Jaganath *et al.*, 2009), which would reduce the pool of phenolic acids available for excretion in Indians (Parkar *et al.*, 2013). For example, Parkar *et al.* (2013) reported an increase in SCFA especially propionate accompanied by the increase of *Bifidobacterium* at 48 h of rutin fermentation. Moreover, as rutin glycosylated with rhamnose-glucose disaccharide, the glycosyl group could add to the SCFA. Rhamnose is particularly propiogenic as it is metabolised to propionic acid via the propane-diol pathway. This also could indicate the higher ability of Indians to metabolise polyphenols compared to Europeans.

Despite the differences between groups, the urinary phenolic acids increased after the high-polyphenol diet in both groups. The same result has been previously reported by Rechner *et al.* (2002); Jaganath *et al.* (2006); Roowi *et al.*, (2010); Olthof *et al.* (2003); and Graefe *et al.* (1999). Most of the bioavailability studies reported inter-individual variations between subjects but none of them reported ethnic differences in their group or studied the effect of ethnicity on the metabolism of polyphenols (Graefe and Veit, 1999, Rechner *et al.*, 2002a, Olthof *et al.*, 2003, Jaganath *et al.*, 2006, Roowi *et al.*, 2010).

Gardana *et al.* (2009) investigated the intestinal microbial transformation of daidzein to equol using anaerobic batch cultures and found that the transformation was subject to a wide inter-individual variability which might be due to dietary habits. They found that the equol-producer group consumed less fibre, vegetables and cereals and more lipids from

animals. Furthermore, Gross *et al*. (2010) studied the effect of the inter-individual variation of gut microbials on the metabolism of black tea and wine/grape juice from ten healthy volunteers using *in-vitro* faecal batch cultures. The study reported that each subject showed a specific metabolite profile differing in composition, time and level of metabolites. Fuhr and Kummert (1995) also reported that between 5-57% of naringin consumed with grapefruit juice was detected in urine in different individuals .

In this study, hippuric acid was a major metabolite in the urine of both groups. Hippuric acid can derive from a diet that is rich in polyphenols such as tea, coffee, wine and grape juice extracts, and green tea (Clifford *et al.*, 2000, Rechner *et al.*, 2002a, Olthof *et al.*, 2003, Roowi *et al.*, 2010, van Dorsten *et al.*, 2010). It can however also derive from amino acids such as aromatic amino acid tryptophan, tyrosine, and phenylalanine (Self *et al.*, 1960, Grumer, 1961, Bridges *et al.*, 1970). Therefore, in the present study the urinary phenolic acid analyses were performed with and without the hippuric acid to eliminate the effect of this acid on the outcome measure. In both results, the sum of the urinary phenolic acids with or without hippuric acid showed that Europeans still significantly excreted more phenolic acid in urine than Indians. In addition, 4-OHPAA was not considered in the sum of all phenolic acids, because it did not increase after the high-polyphenol diet in all participants. It is produced by unrelated routes of the colonic degradation of polyphenols (Roowi *et al.*, 2010).

The inter-individual variation in phenolic acid excretion after the high polyphenol diet was approximately 2.5-fold in the European group, compared to approximately 6-fold in the Indian group. This could be linked to variability in dietary intake and gut microbiota (two participants in the Indian group were vegetarians, and had the lowest extraction of urinary phenolic acid compared to other participants in the same group).

To support the urinary phenolic acid result, an *in-vitro* fermentation of rutin was used to understand the effect of ethnicity on colonic metabolism. The ability of the Indian faecal fluids to metabolize rutin was faster at 6 h compared to Europeans. This could be due to their gut microbiota. In the human colon, the occurrence of bacterial enzymes (β-glucosidases, β-glucuronidases, and α-rhamnosidase) enables the hydrolysis of rutin to release the quercetin aglycone (Aura *et al.*, 2002). Insufficient or lower levels of bacterial enzyme in the colon could be one of the reasons why Europeans formed less phenolic acid than Indians during the faecal fermentation of rutin. Four acids were detected in fermented faecal fluids of rutin in the present study (3,4diOHPAA, 3-OHPAA, 4-OHBA, and

3,4diOHPPA), which were also detected before by Jaganath *et al*. (2009) after the *in-vitro* fermentation of rutin; however, the 3,4diOHBA they reported was not detected in the present study (Jaganath *et al.*, 2009).

Consistent with the urinary phenolic acid (GC-MS), the urinary total phenols measured by Folin-Ciocalteu assay increased in both groups after the high polyphenol diet. However, the difference between the groups was not consistent with the urinary phenolic acid. This could be related to other interfering substances for the Folin-Ciocalteu assay in the urine such as aromatic amines, sulphur dioxide, ascorbic acid, organic acids, Fe (II), and non-phenolic substances (Roura *et al.*, 2006).

The urinary antioxidant activity can be affected by the profile of phenolic compounds excreted in the urine. The FRAP values increased significantly after the high-polyphenol diet in the European participants but not in the Indian participants. This is due to lower phenolic acid in the Indians' urine samples. According to Olthof *et al.* (2003), phenolic acid metabolites have much lower antioxidant activity than their parent compounds. The breakdown of polyphenols into smaller molecules in the colon and liver lower their antioxidant activity; therefore, the conjugated step with glucuronic acid, sulfates, or glycine lowers the antioxidant activity (RiceEvans *et al.*, 1996).

In agreement with the faecal pH and SCFA, Indians had lower fermented faecal slurry pH and produced more SCFA compared to Europeans. Further work is required to determine the mechanism underlying this difference but it may be due to their ability to ferment nutrients more effectively than Europeans, possibly related to the composition of gut microbiota or the effect of dietary fibre. The fermentation of polyphenols alone had no effect on the faecal slurry pH or on the gas production in comparison to the faecal fermentation with raftiline, suggesting an important role for dietary fibre in this activity. A study by Pereira and Gibson (2002) showed that inulin increased gas production because of the simple sugar in the inulin compound which makes it highly fermentable and more available for the bacteria. The faster production of gas in the Indian group at 2 hours may be due to their gut microbiota composition activity and ability to ferment fibre. Unfortunately, the faecal bacteria enzymes were not measured in this study; and thus further research would be advantageous to determine if this provides an explanation for the faster fermentation in Indians.

Faecal ammonia is suggested to have an adverse effect on the health of the colon. Previous studies have reported that it may promote tumorigenesis by stimulation of cell proliferation in the colon and also has a toxic effect that can damage the colonic epithelium and increase the risk of CRC (Birkett *et al.*, 1996). Since the reported rate of CRC is low in Indians, the measurement of faecal ammonia was performed in both groups. The study was unable to detect differences between the groups because it did not have the required statistical power. Moreover, the high polyphenol diet had no effect on the faecal ammonia and this could be due to the short term study of the intervention. A study by Shinohara *et al.* (2010) showed that a decrease in faecal ammonia was detectable after an intake of only two apples for 14 days.

This study was designed as a human cross-over dietary intervention (*in-vivo*) to study the colonic metabolism of dietary polyphenols (after low and high polyphenol diets) between the two groups, focusing on urinary phenolic acid excretion in the light of gut bacterial diversity. The choice of this study design over other possible approaches has a number of inherent strengths and weaknesses. The strengths were perceived to be: 1) using the crossover design has the advantage of reducing the cofounding variable because each crossover subject served as his or her own control; 2) the study's dietary intervention was well controlled, and the same high polyphenol foods were provided to all participants to reduce the variation in food brand and quantity and 3) the faecal fermentation of *in-vitro* rutin provided an assessment of the fermentation ability of each group.

On the other hand, there were some limitations which included the following: 1) the phenolic acid was not measured in faecal samples which could have provided useful information if phenolic compounds did not metabolise in the colon or had been used by the gut bacteria and absorbed in their body; 2) the study did not measure the colonic bacteria enzyme activity such as β- glucosidases, β- glucuronidases, and α-rhamnosidas in the fermented faecal samples. The measurement of these enzymes could have given a better understanding regarding the differences between groups in terms of the formation of phenolic acid; 3) the study was not designed to test the effect of dietary habit, colonic pH, colonic SCFA, and colonic.

In conclusion, the data of this study showed clear differences between groups in terms of the colonic biomarkers of dietary polyphenols and fiber fermentation (pH, SCFA, and phenolic acid). The lower excretion of urinary phenolic acid and higher phenolic acid formation during the fermentation of rutin in the Indian group suggested that Indians have

the ability to absorb and ferment the polyphenols much faster than the Europeans. Another possibility could be due to the effect of fiber on the bacterial growth and their need to use the phenolic as a source of carbon. The high intake of fibre has a direct effect by providing selective substrates for some bacteria and indirectly by reducing the colonic pH which in turn can alter the growth and the composition of gut microbiota. The differences between groups could be due to a variation in colonic microbiota which might be affected by ethnic genetics, environmental reasons including dietary habits or a combination. However, this study did not aim to dissect the effect of these factors on the metabolism of dietary polyphenols. Further studies are needed to distinguish and evaluate the effect of these factors.

The low incidence of CRC in Indians could be a result of the high intake of fruit and vegetables which are known for their high amount of polyphenols and fibre. A diet that is high in fruit and vegetables has the ability to acidify the colon which can protect against colon cancer, while the alkaline faecal pH can induce the carcinogens in the colon and increase the risk of CRC (Mitani *et al.*, 1999).

Chapter Four

Ageing and the Colonic Metabolism of Dietary Polyphenols

4.1 Introduction

The British population is increasingly ageing, with over one third of the UK population expected to be over 65 by 2050 (Cracknell, 2010). The increase in life span has a direct effect on the incidence of age-related disease, including CRC (Biagi *et al.*, 2010), which can put a tremendous strain on the National Health Service (NHS) (Cracknell, 2010).

Aging is known to affect bowel function (Russell, 1992), which may be related to one or a combination of the following factors: 1) reduced gut motility, leading to longer transit time and chronic constipation (Madsen, 1992, Firth and Prather, 2002, Madsen and Graff, 2004); 2) reduced chewing strength, resulting in food choices that tend to be lower in fibre (Brodeur *et al.*, 1993); 3) reduced water consumption (Kenney and Chiu, 2001); 4) reduced physical activity (Wijhuizen *et al.*, 2007). All of these factors can affect dietary intake and alter nutrient metabolism, leading to an inadequate range of food consumption which can slow down the transit time and affect the growth and composition of gut microbiota (O'Toole and Claesson, 2010).

It has been reported that the composition of gut microbiota may be altered in the elderly, with some of the beneficial bacteria, such as *Bifidbacterium*, declining, and potentially harmful bacteria, such as *Enterobacteriaceae* and *Clostridium perfringens*, increasing (Gavini *et al.*, 2001). The changes in the colonic microbiota and its products (such as SCFA, in particular butyrate which has shown an antiproliferative effect on colon cancer and provides energy for colonic epithelial cells) could directly affect the regulation of the gene expression for the cellular growth and proliferation by hyperacetylation cells (Rowland, 2009, Donohoe *et al.*, 2011, Tremaroli and Bäckhed, 2012). Importantly, there is a link between the age-related change in the gut microbiota, colonic inflammation and the risk of CRC (Garagnani *et al.*, 2013).

Plant foods contain a range of bioactive molecules, including polyphenolics, which may have antioxidant, anti-carcinogenic, anti-inflammatory, and antimicrobial properties that could impact on ageing and the risk of chronic diseases such as CRC (Linseisen and Rohrmann, 2008, MacDonald and Wagner, 2012). As discussed in earlier chapters, the colonic bacteria are involved in the metabolism of the majority of these molecules. However, research into the bioavailability and metabolism of a large number of polyphenols, including flavonoids, and in particular rutin, have so far been limited to young adults (Rechner *et al.*, 2002a, Olthof *et al.*, 2003, Jaganath *et al.*, 2006, Mullen *et*

al., 2006). As a result, very little is currently known about the impact of ageing on the metabolic fate of polyphenolics in the gastrointestinal tract and human tissues.

This colonic metabolism of these compounds results in a range of metabolites, including phenolic acids, which can have a beneficial effect on the gastrointestinal tract (Erlund *et al.*, 2000, Manach *et al.*, 2005, Jaganath *et al.*, 2006). Polyphenols found in fruit and vegetables may have protective effects in the gastrointestinal tract by: 1) inhibiting the growth of pathogenic species (e.g. *Clostridium spp*, *Staphylococcus aureus*, and *Bacteroides spp.* (Bialonska *et al.*, 2009); 2) suppressing the adhesion of gut pathogens to human gut cells (Nohynek *et al.*, 2006); 3) enhancing natural killer cell activity and cytokine secretion (Bub *et al.*, 2003), leading to reduced intestinal inflammation. All of these may reduce colonic inflammation and the risk of CRC.

Therefore, the changes in gut microbiota composition associated with ageing are likely to be accompanied by changes in microbial activities, including enzymatic activities (Tiihonen *et al.*, 2010). This may have an impact on the colonic metabolism of polyphenols. It is, therefore, important to understand the effect of ageing on the colonic metabolism of dietary polyphenols before examining their role in disease prevention.

In Chapter three, the metabolism of polyphenols in healthy adults, after a low or high-polyphenolic diet, was examined. We saw an effect of ethnicity on the metabolism of dietary polyphenols. This present study aimed to test whether age (≥ 50 years) affects the colonic metabolism of dietary polyphenols, especially flavonols, known to reach the colon, taking into consideration changes in gut microbiota. Since CRC prevalence increases with age; and flavonol intake and their colonic bioactive metabolic fraction, phenolic acids, are inversely associated with CRC risk (Wild *et al.*, 2006, Simons *et al.*, 2009), we hypothesized that the colonic metabolism of polyphenols would differ between younger and older adults. To test this hypothesis, two age groups were studied, and two study designs were used.

1. A human dietary intervention (*in-vivo*), to study the colonic metabolism of dietary polyphenols (after low and high polyphenol diets) between healthy younger adults and a group over 50 years of age, focusing on urinary phenolic acid excretion and gut bacterial diversity (especially polyphenol-degrading bacteria).

2. *In-vitro* fermentations, using faecal samples collected during the dietary intervention, to study the metabolic capacity of the samples when specific flavonols were fermented.

4.2 Subjects and study design

4.2.1 Subjects and recruitment

Older adults (≥ 50 years) were recruited using local advertisements, printed poster display, and online social networking sites. The younger group (20-42 years) was recruited as part of the ethnicity study (European group; Chapter 3). Exclusion criteria included consuming alcohol (>4 units/day), obesity (BMI>30kg/m^2), taking dietary supplements, pregnancy or at risk of pregnancy, smoking, taking any medication, or having any conditions known to affect bowel function. A full ethical application was prepared for this study and submitted to the University of Glasgow, College of Medical; Veterinary & Life Sciences (approval in Appendix 7). All participants gave informed written consent.

4.2.2 Sample size and power calculation

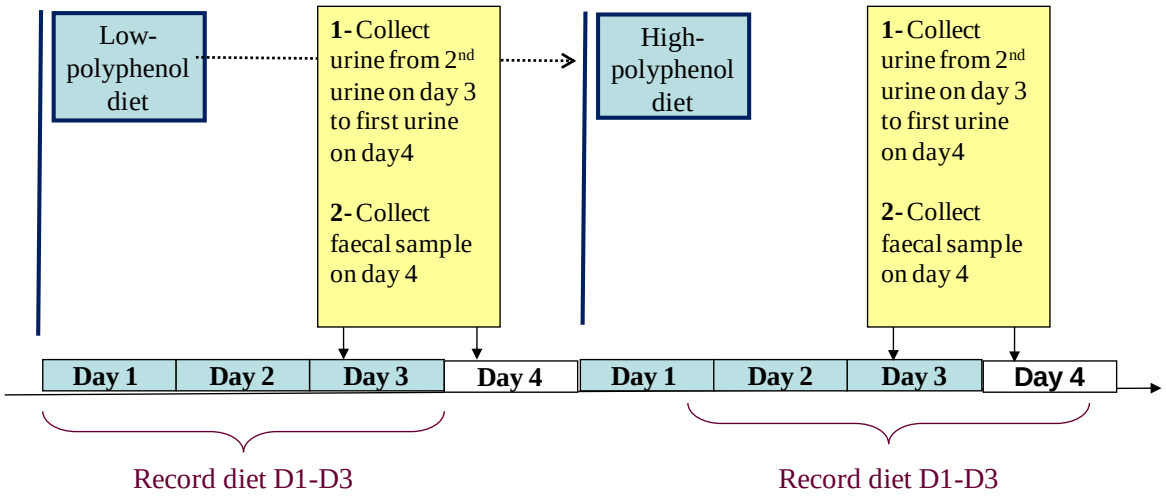
The primary aim of this study was to characterise the difference in colonic metabolism of dietary polyphenols in healthy older adults (≥ 50 years) compared with previous results generated from healthy younger adults (Chapter 3). Urinary phenolic acid excretion in the younger group following a low polyphenol diet was 275.4 ± 110.6 umol/day, versus 1127.8 ± 373.3 umol/day following a high polyphenol diet. The mean difference was 852.4 mg/day with a standard deviation of 337.2. Based on this, a sample size of n= 13 participants will be sufficient to detect (or not) the difference of one standard deviation in urinary phenolic acid excretion between the younger and older adult groups at a power of 80%, $p<0.05$, allowing for a 20% drop-out rate.

4.2.3 Study design and sample collection

All participants were asked to follow a low-polyphenol and a high-polyphenol diet, each lasting three days. Three days for each diet was enough for polyphenol rich foods to be supplied to the colons and fermented over the course of several meals. During the low-polyphenol diet (diet A), participants were asked to avoid all fruits, vegetables, onions, coffee, tea, chocolate, vanilla and similar flavourings, whole meal products, alcohol, spices, and all dietary supplements (vitamins, minerals, and herbal products). During the

high-polyphenol diet (diet B), participants were asked to follow a specific diet including polyphenol-rich foods, which were provided along with cooking guidance and recipe sheets. Examples of foods to be included during the low polyphenol diet, and a detailed menu of the high-polyphenol diet are given in Appendix 4 and 5. Urine and faecal samples were collected after the low-polyphenol diet (day 4) and high-polyphenol diet (day 4) for the human feeding study measurements (Figure 4-1). Stool samples collected after the low-polyphenol diet (day 4) were used in *in-vitro* faecal fermentations. Sociodemographic and anthropometric measurements (height, weight, BMI, and waist circumference), and blood pressure were collected (details described in Chapter 2, page 44).

Figure 4-1: Overall study design and samples collection.



4.3 Dietary assessment

A food frequency questionnaire (FFQ) was used to assess the participants' habitual diet over the past year, as described in Chapter 2, page 68).

4.4 Dietary records

Participants kept a 3-day weighed dietary record for the duration of the low and high-polyphenol diets. Diaries were used to estimate the intake of macronutrients, micronutrients, and flavonoids of each participant during the study and to measure the participants' compliance by reviewing the food types and portion size according to the provided instruction (details described in Chapter 2, page 67).

4.5 Outcome measures for the human feeding and *in-vitro* faecal fermentation studies

Phenolic acids (GC-MS), total phenols (Folin–Ciocalteau), and ferric reducing antioxidant power (FRAP) were measured in urine samples. The pH, short chain fatty acids (GC-FID), and bacterial composition (Taqman real-time quantitative PCR) were measured in the faecal samples.

Phenolic acids, pH, short chain fatty acids, and gas production were measured in the fermentation supernatants (details in Chapter 2).

4.6 Results

4.6.1 Subjects characteristics

Thirteen older adults were recruited, aged between 51-76 years old, to follow a 3-day low and 3-day high-polyphenol diets. They were compared to eight younger adults aged between 23-43 recruited as part of the ethnicity study (European group; Chapter 3). The baseline data for the younger and older groups are presented in Table 4-1. There were no significant differences in anthropometric characteristics between the two groups. The BMI and WC were within the normal cut-off range for younger and older healthy adults. The male to female ratio was similar between groups, with 2 males and 6 females in the younger group and 3 males and 10 females in the older adult group.

Table 4-1: Baseline data in younger (n=8) and older (n=13) participants.

	Younger group (n=8)		Older group (n=13)		p value
	Median	**IQR**	**Median**	**IQR**	
Age (years)	23.0	6.5	61.0	10.0	0.0002
Height (cm)	163.0	19.0	161.0	0.1	0.3
Weight (kg)	63.0	13.3	63.0	16.3	0.4
BMI (kg/m^2)[a]	22.0	5.8	25.1	4.6	0.06
W.C (cm)[b]	80.0	3.0	85.0	19.0	0.1
Systolic BP	122.0	19.0	120.0	20.0	0.2
Diastolic BP	69.0	13.8	86.0	14.0	0.005
	n	**%**	**n**	**%**	
Normal weight	5	62.5	10	77	
Overweight	3	37.5	3	23	
Obese	0	0	0	0	

[a]BMI cut-off points (adult=25, older adult (55-65 years old) = 28); (Heim *et al.*, 2010, Heim *et al.*, 2011).
[b]WC cut-off points (adult women=80 cm, man=94; older women=99, man=106); (Heim *et al.*, 2010, Heim *et al.*, 2011).

Bowel movements (self-reported on the questionnaire as twice daily or more, daily, every 2-3 days or less than twice a week) were not significantly different between younger and older groups (Table 4-2).

Table 4-2: Bowel movement in younger (n=8) and older (n=13) participants.

	Twice daily or more	Daily	Every 2-3 days	Less than twice a week
Younger group	37.5%	37.5%	25%	0%
Older group	25.0%	50%	25%	0%

*Data presented as percentage of frequency

4.6.2 Dietary assessment

4.6.2.1 Estimation of habitual dietary habits

No differences were found between the two groups in terms of their dietary habits, with the exception of potato intake (p=0.01), where a higher consumption was noted in the older group (Table 4-3).

Table 4-3: Estimation of dietary habits (serving/week) using the FFQ in younger (n=8) and older (n=13) participants.

Food item	Younger group		Older group		p value
	Median	IQR	Median	IQR	
Hot Beverages (green tea, black tea, coffee, hot chocolate, and herbal infusion)	15.4	24.4	36.0	24.8	0.6
Milk drinks	3.0	15.2	7.0	4.0	0.9
Milk substitutes (soya, rice, and oat milk)	0.5	0.0	0.5	0.3	0.4
Fruit juices	1.5	6.5	7.5	11.0	0.4
Soft drink	0.4	0.4	0.5	0.3	1.0
Alcoholic	1.9	0.7	1.5	3.3	0.9
Wholemeal products (pasta, rice, and brown rice)	7.6	7.3	8.0	16.2	0.4
Refined products (pasta, rice, and crisps and crackers)	4.9	4.2	6.5	4.9	0.4
Breakfast cereals	3.8	5.1	6.8	4.8	0.1
Fresh fruit	16.0	11.2	23.5	12.7	0.1
Dried fruits	0.5	0.8	3.0	3.9	0.2
Vegetables	10.1	9.1	10.8	11.6	0.6
Potatoes	1.8	2.0	4.0	2.1	0.01
Legumes	2.0	0.6	2.5	7.6	0.2
Onion, tomato, chili, or curry-based products (inc. fresh tomatoes, ketchup, and soup)	11.9	7.9	9.8	8.5	0.6
Meat (beef, chicken, lamb, pork, bacon, and sausages)	2.00	4.25	7.5	3.7	0.4
Seafood (White fish, oil-rich fish, and shellfish)	2.88	1.63	3.5	2.5	0.9
Biscuits, cakes, and sweets	11.0	12.9	20.0	15.0	0.1
Yoghurt	1.8	2.5	6.0	6.8	0.7
Dairy dessert	0.4	0.3	0.5	2.8	0.8
Cheese	2.4	3.4	6.0	2.8	0.4
Eggs	2.0	3.0	1.0	2.5	0.2
Ice cream	0.5	0.8	0.5	0.8	0.9
Fats & oils	6.1	9.6	14.8	20.5	0.4
Seasonings	6.8	19.6	4.0	3.0	0.3
Herbs	6.0	5.9	4.0	12.5	1.0
Spices & Chili	2.5	3.3	1.0	5.3	0.3

4.6.2.2 Macronutrient intake during low and high polyphenol diets

There was no significant difference between the groups in terms of energy, fat, protein, carbohydrate, total sugars, starch, alcohol, or dietary fibre after the low and high-polyphenol diet (Table 4-4). When considering the difference in macronutrient intake (Δ low-high diet), there was no significant difference between the groups.

4.6.2.3 Micronutrient intake during low and high polyphenol diets

There was no significant difference between groups for the intake of vitamins or dietary minerals except for thiamine and copper intake during the high-polyphenol diet, with thiamine ($p=0.05$) higher in the younger group, and copper ($p=0.01$) higher in the older group (Table 4-5).

Table 4-4: Energy and macronutrient intake from the dietary records after low and high-polyphenol diets in younger (n=8) and older (n=13) participants.

Diet	Low-polyphenol diet					High-polyphenol diet				
Group	Younger		Older			Younger		Older		
Nutrient	**Median**	**IQR**	**Median**	**IQR**	**p value**	**Median**	**IQR**	**Median**	**IQR**	**p value**
Energy (KJ)	8016	2466	7768	3277	0.6	8227	2442	7531	2474	0.5
kcal (Kc)	1910	585	1874	785	0.6	1949	586	1794	617	0.5
Fat (g)	95.8	27.8	82.1	56.0	0.9	74.3	25.8	63.8	31.7	0.5
protein (g)	80.4	7.2	94.3	37.7	0.7	54.9	15.6	47.1	22.3	0.7
Carbohydrate (g)	195.0	83.3	195.4	73.0	0.4	247.1	109.5	217.5	78.6	0.4
Total sugars (g)	36.1	26.4	34.3	42.1	0.6	115.9	50.0	92.9	42.1	0.2
Starch (g)	140.5	62.8	149.5	43.9	0.9	70.6	44.2	60.0	35.7	0.9
Alcohol (g)	0.0	0.0	0.0	0.0	0.9	0.0	0.0	0.0	0.5	0.5
Dietary fibre* (g)	9.1	2.0	11.8	8.0	0.3	27.5	3.2	26.5	9.9	0.7

* Non-starch polysaccharide

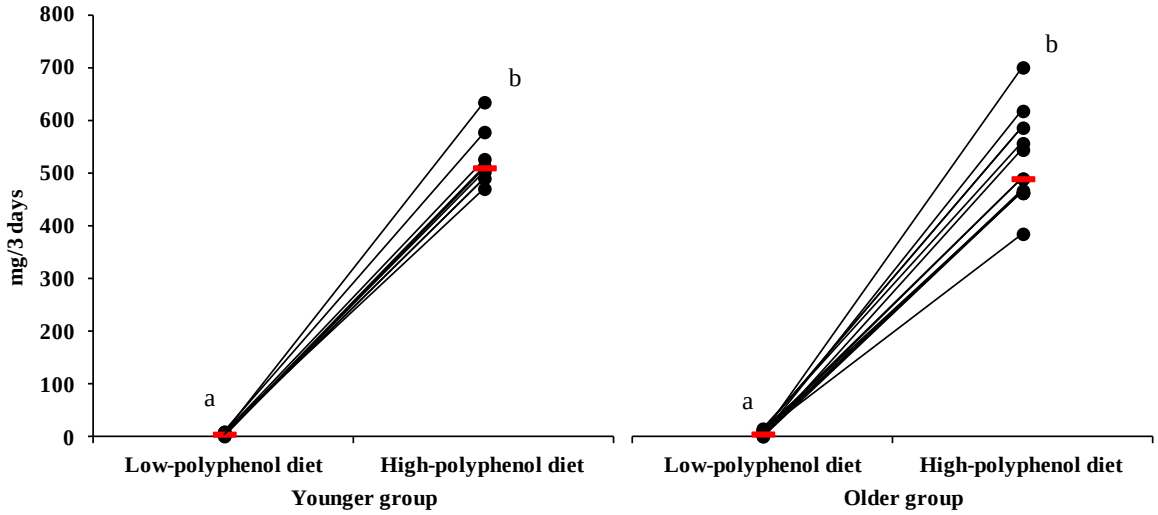
Table 4-5: Estimated micronutrient intake from the dietary records after low and high-polyphenol diets in younger (n=8) and older (n=13) participants.

Diet	Low-polyphenol diet					High-polyphenol diet				
Group	Younger		Older		p value	Younger		Older		p value
Nutrient	Median	IQR	Median	IQR		Median	IQR	Median	IQR	
Vitamin A (µg)	632	201.6	574.0	396.0	0.8	362.2	98.1	383.3	380.3	0.4
Thiamine (mg)	1.2	0.3	1.1	0.5	0.6	0.6	0.3	0.8	0.4	0.05
Riboflavin (mg)	1.7	0.5	1.2	0.9	0.4	0.6	0.4	0.9	0.3	0.2
Niacin (mg)	28.6	4.9	35.9	15.6	0.2	19.5	14.7	16.2	9.5	0.6
Vitamin B6 (mg)	1.5	0.2	1.4	0.9	0.6	0.7	0.5	0.9	0.8	0.5
Vitamin B12 (µg)	5.2	1.3	3.7	9.7	0.9	1.6	2.1	2.0	2.4	0.6
Folic acid (µg)	155.2	70.5	137.0	42.0	0.4	95.5	33.3	125.7	32.3	0.2
Pantothenic acid (mg)	4.6	1.4	5.5	3.9	0.3	2.2	0.5	3.2	1.6	0.2
Biotin (µg)	26.0	5.9	30.9	20.0	1.0	18.5	6.4	22.8	7.1	0.3
Vitamin C (mg)	17.2	8.3	22.0	35.4	0.5	62.4	11.0	75.6	105.0	0.6
Vitamin D (µg)	2.0	1.3	3.4	5.4	0.3	1.2	1.1	0.8	1.0	0.3
Vitamin E (mg)	5.8	3.8	5.4	6.0	0.6	11.7	5.0	7.8	4.0	0.1
Calcium (mg)	1302.3	539.5	909.7	551.7	0.5	358.8	69.7	517.0	223.6	0.08
Magnesium (mg)	182.7	65.8	172.3	132.3	0.9	146.5	55.1	158.3	82.7	0.7
Sodium (mg)	3009.5	1340.0	2831.3	1983.0	0.5	2698.5	713.2	2343.3	323.3	0.3
Potassium (mg)	1934.2	686.1	1887.0	1665.3	0.7	1987.7	680.3	2183.0	990.3	0.7
Chlorine (mg)	4394.5	1702.3	4640.3	3249.3	0.5	2612.2	721.5	2540.7	804.4	0.5
Phosphorus (mg)	1486.7	227.8	1237.0	603.3	0.7	568.2	329.1	681.7	286.3	0.3
Iron (mg)	8.6	2.5	8.3	2.9	0.7	6.2	2.4	7.3	2.3	0.2
Zinc (mg)	8.5	1.6	7.7	3.3	0.5	4.1	1.5	4.1	2.7	0.4
Copper (mg)	0.7	0.3	0.7	0.2	0.7	1.1	0.2	0.8	0.4	0.01
Manganese (mg)	1.3	1.0	1.8	0.8	0.3	2.5	0.5	2.8	1.5	0.8
Selenium (µg)	41.0	9.8	58.0	53.7	0.5	22.5	15.8	16.3	12.0	0.2
Iodine (µg)	198.7	127.9	126.3	166.0	0.8	58.7	57.0	71.0	46.0	0.5

4.6.2.4 Flavonoid intake during low and high polyphenol diets

There was no difference in flavonoid intake between the two age groups over either the 3 days of low or high-polyphenol diet (Figure 4-2). Flavonoid intake during the 3 days low polyphenol diet was 6.0 mg/day (IQR 5.4) in the younger group and 6.3 mg/day (IQR 8.4) in the older group. Flavonoid intake was 510.4 mg/day (IQR 40.1) in the younger group and 489.7 mg/day (IQR 123.1) in the older group after the 3 days of high-polyphenol diet.

Figure 4-2: Median flavonoid intake per day over 3 days low and-high polyphenol diets in younger (n=8) and older (n=13) participants.



Each circle indicates the estimated average daily flavonoid intake for each participant after low and high-polyphenol diets. Median flavonoid intake for each group is indicated by a red horizontal line.
a,b symbols indicate differences within group (low to high-polyphenol diet).

