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Factors affecting gut metabolism and bioavailability of orange juice flavanones in humans

Min Hou

A thesis submitted for the degree of Doctor of Philosophy *to*

School of Medicine,
College of MVLS,
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Dedicated to my families who supported me through my academic education

Abstract

Background: Several studies have supported a beneficial role of dietary flavonoids in reducing the risk/progression of chronic diseases (including hypertension, cardiovascular disease, certain cancers, type-2-diabetes, cognitive dysfunction, age-related bone disease). Their beneficial properties are likely to be affected by their structure, distribution in foods, food matrix, life habits (physical activity). Most dietary polyphenols reach the colon where they are metabolized to phenolic acids by gut bacteria. Lack of knowledge of the factors affecting flavonoid metabolism and bioavailability hinders understanding of their health effects. Therefore, this thesis aimed to investigate the effect of factors on bioavailability and metabolism of dietary polyphenols from orange juices (OJ) in *in vitro* and *in vivo* studies.

Studies: In chapter 3, the variability of orange juice polyphenolic content was assessed. In addition, in vitro models of the human gastrointestinal tract were used to evaluate the release and metabolism of flavanones from orange juices (smooth OJ and OJ with pulp 'bits'). There was no difference in fibre content between smooth OJ and OJ 'with bits'. Total phenols were significantly higher in OJ 'with bits' than smooth OJ (225.9 ± 16.7 µg GAE/ml vs. 208.4 ± 10.7 µg GAE/ml, $p < 0.05$). Hesperidin content correlated with total flavonoids (Pearson correlation coefficient = 0.67) and antioxidant capacity (Pearson correlation coefficient = 0.86). Hesperidin in smooth OJ was more stable in gastric fluid than OJ 'with bits' (28.6 ± 1.3 mg/250 ml vs. 22.0 ± 0.6 mg/250 ml, $p < 0.05$). In the small intestine model, Hesperidin and narirutin were similar in the small intestinal phase. However, narirutin decreased in both juices (OJ; $p < 0.018$ and OJ with bits ($p < 0.009$). Total phenolic content decreased to 104.1 ± 13.5 mg GAE/250 ml and 112.3 ± 0.6 mg GAE/250 ml respectively, and antioxidant capacity decreased by 56.1 % and 41.6 %. In the fermentation model hesperidin was higher for smooth OJ at 2 h (89.6 ± 9.5 µM vs. 77.1 ± 8.9 µM, $p< 0.05$). There was higher production of 3, 4-dihydroxyphenylpropionic acid at 6 h with OJ 'with bits' (8.6 ± 2.8 µM vs. 3.4 ± 1.6 µM, $p < 0.05$). pH reduced to 4.5 after 24 h fermentation with OJ. However, there was no change of total phenol and antioxidant capacity.

In chapter 4, to explain previously published data of reduced urinary phenolic acids after OJ and yoghurt (Y) in humans compared to OJ alone, (Roowi et al., 2009a), *in vitro* models of the human gut were used. OJ or OJ with bits and Y were subjected to gastric, small intestinal and colonic phases. Hesperidin was increased by 1.5 fold in OJ and 1.8 fold in OJ bits after gastric digestion and by 1.4 fold in OJ bits after the small intestinal digestion with Y. In