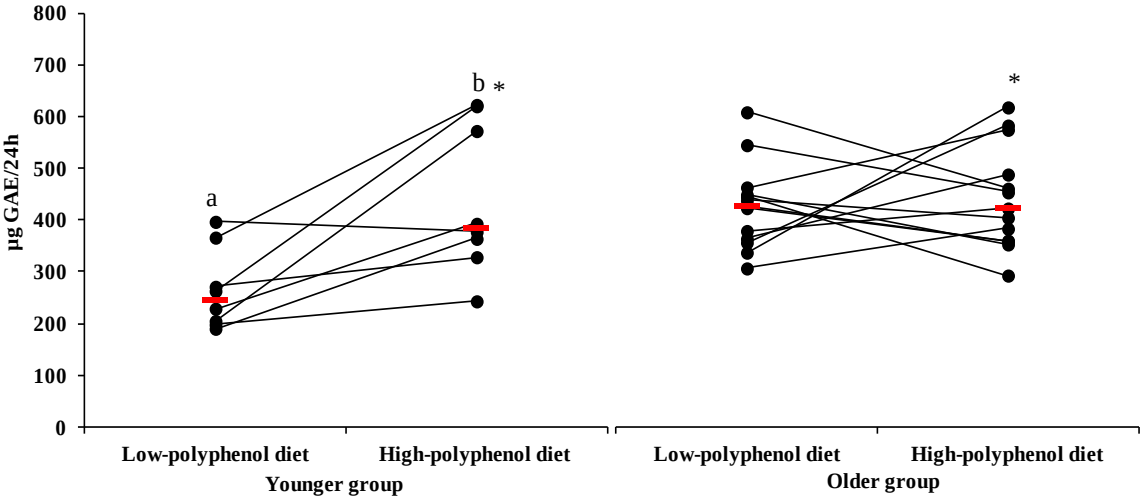
When considering flavonoid intake on day 3 only (since urine samples were collected from the second urine of day 3, including the first urine of day 4), there was no difference between groups during the low-polyphenol diet (5.0 mg (IQR 4.5) versus 8.9 mg (IQR 3.6) for younger and older groups, respectively) or the high-polyphenol diet (553.1 mg (IQR 76.0) versus 496.8 mg (IQR 102.2) for younger and older groups, respectively).

4.6.3 Measurements performed on urine samples

4.6.3.1 Urinary total phenols using Folin-Ciocalteu assay

After the low-polyphenol diet, total urinary phenols was higher in the older group than the younger group ($p<0.001$; Figure 4-3). Total urinary phenols did not increase in the older group after the high polyphenol diet, from 426.5 µg GAE/24h (IQR 85.2) to 422.8 µg GAE/24h (IQR 128.2), while they did in the younger group, from 245.9 µg GAE/24h (IQR 91.9) to 385.4 µg GAE/24h (IQR 229.7; $p=0.02$). When considering the difference in urinary total phenols excretion (Δ low-high diet), it was significantly higher in the younger group (169.3 µg GAE/24h (IQR 227.8) versus -33.4µg GAE/24h (IQR 203.2), $p=0.02$).

Figure 4-3: 24-hour urinary total phenols (µg GAE/24h) after low and high-polyphenol diets in younger (n=8) and older (n=13) participants.

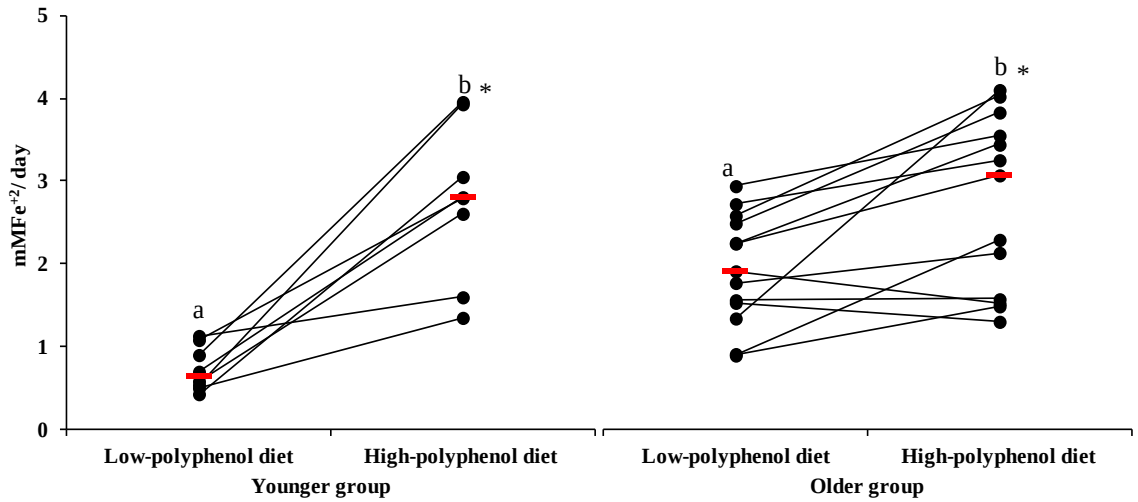


Each circle indicates the measurement of urinary total phenols for each participant after low and high-polyphenol diets. Median urinary total phenols for each group are indicated by a red horizontal line.
a,b symbols indicate differences within group (low to high-polyphenol diet).
*symbol indicates differences in changes (Δ) between groups.

4.6.3.2 Total antioxidant activity (FRAP) of urine samples after low and high polyphenol diets

The urinary FRAP value increased after the high-polyphenol diet in both groups, from 0.6 mM Fe^{+2}/day (IQR 0.4) to 2.8 mM Fe^{+2}/day (IQR 0.9; p=0.0009) in the younger group and from 1.9 mMFe^{+2}/day (IQR 0.1) to 3.1 mMFe^{+2}/day (IQR 2.0; p=0.005) in the older group (Figure 4-4). The difference (Δ low-high diet) in FRAP value was significantly higher (p=0.01) in the younger group (2.07 mM Fe^{+2}/day, IQR 1.24 versus 0.61 mM Fe^{+2}/day, IQR 0.98).

Figure 4-4: 24-hour urinary antioxidant activity (mM Fe^{+2}/day) after low and high-polyphenol diets in younger (n=8) and older (n=13) participants.

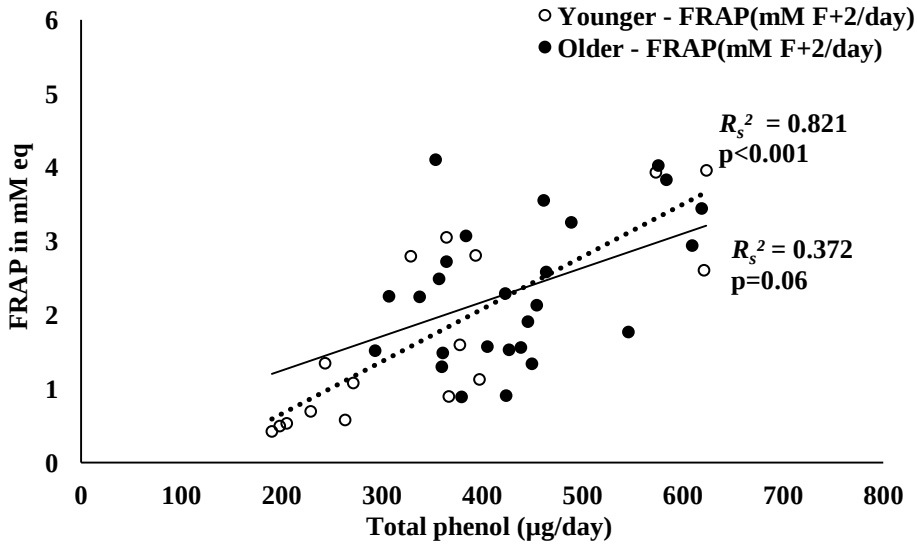


Each circle indicates the measurement of urinary FRAP for each participant after low and high-polyphenol diets. Median urinary FRAP for each group is indicated by a black horizontal line.
a,b symbols indicate differences within group (low to high-polyphenol diet).
*symbol indicates differences in changes (Δ) between groups.

4.6.3.2.1 Correlation between urinary total phenols (measured with Folin-Ciocalteu) and urinary FRAP

The relationship between urinary total phenols (measured with Folin-Ciocalteu) and urinary FRAP was analysed by linear regression (Spearman rank correlation).While the correlation between urinary FRAP and total phenols was good (R_s^2=0.8; p<0.001) in the younger group, it was weak (R_s^2=0.3; p=0.06) in the older group (Figure 4-5).

Figure 4-5: Correlation between urinary FRAP and the urinary total phenols (Folin-Ciocalteu) in younger (n=8) and older (n=13) participants.



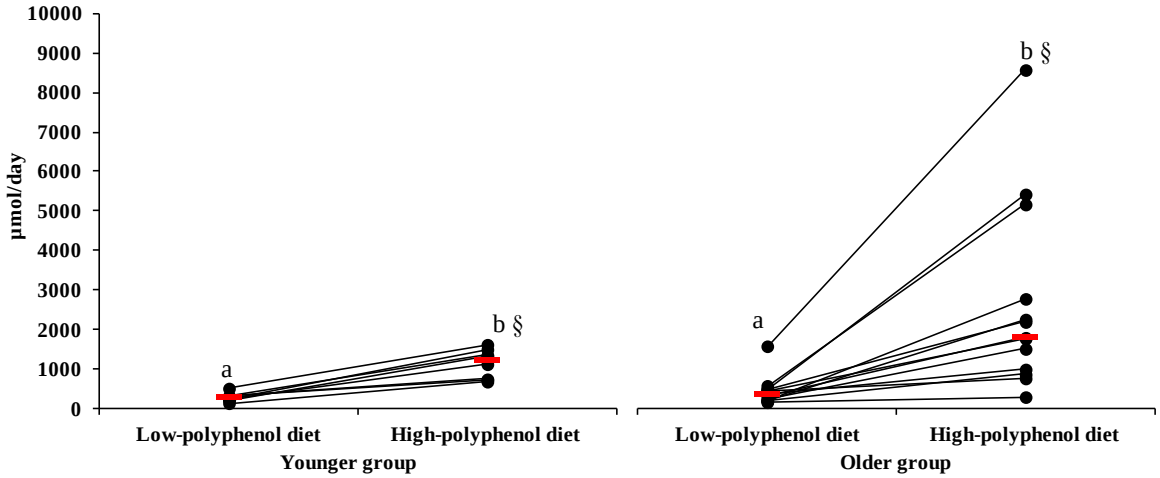
Dotted line indicates younger group; solid line indicates older group.

4.6.3.3 GC-MS measurement of urinary phenolic acid excretion after low and high-polyphenol diets

The sum of the seventeen urinary phenolic acids excreted, significantly increased in the younger group (p<0.001) from 268.2 µmol/day (IQR 89.3) after the low-polyphenol diet to 1220.4 µmol/day (IQR 649.5) after the high-polyphenol diet, and from 349.3 µmol/day (IQR 286.7) to 1789.0 µmol/day (IQR 1788.0) respectively in the older group (p<0.001). The change in urinary excretion (Δ low-high diet) was not different between groups. However, urinary phenolic acid concentration was higher after the high-polyphenol diet in the older group (p=0.04;Figure 4-6).

After correcting for flavonoid intake, the urinary phenolic acid concentration was still higher (p=0.05) in the older group after the high-polyphenol diet compared to the younger group (2860.3 µmol/mg of flavonoid (IQR 2779.6) versus 1629.4 µmol/mg of flavonoid (IQR 1185.3).

Figure 4-6: 24-hour urinary phenolic acid profile excretion (µmol/day) after low and high-polyphenol diets in younger (n=8) and older (n=13) participants.



Each circle indicates the measurement of the urinary phenolic acids profile for each participant after low and high-polyphenol diets. Median urinary phenolic acids for each group are indicated by a red horizontal line.
a,b symbols indicate differences within group (low to high-polyphenol diet).
§ symbol indicates differences between groups (high vs. high).

There were differences between groups for the excretion of individual phenolic acids (Δ low-high diet).The older group excreted less benzoic acid (p=0.04) and vanillic acid (p=0.05) than the younger group (Table 4-6).

Table 4-6: Amount of phenolic acid in 24 h urine (µmol/day) after low and high-polyphenol diet in younger (n=8) and older (n=13) participants.

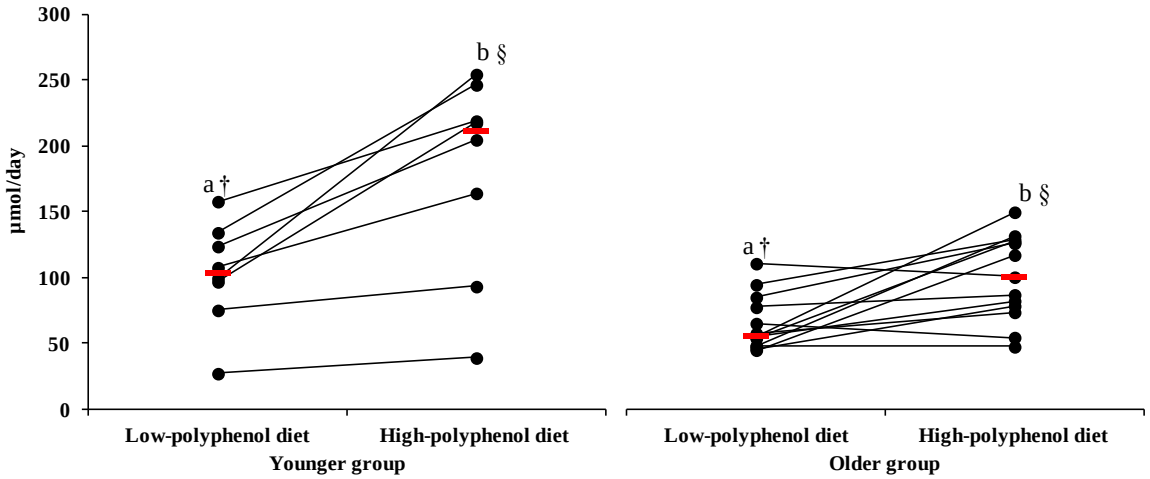
Group	Younger							Older							
Diet	**Low-polyphenol diet**			**High- polyphenol diet**			**Δ**	**Low-polyphenol diet**			**High- polyphenol diet**			**Δ**	**p value**
Acid	**N**	**Median**	**IQR**	**N**	**Median**	**IQR**		**N**	**Median**	**IQR**	**N**	**Median**	**IQR**		
BA	8/8	16.6†	14.3	8/8	31.1§	32.9	**14.5**	13/13	1.1†	1.5	13/13	1.8§	4.3	**0.8**	**0.04**
PAA	8/8	3.2†	2.2	8/8	3.2	4.8	**0.0**	7/13	0.9†	0.4	6/13	1.1	0.7	**0.2**	0.80
MA	8/8	0.7	0.5	8/8	1.1	1.2	**0.4**	13/13	0.3	0.5	13/13	0.5	0.5	**0.2**	0.14
3-OHBA	8/8	0.6*	0.5	8/8	0.9*	1.2	**0.3**	8/13	0.4	1.2	9/13	1.2	1.2	**0.8**	0.27
3-OHPAA	8/8	2.3*	1.3	8/8	19.2*	23.3	**16.9**	13/13	2.3**	1.4	13/13	30.2**	39.1	**27.8**	0.40
4-OHBA	8/8	2.1	1.0	8/8	3.8	1.9	**1.7**	12/13	2.2	2.1	12/13	2.9	2.1	**0.7**	0.17
4-OHPAA	8/8	38.0	21.4	8/8	42.9	15.0	**4.9**	13/13	21.2	20.7	13/13	19.6	14.4	**-1.6**	0.94
4-OHPPA	8/8	0.4	0.4	8/8	0.5	0.9	**0.2**	8/13	0.2	0.6	8/13	0.3	0.5	**0.1**	0.08
VA	8/8	0.7*	0.4	8/8	2.8*	2.7	**2.1**	9/13	0.9	1.1	12/13	1.4	1.2	**0.5**	**0.05**
HVA	8/8	10.8*	4.4	8/8	19.8*	12.7	**9.0**	13/13	7.6**	1.8	4/13	12.4**	5.6	**4.9**	0.71
4-OHMA	8/8	4.9	1.5	8/8	3.9	1.6	**-1.0**	13/13	3.7**	0.9	13/13	2.6**	1.0	**-1.1**	0.84
3,4diOHBA	8/8	1.0*	0.2	8/8	2.1*	1.1	**1.1**	4/13	0.9	0.4	4/13	1.2	0.3	**0.3**	0.20
3,4diOHPAA	8/8	1.5*	0.4	8/8	2.9*	5.3	**1.5**	13/13	1.2**	0.3	13/13	3.2**	3.8	**2.0**	0.91
HA	8/8	155.7*	95.7	8/8	969.8*§	540.2	**814.1**	13/13	301.5**	277.6	13/13	1734.5**§	1704.5	**1433.0**	0.06
Dihydrocaffeic acid	8/8	1.3*	0.9	8/8	2.3*	2.2	**0.9**	6/13	1.3	0.4	9/13	1.5	1.1	**0.2**	0.54
3,4diOHPPA	8/8	0.5*	0.4	8/8	1.6*	1.2	**1.1**	8/13	0.3**	0.6	9/13	0.8**	0.4	**0.5**	0.07
GA	8/8	0.6	0.7	8/8	0.8	1.4	**0.2**	4/13	0.8	0.4	5/13	0.7	0.5	**0.0**	0.25
3-OHhippA	8/8	21.0*	10.2	8/8	68.6*	41.4	**47.6**	13/13	15.1**	10.9	13/13	28.4**	20.0	**13.3**	0.76

N number of participants.
Δ Difference in urinary excretion (high diet minus low diet).
P value is the difference in urinary phenolic acid excretion (Δ low-high diet) between groups.
* Significant increase in younger group after the high-polyphenol diet $p \leq 0.01$
**Significant increase in older group after the high-polyphenol diet $p \leq 0.01$
§Significant difference between groups after the low-polyphenol diet $p \leq 0.05$
†Significant difference between groups after the high-polyphenol diet $p \leq 0.05$

Hippuric acid (HA) was always the most abundant acid in urine samples in both groups (89.2 and 97.9% of the total of all phenolic acids for younger and older, respectively), and was higher in the older group after the high-polyphenol diet compared to low diet (p=0.02). However, the change in excretion, Δ low-high diet, was not different between groups.

The sum of the phenolic acids minus hippuric acid was considered as it is most likely to be formed in the liver by conjugation of benzoic acid and glycine. Dietary sources of benzoic acid and precursors (quinic acid, aromatic amino acid tryptophan, tyrosine, and phenylalanine) should be considered (Self *et al.*, 1960, Grumer, 1961). Other sources of benzoic acid are benzoates (E numbers 210-219) which are commonly used in food, medications, and mouthwashes. An increase was detected after the high-polyphenol diet in both groups; from 103.6 µmol/day (IQR 34.9) to 211.2 µmol/day (IQR 79.6) in the younger group (p=0.003) and from 55.5µmol/day (IQR 29.3) to 100.4 µmol/day (IQR 48.5) in the older group (p=0.007). The difference in urinary excretion (Δ low-high diet) was not different between groups. However, urinary phenolic acid concentration was higher after the low-polyphenol diet (p=0.03) and high-polyphenol diet in the younger group (p=0.02; Figure 4-7).

Figure 4-7: 24-hour urinary phenolic acid profile excretion without hippuric acid (µmol/day) after low and high-polyphenol diets in younger (n=8) and older (n=13) participants.

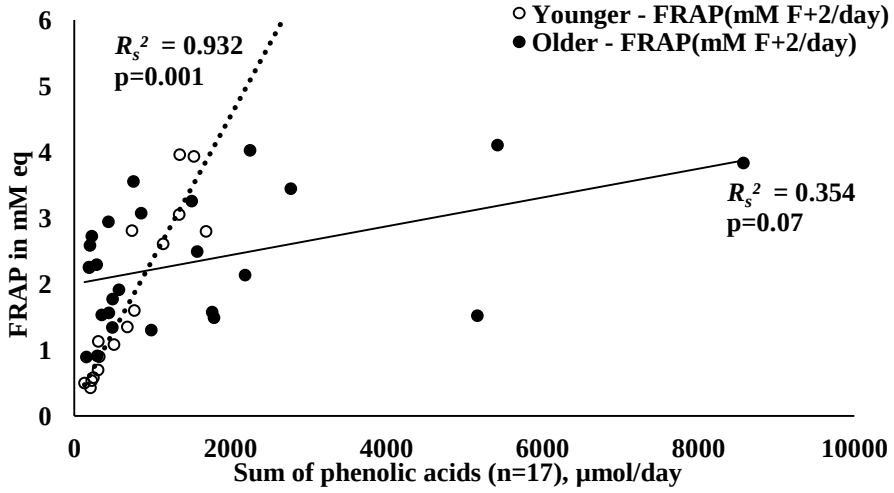


Each circle indicates the measurement of the urinary phenolic acids profile without hippuric acid for each participant after low and high-polyphenol diets. Median urinary phenolic acids profile without hippuric acid for each group is indicated by a red horizontal line.
a,b symbols indicate differences within group (low to high-polyphenol diet).
† symbol indicates differences between groups (low vs. low).
§ symbol indicates differences between groups (high vs. high).

4.6.3.3.1 Correlation between urinary phenolic acid (measured with GC-MS) and urinary FRAP

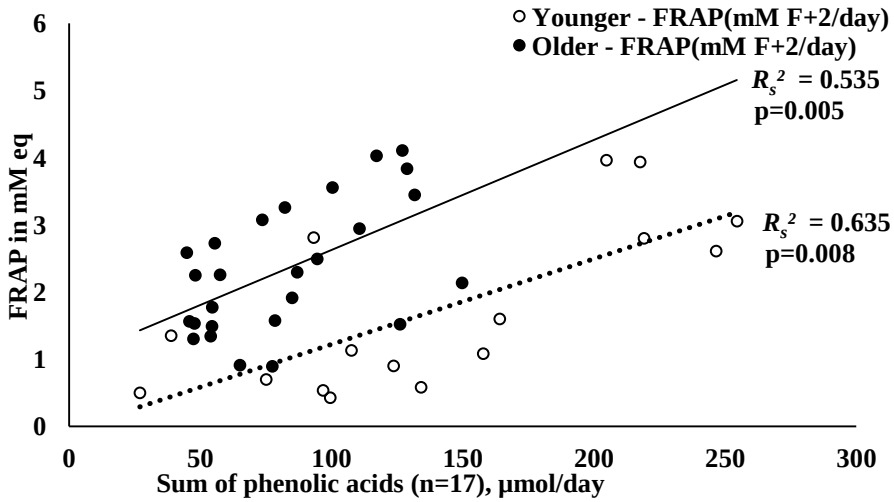
The relationship between urinary phenolic acid (measured with GC-MS) and urinary FRAP was analysed by linear regression (Spearman rank correlation).There was a strong correlation between urinary FRAP and the sum of urinary phenolic acids in the younger group (R_s^2=0.9, p=0.001). However, the association between FRAP and total phenolic acids was very weak in the older group (R_s^2=0.3, p=0.07; Figure 4-8). The correlation without hippuric acid showed gave much stronger association (Figure 4-9).

Figure 4-8: Correlation between urinary FRAP and the phenolic acid (GC-MS) in younger (n=8) and older (n=13) participants.



Dotted line indicates younger group; solid line indicates older group.

Figure 4-9: Correlation between urinary FRAP and phenolic acid without hippuric acid (GC-MS) in younger (n=8) and older (n=13) participants.



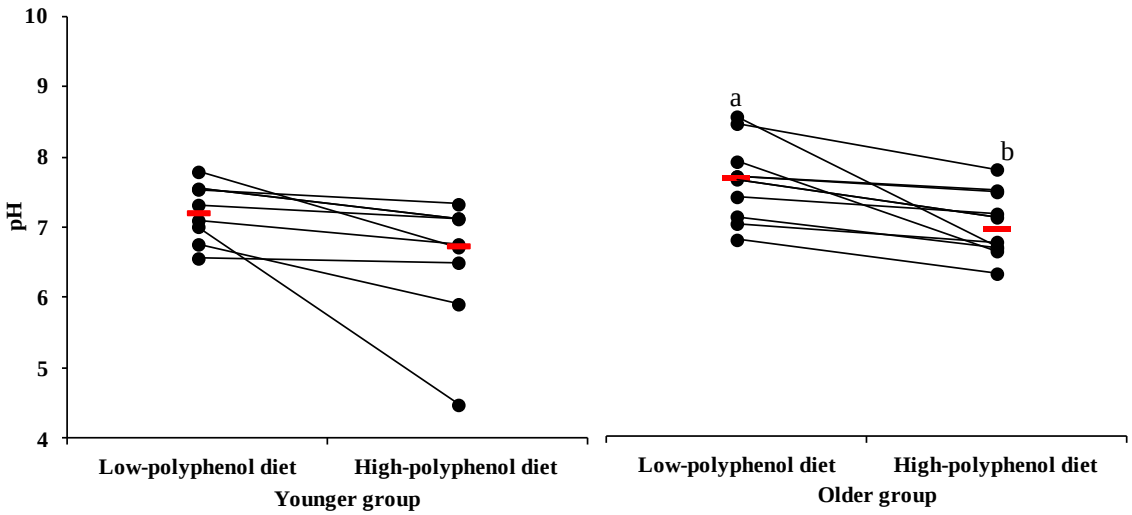
Dotted line indicates younger group; solid line indicates older group.

4.6.4 Measurements performed on faecal samples

4.6.4.1 Faecal pH after low and high-polyphenol diets

The faecal pH decreased significantly in the older group from 7.7 (IQR 0.6) to 6.9 (IQR 0.6; $p<0.01$). However, this was not the case for the younger group (7.2, IQR 0.6 to 6.7, IQR 0.8). Looking at pH change with diet (Δ low-high diet for pH) there was no significant differences between the groups (Figure 4-10).

Figure 4-10: Change in faecal pH after low and high-polyphenol diets in the younger (n=8) and older (n=10) participants.

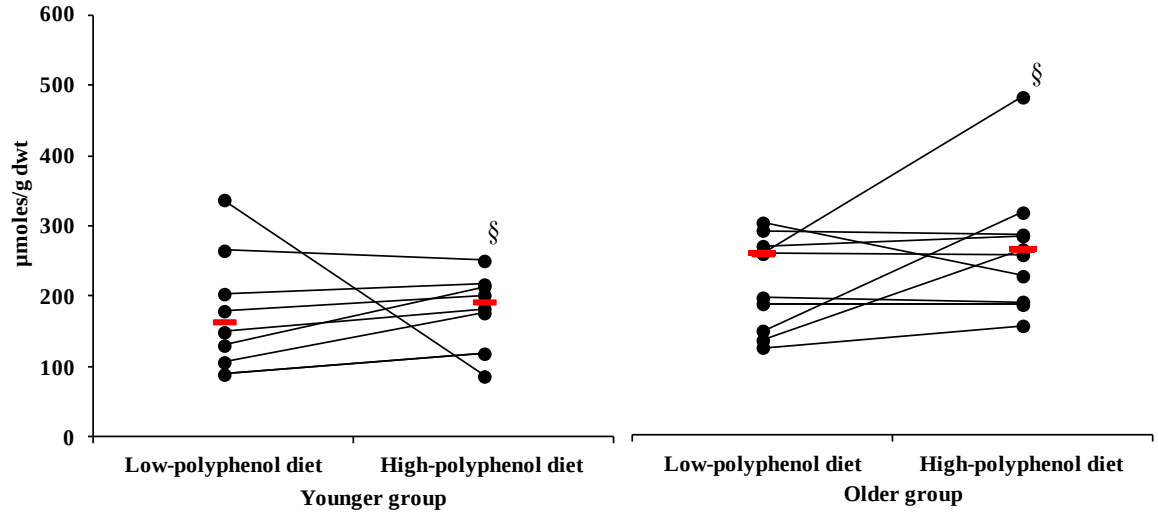


Each circle indicates the measurement of faecal pH for each participant after low and high-polyphenol diets. Median faecal pH for each group is indicated by a red horizontal line.
a,b symbols indicate differences within group (low to high-polyphenol diet).

4.6.4.2 Faecal SCFA after the low and high-polyphenol diets

Total faecal SCFA concentration (sum of all SCFA) did not increase significantly from low to high-polyphenol diet in either group. Total SCFA changed from 164.3 µmoles/g dwt (IQR 94.5) to 192.4 µmoles/g dwt (IQR 52.8) respectively in the younger group, and from 258.4 µmoles/g dwt (IQR 112.3) to 264.5 µmoles/g dwt (IQR 92.7) in the older group. Although there were no significant differences (Δ low-high diet) in either group, the SCFA concentration was significantly higher for the older group than the younger group after the high-polyphenol diet (p=0.01; Figure 4-11).

Figure 4-11: Faecal SCFA (µmoles/g dwt) after low and high-polyphenol diets in younger (n=8) and older (n=11) participants.



Each circle indicates the measurement of faecal SCFA for each participant after low and high-polyphenol diets. Median faecal SCFA for each group is indicated by a red horizontal line.
§ symbol indicates differences between groups (high vs. high).

There were no differences in the change (Δ low-high diet) in each specific acid between groups. However, the absolute levels of acetic acid were higher after both the low (p=0.01) and high-polyphenol diets (p=0.02) in the older group, relative to the younger group. Absolute level of enanthic acid was higher after the high-polyphenol diet in the younger group (p=0.03; Table 4-7).

Table 4-7: Faecal concentration of SCFA (µmol/g dwt) in younger(n=8) and older (n=11) participants after low and high-polyphenol diets.

Group	Younger					Older					
Diet	**Low polyphenol diet**		**High polyphenol diet**			**Low polyphenol diet**		**High polyphenol diet**			**p value**
Acid	**Median**	**IQR**	**Median**	**IQR**	Δ	**Median**	**IQR**	**Median**	**IQR**	Δ	
Acetic acid	97.8§	35.8	130.2†	25.4	**32.4**	146.7§	41.9	185.0†	82.6	**38.3**	0.1
Propionic acid	25.6	25.7	25.9	11.4	**0.3**	34.5	25.2	28.4	10.0	**-6.2**	0.9
Isobutyric acid	3.2	2.9	2.5	0.6	**-0.7**	6.0	3.0	3.2	1.9	**-2.8**	0.8
Butyric acid	19.4	23.0	17.5	8.5	**-1.9**	28.2	29.7	38.4	21.1	**10.2**	0.3
Isovaleric acid	5.7	4.6	3.6	2.2	**-2.1**	9.3	3.8	5.3	2.2	**-3.9**	1
Valeric acid	4.9	3.7	3.3	1.5	**-1.6**	5.0	3.4	3.8	1.3	**-1.2**	0.5
Isocaproic acid	0.0	0.5	0.0	0.0	**0.0**	0.0	0.0	0.0	0.0	**0.0**	0.8
Caproic acid	1.7	2.4	1.1	4.0	**-0.6**	1.1	1.4	2.0	2.6	**0.9**	0.5
Enanthic acid	1.2	2.3	1.8†	4.2	**0.6**	0.0	0.0	0.0†	0.0	**0.0**	0.8
Caprylic acid	1.7	1.6	2.3	3.7	**0.6**	1.1	1.3	1.0	1.3	**-0.1**	0.4
Total SCFA	***161.2***	***102.4***	***188.3***	***61.5***	***27.1***	***258.4***	***112.3***	***264.5***	***92.7***	***6.1***	***0.9***
	Median	**IQR**	**Median**	**IQR**	Δ	**Median**	**IQR**	**Median**	**IQR**	Δ	
Proportional ratio	59.4	16.2	66.2	8.0	**6.8**	62.8	12.1	70.0	5.2	**7.2**	
%Acetic acid	15.6	8.1	13.0	3.5	**-2.6**	12.5	3.9	10.6	3.4	**-1.9**	
%Propionic acid	2.0	0.7	1.3	0.7	**-0.7**	2.2	0.7	1.1	1.3	**-1.1**	
% Isobutyric acid	10.7	6.0	9.7	2.3	**-1.0**	10.9	6.6	12.9	5.3	**2**	
%Butyric acid	3.9	1.4	2.2	0.7	**-1.7**	3.7	1.2	1.9	2.0	**-1.8**	
% Isovaleric acid	2.5	1.6	1.9	1.0	**-0.6**	2.4	0.9	1.5	0.5	**-0.9**	
%Valeric acid	0.0	0.3	0.0	0.0	**0.0**	0.0	0.0	0.0	0.0	**0**	
%Isocaproic acids	1.2	1.5	0.5	1.9	**-0.7**	0.5	0.8	1.1	1.1	**0.6**	
%Caproic acid	0.9	1.0	1.0	2.2	**0.0**	0.0	0.0	0.0	0.0	**0.0**	
%Enanthic acid	0.9	0.7	1.2	0.4	**0.3**	0.5	0.5	0.3	0.5	**-0.2**	

Δdifference in faecal concentration of SCFA (high diet minus low diet).
p values are the difference in urinary excretion (Δ low-high diet) of each acid between groups.
§Significant difference between groups after the low-polyphenol diet $p \leq 0.05$
†Significant difference between groups after the high-polyphenol diet $p \leq 0.05$

4.6.4.3 Concentration of bacterial DNA isolated from faecal samples after low-polyphenol diet

High quality and high yield DNA was obtained from all faecal samples. The purity and yield of the extracted DNA was very high and similar between groups (Table 4-8).

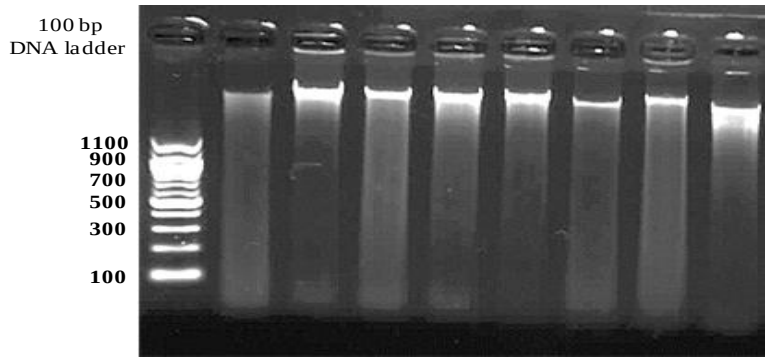
Table 4-8: The DNA concentration and purity measured using Nano-Drop in the younger (n=8) and older (n=12) participants.

Group	Younger			Older		
Measurements	**Median**	**IQR**	**Range**	**Median**	**IQR**	**Range**
DNA concentration (ng/µl)	519.5	98.6	298.0-570.5	463.0	201.4	421.6 - 1031.4
DNA purity 280/260 nm	1.81	0.06	1.7 - 1.9	1.8	0.09	1.7- 2.0
DNA purity 260/230 nm	1.40	0.41	1.1 - 1.8	1.3	0.2	1.0-1.8

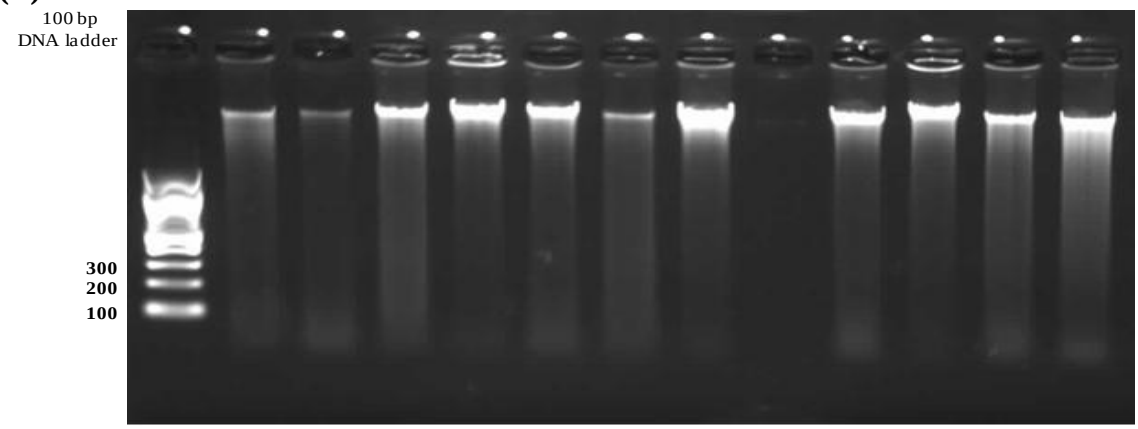
The faecal DNAs appear intact and compact as a high-molecular-weight band when electrophoresed through a 1.5% agarose gel (Figure 4-12).

Figure 4-12:Agarose gel showing the purity of DNA extracted for bacterial qPCR analysis in A) younger (n=8) and B) older (n=12) participants.

(A)



(B)



4.6.4.4 Characteristics of the qPCR run condition

The characteristics of the qPCR runs of each bacterial group and species including the slope, amplification efficiency, and the coefficient of determination (R^2) for both groups are described in Table 4-9. The amplification efficiency was within the normal range (90% - 105%) for the total bacteria, *Bacteroides – Prevotella*, and *Flavonifractor plautii* in both groups; however, efficiency for *Bifidobacterium spp.* was just below the normal range in both groups.

Table 4-9: Characteristics of qPCR runs for each bacteria species/groups in both younger (n=8) and older (n=12) participants.

Bacteria species/groups	Slope		Amplification efficiency (%)		R^2	
	Younger group	Older group	Younger group	Older group	Younger group	Older group
Total Bacteria	-3.444	-3.371	95.1	97.9	0.995	0.994
Bacteroides – Prevotella	-3.629	-3.507	89.1	92.8	0.997	0.998
Bifidobacterium spp.	-3.72	-3.68	86.6	86.9	0.999	0.994
Flavonifractor plautii	-3.581	-3.621	90.2	89.7	0.998	0.999

4.6.4.5 Absolute concentration of bacterial species and groups using qPCR

Absolute levels of *Bifidbacterium*, *Bacteroides*, and *Flavonifractor plautii* did not differ between younger and older groups (Table 4-10).

Table 4-10: Absolute (log^{10}/g) faecal concentrations and relative abundance of bacterial groups/species in younger (n=8) and older (n=12) participants.

Target	Wet weight sample				Dry weight sample*			
	Younger (n=8)		Older (n=12)		Younger (n=8)		Older (n=12)	
Log^{10}/g	Median	IQR	Median	IQR	Median	IQR	Median	IQR
Total bacteria	11.5	0.2	11.4	0.5	11.9	0.3	11.8	0.5
Bifidobacterium spp.	9.7	0.5	9.5	0.9	10.1	0.8	10.1	1.1
Bacteroides+Prevotella	10.5	0.8	10.2	0.6	11.0	1.0	10.7	0.4
Flavonifractor plautii	8.4	0.7	7.7	1.0	8.9	0.8	8.2	1.1
%								
Bifidobacterium spp.	1.6		1.3		1.6		2.0	
Bacteroides+Prevotella	10.0		6.3		12.6		7.9	
Flavonifractor plautii	0.1		0.0		0.1		0.0	

* Calculated using the weight (g) and the moisture percentage (%) of the wet faecal samples.

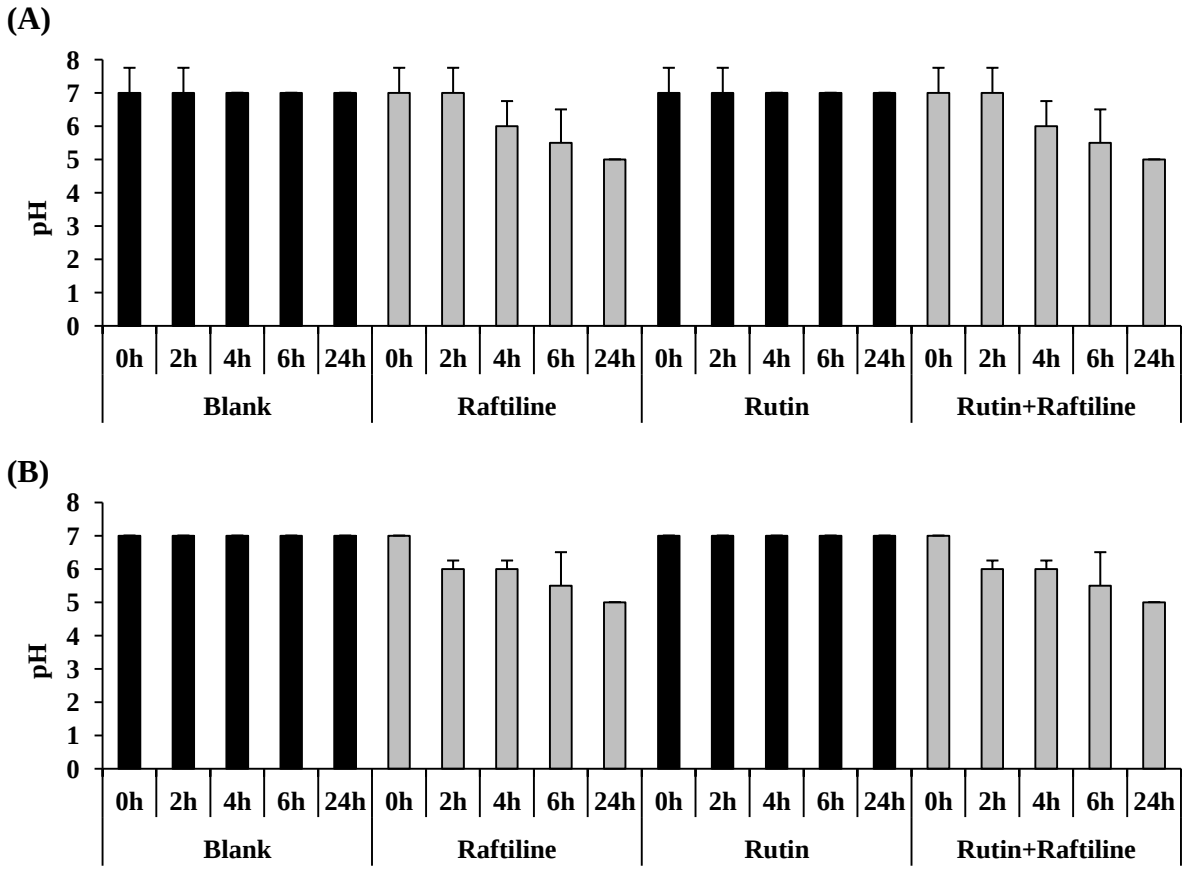
4.6.5 *In-vitro* fermentation of rutin

Ten fermentations were carried out using the stool samples of 6 younger (1 male and 5 females) and 4 older subjects (1 male and 3 females), collected after the low-polyphenol diet. The rutin was fermented for 24 h with or without fibre (raftiline) to test the metabolic capacity of the gut faecal contents, including the microbiota, in relation to ageing.

4.6.5.1 Rutin fermentation and pH of faecal fluids

There was no change in the pH of the fermented faecal fluids containing rutin alone, over time, in either group (Figure 4-13). However, the combination of rutin with raftiline significantly ($p<0.05$) reduced the pH level at the end of the fermentation in both groups. The reduction was not different between groups.

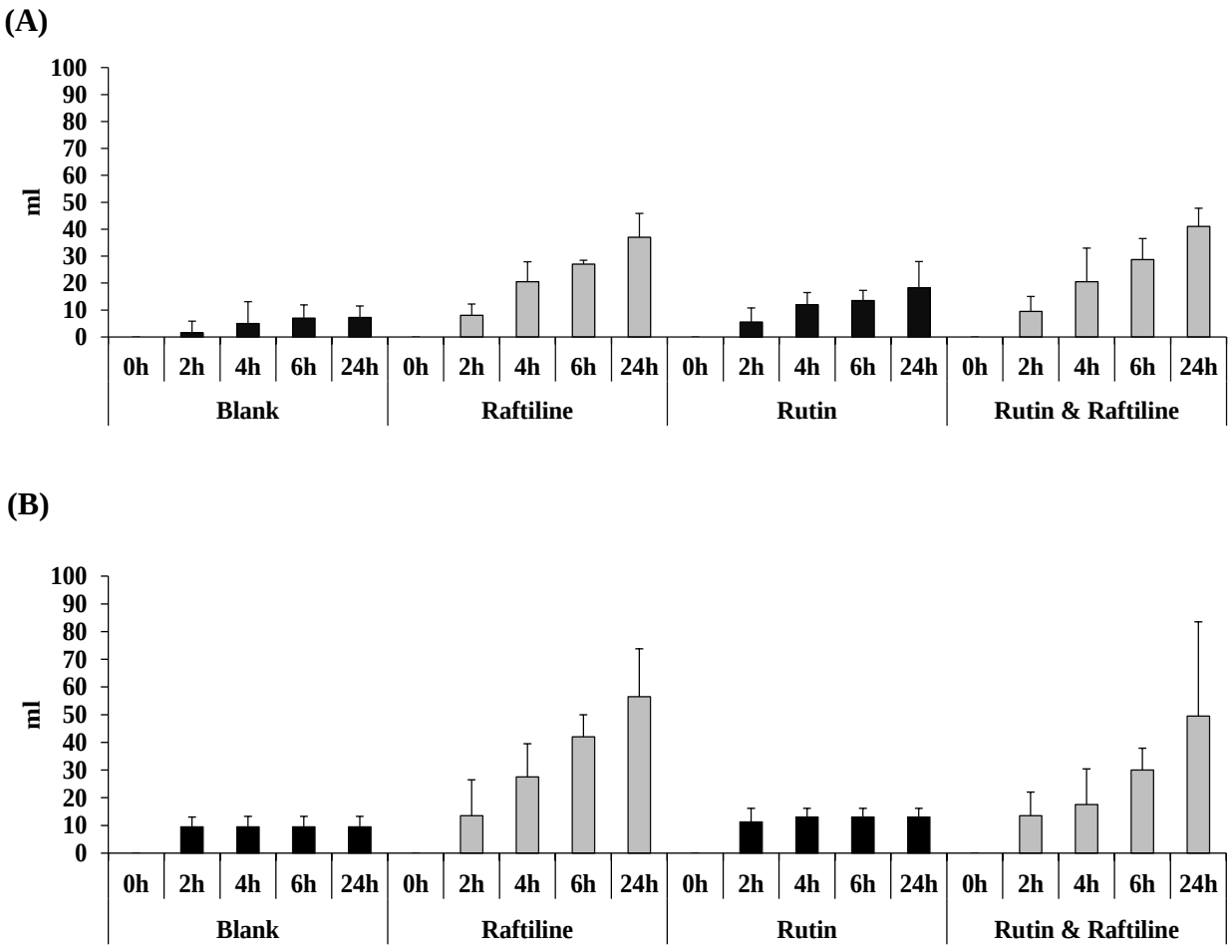
Figure 4-13: Change in pH of fermented faecal fluids over 24 h in A) younger (n=6) and older (n=4) participants. Data presented as median and IQR.



4.6.5.2 Rutin fermentation and gas production

Gas production was higher in older than younger fermented faecal fluids at 2h of rutin fermentation ($p<0.05$), although there was no difference in total gas production between groups after 24 h of fermentation. In both younger and older groups, the combination of rutin with raftiline increased the gas production more than the rutin alone ($p<0.05$). Moreover, the older group produced more gas over the total duration of the fermentation than the younger group when rutin and raftiline together were fermented ($p=0.03$; Figure 4-14).

Figure 4-14: Cumulated gas production from fermented faecal fluids over 24 h of incubation in A) younger (n=6) and older (n=4) participants. Data presented as median and IQR.



4.6.5.3 Metabolism of rutin in faecal fluids and phenolic acids formation

Only seven metabolites were found and identified using GC-MS after the fermentation of rutin with or without raftiline in the fermented faecal fluids of younger and older groups at 0, 6, and 24 hr (as presented previously in Table 3-12, Chapter 3). These seven phenolic acid metabolites were: PAA, 3-OHPAA, 4-OHBA, 3-OHPPA, 4-OHPPA, 3,4diOHPPA, and 3,4diOHPAA.

PAA, 3-OHPPA, and 4-OHPPA were detected in both faeces only fluids and fermented faecal fluids of rutin with or without raftiline. 3,4diOHPAA was detected only in the fermented faecal fluids of rutin with or without raftiline.

4.6.5.3.1 The sum of seven phenolic acid metabolites formed during fermentation of rutin

The sum of the seven phenolic acid metabolites (PAA, 3-OHPAA, 4-OHBA, 3-OHPPA, 4-OHPPA, 3,4diOHPPA, and 3,4diOHPAA) significantly increased over time in the fermented faecal fluids of rutin, and rutin with raftiline, in both groups (rutin: younger ($p<0.01$) and older ($p=0.002$) groups, rutin with raftiline: younger ($p=0.02$) and older ($p=0.03$) groups). The addition of raftiline to the fermentation significantly inhibited the formation of phenolic acids in the younger group only, decreasing it by 7-fold ($p=0.02$; Figure 4-15).

4.6.5.3.2 Individual phenolic acid formed after the fermentation of rutin

PAA was higher in the fermented faecal fluid ("blank" without rutin) in the older group at all time points 0, 6, and 24 h ($p\leq0.05$). PAA increased significantly over time in the fermented faecal fluids with rutin ($p<0.001$) and with the combination of rutin with raftiline ($p\leq0.01$) in both younger and older groups. 3-OHPPA was only significantly increased in the fermented faecal fluids containing rutin in the younger ($p=0.02$) and older ($p=0.01$) groups. In terms of the differences between the groups, the older group formed significantly more PAA in the fermented faecal fluids of rutin ($p=0.008$), and rutin with raftiline ($p=0.003$) compared with the younger group. Also, the formation of 3-OHPPA and 4-OHPPA was higher in the fermented faecal fluids of rutin in the older group ($p<0.05$). There was no difference between groups in the formation of 3-OHPAA and 3,4diHPAA. Lower levels of 4-OHBA and 3,4diOHPPA were detected in both groups (Figure 4-11).

Figure 4-15: The sum of seven phenolic acids (µmol/L) in A) younger (n=6) and B) older (n=4) fermented faecal fluids over 24 h of fermentation. Data presented as median and IQR.

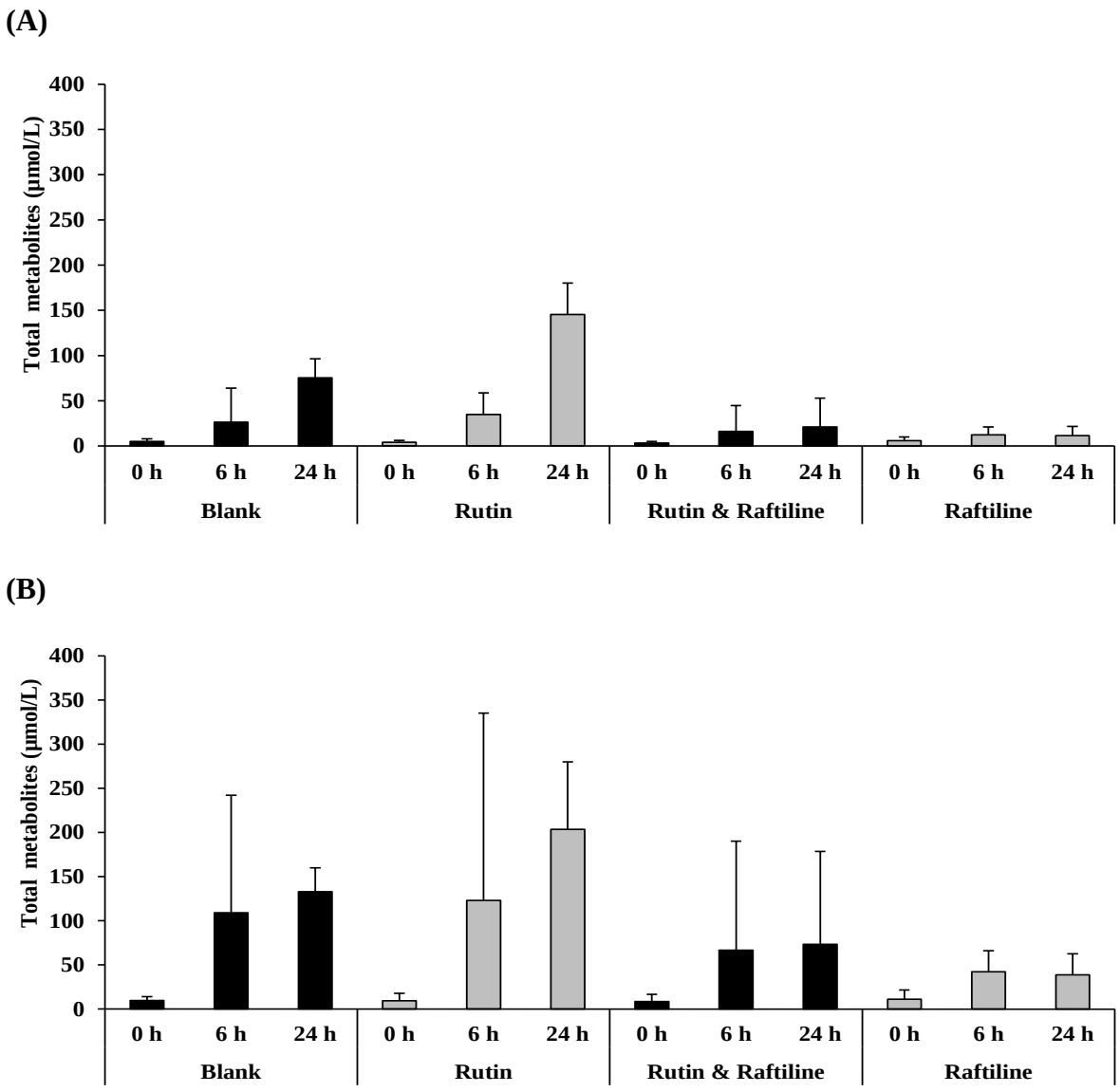


Table 4-11: Accumulation of seven phenolic acids (µmol/L) produced after 0, 6, and 24 h of fermentation in faecal fluids from younger (n=6) and older (n=4) groups.

Metabolite	Group	Substrates	0h		6h		24h	
			Median	IQR	Median	IQR	Median	IQR
PAA	Younger	Blank	3.7	1.0	15.2	22.1	66.1	25.3
		Raftiline	2.7	0.8	8.6	4.5	6.6	6.2
		Rutin	3.3	1.6	6.0	7.8	81.9	51.1
		Rutin+raftiline	4.3	2.3	8.3	1.5	8.9	3.6
	Older	Blank	6.9	1.5	92.8	99.5	88.8	19.9
		Raftiline	7.0	5.1	22.2	26.3	26.6	26.2
		Rutin	6.7	4.6	58.7	98.2	128.3	53.9
		Rutin+raftiline	6.4	4.8	17.5	32.8	25.3	21.9
3-OHPAA	Younger	Blank	0.2	<0.01	0.2	<0.01	0.5	0.2
		Raftiline	0.3	<0.01	0.2	<0.01	0.1	<0.01
		Rutin	0.2	<0.01	1.5	0.9	4.4	23.3
		Rutin+raftiline	0.1	<0.01	1.3	<0.01	1.3	0.8
	Older	Blank	nd	nd	0.1	<0.01	0.1	<0.01
		Raftiline	nd	nd	nd	nd	nd	nd
		Rutin	0.1	<0.01	0.8	0.6	4.2	26.6
		Rutin+raftiline	nd	nd	0.3	0.9	0.6	0.5
4-OHBA	Younger	Blank	nd	nd	nd	nd	nd	nd
		Raftiline	nd	nd	0.1	<0.01	0.1	<0.01
		Rutin	0.1	<0.01	0.1	<0.01	0.1	<0.01
		Rutin+raftiline	nd	nd	0.1	<0.01	0.1	<0.01
	Older	Blank	0.2	<0.01	0.4	11.9	0.3	13.6
		Raftiline	0.2	<0.01	0.2	<0.01	0.1	<0.01
		Rutin	0.3	0.2	0.2	<0.01	0.2	<0.01
		Rutin+raftiline	0.2	<0.01	0.3	<0.01	0.4	0.2

*Continued overleaf

Metabolite	Group	Substrates	0h		6h		24h	
			Median	IQR	Median	IQR	Median	IQR
3-OHPPA	Younger	Blank	1.6	0.8	2.8	1.3	2.9	1.6
		Raftiline	0.5	1.3	0.9	0.5	1.3	1.4
		Rutin	0.4	0.8	1.8	0.8	4.8	8.0
		Rutin+raftiline	0.4	0.9	1.6	4.5	1.2	2.6
	Older	Blank	0.9	1.1	4.2	7.6	3.4	2.7
		Raftiline	1.0	2.3	2.8	4.0	3.4	1.4
		Rutin	0.5	0.8	7.7	23.5	13.4	8.4
		Rutin+raftiline	1.3	1.8	9.5	20.8	9.4	15.8
4-OHPPA	Younger	Blank	0.4	0.3	6.5	6.7	1.2	2.6
		Raftiline	0.6	0.4	3.8	1.2	3.9	1.0
		Rutin	0.5	0.3	1.1	0.8	4.9	6.1
		Rutin+raftiline	0.5	<0.01	1.0	1.3	1.2	1.0
	Older	Blank	0.4	0.6	3.4	12.9	21.6	27.2
		Raftiline	0.3	0.7	1.4	0.6	2.0	0.8
		Rutin	0.8	1.0	1.6	0.8	6.9	9.2
		Rutin+raftiline	0.3	0.7	0.4	0.3	1.9	3.2
3,4diOHPAA	Younger	Blank	nd	nd	nd	nd	nd	nd
		Raftiline	nd	nd	nd	nd	nd	nd
		Rutin	nd	nd	16.1	27.2	32.9	56.8
		Rutin+raftiline	0.2	<0.01	5.0	30.4	7.1	30.0
	Older	Blank	nd	nd	nd	nd	nd	nd
		Raftiline	nd	nd	nd	nd	3.2	<0.01
		Rutin	0.3	<0.01	63.0	34.3	65.8	43.7
		Rutin+raftiline	0.5	<0.01	48.3	23.9	52.0	25.9

*Continued overleaf

Metabolite	Group	Substrates	0h		6h		24h	
			Median	IQR	Median	IQR	Median	IQR
3,4diOHPPA	Younger	Blank	nd	nd	nd	nd	nd	nd
		Raftiline	nd	nd	nd	nd	nd	nd
		Rutin	nd	nd	0.2	<0.01	0.4	0.1
		Rutin+raftiline	nd	nd	nd	nd	nd	nd
	Older	Blank	nd	nd	nd	nd	nd	nd
		Raftiline	nd	nd	nd	nd	nd	nd
		Rutin	0.2	<0.01	1.1	1.0	0.5	<0.01
		Rutin+raftiline	nd	nd	nd	nd	0.6	<0.01

Not detected (nd)

4.6.5.4 Metabolism of rutin in faecal fluids and SCFA production

Ten SCFA were identified and quantified in the fermented faecal fluids after the fermentation of rutin, in the presence or absence of raftiline, in younger and older groups at 0, 2, 4, 6 and 24 hr. They are the same as those measured directly in the faecal samples, (details of the method described in Chapter 2, page 49). Isocaproic, caproic, enanthic, and caprylic acid were either detected in limited amounts or not detected at all.

SCFA concentration increased significantly over time in the fermented faecal fluids of rutin with or without raftiline in the younger and older groups ($p<0.05$). The total SCFA level was not different between groups (Figure 4-16). In the presence of raftiline, significantly higher levels of SCFA were detected in the fluids compared to rutin alone ($p=0.03$ for the younger group; $p<0.05$ for the older group).

Acetic, propionic, and butyric acid significantly increased over time in the fermented faecal fluids of rutin and the combination of rutin with raftiline in both groups ($p<0.05$; Table 4-12). There was no difference in the levels of specific SCFA between groups.

Figure 4-16: SCFA production (µmoles /L) in A) younger (n=6) and B) older (n=4) fermented faecal fluids over 24 h of fermentation. Data presented as median and IQR.

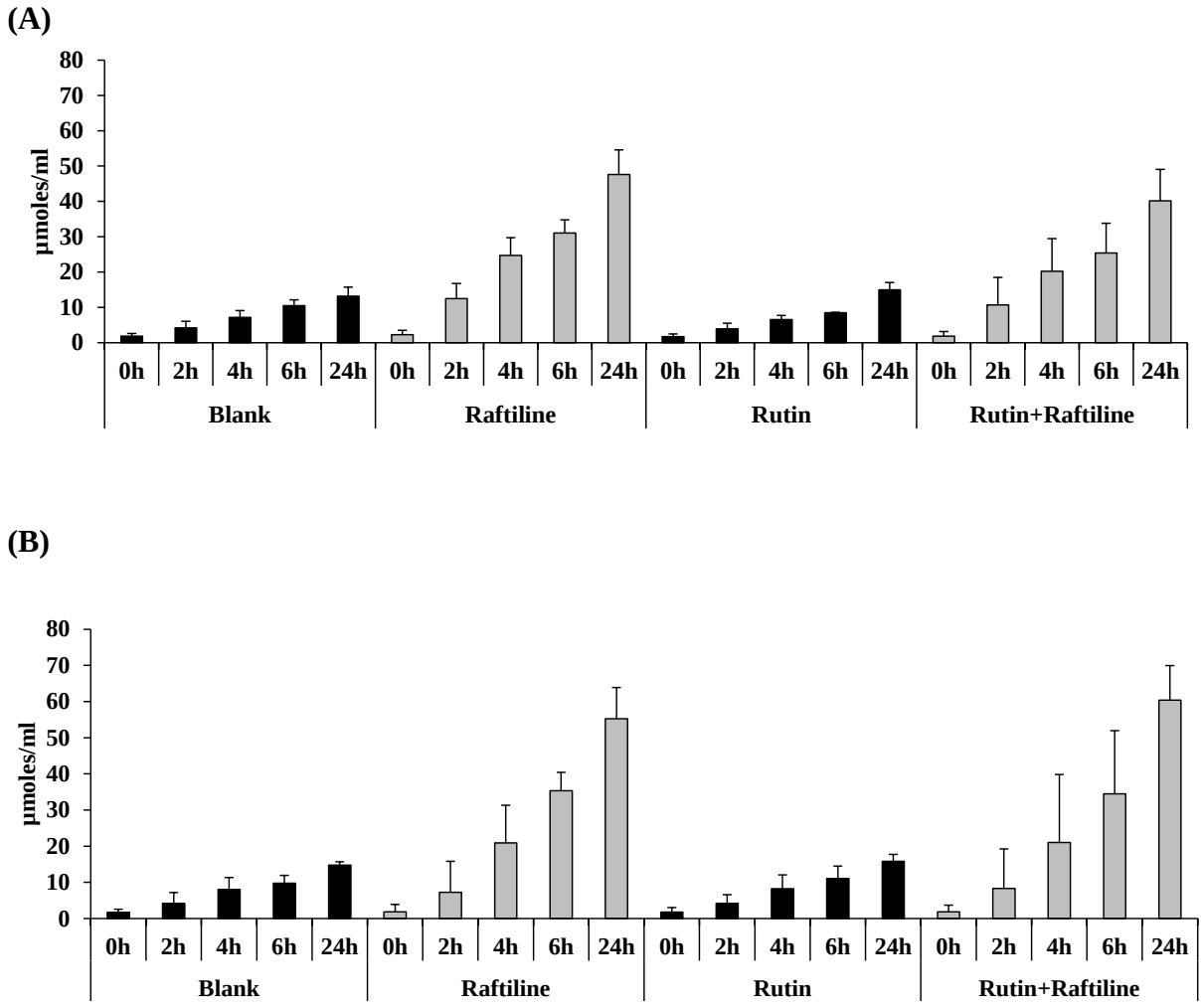


Table 4-12: SCFA concentration (µmole/ml) after 0,2,4,6 and 24 h. of fermentation from younger (n=6) and older (n=4) participants.