addition, Y increased four phenolic acids (3, 4-dihydroxyphenylacetic acid, 3-hydroxy-4-methoxyphenyl propionic acid, 3-methoxy-4-hydroxyphenyl propionic acid, 3, 4-dihydroxyphenylpropionic acid) from 0.6 ± 0.0 mM to 6.1 ± 2.0 mM in the upper GI digestion with OJ bits. FY significantly increased four phenolic acids in OJ during upper GI digestion compared with Y and PY, but did increase phenolic acids in OJ bits compared with PY. Hesperidin degradation reached 44 ± 11 % and 39 ± 15 % respectively from OJ and OJ bits with Y after 24 h fermentation. However, Y did not affect hesperetin, naringenin and phenolic acids. On the other hand, Y increased total phenolic contents from 104.1 ± 13.5 mg GAE/250 ml to 339.4 ± 1.5 mg in OJ and from 112.3 ± 0.6 mg to 352.4 ± 12.0 mg in OJ bits after upper GI digestion ($p < 0.05$). This does not explain the results of the human study. In chapter 5, raftiline (1g /50 ml; selectively used by bacteria) and glucose (0.5/50ml; used by a wide range of bacteria) were tested for effects on metabolism of hesperidin (flavanone not OJ). Raftiline reduced the metabolism of hesperidin by gut bacteria. Hesperidin recovery was 35.2 ± 4.1 µM and 27.6 ± 6.1 µM after 24 h fermentation with or without raftiline respectively and 0.4 ± 0.2 µM and 0.2 ± 0.1 µM of hesperetin production. Raftiline significantly decreased phenolic acids from 220.3 ± 46.8 µM to 126.6 ± 44.1 µM ($p < 0.05$). However, glucose had no effect on phenolic acids at 24h but increased production from 38.0 ± 6.8 µM to 62.1 ± 11.0 µM at 4 h fermentation ($p < 0.02$) suggesting increased rate of metabolism rather than increased total production. Dietary fibre inhibited degradation production of phenolic acids which may change flavanone metabolites in the colon. In chapter 6 after published cross-sectional studies suggested that athletes had different flavonoid bioavailability to sedentary individuals, an intervention study of 4 weeks moderate intensity exercise determined whether exercise affected bioavailability and metabolism of OJ flavanones in healthy sedentary females. Hesperetin metabolites significantly decreased in urine over 0-5 h after juice consumption (21.6 ± 2.8 µmol vs. 14.0 ± 2.0 µm, $p < 0.05$). Hesperidin glucuronide significantly reduced from 10.4 ± 2.9 µmol to 6.2 ± 1.7 µmol after 4 weeks exercise ($p < 0.02$), and 3'-hydrophenylacetic acid reduced from 11.8 ± 3.4 µmol to 6.0 ± 2.3 µmol ($p < 0.05$). Mouth to caecum transit time was prolonged from 3.0 ± 0.2 h to 4.5 ± 0.7 h after exercise. Thus, 4 weeks exercise decreased bioavailability of OJ flavanones probably affecting transit time, but not colonic metabolism of all flavanone metabolites.

Conclusion: The studies in this thesis showed that food sources, food matrix and physical exercise may determine the significant variations in bioavailability and metabolism of flavonoids, seen in a number of studies. These factors could result in differences in

bioactivity and bioefficacy of polyphenols, and need to be taken into account in further studies of the effects of flavanones on disease risk.

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Author's Declaration

I declare that the work in this thesis is original, and is the work of the author Min Hou. I have been solely responsible for the organisation and day to day running of this study as well as the laboratory analysis and data processing, unless otherwise referenced.

Min Hou

Supervisors' declaration

I certify that the work reported in this thesis has been performed by Min Hou and that during the period of study she has fulfilled the conditions of the ordinances and regulations governing the Degree of Doctor of Philosophy, University of Glasgow

Prof. Christine Edwards Dr. Emilie Combet Aspray

Abbreviations

BMI	Body Mass Index
CVD	Cardiovascular Diseases
CHD	Coronary Heart Disease
FY	Strawberry purėe (5 %) containing yoghurt
FRAP	Ferric Reducing Antioxidant Potential
Glu	Glucose
GI	Gastrointestinal
GC-MS	gas chromatography tandem mass spectrometry
HPLC-PDA	high performance liquid chromatography photo-diode array
HPLC-MS	high performance liquid chromatography mass spectrometry
MCTT	Mouth to caecum transit time
NEP	Non-extractable polyphenols
NDC	Non-digestible carbohydrates

OFN	Oxygen-free nitrogen
OJ	Smooth Orange Juice
OJ bits	Orange Juice ‘with bits’
PY	Probiotc containing yoghurt
Raf	Raftiline
Y	Full Fat Natural Yoghurt

CHAPTER 1 *Literature Review*

1.1 Orange juice and Health

Consumption of fruits and vegetables has been reported to reduce the risk of non-communicable diseases such as cardiovascular disease and cancer (WHO, 2014). Fruits and their juices have been increasingly considered an integral part of a healthy diet (Niva, 2007), although juice is not recommended due to its high sugar content (Malik et al., 2006). The WHO report (2015) recommended adults and children to have restrict free sugars to less than 10 % of their total energy intake (Organization, 2015). In the recent SACN report (2015) which used more stringent criteria for evidence, the conclusions on the relationship of sugar containing drinks with chronic disease such as obesity and type II diabetes were less definite but it was recognised that increasing sugar intake increases energy intake in individuals and there is increasing evidence for effects on dental caries and some chronic disease. They recommend that "the population average of free sugars should not exceed 5% of total dietary energy for age groups from 2 years upwards" and "the consumption of sugars-sweetened beverages should be minimised, in both children and adults" (SACN, 2015). From 2000 to 2013, over 10 billion litres of fruit juice and nectar were consumed in the European Union (European Fruit Juice Association 2014 Market Report). By 2019, intake is expected to stabilize at about 9 billion litres consumed by EU countries, among which Germany, France, the UK, Spain and Poland are the top five markets (European Fruit Juice Association 2014 Market Report) (Figure 1-1).