SCFA	Group	Substrates	0h		2h		4h		6h		24h	
			Median	IQR	Median	IQR	Median	IQR	Median	IQR	Median	IQR
C2	Younger	Blank	1.2	0.4	2.2	0.7	4.1	0.7	6.0	1.3	7.8	2.4
		Raftiline	1.5	0.4	8.4	2.6	18.1	5.0	23.1	4.1	30.7	7.2
		Rutin	1.0	0.4	2.3	0.6	3.7	0.5	5.2	0.8	9.3	2.0
		Rutin+raftiline	1.2	0.9	6.8	5.0	14.4	5.6	17.5	8.1	32.1	14.4
	Older	Blank	1.2	0.4	2.8	1.6	4.9	1.3	5.8	0.7	7.8	0.3
		Raftiline	1.3	1.3	5.6	5.8	17.7	5.6	28.1	2.4	35.8	11.6
		Rutin	1.3	0.7	2.9	1.2	5.3	1.7	6.9	0.9	8.3	1.9
		Rutin+raftiline	1.4	1.0	6.3	7.7	14.5	11.3	24.4	6.1	39.2	14.6
C3	Younger	Blank	0.4	0.1	1.1	0.4	1.7	0.7	2.2	0.7	1.8	0.6
		Raftiline	0.4	0.2	2.4	1.6	4.2	0.9	5.3	2.1	5.7	4.8
		Rutin	0.3	0.2	1.2	0.8	1.7	1.0	2.0	1.0	2.4	1.0
		Rutin+raftiline	0.4	0.3	2.5	2.1	4.0	3.0	5.3	2.3	6.8	3.5
	Older	Blank	0.3	0.1	0.6	0.3	1.1	0.1	1.6	0.2	2.2	0.7
		Raftiline	0.2	0.2	0.6	0.8	1.5	1.2	3.1	1.2	5.2	1.9
		Rutin	0.2	0.1	0.5	0.3	1.1	0.2	1.5	0.1	2.4	0.8
		Rutin+raftiline	0.3	0.1	0.6	0.9	1.5	0.8	2.8	1.0	5.6	3.5
iC4	Younger	Blank	0.1	<0.01	0.1	<0.01	0.1	<0.01	0.1	0.1	0.3	0.5
		Raftiline	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
		Rutin	0.1	<0.01	0.2	<0.01	0.1	<0.01	0.1	<0.01	0.6	0.3
		Rutin+raftiline	nd	nd	0.2	<0.01	<0.01	<0.01	0.1	<0.01	0.1	<0.01
	Older	Blank	nd	nd	0.1	<0.01	0.1	<0.01	0.2	0.1	0.7	0.2
		Raftiline	<0.01	<0.01	0.1	<0.01	0.1	<0.01	0.1	<0.01	0.1	<0.01
		Rutin	nd	nd	0.1	<0.01	0.1	<0.01	0.2	<0.01	0.5	0.2
		Rutin+raftiline	nd	nd	0.1	<0.01	0.1	<0.01	0.1	<0.01	0.1	<0.01

*Continued overleaf

SCFA	Group	Substrates	0h		2h		4h		6h		24h	
			Median	IQR	Median	IQR	Median	IQR	Median	IQR	Median	IQR
C4	Younger	Blank	0.3	0.1	0.5	0.3	0.9	0.5	1.3	0.9	1.2	0.7
		Raftiline	0.2	0.1	1.0	0.4	1.8	1.6	2.5	1.4	6.8	5.3
		Rutin	0.2	0.2	0.5	0.2	0.8	0.3	1.0	0.2	1.5	0.5
		Rutin+raftiline	0.2	0.1	0.8	0.7	1.0	1.7	1.6	1.6	3.9	3.3
	Older	Blank	0.2	0.2	0.8	0.3	1.4	0.4	1.5	0.5	2.2	0.4
		Raftiline	0.4	0.2	1.1	1.9	3.1	3.0	4.9	3.9	5.6	6.9
		Rutin	0.2	0.2	0.8	0.3	1.3	0.4	1.7	0.5	2.4	0.4
		Rutin+raftiline	0.4	0.1	1.6	1.8	4.8	2.6	7.8	4.2	7.7	6.6
iC5	Younger	Blank	0.1	0.4	0.1	0.1	0.2	0.1	0.2	0.2	0.8	0.6
		Raftiline	0.4	5.1	0.1	0.2	0.1	0.2	0.1	0.1	0.1	<0.01
		Rutin	0.5	0.4	0.1	<0.01	0.2	0.1	0.2	0.1	1.3	0.4
		Rutin+raftiline	0.1	0.1	0.1	0.2	0.1	0.1	0.1	0.4	0.1	<0.01
	Older	Blank	0.1	<0.01	0.2	<0.01	0.3	0.1	0.4	0.1	1.2	0.4
		Raftiline	0.1	<0.01	0.1	<0.01	0.2	0.1	0.2	0.2	0.2	1.3
		Rutin	0.1	<0.01	0.1	<0.01	0.2	0.1	0.4	0.2	1.0	0.4
		Rutin+raftiline	0.1	<0.01	0.1	<0.01	0.2	<0.01	0.2	0.1	0.2	0.7
C5	Younger	Blank	0.1	0.1	0.1	0.1	0.2	0.1	0.4	0.3	0.5	0.4
		Raftiline	<0.01	<0.01	0.1	<0.01	0.2	0.1	0.2	0.1	0.2	0.3
		Rutin	0.1	<0.01	0.1	0.1	0.1	<0.01	0.2	0.1	0.9	0.3
		Rutin+raftiline	<0.01	<0.01	0.1	0.1	0.1	<0.01	0.1	0.1	0.1	0.1
	Older	Blank	nd	nd	0.3	<0.01	0.8	0.1	0.5	0.8	1.1	0.1
		Raftiline	<0.01	<0.01	0.3	0.1	0.5	0.2	0.6	0.2	0.6	0.2
		Rutin	nd	nd	0.2	<0.01	0.7	<0.01	0.9	0.4	1.0	0.1
		Rutin+raftiline	0.1	<0.01	0.3	<0.01	0.5	0.1	0.6	0.3	0.6	0.3

Not detected (nd), acetic acid (C2), propionic acid (C3), isobutyric acid (iC4), butyric acid (C4), isovaleric acid (iC5), valeric acid (C5).

4.7 Discussion

This study was carried out to test the hypothesis that age influences the metabolism of dietary polyphenols, which may be relevant for gut health and the development of chronic diseases. To our knowledge, this is the first dietary semi-controlled study investigating the colonic metabolism of dietary polyphenols in different age groups using human feeding and *in-vitro* faecal fermentation designs.

The present study showed a difference between younger and older groups in terms of:

1. Urinary excretion of phenolic acid after a low-high polyphenol diet.
2. Phenolic acid formation after the fermentation of rutin with the faecal slurry.

There are potential mechanisms behind the absence of some of the phenolic acids in the urine of some of the participants as well as the low phenolic acid excretion in the urine of the older group.

The first mechanism could involve a decrease in colonic absorption. With ageing, colonic absorption is reduced, the mucosal surface area is diminished, and the activities of intestinal brush border enzymes (e.g., lactase, maltase, and sucrase-isomaltase) are decreased (Montgomery *et al.*, 1978, Holt *et al.*, 1989). In the *in-vitro* faecal fermentation of rutin, a high PAA amount was detected in the faecal only fermented fluid (control) at 0h in the older group. This could suggest that PPA was not absorbed and was eliminated in the faecal sample. Moreover, higher formation of phenolic acid, in particular PAA, 3-OHPPA, and 4-OHPPA accompanied by higher gas production were observed in the older group. This suggests two possibilities: first, the ability of faecal materials from older participants to metabolise rutin and the low urinary phenolic acid excretion is due to the lack of absorption of the phenolic compounds in the colon. Second, high phenolic acid elimination in faecal samples of the older group could contribute to the higher detection of phenolic acid in the fermented faecal fluid. This also could indicate a lack of absorption by the older group.

Secondly, the effect of ageing on the gut microbiota composition and associated microbial and enzymatic activities may be at play. In the human colon, the occurrence of bacterial enzymes (β-glucosidases, β-glucuronidases, and α-rhamnosidase) enables the hydrolysis of rutin to release the quercetin aglycone (Aura et al., 2002). Insufficient or lower levels of bacterial enzymes in the colon could be one of the reasons for the absence and low urinary phenolic acid in the older group. Although these bacterial enzymes were not being measured directly in this present study, there were no differences between groups in the quantity of *Bifidbacterium*, *Bacteroides*, or *Flavonifractor plautii* bacteria. However, as they were the only strains measured in the faecal samples, they may not be representative of all polyphenol metabolism bacteria found in the colon such as *Enterococcus casseliflavus* (Schneider et al., 1999, Schneider and Blaut, 2000) *Butyrivibrio spp* (Krishnam.Hg *et al.*, 1970), and *Bacteroides distasonis* (Bokkenheuser et al., 1987).

The differences in colonic fermentation between groups could also affect the colonic metabolism of dietary polyphenols and lead to differences in urinary phenolic acid excretion. Woodmansey *et al.* (2007) reported that a high faecal pH in the elderly (due to a low fibre intake) may lead to a reduction in SCFA production. In the present study, the faecal pH was close to 8 in the older group after the low-polyphenol diet and above 7 after the high-polyphenol diet; also, the SCFA was higher in the older group after the high-polyphenol diet. pH both above 7.0 could be due to other compounds in the colon known to increase faecal pH such as ammonia (alkaline).Woodmansey *et al.* (2004) found that faecal ammonia was significantly ($P<0.05$) higher in older individuals compared to younger. Higher faecal ammonia could in turn be due to the reduction in fibre intake and increase in proteolytic bacterial species, such as fusobacteria, propionibacteria, and clostridia, which is associated with ageing. Fusobacteria bacteria ferment amino acids, resulting in the production of ammonia and indoles.

Moreover, a higher intake of potato fibre in the older group might be linked to the high production of the SCFA. This finding is supported by Cuervo *et al.* (2013) who looked at the association between the regular intake of potatoes (cellulose, hemicellulose, and pectin) and the production of the SCFA using GC-FID. The study linked regular intake of potatoes with a high concentration of SCFA in elderly people (Cuervo *et al.*, 2013).

On the other hand, older people excreted a higher amount of hippuric acid in their urine, which has not been previously reported. The high amount of hippuric acid could be a result

of polyphenol-rich food consumption as well as of the metabolism of quinic acid, aromatic amino acid tryptophan, tyrosine, and phenylalanine (Self *et al.*, 1960, Grumer, 1961). Moreover, urinary hippuric acid has been shown to correlate with hypertension (Holmes, 2008). In the present study, the older group's blood pressure was collectively higher than the younger group. There was a very high intra-group variability within the older group for the sum of the phenolic acid including the hippuric acid (30-fold change between low and high excreters); however, the variability reduced to 3-fold when hippuric acid was not included in the calculation. Given that the diet was controlled this suggests that the high excretion of hippuric acid could come from a source other than polyphenol rich food metabolism, such as amino acids. In addition, the range of ages in the older group (between 51 and 76 years old) could be contributing to the high intra-group variability. More studies are needed to investigate if the hippuric acid should be excluded when looking at the colonic metabolism of polyphenols in older people. In contrast, the variability increased in the younger group from 2.5-fold to 6.5-fold when hippuric acid was not included in the calculation.

In accordance with the urinary phenolic acid measured by GC-MS, the urinary total phenols measured with the Folin-Ciocalteau assay and urinary antioxidant activity measured by FRAP were different between groups. Surprisingly, the older group had a higher total phenol level following the low-polyphenol diet. This could be related to other interfering substances in the urine such as aromatic amines, sulphur dioxide, ascorbic acid, organic acids, Fe (II), and non-phenolic substances (Roura *et al.*, 2006). In addition, the low urinary antioxidant activity (Δ low-high diet) in the older group could be due to the low amount and diversity of urinary phenolic acid. This is in agreement with Olthof *et al.* (2003) who stated that a higher amount of hippuric acid in urine has no effect on the urinary antioxidant activity (FRAP), due to the lack of hydroxyl moiety on the hippuric molecule.

The strengths of this study included the following 1) the study's dietary intervention was well regulated, and all participants received the same high polyphenol foods so that any variation in food brand and quantity could be kept to a minimum, and 2) the faecal fermentation of *in-vitro* rutin provided an assessment of the fermentation ability of each group. On the other hand this study has some limitations including 1) the phenolic acid was not measured in faecal samples; otherwise, it could have provided useful information regarding the absorption and accumulation of the phenolic compounds in the colon, and 2)

few bacteria were measured in faecal samples; however, looking at other gut bacteria that could contribute to the colonic metabolism of dietary polyphenols is important such as, *Clostridium scindens*, *Eubacterium desmolans*, *Eubacterium ramulus* (Schneider *et al.*, 1999, Simmering *et al.*, 1999, Braune *et al.*, 2001, Schoefer *et al.*, 2003), *Butyrivibrio sp* (Krishnam.Hg *et al.*, 1970), and *Bacteroides distasonis* (Bokkenheuser *et al.*, 1987).

In conclusion, the observed differences between groups in terms of urinary and faecal measurements could be due to the effect of ageing on colonic function and gut microbiota composition. Further research is needed to focus on the effect of ageing on the colonic absorption of the phenolic acid and SCFA as well as the effect of gut physiology parameters on the fermentation and colonic metabolism of dietary polyphenols. This is very important to decipher as it may provide some indication on how to enhance the colonic metabolism and absorption of phenolic compounds to gain maximum benefit from them. In the future such knowledge could improve gut health and reduce the risk of diseases associated with ageing such as CRC.

Chapter Five

Colonic Metabolism of Dietary Polyphenols in the Context of Colorectal Cancer Risk

5.1 Introduction

Colorectal cancer (CRC) is one of the primary causes of cancer-related mortality and morbidity all over the world (Kamangar *et al.,* 2006). Furthermore, it is the third most common cancer in men after lung and prostate cancer and after lung and breast cancer in women (Ferlay *et al.,* 2012). In Scotland, the incidence rate of CRC is higher compared to England (49.7 per 100,000 versus 44.1 per 100,000, respectively) (CRUK, 2010). The aetiology of CRC is still unclear; however, age is a primary risk factor (CRUK, 2010). The majority (73%) of people diagnosed with CRC are over 65 years old (CRUK, 2010). In addition, a family history of CRC and environmental factors such as diet, obesity, alcohol consumption, smoking, and low physical activity levels accompanied with abdominal fatness are strong contributors to an increased risk of CRC (Wiseman, 2008, Center *et al.*, 2009).

In screening for colorectal cancer, there is evidence that using the faecal occult blood test (FOBT) increases the diagnosis of the number of early stage cancers, which therefore reduces cancer mortality (Hardcastle *et al.*, 1996, Kronborg *et al.*, 2004). The early screening process may reduce the incidence of bowel cancer by removing the cancer precursors and dysplastic polyps (Mandel *et al.*, 2001). In response to this evidence, the Scottish Bowel Screening Programme was introduced in 2007 for men and women aged between 50 to 74 years old (ISD, 2013). Any individuals with a positive FOBT result are referred to the local hospital for assessment for a colonoscopy (ISD, 2013).

Poullis et al. (2004) looked at the associations between the environmental factors that are linked to the risk of CRC such as age, fibre intake, obesity, and physical activity, with the level of calprotectin, a gastrointestinal marker for inflammation, in middle-aged (50-70 years old) CRC patients. There was a strong positive association between faecal calprotectin and increasing age, obesity, low physical activity, and an inverse relationship between faecal calprotectin and vegetable and fibre consumption. Bowel inflammation is a risk factor for a number of gastrointestinal diseases and other malignancies (Poullis *et al.*, 2004).

Nutrition contributes to more than one third of cancer deaths, with dietary factors responsible for 70% to 90% of all CRC cases (Ahmed, 2004, Araujo *et al.*, 2011). A diet that is high in processed, heavily browned red meat, or red meat cooked using high

temperature methods may be associated with an increased risk of CRC (Wang *et al.*, 2012): 1) directly via the carcinogenic compounds (heterocyclic amines, polycyclic aromatic hydrocarbons, and N-nitroso compounds) that form during the cooking or processing methods; or 2) indirectly since a diet high in meat and fat may be poor in other dietary components such as fibre and polyphenols from fruit and vegetables (Pericleous *et al.*, 2013).

There is some evidence from the epidemiological (Bobe *et al.*, 2008, Hoensch *et al.*, 2008, Djuric *et al.*, 2012, Johnson *et al.*, 2013, Wang *et al.*, 2013b, Woo and Kim, 2013b), animal (Lodovici *et al.*, 2000, Xiao *et al.*, 2008, Rodriguez-Ramiro *et al.*, 2013), and cell culture (Kim *et al.*, 2005, Park *et al.*, 2005, Ibanez *et al.*, 2012, Valdes *et al.*, 2013) studies that dietary polyphenols, in particular flavonoids, may have a role in protecting against colorectal malignancy. The role of the polyphenols in modulating the risk of CRC is not clear. However, according to the literature it could be due to a number of properties: 1) antioxidant (Kohno *et al.*, 2002, Alia *et al.*, 2006); 2) anti-apoptotic (Reuter *et al.*, 2008), 3) anti-aging (de la Lastra and Villegas, 2005), 4) anti-carcinogenic (Narisawa and Fukaura, 1993, Coates *et al.*, 2007), 5) anti-inflammatory (August *et al.*, 1999, Adams *et al.*, 2006) and 6) anti-cell proliferation (Nomoto *et al.*, 2004, Yi *et al.*, 2005).

The gut microbiota may also play a critical role in maintaining a healthy bowel and lowering the risk of CRC, by metabolizing dietary components such as carbohydrate and dietary polyphenols. In turn, these dietary components can influence gut microbiota activity and composition (Henning *et al.*, 2013). Studies have shown differences in the gut microbiota of patients with or without CRC, but with no clear explanation of how these changes can affect the process of cancer (Moore and Moore, 1995, Wu *et al.*, 2013, Tahara *et al.*, 2014).

The faecal microbiota of different populations (polyp patients, Japanese-Hawaiians, North American Caucasians, rural native Japanese, and rural native Africans) were compared using the culture method technique. The total amount of Bacteroides species, and surprisingly Bifidobacterium species, were associated with an increased risk of colon cancer in polyp patients and Japanese-Hawaiians, while Lactobacillus species and *Eubacterium aerofaciens* showed the closest association with low colon cancer risk in rural native Japanese and rural native Africans (Moore and Moore, 1995). There is limited knowledge about the role of these bacteria in the colonic metabolism of polyphenols.

However, studies have reported that polyphenols can act as prebiotic and anti-microbial components and have a direct effect on the balance of the major groups of gut microbiota contributing to colonic health such as *Bifidobacterium spp., Bacteroidetes*, and *Firmicutes*. The colonic fermentation of polyphenols stimulated proliferation of bifidobacteria and decreased the Firmicute to Bacteroidetes ratio (Lee *et al.*, 2006, Parkar *et al.*, 2008, Tzounis *et al.*, 2008, Parkar *et al.*, 2013). Therefore, the risk of CRC which may impact on gut function and gut microbiota colonisation, should be considered as it might influence the colonic metabolism of non-digestible carbohydrate and non-nutritive compounds such as polyphenols.

Despite all available evidence that links the dietary polyphenols with anti-carcinogenic activity, our understanding of this complex relationship remains incomplete. A number of studies have yet to be performed:

1. Examining dietary polyphenol metabolism in patients diagnosed with colorectal adenoma or carcinoma. Most previous studies have employed *in-vitro* models with purified components from plants, using concentrations often beyond physiological load.

2. Looking at the bioavailability of polyphenols in patients who were diagnosed with colorectal adenoma or carcinoma.

3. Considering the differences in gut microbiota between healthy individuals and those who are at risk of CRC.

In Chapter Four, the metabolism of polyphenols in healthy older adults after a low or a high-polyphenolic diet was examined. We saw differences between younger and older groups in the metabolism of dietary polyphenols. This next study aims to test whether patients aged 50 -75 years old who have had histological colorectal adenomas (at risk of CRC) removed, have different colonic metabolism of dietary polyphenols, especially flavonols known to reach the colon, compared with those healthy adults aged ≥ 50 years from the previous study (Chapter 4). Since the gut microbiota composition is shown to change with a developing risk of CRC (Moore and Moore, 1995), we hypothesized that the colonic metabolism of polyphenols would differ between polypectomy patients and healthy older adults.

To test this hypothesis, two older age groups were studied, and two study designs were used:

1. A human feeding study (*in-vivo*) to test the hypothesis that people who are at risk of CRC (over 50 years old and have a history of adenomas removed) will be less able to metabolise dietary polyphenols than healthy older adults, due to changes in colonic microbiota composition.

2. *In-vitro* fermentations, using faecal samples collected during the dietary intervention, to study the metabolic capacity of the samples when specific flavonols are provided.

5.2 Subjects and study design

5.2.1 Subjects and recruitment

Potential participants were recruited from the Scottish Bowel Screening Programme at the endoscopy clinics at three sites (Glasgow Royal Infirmary, Stobhill Hospital, and Gartnavel General Hospital), prior to colonoscopy. Initial consent was taken for contact to be established within four weeks after the colonoscopy, when the study protocol and consent forms were presented. They were compared to healthy control adults aged between 51-76 years old recruited as part of the ageing study (older group; Chapter 4). Exclusion criteria included: consuming alcohol (>4 units/day), obesity (BMI>30kg/m^2), taking supplements, smoking, taking any medication, or having any conditions known to affect bowel function, other than related to the present colonoscopy. Patients who had one or more histologically-confirmed colorectal adenomas (CRA) removed through polypectomy and had no other types of polyps (hyperplasic polyps, FAP, and HNPCC) were recruited. A full ethical application was prepared for this study and submitted to the University of Glasgow, College of Medical, Veterinary & Life Sciences, and the West of Scotland Research Ethics Committee and the NHS GG&C Research and Development department (approval in Appendix 8).

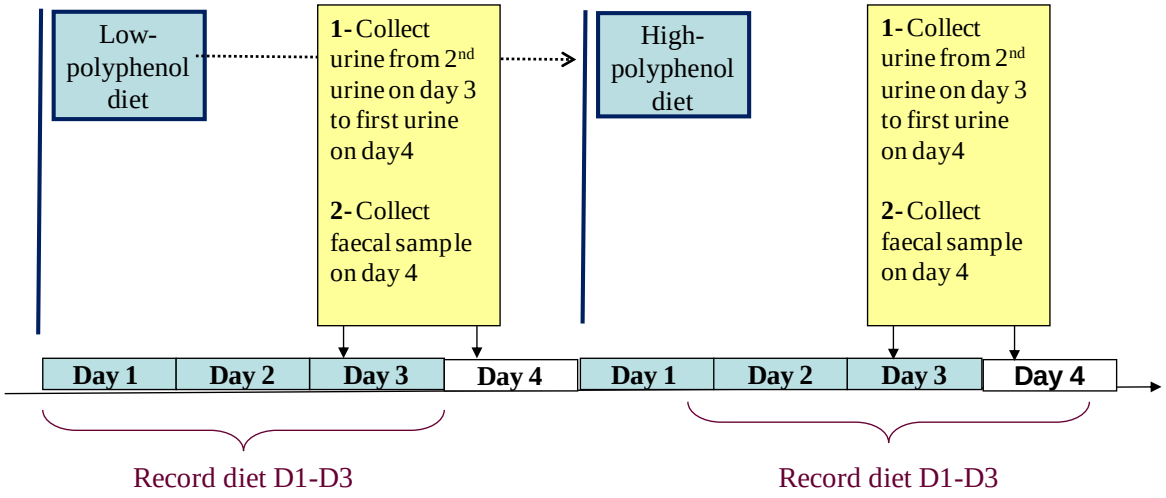
5.2.2 Sample size and power calculation

The primary aim of this pilot study was to characterise the difference in the colonic metabolism of dietary polyphenols in people with colorectal adenomas compared to our previous results in healthy older adults aged over 50 (Chapter 4). Urinary phenolic acid excretion of ten acids (significantly increased after a high-polyphenol diet) in healthy older adults (aged over 50 years) following a low polyphenol diet was significantly lower (64.7 ± 0.9 μmole/day) than those following a high polyphenol diet (100.3 ± 32.3 μmole/day). The mean difference was 35.6 μmole/day with the standard deviation of the differences SD= 35.7. Based on these results it was calculated that a sample size of 13 participants for each group will be sufficient to detect (or not) a difference of 1.3 standard deviation in urinary phenolic acid excretion between the CRC groups and the healthy older group at a power of 80%, $p<0.05$, allowing for a 20% drop-out rate.

5.2.3 Study design and sample collection

All participants were asked to follow a low-polyphenol and a high-polyphenol diet, each lasting three days. Three days for each diet was enough for polyphenol rich foods to be supplied to the colons and fermented over the course of several meals. During the low-polyphenol diet (diet A), participants were asked to avoid all fruit, vegetables, onions, coffee, tea, chocolate, vanilla and similar flavourings, whole meal products, alcohol, spices, and all dietary supplements (vitamins, minerals, and herbal products). During the high-polyphenol diet (diet B), participants were asked to follow a specific diet including polyphenol-rich foods, which were provided along with cooking guidance and recipe sheets. Examples of foods to be included during the low polyphenol diet, and a detailed menu of the high-polyphenol diet are given in Appendix 4 and 5. Urine and faecal samples were collected after the low-polyphenol diet (day 4) and high-polyphenol diet (day 4) for the human feeding study measurements (Figure 5-1). Stool samples collected after the low-polyphenol diet (day 4) were used in *in-vitro* faecal fermentations. Sociodemographic and anthropometric measurements (height, weight, BMI, and waist circumference), and blood pressure were collected (details described in Chapter 2, page 44).

Figure 5-1: Overall study design and samples collection.



5.3 Dietary assessment

A food frequency questionnaire was used to assess the participants' habitual diet over the past year, as described in Chapter 2, page 68.

5.4 Dietary records

Participants kept a 3-day weighed dietary record for the duration of the low and high-polyphenol diets. Diaries were used to estimate the intake of macronutrients, micronutrients, and flavonoids of each participant during the study and to measure the participants' compliance by reviewing the food types and portion size according to the provided instruction (details in Chapter 2, page 67).

5.5 Outcome measures for the human feeding and *in-vitro* faecal fermentation studies

Phenolic acids (GC-MS), total phenols (Folin–Ciocalteu), and ferric reducing antioxidant power (FRAP) were measured in urine samples. The pH, short chain fatty acids (GC-FID), and bacterial composition (Taman real-time quantitative PCR), and calprotectin (ELISA) were measured in the faecal samples.

Phenolic acids, pH, short chain fatty acids, and gas production were measured in the fermentation supernatants (details in Chapter 2).

5.6 Results

5.6.1 Subjects characteristics

Sixteen polypectomy patients were recruited (51- 75 years old), however, only half of these completed the study. Thirteen healthy control participants aged between 51-76 years old were recruited as part of the ageing study (Chapter 4). Both groups followed 3-day low then 3-day high-polyphenol diets. The baseline data for both groups are presented in Table 5-1. There were no differences between groups for anthropometric measurements, with the exception of waist circumference which was higher in the polypectomy group ($p=0.01$). The group average body mass index and waist circumference were within the normal range. The male to female ratio was similar between groups, with two males and six

females in the polypectomy group and three males and ten females in the healthy older adults.

Table 5-1: Baseline data in healthy control (n=13) and polypectomy (n=8) participants.

	Healthy control group (n=13)		Polypectomy group (n=8)		p value
	Median	**IRQ**	**Median**	**IRQ**	
Age (years)	61.0	10.0	57.5	4.5	0.6
Height (cm)	161.0	0.1	177.5	26.3	0.1
Weight (kg)	63.0	16.3	80.9	19.9	0.1
BMI (kg/m^2)[a]	25.1	4.6	27.2	4.4	0.5
W.C (cm)[b]	85.0	19.0	100.5	6.0	0.01
Systolic BP	120.0	20.0	126.0	5.5	0.9
Diastolic BP	86.0	14.0	81.0	7.3	0.6
	n	**%**	**n**	**%**	
Normal weight	10	77	5	62.5	
Overweight	3	23	3	37.5	
Obese	0	0	0	0	

aBMI cut-off points (55-65 years old= 28;Heim et al., 2011).
b WC cut-off points (older women=99, older man=106; Heim et al., 2011).

Bowel movements (self-reported on the questionnaire as twice daily or more, daily, every 2-3 days or less than twice a week) were not significantly different between healthy control and polypectomy groups (Table 5-2).

Table 5-2: Bowel movement in healthy control (n=13) and polypectomy (n=8) participants.

Group	Twice daily or more	Daily	Every 2-3 days	Less than twice a week
Healthy control	25.0%	50%	25%	0%
Polypectomy	37.5	62.5	0%	0%

*Data presented as percentage of frequency

5.6.2 Dietary assessment

5.6.2.1 Estimation of habitual dietary habits

The healthy control group consumed significantly more fruit juices (p=0.02), breakfast cereal (p=0.01), and biscuits, cakes, and sweets (p=0.03) than the polypectomy group (Table 5-3).

Table 5-3: Estimation of dietary habits (serving/week) using the FFQ in healthy control (n=13) and polypectomy (n=8) participants.

Food item	Healthy control group		Polypectomy group		p value
	Median	IQR	Median	IQR	
Hot beverages (green tea, black tea, coffee, hot chocolate, and herbal infusion)	36.0	24.8	22.1	19.8	0.5
Milk drinks	7.0	4.0	5.0	8.8	0.8
Milk substitutes (soya, rice, and oat milk)	0.5	0.3	0.5	0.0	0.2
Fruit juices	7.5	11.0	1.3	0.8	0.02
Soft drink	0.5	0.3	3.0	5.6	0.2
Alcohol	1.3	3.0	3.8	4.7	0.6
Wholemeal products (pasta, rice, and brown rice)	8.0	16.2	3.5	5.8	1.3
Refined products (pasta, rice, and crisps and crackers)	6.5	4.9	12.3	13.8	0.2
Breakfast cereals	6.8	4.8	2.5	3.8	0.01
Fresh fruit	23.5	12.7	24.1	8.8	0.8
Dried fruits	3.0	3.9	0.3	0.9	0.2
Vegetables	10.8	11.6	13.9	7.8	0.7
Potatoes	4.0	2.1	3.4	0.9	0.4
Legumes	2.5	7.6	1.8	1.0	0.1
Onion, tomato, chili, or curry-based products (inc. fresh tomatoes, ketchup, and soup)	9.8	8.5	12.6	8.4	0.3
Meat (beef, chicken, lamb, pork, bacon, and sausages)	7.5	3.7	8.6	3.0	0.3
Seafood (white fish, oil-rich fish, and shellfish)	3.5	2.5	2.4	1.8	0.9
Biscuits, cakes, and sweets	20.0	15.0	8.5	8.6	0.03
Yoghurt	6.0	6.8	4.9	5.1	0.8
Dairy dessert	0.5	2.8	0.3	0.1	0.2
Cheese	6.0	2.8	3.3	0.5	0.2
Eggs	1.0	2.5	3.0	2.8	0.2
Ice cream	0.5	0.8	0.4	2.8	1.0
Fats & oils	14.8	20.5	6.5	12.0	0.3
Seasonings	4.0	3.0	4.5	0.9	0.5
Herbs	4.0	12.5	1.6	2.3	0.1
Spices &Chili	1.0	5.3	1.8	2.9	0.7

5.6.2.2 Macronutrients intake during low and high polyphenol diets

There were no significant differences between groups in terms of energy, fat, protein, carbohydrate, total sugars, starch, alcohol, and dietary fibre consumption after the low and high-polyphenol diets (Table 5-4). When considering the difference in macronutrient intake (Δ low-high diet), it was not significantly different between the groups.

5.6.2.3 Micronutrient intake during low and high polyphenol diets

There was no significant difference between groups for the intake of vitamins or dietary minerals (Table 5-5) with the exception of calcium and iodine intakes during the high-polyphenol diet, with calcium ($p=0.02$) and iodine ($p=0.001$) higher in the healthy control group.

Table 5-4: Energy intake and macronutrients from the dietary records after low and high-polyphenol diets in healthy control (n=13) and polypectomy (n=8) participants.

Group	Low-polyphenol diet					High-polyphenol diet				
	Healthy control group		Polypectomy group		p value	Healthy control group		Polypectomy group		p value
Nutrient	**Median**	**IQR**	**Median**	**IQR**		**Median**	**IQR**	**Median**	**IQR**	
Energy (KJ)	7768	3277	6996	2360	0.6	7531	2474	6461	1840	0.5
kcal (Kc)	1874	785	1905	623	0.6	1794	617	1527	422	0.5
Fat (g)	82.1	56.0	77.8	13.9	0.9	63.8	31.7	55.0	27.6	0.5
Protein (g)	94.3	37.7	90.1	30.8	0.7	47.1	22.3	52.1	26.2	0.7
Carbohydrate (g)	195.4	73.0	185.1	99.8	0.4	217.5	78.6	209.3	65.2	0.4
Total sugars (g)	34.3	42.1	41.0	28.2	0.6	92.9	42.1	84.9	35.1	0.2
Starch (g)	149.5	43.9	108.4	20.8	0.9	60.0	35.7	74.1	31.5	0.9
Alcohol (g)	0.0	0.0	0.0	0.0	0.9	0.0	0.5	0.0	0.0	0.5
Dietary fibre*(g)	11.8	8.0	8.9	3.2	0.3	26.5	9.9	25.9	6.7	0.7

* Non-starch polysaccharide

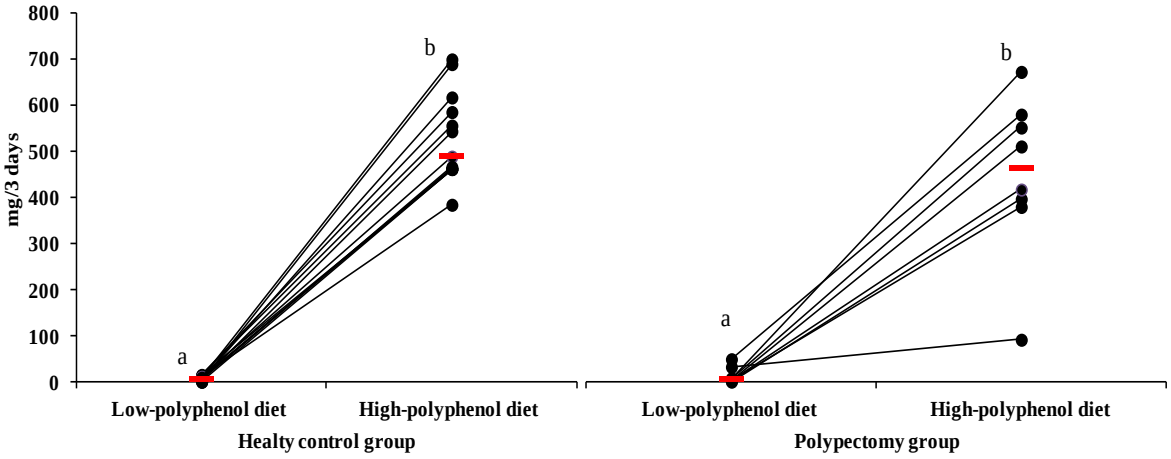
Table 5-5: Micronutrients intake from the dietary records after low and high-polyphenol diets in healthy control (n=13) and polypectomy (n=8) participants.

Diet	Low-polyphenol diet					High-polyphenol diet				
Group	Healthy control		Polypectomy			Healthy control		Polypectomy		
Nutrient	Median	IQR	Median	IQR	p value	Median	IQR	Median	IQR	p value
Vitamin A (µg)	574.0	396.0	460.7	208.1	0.2	383.3	380.3	256.3	219.7	0.2
Thiamine (mg)	1.1	0.5	1.2	0.5	1.0	0.8	0.4	0.5	0.3	0.3
Riboflavin (mg)	1.2	0.9	1.7	0.9	0.6	0.9	0.3	0.5	0.5	0.1
Niacin (mg)	35.9	15.6	34.5	13.8	0.5	16.2	9.5	18.4	12.7	0.8
Vitamin B6 (mg)	1.4	0.9	1.9	0.6	0.3	0.9	0.8	0.7	0.2	0.2
Vitamin B12 (µg)	3.7	9.7	7.9	6.5	0.3	2.0	2.4	0.2	1.8	0.08
Folic acid (µg)	137.0	42.0	163.0	84.9	0.3	125.7	32.3	99.5	42.9	0.2
Pantothenic acid (mg)	5.5	3.9	5.2	2.8	1.0	3.2	1.6	1.9	1.1	0.4
Biotin (µg)	30.9	20.0	24.4	10.9	0.7	22.8	7.1	14.1	11.9	0.3
Vitamin C (mg)	22.0	35.4	25.0	19.3	0.4	75.6	105.0	56.2	14.1	0.3
Vitamin D (µg)	3.4	5.4	2.9	3.4	1.0	0.8	1.0	0.1	0.5	0.1
Vitamin E (mg)	5.4	6.0	3.5	0.8	0.4	7.8	4.0	7.1	2.9	0.3
Calcium (mg)	909.7	551.7	1176.8	559.7	0.9	517.0	223.6	243.0	238.8	0.02
Magnesium (mg)	172.3	132.3	168.5	64.6	0.4	158.3	82.7	143.0	75.6	0.3
Sodium (mg)	2831.3	1983.0	2877.3	523.3	0.7	2343.3	323.3	2207.7	1321.3	0.9
Potassium (mg)	1887.0	1665.3	2160.0	918.0	0.4	2183.0	990.3	1544.3	937.7	0.3
Chlorine (mg)	4640.3	3249.3	3908.8	1375.6	0.6	2540.7	804.4	2087.0	1830.8	0.9
Phosphorus (mg)	1237.0	603.3	1328.0	423.1	0.9	681.7	286.3	466.7	408.8	0.2
Iron (mg)	8.3	2.9	7.5	3.1	0.2	7.3	2.3	4.8	2.7	0.08
Zinc (mg)	7.7	3.3	8.4	3.3	0.8	4.1	2.7	3.2	3.0	0.4
Copper (mg)	0.7	0.2	0.7	0.2	0.4	0.8	0.4	0.9	0.2	0.2
Manganese (mg)	1.8	0.8	1.2	0.4	0.06	2.8	1.5	2.8	1.2	0.8
Selenium (µg)	58.0	53.7	51.7	31.2	1.0	16.3	12.0	18.8	7.3	0.5
Iodine (µg)	126.3	166.0	24.4	10.9	0.002	71.0	46.0	14.1	11.9	0.001

5.6.2.4 Flavonoid intake during low and high polyphenol diets

There was no significant difference in flavonoid intake between groups over either the three days of low or high-polyphenol diets (Figure 5-2). The flavonoid intake during the 3 days long low polyphenol diet was 6.3 mg/day (IQR 8.3) in the healthy control group and 6.8 mg/day (IQR 10.7) in the polypectomy group. The flavonoid intake was 489.7 mg/day (IQR 123.1) in the healthy control group and 465.0 mg/day (IQR 166.2) in the polypectomy group after the 3-day high-polyphenol diet. When considering flavonoid intake on day 3 only (since urine samples were collected from the second urine of day 3, and including the first urine of day 4), there was no difference between groups during the low-polyphenol diet (9.0 mg (IQR 3.6) versus 4.8 mg (IQR 8.7) for healthy control and polypectomy groups, respectively) or the high-polyphenol diet (496.8 mg (IQR 102.2) versus 475.4 mg (IQR 130.5) for healthy control and polypectomy groups, respectively).

Figure 5-2: Median flavonoid intake per day over 3 days after low and-high polyphenol diets in healthy control (n=13) and polypectomy (n=8) participants.



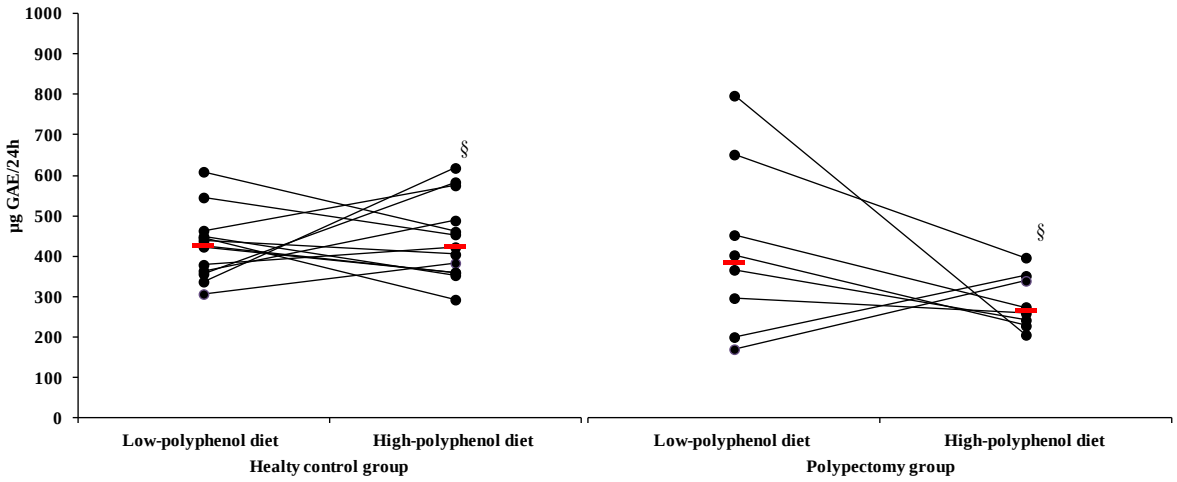
Each circle indicates the estimated average daily flavonoid intake for each participant after low and high-polyphenol diets. Median flavonoid intake for each group is indicated by a red horizontal line.
a,b symbols indicate differences within group (low to high-polyphenol diet).

5.6.3 Measurements performed on urine samples

5.6.3.1 Urinary total phenols using Folin-Ciocalteu assay

Urinary total phenols did not increase in either group after the high polyphenol diet, going from 426.5 µg GAE/24h (IQR 85.2) to 422.8 µg GAE/day 24h (IQR 128.1) in the healthy control group and from 384.8 µg GAE/day24h (IQR 229.7) to 266.9 µg GAE/day 24h (IQR 103.6) in the polypectomy group (Figure 5-3). The difference in urinary total phenols excretion (Δ low-high diet) was not different between groups. However, urinary total phenols were higher in the healthy group urine than the polypectomy group after the high polyphenol diet ($p<0.001$).

Figure 5-3: 24-hour urinary total phenols (µg GAE/24h) after low and high-polyphenol diets in the healthy control (n=13) and polypectomy (n=8) participants.

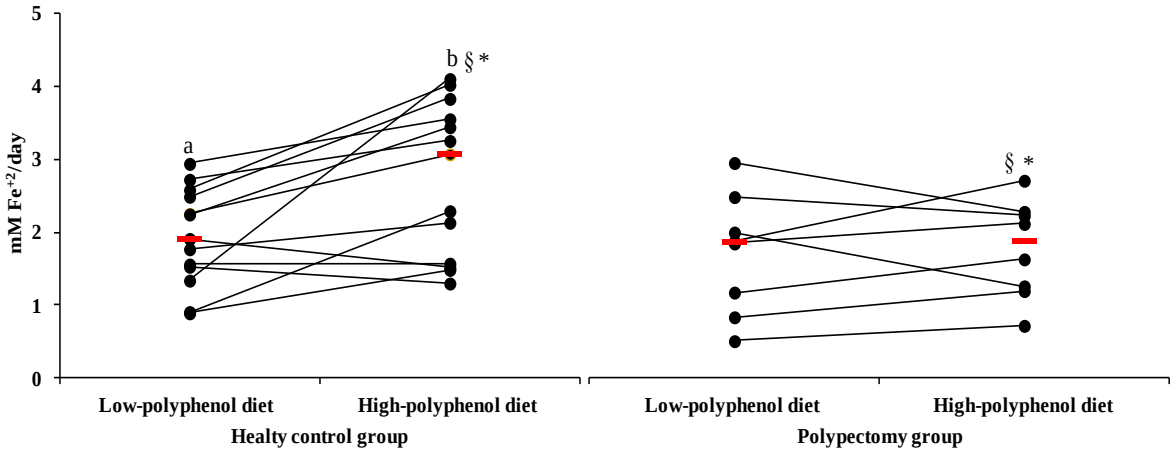


Each circle indicates the measurement of urinary total phenols of each participant after low and high-polyphenol diets. Median urinary total phenols for each group is indicated by a red horizontal line.
§ symbol indicates differences between groups (high vs. high).

5.6.3.2 Total antioxidant activity (FRAP) of urine samples after low and high polyphenol diets

The urinary FRAP value increased following the high-polyphenol diet, from 1.9 mMFe^{+2}/day (IQR 0.1) to 3.1 mMFe^{+2}/day (IQR 2.0; p=0.005) in the healthy control group, but not in the polypectomy group (Figure 5-4). The difference (Δ low-high diet) in FRAP value was higher (p=0.02) in the healthy control group (0.7 mMFe^{+2}/day; IQR 0.7) than the polypectomy group (0.2 mMFe^{+2}/day; IQR 0.7).

Figure 5-4: 24-hour urinary antioxidant activity (mM Fe^{+2}/day) after low and high-polyphenol diets in the healthy (n=13) and CRC (n=8) participants.

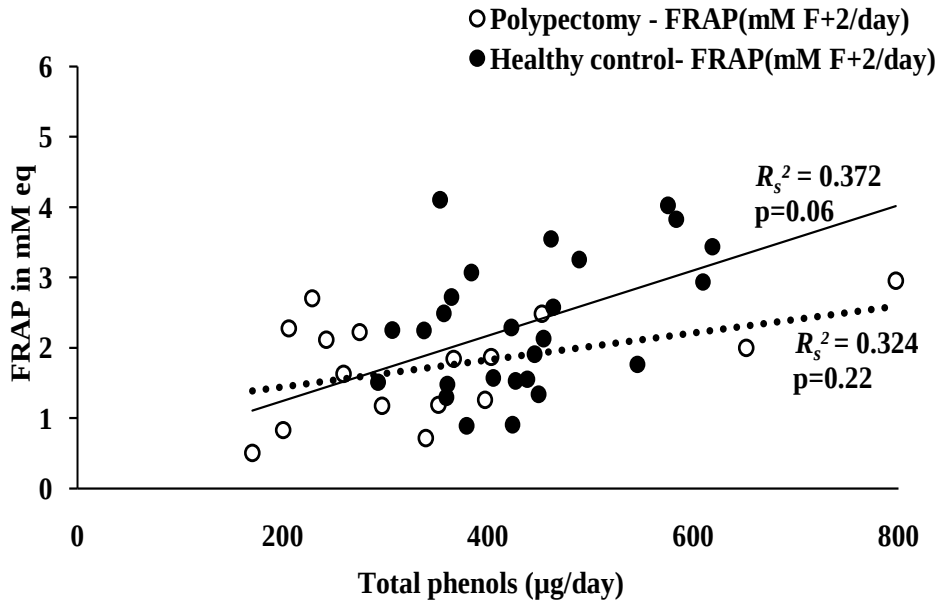


Each circle indicates the measurement of urinary FRAP for each participant. Median urinary FRAP for each group is indicated by a red horizontal line.
a,b symbols indicate differences within group (low to high-polyphenol diet).
§ symbol indicates differences between groups (high vs. high).
*symbol indicates differences in changes (Δ) between groups.

5.6.3.2.1 Correlation between urinary total phenols (measured with Folin-Ciocalteu) and urinary FRAP

The relationship between urinary total phenols (measured with Folin-Ciocalteu) and urinary FRAP was analysed by linear regression (Spearman rank correlation). FRAP and total phenols were not significantly correlated in the healthy group (R_s^2=0.4; p=0.06) or polypectomy group (R_s^2= 0.3; p=0.2) (Figure 5-5).

Figure 5-5: Correlation between urinary FRAP and the urinary total phenols (Folin-Ciocalteu) in healthy (n=13) and polypectomy (n=8) participants.

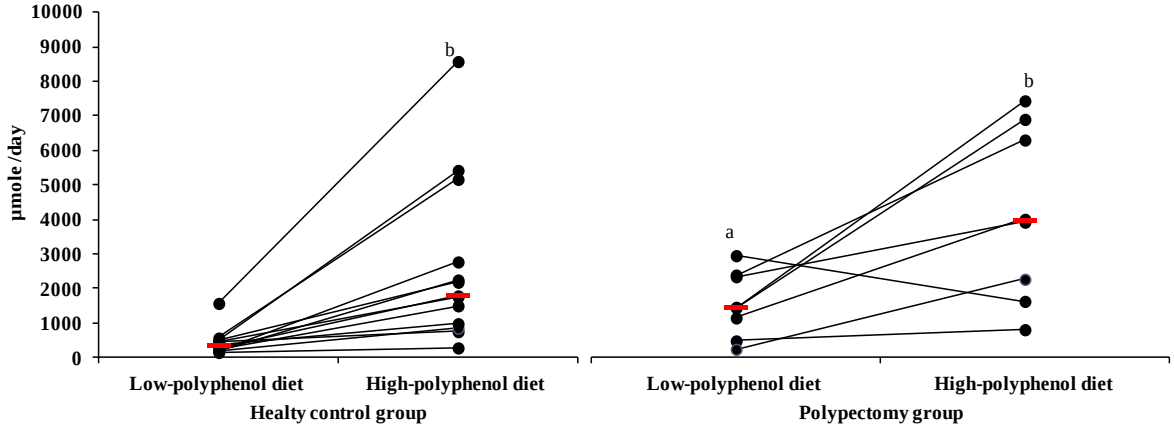


Dotted line indicates polypectomy; solid line indicates healthy control.

5.6.3.3 GC-MS measurement of phenolic acid excretion after low and high-polyphenol diets

The sum of the seventeen urinary phenolic acids significantly increased after the high-polyphenol diet from 349.3 µmole/day (IQR 286.7) to 1789.0µmole/day (IQR 1788.8) in the healthy control group (p=0.0002) and from 1454.4µmole/day (IQR 1373.1) to 3966.5µmole/day (IQR 4342.3) in the polypectomy group (p=0.04). The difference in excretion (Δ low-high diet) was not different between groups (Figure 5-6).

Figure 5-6: 24-hour urinary phenolic acids profile excretion (µmole /day) after low and high-polyphenol diets in healthy control (n=13) and polypectomy (n=8) participants.



Each circle indicates the measurement of urinary phenolic acids profile for each participant after low and high-polyphenol diets. Median urinary phenolic acids profile for each group is indicated by a red horizontal line.
a,b symbols indicate differences within group (low to high-polyphenol diet).

After correction for flavonoid intake, the urinary phenolic acid concentration remained similar after high polyphenol diet between the healthy control group (2860.3µmole/mg of flavonoids (IQR 2779.6) and the polypectomy group (6666.2 µmole/mg of flavonoids (IQR 5316.9).

There were no differences between groups for the excretion of individual phenolic acid (Δ low-high diet); however, the polypectomy group excreted higher hippuric acid after the low-polyphenol diet compared to health control group (Table 5-6).

Table 5-6: Amount of phenolic acid in 24 h urine (µmole/day) after low and high-polyphenol diets in healthy control (n=13) and polypectomy (n=8) participants.

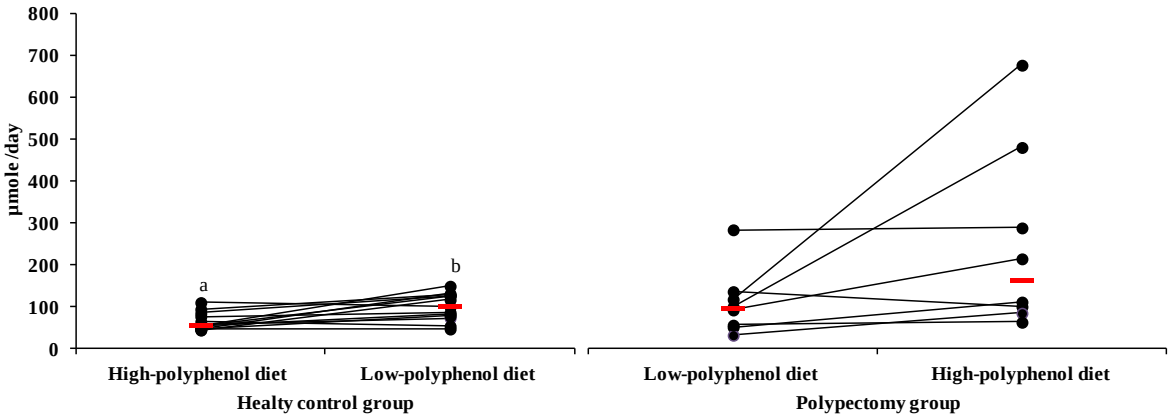
Group	Healthy control							Polypectomy							
Diet	Low-polyphenol diet			High- polyphenol diet			Δ	Low-polyphenol diet			High- polyphenol diet			Δ	P
Acid	N	Median	IQR	N	Median	IQR		N	Median	IQR	N	Median	IQR		value
BA	13/13	1.1	1.5	13/13	1.8	4.3	**0.8**	5/8	1.8	3.9	5/8	1.6	5.0	**-0.2**	0.8
PAA	7/13	0.9	0.4	6/13	1.1	0.7	**0.2**	0/8	nd	nd	0/8	nd	nd	**nd**	-
MA	13/13	0.3	0.5	13/13	0.5	0.5	**0.2**	8/8	0.7	0.4	8/8	1.5	1.8	**0.4**	0.1
3-OHBA	8/13	0.4	1.2	9/13	1.2	1.2	**0.8**	2/8	0.6	0.4	3/8	0.2	0.2	**-0.3**	0.2
3-OHPAA	13/13	2.3*	1.4	13/13	30.2*	39.1	**27.8**	8/8	5.0	6.1	8/8	20.3	126.2	**15.2**	0.9
4-OHBA	12/13	2.2	2.1	12/13	2.9	2.1	**0.7**	8/8	3.0	2.2	8/8	2.5	5.1	**-0.5**	0.8
4-OHPAA	13/13	21.2§	20.7	13/13	19.6†	14.4	**-1.6**	8/8	59.9§	53.6	8/8	75.2†	179.6	**15.3**	0.1
4-OHPPA	8/13	0.2	0.6	8/13	0.3	0.5	**0.1**	3/8	0.2	0.1	3/8	0.2	0.5	**0.0**	0.2
VA	9/13	0.9	1.1	12/13	1.4	1.2	**0.5**	2/8	0.5	0.0	5/8	1.1	1.2	**0.6**	0.7
HVA	13/13	7.6*	1.8	4/13	12.4*	5.6	**4.9**	6/8	8.8	16.8	8/8	12.8	8.3	**4.0**	0.3
4-OHMA	13/13	3.7*	0.9	13/13	2.6*	1.0	**-1.1**	8/8	4.5	5.8	8/8	5.9	5.4	**1.5**	0.3
3,4diOHBA	4/13	0.9	0.4	4/13	1.2	0.3	**0.3**	0/8	nd	nd	0/8	nd	nd	**nd**	-
3,4diOHPAA	13/13	1.2*	0.3	13/13	3.2*	3.8	**2.0**	7/8	1.6	1.2	8/8	2.9	9.5	**1.2**	0.6
HA	13/13	301.5*§	277.6	13/13	1734.5*	1704.5	**1433.0**	8/8	1348.7**§	1234.2	8/8	3581.4**	4206.7	**2232.6**	0.8
Dihydrocaffeic acid	6/13	1.3	0.4	9/13	1.5	1.1	**0.2**	1/8	2.4	0.0	3/8	5.5	3.4	**3.1**	-
3,4diOHPPA	8/13	0.3*	0.6	9/13	0.8*	0.4	**0.5**	8/8	nd	nd	8/8	2.7	0.8	**nd**	-
GA	4/13	0.8	0.4	5/13	0.7	0.5	**0.0**	0/8	nd	nd	0/8	nd	nd	**nd**	-
3-OHhippA	13/13	15.1*	10.9	13/13	28.4*	20.0	**13.3**	4/8	33.7	34.5	4/8	44.9	22.7	**11.2**	0.3

N number of participants (present/total).
Δ Difference in urinary excretion (high diet minus low diet).
P value is the difference in urinary phenolic acid excretion (Δ low-high diet) between groups.
* Significant increase in healthy control group after the high-polyphenol diet $p \leq 0.01$
** Significant increase in polypectomy group after the high-polyphenol diet $p \leq 0.01$
§ Significant difference between groups after the low-polyphenol diet $p \leq 0.05$
† Significant difference between groups after the high-polyphenol diet $p \leq 0.05$
Nd not detected

Hippuric acid (HA) was always the most abundant acid in urine samples in both groups (97.9% and 97.3% of the total of all phenolic acids for healthy control and polypectomy groups, respectively).

The sum of the phenolic acids minus hippuric acid was considered as it is most likely to be formed in the liver by conjugation of benzoic acid and glycine. Dietary sources of benzoic acid and precursors (quinic acid, aromatic amino acid tryptophan, tyrosine, and phenylalanine) should be considred (Self *et al.*, 1960, Grumer, 1961). An increase was seen after the high-polyphenol diet only in the healthy control group, from 55.5 µmol/day (IQR 29.3) to 100.4 µmol/day (IQR 48.5; p=0.007). However, no significant increase was found in the polypectomy group when hippuric acid was not considered (from 96.9µmol/day; IQR 67.4to 163.5 µmol/day; IQR 240.0). The difference in urinary excretion (Δ low-high diet) was not different between groups (Figure 5-7).

Figure 5-7: 24-hour urinary phenolic acids profile excretion without hippuric acid (µmole/day) after low and high-polyphenol diets in healthy (n=13) and polypectomy (n=8) participants.

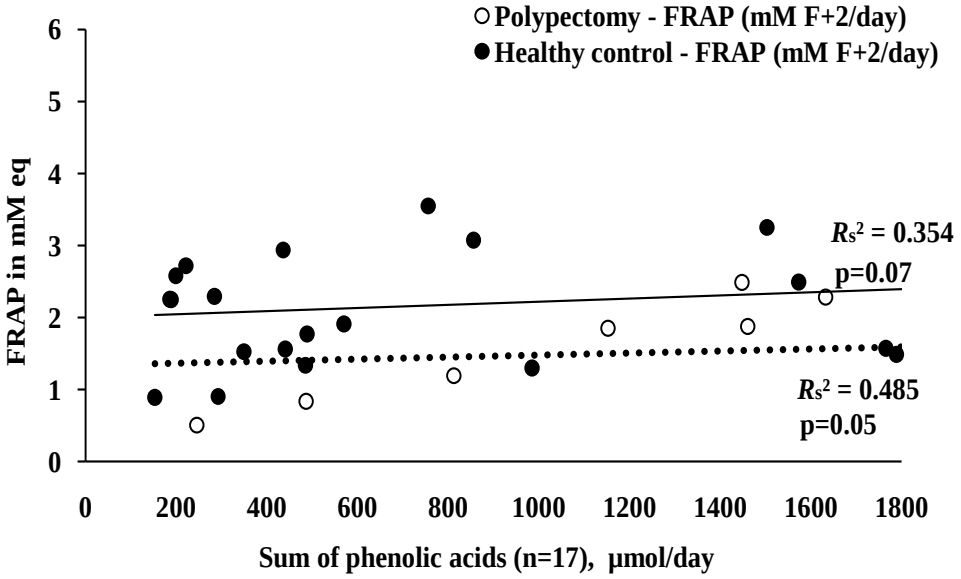


Each circle indicates the measurement of urinary phenolic acids profile without hippuric acid for each participant after low and high-polyphenol diets. Median urinary phenolic acids profile without hippuric acid for each group is indicated by a red horizontal line.
a,b symbols indicate differences within group (low to high-polyphenol diet).

5.6.3.3.1 Correlation between urinary phenolic acid (measured with GC-MS) and urinary FRAP

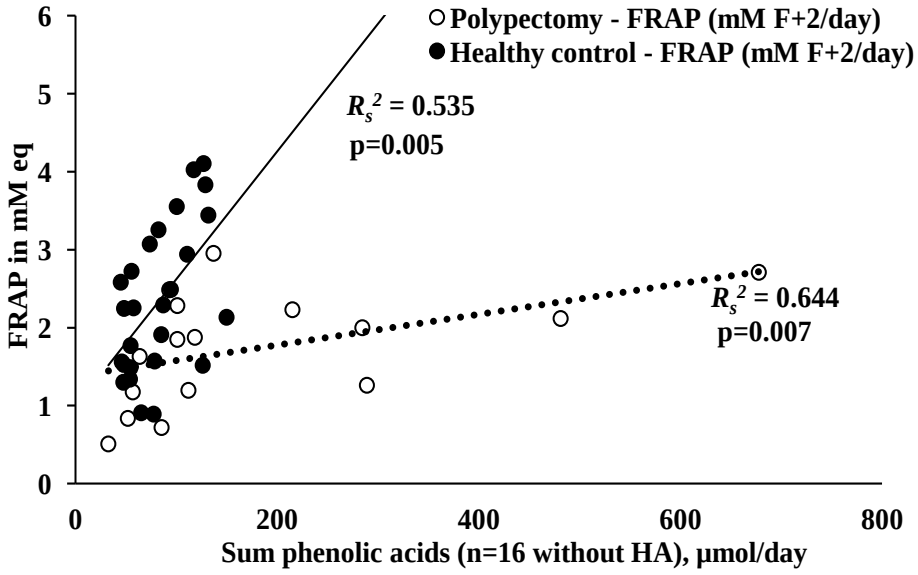
The relationship between urinary phenolic acid (measured with GC-MS) and urinary FRAP was analysed by linear regression (Spearman rank correlation). FRAP and the sum of all urinary phenolic acid (including hippuric acid) were not significantly correlated in the healthy group (R_s^2=0.4; p=0.07) and moderately correlated in the polypectomy group (R_s^2= 0.5; p=0.05) (Figure 5-8). The correlation without hippuric acid gave a much stronger association between FRAP and the sum of phenolic acids (Figure 5-9).

Figure 5-8: Correlation between urinary FRAP and the phenolic acid (GC-MS) in healthy control(n=13) and polypectomy (n=8) participants.



Dotted line indicates polypectomy; solid line indicates healthy control.

Figure 5-9: Correlation between urinary FRAP and the phenolic acid without hippuric acid (GC-MS) in healthy control (n=13) and polypectomy (n=8) participants.



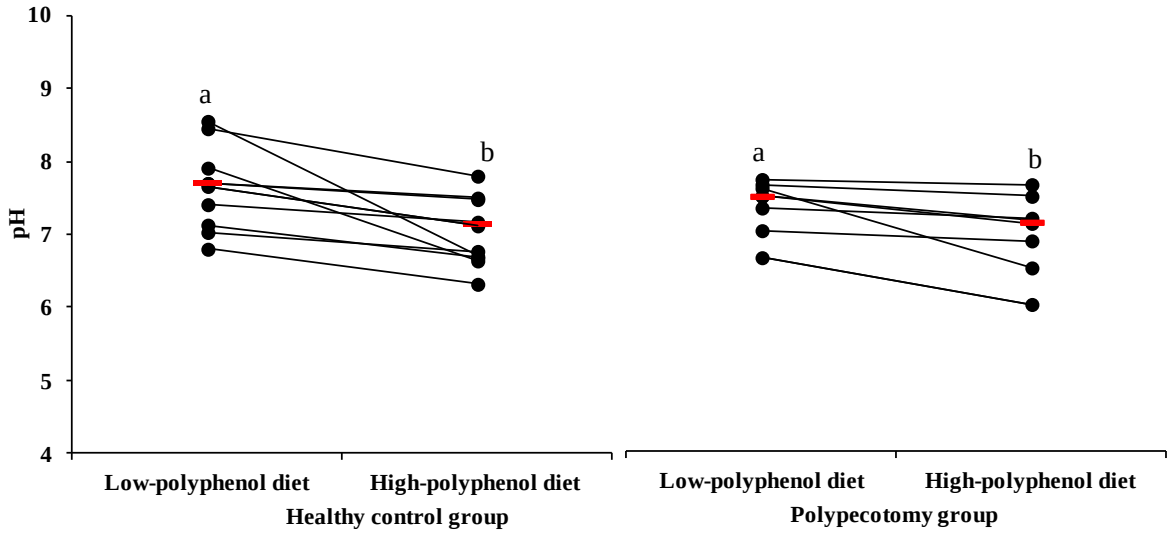
Dotted line indicates polypectomy; solid line indicates healthy control.

5.6.4 Measurements performed in faecal samples

5.6.4.1 Faecal pH after low and high-polyphenol diets

The faecal pH decreased in both groups after the high-polyphenol diet (Figure 5-10), from 7.7 (IQR 0.6) to 6.9 (IQR 0.6) in the healthy control group (p=0.006) and from 7.5 (IQR 0.4) to 7.1 (IQR 0.5) in the polypectomy group (p=0.01).The difference in faecal pH change (Δ low-high diet) was not different between groups.

Figure 5-10: Change in faecal pH after low and high-polyphenol diets in the healthy (n=10) and polypectomy (n=8) participants.