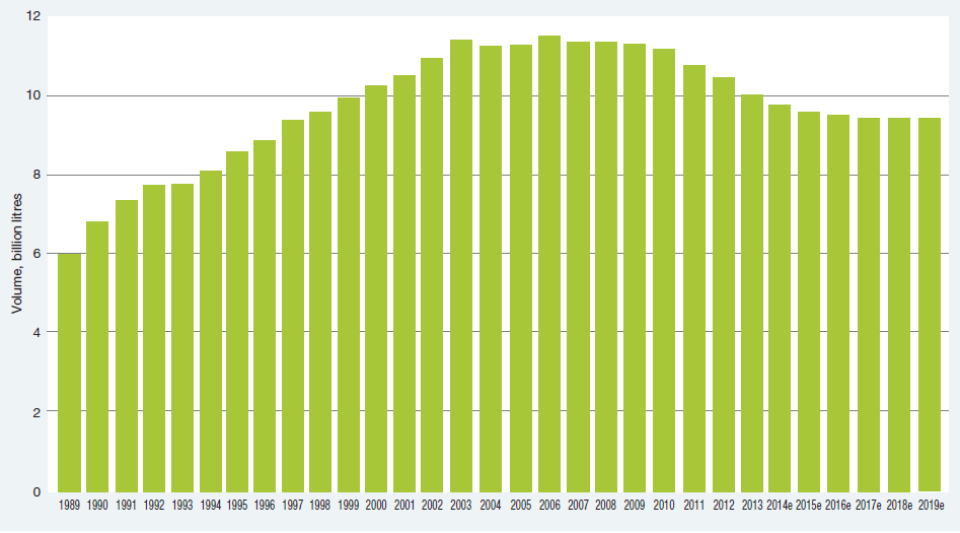


Figure 1-1 Total EU fruit juice and nectar consumption from 1989 to 2019e

(Source: European Fruit Juice Association 2014 Market Report)

These fruits and juices are a source of polyphenols that have been shown to have anti-inflammatory (Coelho et al., 2013, Jung et al., 2009, Marchi et al., 2014, Murakami et al., 2000), anti-oxidative (Dussossoy et al., 2011, Kuntz et al., 2014), anti-hypertensive (Asgary et al., 2014), and lipid-lowering actions (Aptekmann and Cesar, 2013, Ravn-Haren et al., 2013) in clinical and experimental studies and their consumption has been associated with reduced risk of chronic diseases in epidemiological studies, such as cardiovascular disease (Bahorun et al., 2012, Lai et al., 2015), some cancers (Gates et al., 2007, Kolonel et al., 2000, Simons et al., 2009, Zamora-Ros et al., 2014), type 2 diabetes (Jacques et al., 2013, Wedick et al., 2012), Parkinson disease(Gao et al., 2012), and aging (Maras et al., 2011).

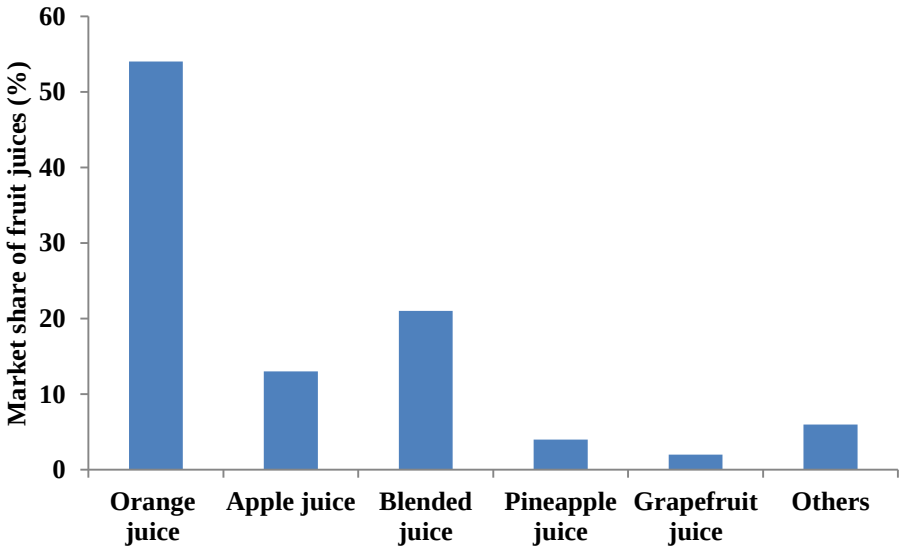


Figure 1-2 Market share of fruit juice in the United Kingdom in 2013

(Zenith International Statista 2014)