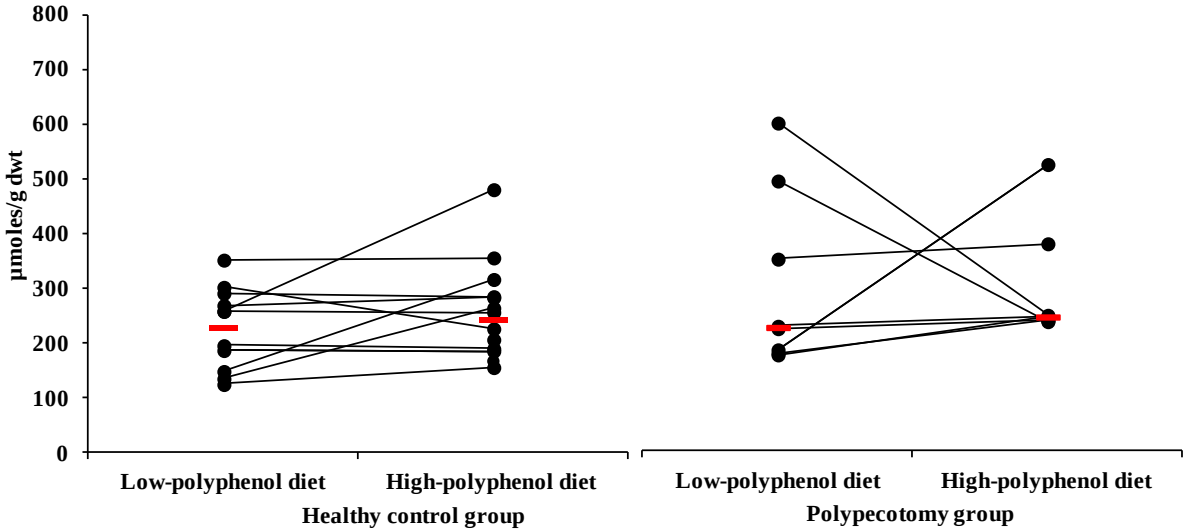


Each circle indicates the measurement of faecal pH for each participant after low and high-polyphenol diets.
Median faecal pH for each group is indicated by a red horizontal line.
a,b symbols indicate differences within group (low to high-polyphenol diet).

5.6.4.2 Faecal SCFA after low and high-polyphenol diets

Faecal SCFA concentration did not significantly increase from low to high-polyphenol diet in either group, going from 258.4 µmoles/g dwt (IQR 112.3) to 264.5 µmoles/g dwt (IQR 92.7) in the healthy control group, and from 226.0 µmoles/g dwt (IQR 202.7) to 245.1 µmoles/g dwt (IQR 41.1) in the polypectomy group. There were no significant differences (Δ low-high diet) in either group (Figure 5-11).

Figure 5-11: Faecal SCFA after low and high-polyphenol diets in the healthy control (n=11) and polypectomy (n=8) participants.



Each circle indicates the measurement of faecal SCFA for each participant after low and high-polyphenol diets. Median faecal SCFA for each group is indicated by a red horizontal line.

There were no differences in the change (Δ low-high diet) for each specific acid between groups (Table 5-7).

Table 5-7: Faecal concentration of SCFA (µmol/g dwt) in the healthy control (n=11) and polypectomy (n=8) groups after low and high-polyphenol diets.

Group	Healthy					Polypectomy					
Diet	Low polyphenol diet		High polyphenol diet		Δ	Low polyphenol diet		High polyphenol diet		Δ	p value
Acid	Median	IQR	Median	IQR		Median	IQR	Median	IQR		
Acetic acid	146.7	41.9	185.0	82.6	**38.3**	138.5	150.5	187.8	19.0	**49.3**	0.5
Propionic acid	34.5	25.2	28.4	10.0	**-6.2**	33.4	21.4	28.4	2.2	**-5.0**	1.0
Isobutyric acid	6.0	3.0	3.2	1.9	**-2.8**	5.4	3.6	3.4	1.7	**-2.0**	0.6
Butyric acid	28.2	29.7	38.4	21.1	**10.2**	37.0	33.2	24.6	23.9	**-12.4**	0.4
Isovaleric acid	9.3	3.8	5.3	2.2	**-3.9**	7.9	5.3	4.5	3.3	**-3.5**	0.9
Valeric acid	5.0	3.4	3.8	1.3	**-1.2**	5.8	4.8	3.7	2.8	**-2.1**	1.0
Isocaproic acid	0.0	0.0	0.0	0.0	**0.0**	0.0	0.0	0.0	0.0	**0.0**	-
Caproic acid	1.1	1.4	2.0	2.6	**0.9**	0.0	0.8	0.0	2.7	**0.0**	0.8
Enanthic acid	0.0	0.0	0.0	0.0	**0.0**	0.0	0.0	0.0	0.0	**0.0**	0.5
Caprylic acid	1.1	1.3	1.0	1.3	**-0.1**	0.3	0.5	0.1	0.7	**-0.2**	0.2
Total SCFA	***258.4***	***112.3***	***264.5***	***92.7***	***6.1***	***226.0***	***202.7***	***245.1***	***41.1***	***19.1***	0.8
Proportional ratio	**Median**	**IQR**	**Median**	**IQR**	Δ	**Median**	**IQR**	**Median**	**IQR**	Δ	
%Acetic acid	62.8	12.1	70.0	5.2	**7.2**	59.9	14.2	72.6	10.5	**12.7**	
%Propionic acid	12.5	3.9	10.6	3.4	**-1.9**	14.8	3.9	11.4	1.2	**-3.4**	
% Isobutyric acid	2.2	0.7	1.1	1.3	**-1.1**	2.4	1.3	1.3	0.6	**-1.2**	
%Butyric acid	10.9	6.6	12.9	5.3	**2.0**	12.8	9.2	10.2	7.4	**-2.7**	
% Isovaleric acid	3.7	1.2	1.9	2.0	**-1.9**	3.4	1.6	1.8	1.3	**-1.7**	
%Valeric acid	2.4	0.9	1.5	0.5	**-0.9**	2.3	1.4	1.5	0.4	**-0.8**	
%Isocaproic acids	0.0	0.0	0.0	0.0	**0.0**	0.0	0.0	0.0	0.0	**0.0**	
%Caproic acid	0.5	0.8	1.1	1.1	**0.6**	0.0	0.4	0.0	1.1	**0.0**	
%Enanthic acid	0.0	0.0	0.0	0.0	**0.0**	0.0	0.0	0.0	0.0	**0.0**	
%Caprylic acid	0.5	0.5	0.3	0.5	**-0.2**	0.1	0.2	0.0	0.2	**0.0**	

5.6.4.3 Faecal calprotectin concentration after low-polyphenol diet

There was no difference in faecal calprotectin levels between the healthy control and the polypectomy groups. All polypectomy patients were within the positive or active symptomatic inflammatory bowel disease range (90.9 – 1118.3 mg/kg wet faeces) except for one patient (29.4 mg/kg wet faeces weight), who was within the normal range. In the healthy control group, the calprotectin concentrations were within the normal range (5-50 mg/kg) in 4 participants, within the positive range (> 50 mg/kg) in 7 participants, and within the active symptomatic inflammatory bowel disease range (200 – 40,000 mg) in one participant (Table 5-8).

Table 5-8: Faecal calprotectin concentrations (mg/kg) in healthy control (n=12) and polypectomy (n=8) groups after low-polyphenol diet.

Group	Median	IQR	Range (mg/kg)
Healthy control	52.9	42.2	26.5-445.9
Polypectomy	111.5	115.3	29.4-1118.3

The relationship between the sum of urinary phenolic acid (measured with GC-MS) and faecal calprotectin was analysed by linear regression (Spearman rank correlation). There was no correlation between faecal calprotectin and the sum of all urinary phenolic acid with or without hippuric acid (R_s^2=0.1, p=0.7 and R_s^2=0.4, p=0.5; respectively).

5.6.4.4 Concentration of bacterial DNA isolated from faecal samples after low-polyphenol diet

High quality and high yield DNA was obtained from all faecal samples, with a similar concentration and purity of the extracted DNA in both groups (Table 5-9).

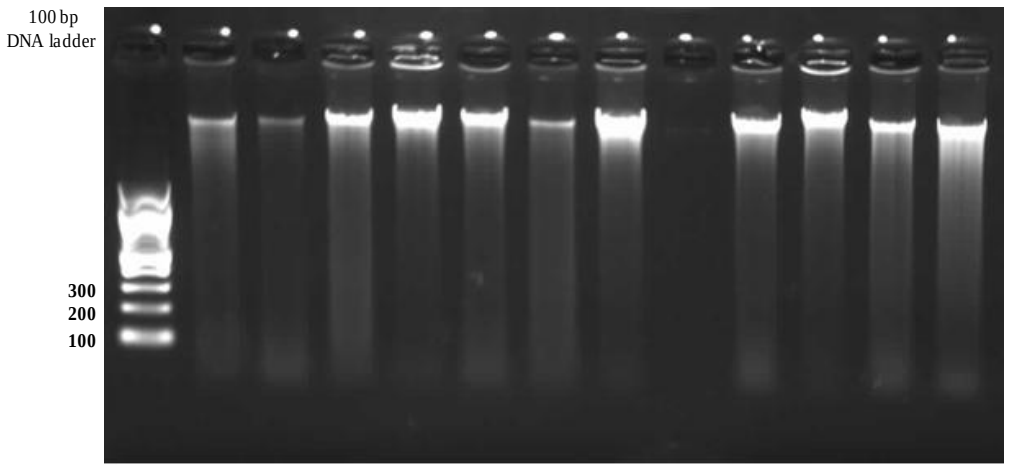
Table 5-9: The DNA concentration and purity in the healthy control (n=12) and polypectomy (n=8) groups.

Group	Healthy control			Polypectomy		
Measurements	**Median**	**IQR**	**Range**	**Median**	**IQR**	**Range**
DNA concentration ng/µl	463	201.4	421.6-1031.4	429.7	99.7	271.6-480.7
DNA purity 280/260 nm	1.8	0.09	1.7-2.0	1.7	0.1	1.7-1.8
DNA purity 260/230 nm	1.3	0.2	1.0-1.8	1.3	0.2	1.0-1.5

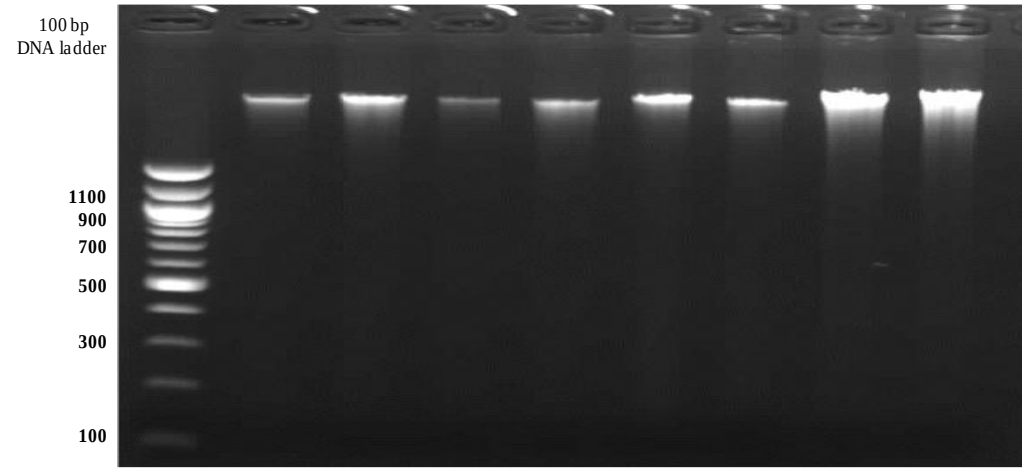
The faecal DNAs were intact and appear as a high-molecular-weight band in 1.5% agarose gel (Figure 5-12).

Figure 5-12: Agarose gel showing the purity of DNA extracted for bacterial qPCR analysis in A) healthy control (n=12) and B) polypectomy (n=8) groups.

(A)



(B)



5.6.4.5 Characteristics of the qPCR run condition

The characteristics of the qPCR runs of each bacterial group and species including the slope, amplification efficiency, and the coefficient of determination (R^2) for both groups are described in Table 5-10. The amplification efficiency was within the normal range (90% - 105%) for the total bacteria, *Bacteroides – Prevotella* and *Flavonifractor plautii* in both groups; however, the efficiency for *Bifidobacterium spp.* was just below the normal range in both groups.

Table 5-10: Characteristics of qPCR runs for each bacteria species/groups in healthy control (n=12) and polypectomy (n=8) groups.

Bacteria species/groups	Slope	Amplification efficiency (%)	R^2
Total Bacteria	-3.371	97.9	0.994
Bacteroides – Prevotella	-3.507	92.8	0.998
Bifidobacterium spp.	-3.68	86.9	0.994
Flavonifractor plautii	-3.621	89.7	0.999

5.6.4.6 Absolute concentration of bacterial species and groups using qPCR

Absolute levels of total bacteria, *Bacteroides*, *Bifidbacterium*, and *Flavonifractor plautii* did not differ between healthy control and polypectomy groups (Table 5-11).

Table 5-11: Absolute log_{10}faecal concentrations and relative abundance of bacterial groups/species in healthy control (n=12) and polypectomy (n=8) participants.

Target	Wet weight sample				Dry weight sample*			
	Healthy control (n=12)		Polypectomy (n=8)		Healthy control (n=12)		Polypectomy (n=8)	
Log_{10} /g	**Median**	**IQR**	**Median**	**IQR**	**Median**	**IQR**	**Median**	**IQR**
Total bacteria	11.4	0.5	10.9	0.3	11.8	0.5	11.4	0.3
Bifidobacterium spp.	9.5	0.9	9.1	0.4	10.1	1.1	9.6	0.3
Bacteroides + Prevotella	10.2	0.6	9.7	0.7	10.7	0.4	10.1	0.7
Flavonifractor plautii	7.7	1.0	7.9	0.9	8.2	1.1	8.4	0.9
%								
Bifidobacterium spp.	1.26		1.58		2.00		1.58	
Bacteroides + Prevotella	6.31		6.31		7.94		5.01	
Flavonifractor plautii	0.02		0.10		0.03		0.10	

* Calculated using the weight (g) and the moisture percentage (%) of the wet faecal samples.

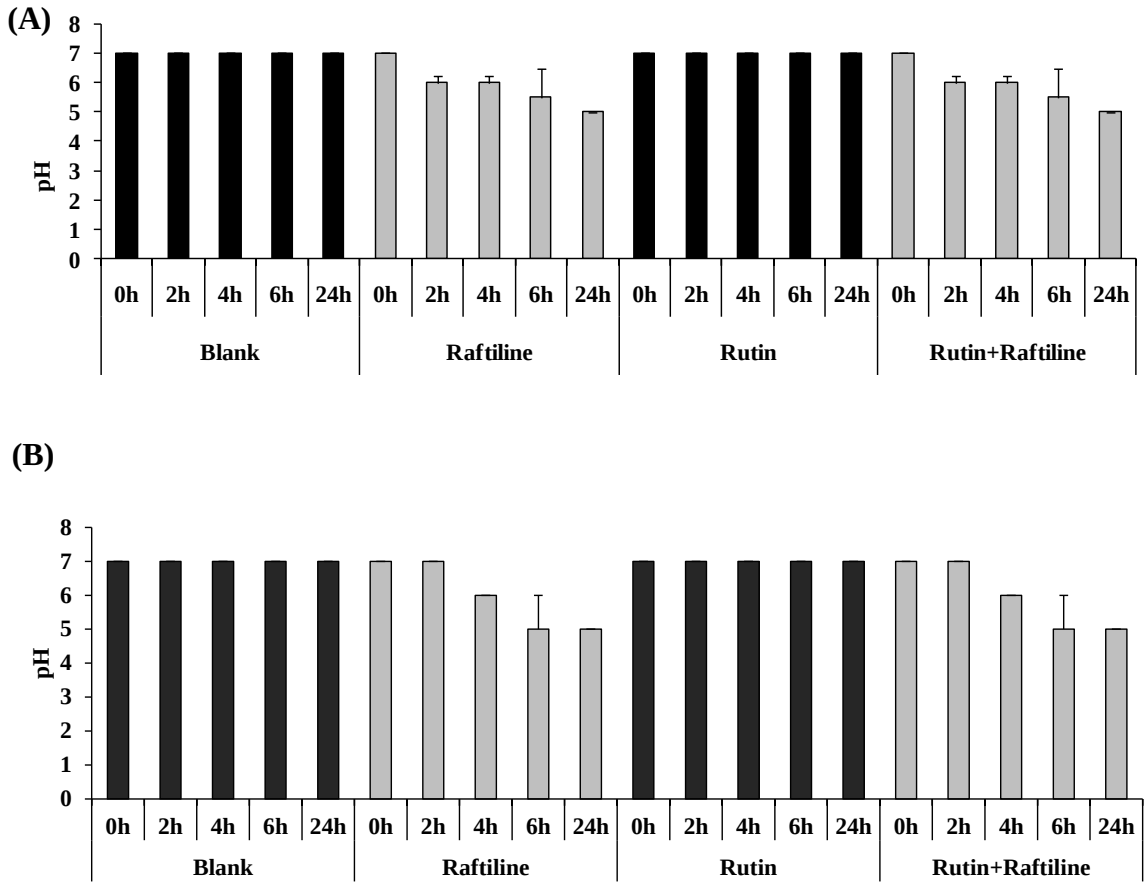
5.6.5 *In-vitro* fermentation of rutin

Nine sets of fermentation were carried out using the stool samples of four healthy adults (one male and three female) and five patients who underwent polypectomy (one female and four males), collected after the low-polyphenol diet. The flavonol rutin was fermented for 24 h with or without fibre (raftiline) to test the metabolic capacity of the gut faecal contents, in the context of gut health and the risk of developing CRC.

5.6.5.1 Rutin fermentation and pH of faecal fluids

There was no change in the pH of the fermented faecal fluids containing rutin alone, over time, in either group. However, the combination of rutin with raftiline significantly ($p<0.01$) reduced the pH at the end of the fermentation in both groups. While the overall reduction was not different between groups, the reduction in pH started after two hours of fermentation in the polypectomy group and after the zero hour time point in the healthy group (Figure 5-13).

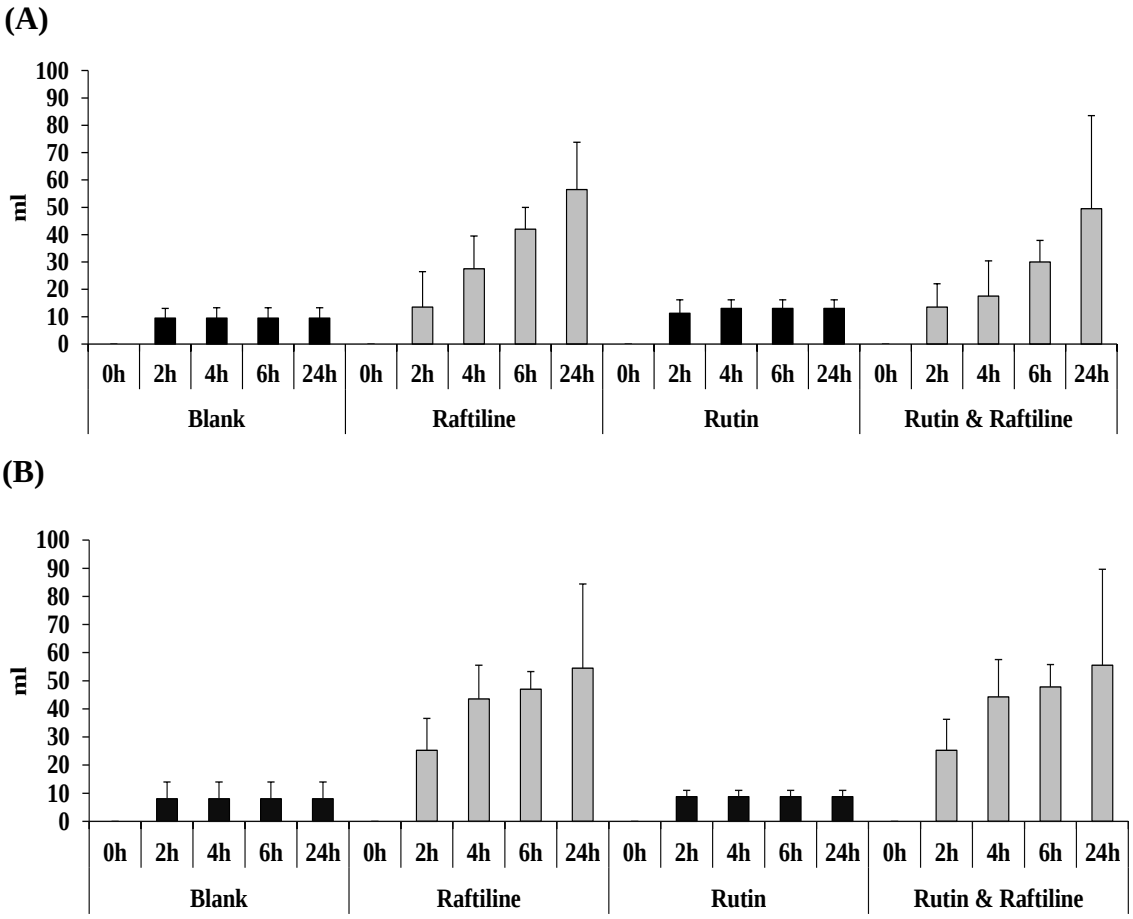
Figure 5-13: Change in pH of fermented faecal fluids over 24 h in A) healthy control (n=4) and B) polypectomy (n=5) participants. Data presented as median and IQR.



5.6.5.2 Rutin fermentation and gas production

In both healthy control and polypectomy groups, the combination of rutin with raftiline increased gas production more than the rutin alone ($p<0.05$). The accumulation of gas over the 24h fermentation period was not different between groups (Figure 5-14).The addition of rutin to raftiline did not significantly decrease the gas production in either group.

Figure 5-14: Cumulated gas production from fermented faecal fluids over 24 h of incubation in A) healthy control (n=4) and polypectomy (n=5) groups. Data presented as median and IQR.



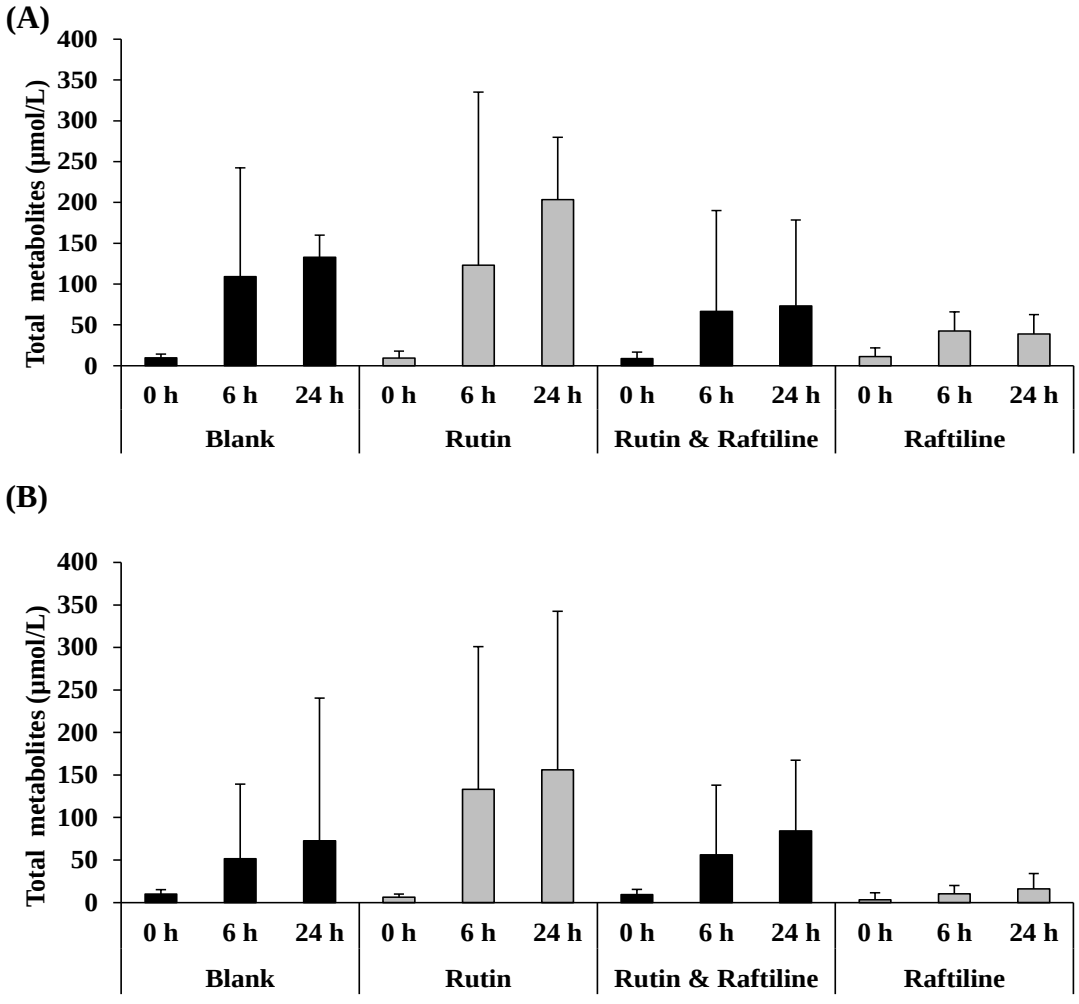
5.6.5.3 Metabolism of rutin in faecal fluids and phenolic acids formation

Only seven metabolites were found and identified using GC-MS after the fermentation of rutin with or without raftiline in the fermented faecal fluids of healthy control and polypectomy groups at 0, 6, and 24 h (as presented previously in Table 3-12, Chapter 3). PAA, 3-OHPAA, 3-OHPPA, 4-OHPPA, and 3,4diOHPPA were detected in both faecal only fluids and fermented faecal fluids of rutin with or without raftiline. 3,4diOHPAA was detected only in the fermented faecal fluids of rutin with or without raftiline. 4-OHBA was not detected in the fluids from the polypectomy group at all (Table 5-12).

5.6.5.3.1 Total phenolic acid metabolites formed after the fermentation of rutin

The sum of the seven metabolites significantly increased over time in the fermented faecal fluids of rutin in the healthy control (p=0.002) and polypectomy (p=0.02) groups (Figure 5-15). There were no significant differences between groups.

Figure 5-15: Total phenolic acids (µmol/L) in A) healthy group (n=4) and B) polypectomy (n=5) fermented faecal fluids over 24 h of fermentation. Data presented as median and IQR.



5.6.5.3.2 Individual phenolic acid formed after the fermentation of rutin

PAA increased significantly over time in the fermented faecal fluids of rutin in the healthy control (p=0.01) and polypectomy (p=0.01) groups. 3-OHPPA and 4-OHPAA significantly increased only in the fermented faecal fluids of rutin in the healthy control group (p=0.01; p=0.04, respectively; Table 5-12). No differences were found between groups in terms of the formation of the individual acids.

Table 5-12: Accumulation of seven phenolic acids (µmol/L) produced after 0, 6, and 24 h of fermentation in faecal fluids from healthy (n=4) and polypectomy (n=5) groups.

Metabolite	Group	Substrates	0h		6h		24h	
			Median	IQR	Median	IQR	Median	IQR
PAA	Healthy control	Blank	6.9	1.5	92.8	99.5	88.8	19.9
		Raftiline	7.0	5.1	22.2	26.3	26.6	26.2
		Rutin	6.7	4.6	58.7	98.2	128.3	53.9
		Rutin+raftiline	6.4	4.8	17.5	32.8	25.3	21.9
	Polypectomy	Blank	9.5	4.1	32.8	60.8	69.7	145.2
		Raftiline	6.2	6.7	11.5	8.0	17.9	9.7
		Rutin	6.3	1.8	34.2	56.5	113.8	143.1
		Rutin+raftiline	7.3	4.4	12.8	18.5	18.5	30.7
3-OHPAA	Healthy control	Blank	nd	nd	0.1	<0.01	0.1	<0.01
		Raftiline	nd	nd	nd	nd	nd	nd
		Rutin	0.1	<0.01	0.8	0.6	4.2	26.6
		Rutin+raftiline	nd	nd	0.3	0.9	0.6	0.5
	Polypectomy	Blank	nd	nd	nd	nd	7.5	<0.01
		Raftiline	nd	nd	nd	nd	nd	nd
		Rutin	nd	nd	1.6	<0.01	3.0	4.2
		Rutin+raftiline	nd	nd	0.3	0.2	0.5	0.4
4-OHBA	Healthy control	Blank	0.2	<0.01	0.4	11.9	0.3	13.6
		Raftiline	0.2	<0.01	0.2	<0.01	0.1	<0.01
		Rutin	0.3	0.2	0.2	<0.01	0.2	<0.01
		Rutin+raftiline	0.2	<0.01	0.3	<0.01	0.4	0.2
	Polypectomy	Blank	nd	nd	nd	nd	nd	nd
		Raftiline	nd	nd	nd	nd	nd	nd
		Rutin	nd	nd	nd	nd	nd	nd
		Rutin+raftiline	nd	nd	nd	nd	nd	nd

*Continued overleaf

Metabolite	Group	Substrates	0h		6h		24h	
			Median	IQR	Median	IQR	Median	IQR
3-OHPPA	Healthy control	Blank	0.9	1.1	4.2	7.6	3.4	2.7
		Raftiline	1.0	2.3	2.8	4.0	3.4	1.4
		Rutin	0.5	0.8	7.7	23.5	13.4	8.4
		Rutin+raftiline	1.3	1.8	9.5	20.8	9.4	15.8
	Polypectomy	Blank	0.3	2.2	1.1	1.4	1.7	1.5
		Raftiline	1.2	1.2	1.2	0.8	1.3	1.6
		Rutin	1.6	1.5	4.2	7.8	4.1	9.3
		Rutin+raftiline	0.2	1.5	1.6	3.9	1.7	6.0
4-OHPPA	Healthy control	Blank	0.4	0.6	3.4	12.9	21.6	27.2
		Raftiline	0.3	0.7	1.4	0.6	2.0	0.8
		Rutin	0.8	1.0	1.6	0.8	6.9	9.2
		Rutin+raftiline	0.3	0.7	0.4	0.3	1.9	3.2
	Polypectomy	Blank	0.1	0.5	1.3	19.8	13.5	29.0
		Raftiline	0.7	<0.01	1.2	1.5	1.0	1.8
		Rutin	0.4	0.4	5.8	10.5	4.8	14.2
		Rutin+raftiline	0.7	<0.01	1.1	1.7	0.4	0.6
3,4diOHPAA	Healthy control	Blank	nd	nd	nd	nd	nd	nd
		Raftiline	nd	nd	nd	nd	3.2	<0.01
		Rutin	0.3	<0.01	63.0	34.3	65.8	43.7
		Rutin+raftiline	0.5	<0.01	48.3	23.9	52.0	25.9
	Polypectomy	Blank	nd	nd	nd	nd	nd	nd
		Raftiline	nd	nd	nd	nd	nd	nd
		Rutin	0.2	<0.01	74.0	0.6	27.7	18.5
		Rutin+raftiline	0.3	<0.01	42.9	40.0	51.6	30.2

*Continued overleaf

Metabolite	Group	Substrates	0h		6h		24h	
			Median	IQR	Median	IQR	Median	IQR
3,4diOHPPA	Healthy control	Blank	nd	nd	nd	nd	nd	nd
		Raftiline	nd	nd	nd	nd	nd	nd
		Rutin	0.2	<0.01	1.1	1.0	0.5	<0.01
		Rutin+raftiline	nd	nd	nd	nd	0.6	<0.01
	Polypectomy	Blank	nd	nd	nd	nd	nd	nd
		Raftiline	nd	nd	nd	nd	nd	nd
		Rutin	nd	nd	1.3	<0.01	nd	nd
		Rutin+raftiline	nd	nd	0.1	<0.01	nd	nd

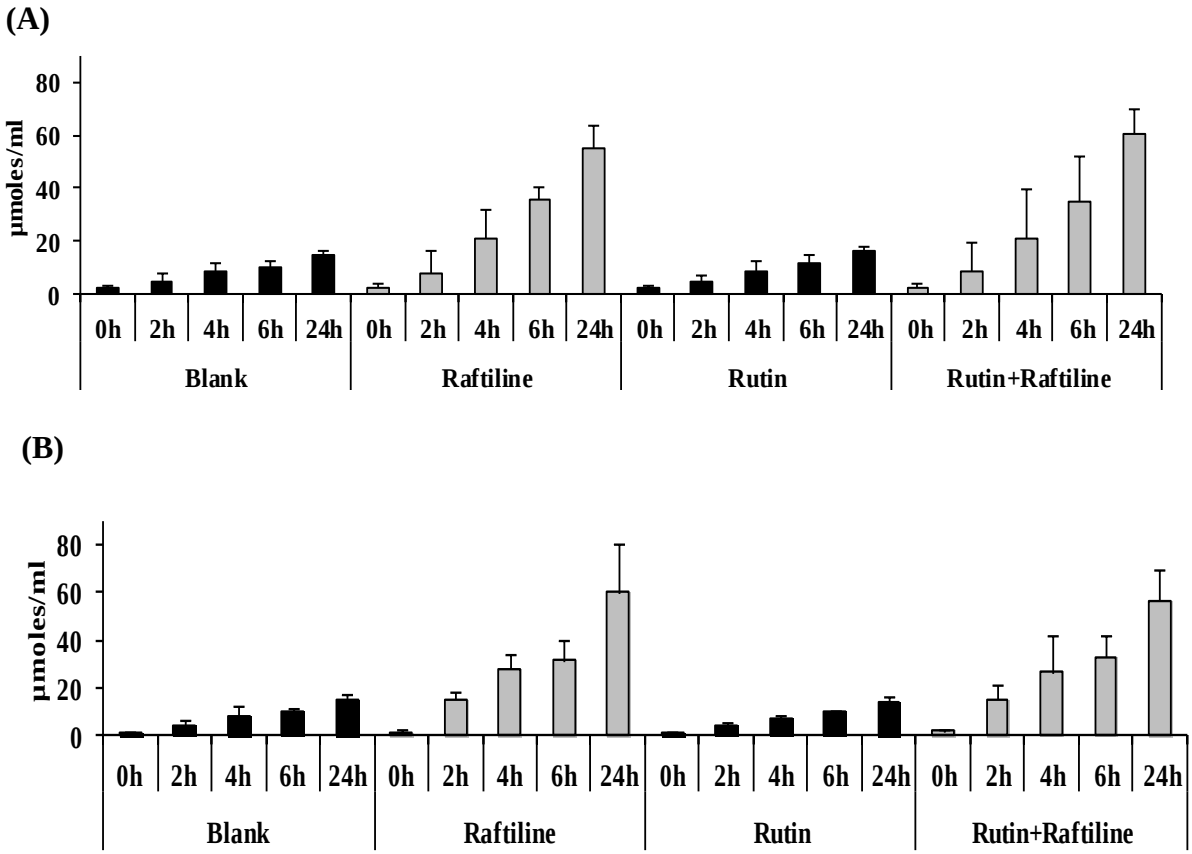
Not detected (nd)

5.6.5.4 Metabolism of rutin in faecal fluids and SCFA production

Ten SCFA were identified and quantified in the fermented faecal fluids after the fermentation of rutin in the presence or absence of raftiline in healthy control and polypectomy groups at 0, 2, 4, 6 and 24 h .They are the same as those measured directly in the faecal samples (Table 2-1; Chapter 2). Isocaproic, caproic, enanthic, and caprylicacid were either detected in limited amounts or not detected at all.

The total SCFA concentration significantly increased over time in the fermented faecal fluids of rutin with or without raftiline in healthy control and polypectomy groups ($p<0.05$). With raftiline, significantly higher levels of SCFA were detected in the fluids compared to rutin alone ($p<0.05$) for the healthy control group; $p=0.002$ for the polypectomy group). Total SCFA levels were not different between groups (Figure 5-16).

Figure 5-16: SCFA production (µmol/ml) in A) healthy control (n=4) and B) polypectomy (n=5) groups fermented faecal fluids over 24 h of fermentation. Data presented as median and IQR.



Acetic, propionic, and butyric acid significantly increased over time in the fermented faecal fluids of rutin and the combination of rutin with raftiline in both groups ($p<0.05$; Table 5-13). There was no difference in the levels of specific SCFA between groups.

Table 5-13: SCFA concentration (µmole/ml) after 0,2,4,6 and 24 h. of fermentation from healthy control (n=4) and polypectomy (n=5) participants.

SCFA	Group		0h		2h		4h		6h		24h	
		Substrates	Median	IQR	Median	IQR	Median	IQR	Median	IQR	Median	IQR
C2	Healthy control	Blank	1.2	0.4	2.8	1.6	4.9	1.3	5.8	0.7	7.8	0.3
		Raftiline	1.3	1.3	5.6	5.8	17.7	5.6	28.1	2.4	35.8	11.6
		Rutin	1.3	0.7	2.9	1.2	5.3	1.7	6.9	0.9	8.3	1.9
		Rutin+raftiline	1.4	1.0	6.3	7.7	14.5	11.3	24.4	6.1	39.2	14.6
	Polypectomy	Blank	1.0	0.2	2.8	1.4	4.6	2.4	5.9	0.7	7.6	1.5
		Raftiline	1.0	0.3	11.4	3.3	18.6	7.2	24.8	8.1	28.0	14.4
		Rutin	1.1	0.3	2.9	0.6	4.5	0.6	6.4	0.7	7.6	0.7
		Rutin+raftiline	1.3	0.4	10.5	4.0	18.5	10.5	21.3	10.0	33.9	11.2
C3	Healthy control	Blank	0.3	0.1	0.6	0.3	1.1	0.1	1.6	0.2	2.2	0.7
		Raftiline	0.2	0.2	0.6	0.8	1.5	1.2	3.1	1.2	5.2	1.9
		Rutin	0.2	0.1	0.5	0.3	1.1	0.2	1.5	0.1	2.4	0.8
		Rutin+raftiline	0.3	0.1	0.6	0.9	1.5	0.8	2.8	1.0	5.6	3.5
	Polypectomy	Blank	0.1	0.1	0.5	0.8	1.5	0.7	1.8	0.8	2.2	1.3
		Raftiline	0.4	0.1	1.9	0.7	4.4	1.1	4.7	1.4	5.1	2.1
		Rutin	0.4	<0.01	0.7	0.4	1.3	0.5	1.7	0.7	2.0	1.4
		Rutin+raftiline	0.4	0.1	2.6	1.8	4.0	0.9	4.4	0.4	6.3	1.0
iC4	Healthy control	Blank	nd	nd	0.1	<0.01	0.1	<0.01	0.2	0.1	0.7	0.2
		Raftiline	<0.01	<0.01	0.1	<0.01	0.1	<0.01	0.1	<0.01	0.1	<0.01
		Rutin	nd	nd	0.1	<0.01	0.1	<0.01	0.2	<0.01	0.5	0.2
		Rutin+raftiline	nd	nd	0.1	<0.01	0.1	<0.01	0.1	<0.01	0.1	<0.01
	Polypectomy	Blank	nd	nd	nd	nd	0.2	<0.01	0.1	0.1	0.7	0.1
		Raftiline	nd	nd	nd	nd	nd	nd	nd	nd	<0.01	<0.01
		Rutin	nd	nd	nd	nd	0.1	<0.01	0.1	0.1	0.6	0.2
		Rutin+raftiline	nd	nd	nd	nd	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

*Continued overleaf

SCFA	Group		0h		2h		4h		6h		24h	
		Substrates	Median	IQR	Median	IQR	Median	IQR	Median	IQR	Median	IQR
C4	Healthy control	Blank	0.2	0.2	0.8	0.3	1.4	0.4	1.5	0.5	2.2	0.4
		Raftiline	0.4	0.2	1.1	1.9	3.1	3.0	4.9	3.9	5.6	6.9
		Rutin	0.2	0.2	0.8	0.3	1.3	0.4	1.7	0.5	2.4	0.4
		Rutin+raftiline	0.4	0.1	1.6	1.8	4.8	2.6	7.8	4.2	7.7	6.6
	Polypectomy	Blank	0.2	<0.01	0.4	0.3	0.9	0.2	1.0	0.6	1.6	0.4
		Raftiline	0.2	<0.01	1.3	0.5	3.5	2.2	3.3	1.9	12.4	9.0
		Rutin	0.1	0.1	0.4	0.2	0.8	0.2	1.1	0.3	1.9	0.4
		Rutin+raftiline	0.2	0.1	1.0	1.0	2.6	2.2	4.5	1.3	15.0	7.5
iC5	Healthy control	Blank	0.1	<0.01	0.2	<0.01	0.3	0.1	0.4	0.1	1.2	0.4
		Raftiline	0.1	<0.01	0.1	<0.01	0.2	0.1	0.2	0.2	0.2	1.3
		Rutin	0.1	<0.01	0.1	<0.01	0.2	0.1	0.4	0.2	1.0	0.4
		Rutin+raftiline	0.1	<0.01	0.1	<0.01	0.2	<0.01	0.2	0.1	0.2	0.7
	Polypectomy	Blank	nd	nd	0.1	0.1	0.2	0.0	0.4	0.2	1.3	0.5
		Raftiline	nd	nd	0.1	<0.01	0.2	0.0	0.1	0.0	0.1	0.0
		Rutin	nd	nd	0.1	<0.01	0.1	0.1	0.2	0.2	1.2	0.2
		Rutin+raftiline	nd	nd	<0.01	<0.01	0.0	0.1	0.1	0.0	0.0	0.1
C5	Healthy control	Blank	nd	nd	0.3	<0.01	0.8	0.1	0.5	0.8	1.1	0.1
		Raftiline	<0.01	<0.01	0.3	0.1	0.5	0.2	0.6	0.2	0.6	0.2
		Rutin	nd	nd	0.2	<0.01	0.7	<0.01	0.9	0.4	1.0	0.1
		Rutin+raftiline	0.1	<0.01	0.3	<0.01	0.5	0.1	0.6	0.3	0.6	0.3
	Polypectomy	Blank	nd	nd	0.1	<0.01	0.4	0.2	0.7	0.3	0.9	0.0
		Raftiline	nd	nd	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.1
		Rutin	nd	nd	0.1	<0.01	0.2	0.2	0.5	0.3	0.9	0.1
		Rutin+raftiline	nd	nd	0.2	<0.01	0.2	0.1	0.2	0.1	0.2	0.1

Not detected (nd), acetic acid (C2), propionic acid (C3), isobutyric acid (iC4), butyric acid (C4), isovaleric acid (iC5), valeric acid (C5).

5.7 Discussion

This current study was carried out to test the hypothesis that older people who are at risk of CRC (colorectal adenomas removed) are less able to metabolise dietary polyphenols than healthy adults due to changes in their gut function and colonic microbiota composition. To our knowledge, this is the first semi-controlled dietary study investigating the colonic metabolism of dietary polyphenols in polypectomy patients using human feeding and *in-vitro* faecal fermentation designs.

In the present study, there were no significant differences detected between the healthy control and polypectomy groups in terms of 1) sum urinary phenolic acid excretion, and 2) sum phenolic acid formation in the faecal fluids. However, three phenolic acids were absent in the urine of the polypectomy group, while some phenolic acids were not detected in certain participants in the older group. This finding could be limited to the small size of the polypectomy group. A post-hoc calculation of the sum urinary phenolic acid using G-power software showed that the urinary phenolic acid data of the present study achieved a power of 50%. To achieve a power of 80%, six more participants in the healthy control group and eleven more patients in the polypectomy group would be required to detect any differences. The recruitment for this study started on the 1st of August 2012 until March 2013; however, due to 50% of the recruited participants dropping out and the PhD timeline, we included this data as preliminary information.

There is no clear explanation regarding the absence of the PAA, 3,4diOHBA, and GA in the urine samples of all polypectomy patients and 3-OHBA in the fermented faecal fluids. Several proposed metabolic pathways of rutin and tea in humans and *in-vitro* and faecal fermentation showed a production of these acids (Olthof *et al.*, 2003, Jaganath *et al.*, 2009, Serra *et al.*, 2012). The absence of these acids could be a result of changes in gut microbiota composition, function, or/and gut physiology associated with the development of colorectal adenoma/cancer. A study by Moore and Moore (1995) showed that patients with polyps have different gut microbiota composition compared with healthy individuals.

The change in gut microbiota is linked to the derived microbial and enzymatic activities. In the human colon, the occurrence of bacterial enzymes (β-glucosidases, β-glucuronidases, and α-rhamnosidase) enables the hydrolysis of rutin to release the quercetin aglycone (Aura *et al.*, 2002). Insufficient or lower levels of bacterial enzyme in the colon could be one of the reasons for the absence and low urinary phenolic acid in the polypectomy group.

This may have consequences on the colonic health as a high local concentration of gut microbial metabolites such as, phenolic acids, accumulating in the colon could have positive biological activities (Dorsten *et al.,* 2012).

In this present study there were no differences between groups in the quantity of the bacteria known to modulate by phenolic compounds such as *Bifidbacterium* and *Bacteroides,* or *Flavonifractor plautii* (a known flavonoid degrader). However, these were the only strains measured in the faecal samples, and they may not be representative of all polyphenol metabolism bacteria that are found in the colon. There are a number of human faecal bacteria species that have been identified to degrade the C-ring system of flavonoids, such as, *Clostridium scindens, Eubacterium desmolans, Eubacterium ramulus* (Schneider *et al.,* 1999, Simmering *et al.,* 1999, Braune *et al.,* 2001, Schoefer *et al.,* 2003), *Butyrivibrio sp* (Krishnam.Hg *et al.,* 1970), and *Bacteroides distasonis* (Bokkenheuser *et al.,* 1987). Yet, there remains a lack of information on the type and occurrence of the flavonoid-degrading bacteria in the human intestinal tract in relation to health and disease. Moreover, the levels of bacterial enzyme and activity were not measured in this study which limits our knowledge of understanding the difference in the microbial profile of polyphenols between the healthy control and polypectomy groups.

The current study observed a very large inter-individual variation in urinary phenolic acids within the healthy control group (30-fold) compared with the polypectomy patients (9-fold). However, the variation reduced to 3-fold within the healthy control group and increased to 10-fold within the polypectomy group once hippuric acid had been removed from the calculation. Urinary hippuric acid is the glycine hepatic conjugate of benzoic acid (Phipps *et al.*, 1998). It was the main acid contributing ~ 97% of phenolic acid excretion in the healthy control and polyectomy groups. Hippuric acid is most likely to be formed in the liver by conjugation of benzoic acid and glycine. Dietary sources of benzoic acid and precursors (quinic acid, aromatic amino acid tryptophan, tyrosine, and phenylalanine) should be considered (Self *et al.*, 1960, Grumer, 1961). Other sources of benzoic acid are benzoates (E numbers 210-219) which are commonly used in food, medications, and mouthwashes.

In the present study, the hippuric acid was higher in the polypectomy group after the low-polyphenol diet and tended to be higher after the high-polyphenol diet in the polypectomy group compared to the healthy group (1734.5, IQR 1704.5 vs 3581.4, IQR 4206.7). The high levels of hippuric acid could be due to several reasons: 1) altered gut microbiota.

Williams *et al.* (2010) reported an association between altered gut microbiota and the urinary excretion of hippuric acid in Crohn's disease; 2) the high amount of *Clostridia* spp. in the polypectomy group. Li *et al.* (2008) found a positive association between *Clostridia spp.* and the level of hippuric acid; 3) the high amount of hippuric acid could be a result of amino acid metabolism such as that of aromatic amino acids tryptophan, tyrosine, and phenylalanine (Self *et al.*, 1960, Grumer, 1961). The high level of hippuric acid could be considered an objective marker of fruit and vegetable intake; however, the non-dietary factors that increase hippuric acid should be taken into consideration.

The higher amount of hippuric acid in the urine of the polypectomy group had no effect on the urinary antioxidant activity (FRAP). According to Olthof *et al.* (2003) dietary phenols are strong antioxidants in vitro but not in vivo because they are metabolized extensively. He found that the urinary hippuric acid which is the most important metabolite of chlorogenic acid and tea phenols has no antioxidant activity using FRAP assay, because it has no hydroxyl group. There is no clear evidence why the urinary FRAP did not increase after the high-polyphenol diet in the polypectomy group. However, it could be due to the absence of some phenolic acids in the urine of the polypectomy group, possibly associated with the changes in gut microbiota composition or/and function and gut physiology.

In contrast to the sum of urinary phenolic acid, the urinary total phenols measured with the Folin-Ciocalteu assay did not increase after the high-polyphenol diet in the healthy control and polypectomy groups. This may be attributed to lack of hydroxyl moiety on some of the phenolic acid such as hippuric molecule or/and differences in the urinary phenolic acid reactivity to the Folin reagent. A recent cohort study evaluated the association between total urinary polyphenols using Folin-Ciocalteu assay and all-cause mortality during a 12-year period among 807 men and women living in Italy. This study suggested that older people with low total urinary phenol concentration are at high risk of overall mortality. The excretion of total urinary phenols tended to be higher among survivors than those who died within the 12 year study period (Zamora-Ros *et al.*, 2013c).

In view of the biomarkers of colonic fermentation (SCFA) in the human feeding and fermentation models of the present study, it is suggested that polypectomy patients have similar fermentation ability to their healthy counterparts. This is in agreement with Clausen *et al.* (1991) who found that the faecal concentrations of total SCFA and the concentrations

and ratios of the individual SCFA using the gas-liquid chromatography did not differ between 16 healthy controls, 17 patients with colonic adenomas, and 17 patients with colonic cancer. On the other hand, Monleon *et al.* (2009) reported that the metabolic profile of faecal water using the nuclear magnetic resonance spectroscopy of colorectal cancer patients is low in SCFA concentration, particularly acetate and butyrate, which has been associated with the risk of CRC. The inconsistency between these results could be due to differences in the sample size employed as well as the technique used to measure the SCFA. In our study, the polypectomy group habitually consumed less breakfast cereal than the healthy group. However, there was no difference between the two groups for pH and SCFA in faecal samples (direct measurements) or fermented faecal fluids.

The reduction in faecal pH in both groups after the high polyphenol diet may be due to the effect of the dietary fibre in the high-polyphenol diet more than the effect of polyphenols which had been reported before by Btavo *et al.* (1994) and Nordlund *et al.* (2012). In the *in-vitro* fermentation model of this study, the pH of fermented faecal fluids decreased only when raftiline had been added to the rutin. The reduction in pH during the fermentation is a result of the production of SCFA when raftiline was fermented. The fermentation of flavonols alone did not influence the pH of the faecal fluids.

No study has yet examined whether colonic inflammation has a direct effect on the metabolism of dietary polyphenols. In the present study, a calprotectin assay was used to detect differences between the groups in terms of their colonic inflammation. However, most participants in the polypectomy group were within the active systemic range. A study by Shitrit *et al.* (2007) showed that calprotectin levels were significantly higher in patients with abnormal colonic histology compared to patients with normal colonoscopy. This result suggested the use of faecal calprotectin as a predictive marker for colonic polyps and colonic cancer not only for Crohns disease but also for IBD. With no correlation between urinary phenolic acids and faecal calprotectin levels, as well as no significant differences between groups, it is hard to make any conclusion about the possible link between inflammation of the colon and the colonic metabolism of dietary polyphenols.

Polyphenols have been shown to reduce colonic inflammation and modulate the signalling pathways in the intestinal cells by interfering with inflammatory mediator production and the signalling pathways of NF-kB (Ahmad *et al.*, 2000) and MAPK (Balasubramanian *et al.*, 2002). In the present study, one of the patients in the polypectomy group had a very

low calprotecin level even when compared to the healthy group. According to the initial visit and the FFQ of the patient, it was found that he consumed six cups of green tea every day. However, all of his urinary and faecal measurements were within the range of the rest of his group.

To our knowledge this is the first human feeding and *in-vitro* faecal fermentation study which investigates the colonic metabolism of dietary polyphenols in patients who were recruited from the *Scottish Bowel Screening Programme*. The current study provides preliminary data to establish a longer term study on the effect of a high-polyphenol diet on the inflammatory and immunity colonic biomarkers. This study does, however, have some limitations: 1) due to a high drop-out rate the final sample size was small and thus is of low statistical power; and 2) the study groups were not matched for gender, meaning that the results could be affected by different colonic transit times. However, the bowel movements data of this study showed no significant differences between the groups. Moreover, this is unlikely to be a major factor in this study looking at participants over 50 years of age, as these differences were not observed to occur between men and post-menopausal women (Madsen, 1992).

In conclusion, no significant differences were detected between the healthy control and polypectomy groups in terms of the sum urinary phenolic acid excretion and phenolic acids formation in the faecal fluids. However, three phenolic acids (PAA, 3,4diOHBA, and GA) were not detected in the urine of the polypectomy group as well as one acid (3-OHBA) in the faecal fermentation fluids, while some of the acids were not detected in some participants in the healthy control group. Moreover, no significant differences were observed between the groups in terms of the markers of colonic fermentation and inflammation. This could be due to the small sample size; the result of this study, especially the urinary phenolic acid, is considered as preliminary data that will help to plan subsequent studies in the future. In addition, the result of this study is for patients with colorectal polyps; however, the colonic metabolism of polyphenols in CRC patients may be different. Long term studies lasting at least eight weeks using a large number of polypectomy patients are needed to understand the colonic metabolism dietary polyphenol in patients who are at risk of CRC. These types of studies can help to understand the local and systemic biological activities of polyphenols to be able to generate optimal dietary recommendations for prevention and treatment.

Chapter Six

General discussion

6.1 Introduction

The last decade has witnessed a significant increase in dietary polyphenol research for the prevention of diseases, such as cancer and cardiovascular disease, owing to their well-known anti-oxidant and anti-inflammatory activities (Kim *et al.*, 2013, Macready *et al.*, 2014). However, most of the evidence has been derived primarily from epidemiological studies (Cassidy, 2010, Rossi *et al.*, 2010).

Experimental studies have predominantly examined dietary polyphenols using either: 1) *in-vitro* or animal studies, investigating specific phenolic degradation pathways or testing higher concentrations of polyphenols than those usually found in the diet (Gonthier *et al.*, 2006, Gardana *et al.*, 2009, Jaganath *et al.*, 2009, Gross *et al.*, 2010, Ostertag *et al.*, 2011, Pae *et al.*, 2012); or 2) *in-vivo* studies involving healthy volunteers to evaluate the bioavailability of polyphenols or their effects on the markers of oxidative stress, inflammation and immunity (O'Byrne *et al.*, 2002, Cerda *et al.*, 2005, Karlsen *et al.*, 2007, Chun *et al.*, 2008, Stalmach *et al.*, 2011, Henning *et al.*, 2013). However, the majority of *in-vitro* and polyphenol bioavailability studies have reported large inter-individual variations among participants in terms of polyphenol metabolites (phenolic acids) (Cerda *et al.*, 2005, Gardana *et al.*, 2009, Jaganath *et al.*, 2009, Gill *et al.*, 2010, Gross *et al.*, 2010). To the best of our knowledge, none of these studies investigated the reasons behind these variations. The inter-individual variation of polyphenol metabolism can result in bioavailability variation and, in turn, differences in the biological activity of polyphenol metabolites among individuals and the subsequent health benefits (Jaganath *et al.*, 2009).

The problem is further complicated by the scarcity of information regarding variations in dietary polyphenol metabolism and bioavailability. Not only does this complicate the understanding of the relationship between polyphenol metabolism variations and disease incidence, but also it makes it difficult to determine optimal polyphenol intake requirements for disease prevention and health promotion, especially in older populations and those at higher risk of chronic disease. Consequently, it is challenging to establish a dietary guideline for polyphenols. As a result, it is crucial to evaluate the factors that could contribute to the variations in polyphenol metabolism and influence their absorption and bioavailability.

As highlighted in the previous chapters of this thesis, some of the proposed factors contributing to the variations in polyphenol metabolism include dietary habits, gut

microbiota, and/or food matrix interactions which are further dependent on ethnicity, age and colonic health. Therefore, the work in this thesis has focused on the inter-individual variation in relation to ethnicity, ageing, and colonic health status (colorectal cancer risk patients) using *in-vivo* and *in-vitro* faecal fermentation designs. Patients who are at risk of CRC were selected because the majority of polyphenols are metabolised in the colon by bacterial enzymes, and several cell culture studies have linked the microbial metabolites of polyphenols with CRC prevention (Kim *et al.*, 2005, Araujo *et al.*, 2011, MacDonald and Wagner, 2012).

6.2 Summary of findings

The work of this thesis showed variations in terms of the phenolic acids profile in the urine and fermented faecal fluid samples among the study groups as listed below:

1. The result of the first study (Chapter 3) showed that faecal material from Indians was more capable and faster at metabolizing rutin in the *in-vitro* model than that from Europeans, despite a lower excretion of urinary phenolic acid after a high-polyphenols diet.

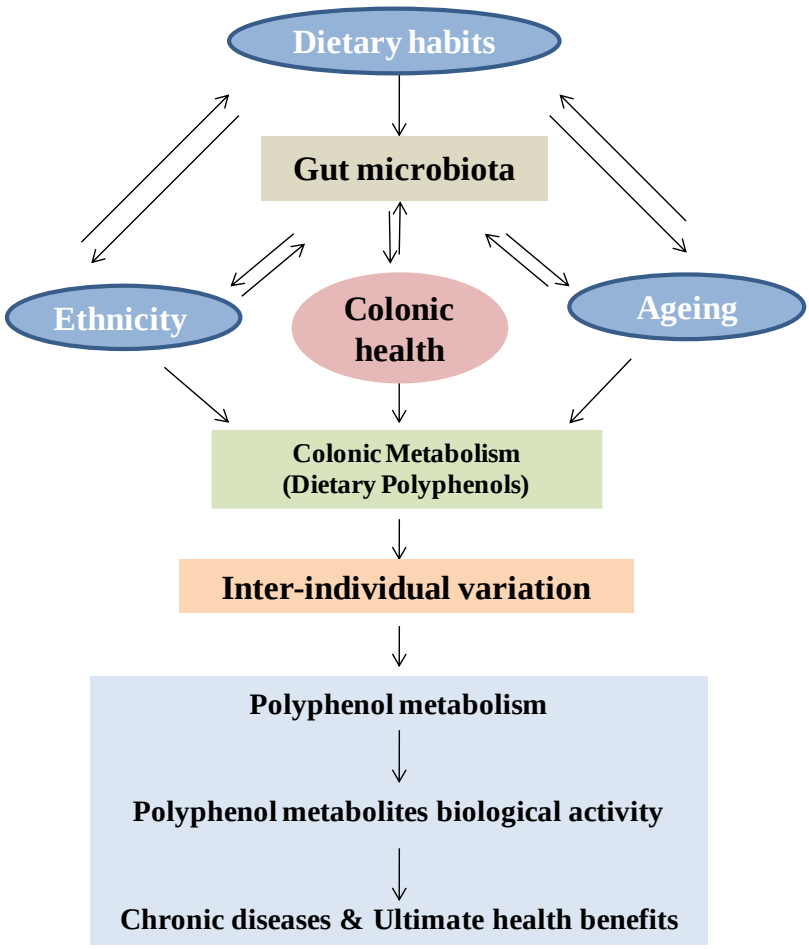
2. The second study (Chapter 4) shows that the older group excreted less urinary phenolic acid (lower amount and lower diversity / fewer acids) compared to the younger group. The sum of the phenolic acid formed after faecal fermentation of rutin was however not significantly different between groups.

3. No significant differences were detected between the healthy control and polypectomy groups for the sum urinary phenolic acid excretion or phenolic acids formation in the faecal fluids (Chapter 5). This is potentially due to the small size of the polypectomy group in the last study. However, three phenolic acids (PAA, 3,4diOHBA, and GA) were not detected in the urine of the polypectomy group as well as one acid (3-OHBA) in the faecal fermentation fluids.

6.3 Possible causes of variation in polyphenol metabolism

The variation between and within groups in urinary phenolic acid excretion and phenolic acids formation in the faecal fluids could be due to several factors such as: 1) the influence of dietary habit, ethnic food, and ageing on gut microbiota composition and function (Wu *et al.*, 2011); and 2) differences in gut health and its effect on gut physiology, such as fermentation, absorption, and transit time, as these have been shown to be affected by ageing and gastro-intestinal disease (Chapter 4 and 5). These factors could result in bioavailability variation and sequential differences in the biological activity of polyphenol metabolites causing differences in optimal health among individuals (Figure 6-1).

Figure 6-1: An overview of the factors that contribute to the inter-individual variation of dietary polyphenol metabolism and their effect on polyphenol bioavailability and biological activities and in turn on chronic disease incidence and health benefit.



In the first study (Chapter 3) the low urinary excretion by Indians with higher and faster formation of phenolic acids in the fermented faecal fluids suggests some possible mechanisms. First, it could be linked to genetics and its impact on gut microbiota composition and diversity. Second, differences in dietary habits and its impact on gut

microbiota and colonic function. The FFQ showed that Indians significantly consumed more yogurt, onion, tomato, chilli, and curry- based products, and spices than Europeans. Regular intake of yogurt, onion, tomato, and spices could be one of the reasons behind the low faecal pH and higher quantity of *Bifidobacterium* in Indians due to their prebiotic and probiotic effects of these dietary components.

Fermented dairy products (such as yogurt), act as probiotic in the human colon as they naturally contain a number of the beneficial bacteria species that are present in the colon such as *Lactobacilli* and *Bifidobacteria*, which are believed to improve intestinal health through the prevention and treatment of diarrhoea, inflammatory bowel disease, and irritable bowel syndrome (Pashapour and Iou, 2006, Shadnoush *et al.*, 2013). Palaria *et al.*(2012) found that the intake of prepared yogurt with *Streptococcus thermophilus*, *Lactobacillus bulgaricus*, and *Bifidobacterium animalis* significantly increase the total faecal bifidobacterial numbers ($P<0.001$) after a feeding period of three weeks.

In addition, a diet that is high in onion, tomatoes, and spices will be rich in polyphenols that could act as prebiotic. This has been supported by several studies including the recent study by Parkar *et al.* (2013) which reported that pure polyphenols such as rutin and quercetin as well as individual phenolic acid such as 3OHPAA, 3OHPPA, and 4OHPPA significantly increased the proliferation of *Bifidobacterium longum* in an *in-vitro* fermentation model. Thus, the low urinary excretion of phenolic acid by Indians could be due to the prebiotic and probiotic effects of their diet, which lead to high amounts of bifidobacteria. This could have an impact on the colonic metabolism of dietary polyphenols, either by using them as a source of carbon for their growth or by improving their metabolism leading to faster absorption and tissue uptake by Indians.

As *Bifidobacterium adolescentis* has been shown to inhibit the proliferation of colon cancer cell lines and harmful bacterial enzyme activity(Kim *et al.*, 2008); and as the low colonic pH was linked to low CRC (Walker *et al.*, 1992, Kim *et al.*, 2008), therefore the low risk of CRC in Indians could be due to the contribution of these factors.

Third, the influence of the food matrix may have affected the outcome of the study (Chapter 1) as the dietary intervention was semi-controlled, and participants could add their own food to the diet. However, at this stage of understanding it is not possible to link the intake of macronutrients with the urinary excretion of phenolic acid. On the other hand,

dietary fibre could have an effect on the urinary excretion of the phenolic acid; though, dietary records showed no differences between groups in terms of fibre intake.

In the second study (Chapter 4) the low urinary phenolic acid excretion in the older group had a different story to the low phenolic acid excretion in the Indian group. This is because all urinary phenolic acids were detected in Indians but in much lower amounts than the Europeans, which suggested either faster absorption or the acids were used as a source of carbon for the colonic microbiota growth. However, according to the results in Chapter 4 the absence of some of the urinary phenolic acid in the older group suggested a lack of absorption of some phenolic acids.

There is a need to validate if the low phenolic acid excretion in older people is due to the lack of absorption, in a large well-designed study. Ageing has been shown to decrease the colonic absorption, diminish the mucosal surface area, and decrease the activities of intestinal brush border enzymes (Montgomery *et al.*, 1978, Holt *et al.*, 1989). Therefore, if the low phenolic acid excretion is due to the lack of absorption, this could contribute to the risk of chronic diseases including CRC. This is because CRC risk has been reported to increase with ageing with a possible risk reduction following a diet rich in polyphenols (Bobe *et al.*, 2008, Araujo *et al.*, 2011, Cappellani *et al.*, 2013). In this study the faecal phenolic acids were not measured; otherwise, it could have provided the information of whether the phenolic acid was quickly absorbed or accumulated in the colon and eliminated. However, the control fermented faecal fluids were used for this purpose.

Looking from another angle, could the accumulation of phenolics in the colon increase the interaction between phenolics and colonic cells, resulting in colonic health improvement? No conclusive answer can be made at this stage as data is still lacking as to whether the accumulation of phenolic acid over time could have an effect on bioactivity. More studies are needed in this area to clarify the association between the length of interaction between phenolic and colonic cells.