Orange juice is a key source of fruit juice rich in flavonoids, especially flavanones (Kelebek et al., 2009) (Figure 1-3). Flavanones have been shown to have the properties mentioned above in clinical and experimental studies, and consumption of orange juice has been associated with reduced risk of chronic diseases in epidemiological studies. For example, consumption of orange juice or flavanone intake has been associated with reduced risk of cardiovascular diseases and mortality (Mink et al., 2007b, Vinson et al., 2002), certain cancers (colon and ovarian cancer (Cassidy et al., 2014, Miyagi et al., 2000), enhanced cognitive function (Kean et al., 2015b), modulated inflammatory markers, such as interleukin-1β (IL-1β), tumour necrosis factor-α (TNF-α) and monocyte chemoattractant protein-1 (MCP-1) (Choi and Lee, 2010), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-10 (IL-10), inactivated nuclear factor-kappaB and mitogen-activated protein

kinases (Park et al., 2012), reduced diastolic blood pressure and LDL-cholesterol (Aptekmann and Cesar, 2013), improved postprandial microvascular endothelial reactivity (Morand et al., 2011), and increased antioxidant capacity (Constans et al., 2014, Rangel-Huerta et al., 2015b), inhibited bone loss and decreased serum and hepatic lipids (Chiba et al., 2003), affected leukocyte gene expression (Milenkovic et al., 2011). The evidence for these potential actions is discussed in detail later in this chapter (Section 1.9.2 and Tables 1-4, 1-5). Thus ingestion of orange juice may be a good way to prevent disease but we need to ensure the most efficient effects without increasing sugar intake which may instead increase risk, since caloric intake has been linked to oxidative and inflammatory stress (Dandona et al., 2010). Thus this thesis explores factors, such as food source, food matrix and physical activity, which influence the bioavailability of flavonoids from orange juice and their resultant phenolic acids produced in the gut to potentially enhance benefits of orange juice flavanones in human health.

1.2 Flavonoids

Plant polyphenols are common secondary metabolites involved in inhibition of pathogens and predators in plants (Bravo, 1998), and protection of the plant from other damaging exposures (Sharma et al., 2012). Flavonoids are the most studied due to their bioactive properties in humans, and belong to one subclass of polyphenols with common structural features (C15 phenylchromane core, composed of a C6-C3-C6 backbone) (Figure 1-3). Fruits, vegetables, grains and their processed products are main sources of flavonoids in the human diet (Middleton Jr, 1998). These flavonoids are categorized into subclasses based on their molecular structure. Main sources of specific flavonoids are illustrated in Table 1-1.

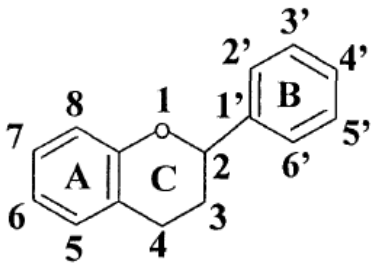


Figure 1-3 General structure of flavonoids

(Cook and Samman, 1996a).