Moreover, the effect of ageing on gut microbiota and physiological colonic functions (Biagi *et al.*, 2010) could play an important role in the differences in the colonic metabolism of dietary polyphenols between older and younger groups. However, of the few strains measured (*Bifidbacterium*, *Bacteroides*, and *Flavonifractor plautii*) no differences were seen between the groups. However, other colonic microbiotas, which were not measured, could be involved in the colonic metabolism polyphenols. These

bacteria could include *Enterococcus casseliflavus*, *Eubacterium ramulus* (Schneider *et al.*, 1999, Schneider and Blaut, 2000) *Butyrivibrio sp* (Krishnam.Hg *et al.*, 1970), and *Bacteroides distasonis* (Bokkenheuser *et al.*, 1987).

As the results of the first study and other studies suggested that the intake of yogurt can improve gut health, including increasing the *Bifidbacterium*, this could have important implications for those persons wanting to improve their colonic metabolism of polyphenols. However, further studies are advised to confirm and additionally quantify the suggested improvement of yogurt on the colonic metabolism of polyphenols.

In the last study (Chapter 5), due to the small sample size, there is no clear explanation behind the absence of PAA, 3,4diOHBA, and GA in the urine of the polypectomy group. Moreover, the secondary measurement in urine samples (total phenols and FRAP) and in faecal samples (pH, SCFA, calprotectin, absolute concentration of total of signal bacteria) did not provide any further insight regarding this result.

However, as the provided diet was high in flavonoids that reach the colon, any alteration in the colonic microbiota could have an effect on the urinary excretion of the phenolic acid in the polypectomy group. Many studies reported changes in gut microbiota in patients with colorectal adenoma and CRC. For example, Chan *et al.* (2013) reported differences in the faecal microbiota between colorectal adenoma patients and healthy controls. *Clostridium*, *Roseburia*, and *Eubacterium spp.* were significantly lower, while *Enterococcus* and *Streptococcus* spp. were more prevalent in colorectal adenoma patients. Still there remains limited information regarding the contribution of these bacteria to the metabolism of polyphenols.

It is important to study the colonic metabolism, absorption, and bioavailability of polyphenols in patients who are at risk of CRC for several reasons. Firstly, there is increasing evidence that gut microbiota plays a role in the pathogenesis of the development from colorectal adenoma to CRC. Secondly, the risk could be reduced by increasing the intake of dietary polyphenols due to their biological activities involving antioxidant, anti-carcinogenic, anti-inflammatory, and antimicrobial properties (Araujo *et al.*, 2011). Millen *et al.* (2007) found a modest association between a diet high in fruit and deep-yellow vegetables, dark-green vegetables, onions, and garlic and reducing the risk of colorectal adenoma and cancer. These types of food are high in polyphenols, which have accumulating evidence showing their protective biological activities.

At this stage, it is still too early to speculate that the changes in gut morphology and physiology with colorectal adenoma could have an effect on the colonic metabolism of polyphenols. However, according to Scanlan *et al.* (2008) any changes in gut microbiota diversity, composition, or function could affect the regular physiological processes of colonic epithelium with potential impact on microbiota metabolic end-products and subsequently potential risk for disease development. Therefore, there is a need for further studies to understand the effect of gut morphology and physiology changes in the colonic metabolism of dietary polyphenols.

In the three studies undertaken here, the measurements of polyphenol metabolites in plasma could help in understanding the variation in the polyphenol metabolites absorption rate in each group; however, this would require a bioavailability study alongside the human feeding and *in-vitro* studies. The result of this thesis should be utilized to understand the differences in bioavailability between groups.

6.4 Reflections on methodology

The *in-vitro* faecal fermentation experiments provided interesting data regarding the inter-individual variation of each study's groups in terms of their ability to ferment rutin, known to reach the colon. The setup of the *in-vitro* faecal fermentation, which used the physiological concentration (28 μmol), measured the ileal fluid 0‑24 h after the intake of tomato juice supplemented with rutin by healthy volunteers (Jaganath *et al.*, 2006). Moreover, the measurement of the phenolic acid formation (GC-MS) was the best option to detect the differences between and within the groups.

Apart from the data that the *in-vitro* faecal fermentation experiments provided, they also supported the human feeding studies of this thesis by identifying the type of phenolic acid that formed in the colon. The identification of the main colonic metabolites of rutin metabolism need to be further investigated in terms of their colonic bioactivity in maintaining gut health involving modulating the gut microbiota and reducing colonic disease, including CRC.

A limitation of *in-vitro* faecal fermentation is that the build-up of inhibitors (degradation products) which are not representative of the colonic environment, can occur, as normally these inhibitors are reduced by colonic absorption. Raftiline (fibre) was thus added to the

fermentation medium to help mimic *in-vivo* conditions. Overall, the *in-vitro* faecal fermentation model provided information with a quick, reliable, and affordable approach.

Due to the time limitations of the PhD, the measurement of the bacterial enzymes activity or the composition of the bacteria in the *in-vitro* fermented faecal fluids was not performed. This type of measurement could have provided more information about the differences between groups in terms of their gut microbiota activity and ability to metabolise rutin. However, this could be a future project for a graduate student to test the differences between groups in terms of their bacterial enzyme activities.

The human feeding studies conducted in this thesis using the high-polyphenol diet successfully detected the difference between the groups in terms of the polyphenol intake biomarkers (urinary phenolic acid). The use of a high-polyphenol diet for three days gives a closer picture of a natural diet than using a high amount of one food item rich in polyphenols or polyphenol supplementation. As the short term period of the human feeding studies showed differences between groups, a long term study (> 8 weeks) could provide more information about the effect of the high-polyphenol intake on: 1) the pattern of the inter-individual variations; 2) the gut microbiota composition and activities; 3) effective duration of polyphenol intervention that could modulate the balance of the gut microbiota; and 4) the markers of the oxidative stress, inflammation and immunity.

The measurement of colonic fermentation biomarkers such as the faecal pH, faecal SCFA, and faecal bacterial compositions (qPCR) provide supportive evidence that the colonic conditions could have a role in the colonic metabolism of dietary polyphenols. Therefore, these measurements should be considered in all studies looking at the effect of polyphenols that usually metabolise in the colon.

The use of the Phenolic Explorer database to estimate flavonoid intake was, in our opinion, the best option for this thesis (Neveu *et al.*, 2010). This database has recent data on the influences of food processing on polyphenol contents. It also has more than 100 foods, including 161 polyphenols or groups of polyphenols before and after processing, which makes it the best tool to use for the human feeding study. However, the use of WinDiet software was more problematic. As with most dietary analysis software there was a need to enter the ingredients of recipes and different types of branded food from their manufacturer websites. This could have generated some errors.

Besides the human feeding studies and the faecal fermentation *in-vitro* experiments in this thesis, colonic biopsies could greatly contribute to our understanding about the differences between groups in terms of morphological differences and their effect on the absorption of the polyphenol metabolites. However, this will be challenging due to the ethical approval and the willingness of the participants.

6.5 Strengths

The human feeding studies provided much information regarding the influences of ethnicity, ageing, and the risk of CRC on the colonic metabolism of polyphenol biomarkers including the urinary phenolic acid (GC-MS) and urinary total phenols (Folin–Ciocalteau).The *in-vitro* faecal fermentation model afforded supportive information that rutin was metabolised by the colonic microbiota and it also identified the type of phenolic acids formed in the colon. Moreover, the effects of fibre (food matrix) and pH on the phenolic acids formation have been observed. In addition to this, the dietary habits of the participants using the FFQ, and colonic fermentation biomarkers such as the faecal pH, faecal SCFA, and faecal bacterial compositions (qPCR) were useful tools contributing to the understanding of variation in the phenolic acid urinary excretion and formation in the fermented faecal fluids.

6.6 Limitations

There were some limitations and challenges in conducting the human feeding studies, which involved recruitment, sample size, and measurements. The recruitment for the ethnicity study took longer than expected as it was difficult to find Indian people who have been in Glasgow for less than five years and were able to keep a record of their dietary habits. Indian men were more willing to participate in the study than women, which may be due to cultural barriers. Moreover, the recruitment of older healthy people took longer than expected as it was hard to find people who liked and were willing to follow a high-polyphenol diet. Many potential participants refused to participate in the study because of the high intake of fruit and vegetables especially tomatoes. In addition, the recruitment of the polypectomy patients from the endoscopy clinics was most challenging. These patients were usually stressed about their diagnosis, and as a consequence recruitment had to be expanded to include another two sites to find the requisite number of participants. In general, for all studies, the two main reasons for refusing to participate in the study were

having to collect entire bowel movements and having to follow the low-polyphenol diet for three days.

The sample size for all studies was powered to detect the differences between the groups in terms of the urinary phenolic acids and did not take into account the secondary measurements such as faecal pH, faecal SCFA, faecal ammonia, faecal bacterial compositions, and faecal calprotectin. The results of the three studies are considered as preliminary data, which may contribute to possible future research in bioavailability of polyphenols and health. Moreover, the studies limited the colonic metabolism biomarkers to the urinary phenolic acid while the measurement of the phenolic acid in the urine and faecal samples could have enhanced the overall results knowledge.

6.7 Implications for disease prevention and clinical practice

Despite the results of the studies pointed out in Chapter One, it is still too early to advise on dietary polyphenol intake as a strategy for CRC prevention. This is because of: 1) large inter-individual variation in polyphenol metabolism with no information regarding the relationship between these variations and the CRC risk; 2) limited evidence from the clinical trial studies on the intake of polyphenols and the risk of CRC (see Chapter 1); 3) No studies having looked at the bioavailability of polyphenols in individuals at risk or with CRC; and 4) the suggested evidence from the epidemiological studies that a high intake of fruit and vegetables could reduce the risk of CRC due to polyphenols, vitamins, and salicylate content (Cho *et al.*, 2004, Gorham *et al.*, 2007, Wood *et al.*, 2011, Jin *et al.*, 2012a, Woo and Kim, 2013a)

India has the lowest CRC incidence in the world. This could be attributed to their consumption of food rich in polyphenols such as tomatoes, onions, garlic, and spices (curry, cumin, turmeric, etc.) (Wood *et al.*, 2011). However, it needs to be further assessed through well designed clinical and epidemiological studies whether this decreased risk is associated with specific polyphenols, a combination of polyphenols or other factors such as salicylate which is high in fruit and vegetables. Epidemiologic evidence reported that regular and long-term use of salicylate significantly reduces the risk of CRC via its analgesic and anti-inflammatory properties (Tougeron *et al.*, 2014). Looking from another dimension, would the sources of salicylate matter? A study by Blacklock *et al.* (2001) reported that there are higher serum concentrations of salicylic acid in vegetarians than in non-vegetarians, and there was overlapping in the serum concentrations between

vegetarians and those taking aspirin (75 mg daily). On this basis, the chemo-preventive action of aspirin or dietary salicylates can have the same effect; however, the natural salicylates contribute to the other known benefits of a healthy diet.

Unlike the Indian diet, the Scottish population has a low fruit and vegetable consumption as well as low salicylate intake. The inadequate intake of fruit and vegetables as a rich source of fibre and polyphenols could be one of the reasons for the high CRC incidence in Scotland (Wood *et al.*, 2011).

Currently, due to the limited research on the intake of polyphenols and CRC, education of patients by healthcare professionals as to the benefits of following a healthy, balanced diet high in fruit and vegetables is an important strategy in the reduction of the risk of CRC and chronic disease. On the other hand, there is a need for a clinical trial to examine all nutrients that could reduce the risk of CRC including dietary polyphenols as well as their metabolism, absorption, and bioavailability both in healthy people and in patients who are at risk of or have CRC. This will help identify the potential compounds that could help reduce and prevent the recurrence of CRC and possibly other chronic diseases.

6.8 Implications for nutrition research and food industry

The data in Chapter 3 suggested that Indians could be more capable of metabolising polyphenols compared to Europeans. However, there is no available information regarding the type or amount of polyphenols that are usually consumed by the Indian population. Subsequently, further research is needed to estimate the daily intake of polyphenols in Indians to be able to compare the Indian intake with other European countries. This type of study will not only provide information to possibly incorporate polyphenols as part of pharmaceutical and nutraceutical agents but also to meet optimum polyphenol daily requirements.

The work of Chapter 4 showed ageing should be taken into consideration in all nutritional studies as a factor that could influence polyphenol bioavailability and potential therapeutic or disease-preventing effects. Moreover, the establishment of the dietary guideline for the dietary polyphenols should take into account the age range, as older people might need higher polyphenol intake compared to younger adults, especially those who are at risk of disease. Even though there is no conclusive data showing the need for older people to increase their polyphenol intake, it would be advisable for the food industry to modify and

develop their product by adding and increasing the amount of fruit and vegetables as a strategy to increase health benefits due to their high content of fibre, polyphenols and vitamins.

Nowadays, there are only a few products in the UK that claim high polyphenol content in their labels such as: 1) POM wonderful pomegranate juice which states that it contains polyphenol antioxidants which help protect the body against free radicals that can cause damage to people's bodies over time; 2) Welch's 100% purple grape juice which has been officially recognised by the HEART UK- Cholesterol charity for promoting heart health due to its contents of nutrients including polyphenols; 3) NESCAFÉ Green Blend coffee which claims that high polyphenol levels have health supporting effects to protect the body against everyday cell damage; 4) CocoaVia cocoa extract supplement which mentions that it delivers the highest concentration of flavonols per serving in a dietary supplement and is scientifically proven to help support healthy circulation, important for cardiovascular and heart health, cognitive health, skin health, blood flow, and exercise performance.

However, the question is do we need more polyphenolic products in the market? To answer this question, more clinical trial studies on the effect of polyphenol compounds on improving one's health are essential to confirm this need.

6.9 Implications for public health

This thesis further indicates a large variation in the colonic metabolism of polyphenols between individuals, as reported in previous studies. With no available dietary guideline for dietary polyphenol intake, the target intake of polyphenols needs to be based on the amount of the polyphenols achievable through the 5-A-Day fruit and vegetable programme (similar to the Eat Well programme supported by the Food Standards Agency in the UK) (Williamson and Holst, 2008). The USDA database estimated that 5-A-Day would lead to over 500 mg of polyphenols per day based on the intake of five selected key fruits and vegetables: oranges, red onions, blueberries, strawberries, and apples (Williamson and Holst, 2008). However, certain fruit and vegetables, especially those low in polyphenols such as banana, cucumber, zucchini, or sweet peppers, may provide less than 500 mg of polyphenols per day.

It could be helpful to increase individual polyphenol intake by promoting these programmes to the public, in particular the dark colour fruit and vegetables as tools for

healthier lives. However, the health benefits of five portions a day could vary between individuals due to: 1) different selection of the fruit and vegetables among individuals which will provide different types and amounts of polyphenols; 2) different ability of metabolising the bioactive compounds in the fruit and vegetables. Fruit and vegetable public health programs to increase polyphenol intake are not necessarily effective for low income people, as most of the high polyphenol fruit is expensive such as blueberries, strawberries, red grapes and pomegranates. Other cheap sources of polyphenols that showed a beneficial health effect such as cocoa, green tea, black tea, and coffee could be considered.

6.10 Future research

Based on the results of this thesis, the following further research is needed:

6.10.1 Short term studies:

1. To conduct a dietary intervention to evaluate different types of dietary polyphenols in terms of their potential treatment to reduce the risk of CRC by measuring colonic inflammatory and immunity markers.

2. To identify the bacteria involved in the colonic metabolism of polyphenols.

3. To set up a database for microbial metabolites of polyphenols according to their potential action in the colon.

6.10.2 Long term studies:

1. To launch a longitudinal study taking into account all possible factors that may have an effect on the colonic metabolism of dietary polyphenols such as ethnicity, ageing, colonic health, obesity, or/ and smoking, to be able to draw an association between the intake of dietary polyphenols, health, and chronic diseases including CRC.

2. To establish more clinical trials on the effect of dietary polyphenols known to metabolise in the colon for the prevention of CRC recurrence.

6.11 Conclusion

The data of this thesis demonstrated that ethnicity, ageing, and colonic health could be contributing to the large variation between individuals in the metabolism of dietary polyphenols which has been previously reported by *in-vivo* and *in-vitro* studies. There is a need to understand if there is a relationship between the inter-individual variation of polyphenol metabolism and the incidence of chronic disease including CRC among individuals. This is because the biological activities of dietary polyphenols are depending on their metabolism, absorption, and bioavailability. Without knowing if there is a link between the inter-individual variation of polyphenol metabolism and the risk of chronic disease including CRC, it will not be feasible to relate the inter-individual variations in polyphenol metabolism to differences in disease incidence.

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Appendices

Appendix 1: Collecting 24-hour urine samples - instructions

1. Please collect and keep all urines passed in the container provided as following:
   - from the second urine on the first day of collection
   - to the first urine passed in the morning of the following day

   This will be repeated twice during the first part of the study (after each diet), and three times during the second part of the study (beginning, middle and end of the dietary intervention)
2. Please keep the container in the cool-pack with the ice blocks provided throughout the duration of collection.
3. Collect all urine each time you urinate
4. Please write down any "mistakes" that may have occurred (such as accidentally throwing out urine) in the error form.
5. Bring all bottles to the Department of Human Nutrition at Yorkhill hospital. If you are not able to come, please contact us to arrange a time for collection.

Collecting stool samples - instructions

Please collect your stool sample in the special pot provided

This will be repeated twice during the first part of the study (after each diet), and three times during the second part of the study (beginning, middle and end of the dietary intervention) on the morning following 24-hour urine collection.

1. Place the plastic bag inside the pot and then put the pot on the top of the cardboard support (frame).
2. Place the cardboard frame on the top of the toilet seat place the pot inside the frame
3. Make sure to expel excess air from the plastic bag before closing it.
4. Fold the plastic bag over the sample, still inside the pot
5. Remove the special "Anaerocult" paper sachet from the foil pouch and then place it in the pot, on top of the plastic bag
6. Close the pot and place it in the cool bag with ice blocks.
7. Bring the sample to the Department of Human Nutrition at Yorkhill hospital. If you are not able to come, please contact us as soon as possible.

Appendix 2: Dietary record instruction

- In the first dietary intervention, please keep the food diary record with you for the 3 days of each study period.
- **Please write down ALL food or beverage that you have consumed.** Include all meals, snacks, treats between meals such as a piece of candy, and beverages.
- **Don't depend on your memory!** Record everything you eat as you go through the day.
- **Be specific.** Make sure you write down the ***extras***, for instance sauce on your spaghetti, cheese in your sandwich or vegetables, butter, and salad dressings.
- **Weigh your foods.** Measure them using the food scales provided. If it is not convenient, use the portion guide overleaf.
- **List all ingredients in mixed dishes**, like salad or soup, and try to write down all of the ingredients and estimated amounts.
- **In column 1**, write in detail about each food item such as:
    - Food brand or type
    - Fresh, dried, canned, frozen, or salted, etc.
    - Write the method of cooking (fried, baked, boiled, etc.)
- **In column 2**, please write the portion size weight of your foods.
- **In column 3**, please record the time of each meal and snack.

4.

5. In the second dietary intervention, please record your dietary intake for 3 periods (beginning, middle, and end of the study), each period last 3 days. The diet that you will follow over the first three days will need to be repeated during the following two diet-recording periods to limit variability. Make sure that you consume food that you are used to, to enable easy replication.

Dietary record form

Participant study number:
Diet / Part:
Date:

	Food / drink	Portion size / volume / weight	Time
Breakfast			
Snack			
Lunch			
Snack			
Dinner			
Snack			

Appendix 3: Food frequency questionnaire

To be filled by researcher:
Date:

Researcher:

Participant Number: □□□

To be filled by participant:

Gender: □ **Male** □ **Female**
Date of Birth: ____/____/___
Home Postcode: _____

Are you vegetarian: □ **Yes** □ **No**
Are you vegan: □ **Yes** □ **No**
Ethnicity: □ **White European**
□ **Mixed**
□ **Asian or Asian British**
□ **Black or Black British**
□ **Other Ethnic Groups, please specify:**

_____

2. Do you move your bowels?

Twice daily or more	Daily	Every 2-3 days	Less than twice a week

3. How often do you drink these beverages? Tick only <u>one</u> column per row.

	Per day (times)				Per week			Per month	
	6+	4-5	2-3	once	5-6	2-4	once	1-3	Less than once
Green tea									
Black tea									
Coffee									
Hot chocolate, cocoa									
Herbal infusions									
Other hot drink (maté tea)									
Milk drinks (including milk in breakfast cereals)									
Soya milk									
Rice or Oat milk									
Cola and fizzy drinks									
Cordial and flavoured waters									
Orange juice									

Fruit smoothies										
Other fruit juices										
Red wine										
White wine										
Beers										
Liquors										
Energy drinks										

	Per day (times)				Per week			Per month	
FRUITS &VEGETABLES	6+	4-5	2-3	once	5-6	2-4	once	1-3	Less than once
Tomatoes									
Onions									
Carrots									
Broccolli									
Brussel sprouts, cabbage, kale									
Peas, mangetout									
Green beans, runner beans									
Marrow, courgette									
Spinash									
Cauliflower									
Parsnip, turnips, sweede									
Leeks									
Mushrooms									
Sweet peppers									
Bean sprouts									
Sweetcorn									
Avocado									
Lentils and dried beans									
Soya, tofu									
Apples									
Pear									
Oranges, satsumas, mandarins									
Grapefruit									
Banana									
Grapes (handful)									
¼ Melon									
Apricot, peaches									
Berries (strawberries, raspberries, blackberries)									
Other fresh fruits									
Dried fruits (e.g. raisins, apricots, dates)									

	Per day (times)				Per week			Per month	
PREPARED MEALS	6+	4-5	2-3	once	5-6	2-4	once	1-3	Less

(homemade or bought)									than once
Onion or tomato-based dishes (pizza, pasta bakes, lasagne, moussaka…)									
Onion or tomato-based curries									
Chili con carne									
Tomato or onion soup									
Other soup									
Soya and bean-burgers									

	Per day (times)				Per week			Per month	
EGGS, FISH & MEATS	6+	4-5	2-3	once	5-6	2-4	once	1-3	Less than once
Eggs (boiled, fried, or omelette)									
White fish, non-fried									
White fish, fried (fish & chips, fish fingers)									
Oil-rich fish (canned and fresh, eg tuna, salmon)									
Shellfish (eg. crab, mussel, oysters)									
Beef									
Chicken									
Pork and pork products (e.g. bacon, sausages)									
Lamb									

	Per day (times)				Per week			Per month	
POTATOES, PASTA, RICE, BREADS	6+	4-5	2-3	once	5-6	2-4	once	1-3	Less than once
Wholemeal pasta									
White pasta									
Brown rice									
White rice									
Fried potatoes									
Boiled potatoes									
Muesli									
Porridge									
Other breakfast cereal									
Crisps and crackers									
Wholewheat / brown bread									
White bread									

	Per day (times)	Per week	Per month

SWEETS & TREATS	6+	4-5	2-3	once	5-6	2-4	once	1-3	Less than once
Almonds and other nuts									
Peanut butter, tahini									
Chocolate spread (e.g. nutella)									
Chocolate (sweets)									
Jam, marmelade									
Plain biscuits, cakes									
Chocolate or fruit-based biscuits, cakes									
Other sweets									

	Per day (times)				Per week			Per month	
DAIRY & FATS	6+	4-5	2-3	once	5-6	2-4	once	1-3	Less than once
Full fat / greek yoghurt									
Low fat yoghurts									
Live yoghurts (e.g. Activia, Actimel)									
Dairy dessert									
Hard cheese									
Soft cheese, fromage frais									
Ice cream									
Butter (1 teaspoon)									
Lard (1 teaspoon)									
Margarine (1 teaspoon)									
Cream (single or double)									

	Per day (times)				Per week			Per month	
SEASONINGS & SAUCES	6+	4-5	2-3	once	5-6	2-4	once	1-3	Less than once
Pepper (black, white)									
Pesto									
Tomato ketchup									
Garlic									
Tomato sauce									
Fresh herbs (mint, parsley, thyme, basil, coriander)									
Dried herbs									
Mustard, horseradish									
Vinegar									
Olive oil									
Olives									
Capers									
Spices (turmeric, cumin seeds, fennel seeds, etc…)									
Chili powder or fresh chilli									
Pickles, chtuney									

Marmite, Bovril											
Ginger											

4 In summary:

(a) how many times do you eat fruit and vegetables (not including potatoes)

☐☐ per day OR ☐☐ per week OR ☐☐ per month

(b) how many times do you drink fruit juices?

☐☐ per day OR ☐☐ per week OR ☐☐ per month

(b) how many times do you drink tea or coffee?

☐☐ per day OR ☐☐ per week OR ☐☐ per month

(b) how many times do you eat spicy food?

☐☐ per day OR ☐☐ per week OR ☐☐ per month

Thank you for filling this questionnaire.

Appendix 4: Low polyphenol diet

Meals	Examples of food items
Breakfast	Cheese Sausage, Bacon Fish Toast, bread, Croissant (NO chocolate), Scones Waffles, pancakes with butter and sugar (NO jam) Butter Milk Biscuits (NOT wholemeal) Rice based cereals (NOT Coco pops)
Lunch & Dinner All meats, fish, eggs, cheese and spreads. White rice Pasta Noodles Potatoes without skin	Tuna, chicken and egg sandwiches (mayonnaise ok) Burger and chips (NO ketchup, relish, gherkins) Sausage rolls White pasta and cheese / cream Chicken / sausages and mashed potatoes (NO gravy) Omelette (with cheese, ham) Potatoes without skin Meats (NO ketchup, brown sauce) Cheese and cream cheese (NO garlic or onion-based cheeses) Fish and chips with salt and vinegar (NO ketchup) Sardines on white toast (no tomato sauce) Chicken fried rice with eggs and oyster sauce (NO soya Products or vegetables) Roast Chicken/ prawns with white rice Chicken nuggets and chips Roast Beef Sandwich with crisps Salmon with white rice Steak and mashed potatoes
Snacks	Biscuits (NOT wholemeal, chocolate, or fruit contains) Shortbreads Custard rice puddings Crisps (ready salted), Salted rice crackers Plain Frozen Yoghurt Cheese and crackers Plain donut without chocolate or fruit fillings Rice cakes
Drinks	Water, Milk, and Irn-bru

Appendix 5: High polpyhenol diet

	Day 1	Day 2	Day 3
Breakfast	1 plum 1 glass of purple grape juice 1 cup of black tea	1 plum 1 glass of purple grape juice 1 cup of black tea	1 plum 1 glass of purple grape juice 1 cup of black tea
Snack	One line of dark chocolate (85%) 1 cup of black tea	One line of dark chocolate (85%) 1 cup of black tea	One line of dark chocolate (85%) 1 cup of black tea
Lunch	5 Cherry tomatoes 1 can of tomato soup 1 plum	5 Cherry tomatoes 1 can of tomato soup 1 plum	9 Cherry tomato 1 can of fresh onion soup 1 plum
Snack	1 cup of black tea One line of dark chocolate (85%)	1 cup of black tea One line of dark chocolate (85%)	1 cup of black tea One line of dark chocolate (85%)
Dinner	20 g Sun dried tomatoes (~5) 5 black olives Pasta and tomato sauce (see recipe) 1/3 punnet of raspberry Glass of Purple grape juice	20 g Sun dried tomatoes (~5) 5 black olives Chicken Balti ready meal 1/3 punnet of raspberry Glass of purple grape juice	20 g Sun dried tomatoes (~5) 5 black olives Rice & tomato sauce 1/3 punnet of raspberry Glass of purple grapes juice
Snack	Cup of black tea One line of dark chocolate (85%)	Cup of black tea One line of dark chocolate (85%)	Cup of black tea One line of dark chocolate (85%)

Appendix 6: Ethical application for chapter three (Ethnicity study)

Dr Emilie Combet
Section of Human Nutrition, Above A&E
Yorkhill Hospital
Dalnair Street
Glasgow
G3 8SJ

Dear Dr Combet

Medical Faculty Ethics Committee

Project Title: ***Colonic metabolism of dietary polyphenols: A 'pilot' study to understand inter-individual variations in the colonic metabolism of dietary polyphenols.***

Project No.: ***FM04509***

The Faculty Ethics Committee has reviewed your application and has agreed that there is no objection on ethical grounds to the proposed study now that the requested revisions have been incorporated. They are happy therefore to approve the project, subject to the following conditions:

- The research should be carried out only on the sites, and/or with the groups defined in the application.
- Any proposed changes in the protocol should be submitted for reassessment, except when it is necessary to change the protocol to eliminate hazard to the subjects or where the change involves only the administrative aspects of the project. The Ethics Committee should be informed of any such changes.
- If the study does not start within three years of the date of this letter, the project should be resubmitted.
- You should submit a short end of study report to the Ethics Committee within 3 months of completion.

Yours sincerely

Dr Una MacLeod
Faculty Ethics Officer

Dr U MacLeod
Clinical Senior Lecturer

General Practice & Primary Care, Division of Community Based Sciences, University of Glasgow, 1 Horselethill Road, Glasgow, G12 9LX

Tel: 0141 330 8328
E-mail: u.macleod@clinmed.gla.ac.uk

Appendix 7: Ethical application for chapter four (Ageing study)

Miss Areej Alkhaldy
Section of Human Nutrition, Above A&E
Yorkhill Hospital
Dalnair Street
Glasgow, G3 8SJ

Dear Miss Alkhaldy

Medical Faculty Ethics Committee

Project Title: Colonic Metabolism of Polyphenol Components in Older Adults.

Project No.: FM03110

The Faculty Ethics Committee has reviewed your application and has agreed that there is no objection on ethical grounds to the proposed study now that the requested revisions have been incorporated. They are happy therefore to approve the project, subject to the following conditions:

- The research should be carried out only on the sites, and/or with the groups defined in the application.
- Any proposed changes in the protocol should be submitted for reassessment, except when it is necessary to change the protocol to eliminate hazard to the subjects or where the change involves only the administrative aspects of the project. The Ethics Committee should be informed of any such changes.
- If the study does not start within three years of the date of this letter, the project should be resubmitted.
- You should submit a short end of study report to the Ethics Committee within 3 months of completion.

Yours sincerely

Dr David Shaw
Faculty Ethics Officer

Dr D Shaw
Lecturer in Ethics & Ethics Officer

School of Medicine, University of Glasgow, 378 Sauchiehall Street, Glasgow, G2 3JZ

Tel: 0141 211 9755
E-mail: david.shaw@glasgow.ac.uk

Appendix 8: Ethical application for chapter five (CRC study)

Coordinator: NSIBR
Telephone Number: 0141-211-8544
E-Mail: Nathaniel.Britain@ggc.scot.nhs.uk
Website: www.nhsggc.org.uk/r&d

NHS
Greater Glasgow and Clyde

R&D Management Office
Western Infirmary
Tennent Institute
1st Floor 38 Church Street
Glasgow, G11 6NT

9 July 2012

Dr Campbell Roxburgh
Academic Unit of Surgery
4th floor, Walton Building
Glasgow Royal Infirmary
84 Castle Street
Glasgow
G4 0SF

NHS GG&C Board Approval

Dear Dr Roxburgh

Study Title:	**Colonic Metabolism of Dietary Polyphenols in Patients diagnosed with colorectal adenomas and adenocarcinomas: Anti-inflammatory effects and impact on oxidative stress.**
Principal Investigator:	**Dr Campbell Roxburgh**
GG&C HB site	**Glasgow Royal Infirmary**
Sponsor	**NHS Greater Glasgow & Clyde**
R&D reference:	**GN12SU184**
REC reference:	**12/WS/0123**
Protocol no: (including version and date)	**Version 1 dated 05/04/2012**

I am pleased to confirm that Greater Glasgow & Clyde Health Board is now able to grant **Approval** for the above study.

Please note that **CRF approval is still pending, and Miss Areej Alkhaldy and Dr Emilie Combet are unable to work on the study until their Research Passport Applications have been processed, and Honorary Research Contracts issued** by Greater Glasgow & Clyde Health Board.

Conditions of Approval

1. **For Clinical Trials** as defined by the Medicines for Human Use Clinical Trial Regulations, 2004
   a. During the life span of the study GGHB requires the following information relating to this site
      i. Notification of any potential serious breaches.
      ii. Notification of any regulatory inspections.