Table 1-1 Structures and food sources of flavonoids

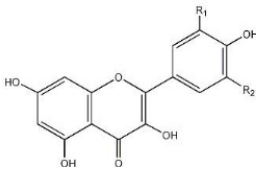
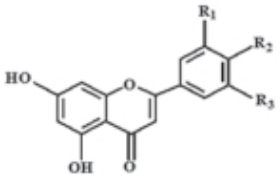
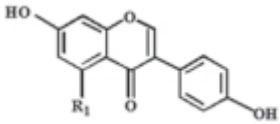
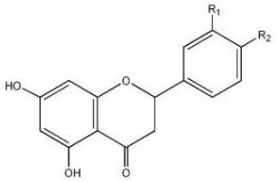
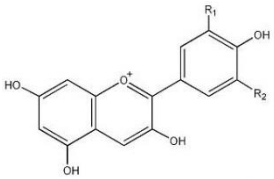
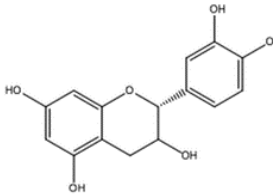
Flavonoids	**Structure**	**Compounds**	**Source**
Flavonols		Keampferol (R_2=OH) Quercetin (R_1=R_2=OH) Isorhamnetin (R_1=OCH_3, R_2=OH) Myricetin (R_1=R_2=R_3=OH)	Leak, Broccoli, Lettuce, Tomato, Blueberry, black currant, Apple, Beans, Red wine
Flavones		Apigenin (R_2=OH) Luteolin (R_1=R_2=OH)	Parsley, Celery
Isoflavones		Daidzein (R_1=H) Genistein (R_1=OH) Glycitein (R_1=OCH_3)	Soybean
Flavanones		Hesperetin (R_1=OH, R_2=OCH_3) Naringenin (R_2=OH); Eriodictyol (R_1=R_2=OH)	Citrus fruits and juices
Anthocyanidins		Cyanidin (R_1=OH) Pelargonidin (R_1=R_2=H) Peonidin (R_1=OCH_3) Dlphinidin (R_1=R_2=OH) Petunidin (R_1=OCH3,R_2=OH) Malvidin (R_1=R_2=OCH_3)	Blackberry, Blueberry, Black grape, Cherry, Rhubarb, Strawberry, Plum, Red wine, Cocoa
Flavan-3-ols		Catechin Epicatechin	Cocoa, Grape, Peach Apricot, Apple, Blackberry, Green tea, Black tea, Red wine, Cider

Table was drawn based on previous studies (Tripoli et al., 2007b, Manach et al., 2004)

Flavonoids are usually present in food as glycosides. The common sugar moieties include D-glucose and L-rhamnose. O-glycosides are major glycosides bound to the hydroxyl group at C-7 or C-3. The sugar moiety influences absorption and bioavailability of flavonoids in the

host. Quercetin glucoside was 20 times higher than quercetin rutinoside in terms of maximal concentration of quercetin (C_{max}) in plasma, and 10 times faster to reach C_{max} (T_{max}) in men, and bioavailability of quercetin rutinoside was 5 times lower than quercetin glucoside (Hollman et al., 1999). Some researchers have applied enzymatic treatments to enhance the bioavailability. For example, bioavailability as measured by urinary excretion of flavanone metabolites was increased from 7 % to 47 % by treatment of orange juice with α-rhamnosidase (Bredsdorff et al., 2010). The concentration of hesperetin metabolites in plasma was enhanced by two fold in humans through treatment of orange juice with hesperidinase (Nielsen et al., 2006a).

1.2.1 Flavanones in citrus fruits and juices

Citrus fruits and juices contain several potentially bioactive molecules including vitamin C, folate, dietary fibre and flavonoids, especially flavanones (Tripoli et al., 2007a). It is important to consider potential confounding factors when studying possible effects of flavonoids on health. The most abundant flavanone is hesperidin, followed by narirutin and didymin, which are conjugated with rutinosides (Gattuso et al., 2007). Other flavanones are found in very low concentration. Hesperidin and narirutin are naturally present as hesperetin-7-*O*-rutinosides and naringenin-7-*O*-rutinosides (Figure 1-4).

Citrus peel and seeds also contain a great number of phenolic acids and flavonoids (Molina-Calle et al., 2015),including non-extractable polyphenols that are closely associated with cell wall constituents (polysaccharides and protein) (Arranz et al., 2010, Hasni et al., 2011, Yuksel et al., 2010), and are not easier to detect by the usual analytical procedures (Perez-Jimenez et al., 2013). Hesperidin and narirutin are abundant in common sweet oranges (Sawalha et al., 2009). Their chemical structures are illustrated in Figure 1-4. Lemon juices and sour oranges contain more eriocitrin and neoeriocitrin respectively (Sawalha et al., 2009). Flavanone rutinosides are tasteless, whereas flavanone neohesperidoside, such as hesperetin-7-O-neohesperidoside (neohesperidin) in bitter orange and naringenin-7-O-neohesperidoside (naringin) in grapefruit, are intensely bitter (Tomás-Barberán and Clifford, 2000). Bitterness of grapefruit fruits and juices is attributed to naringin and neohesperidin (Davis, 1947, Fisher and Wheaton, 1976).

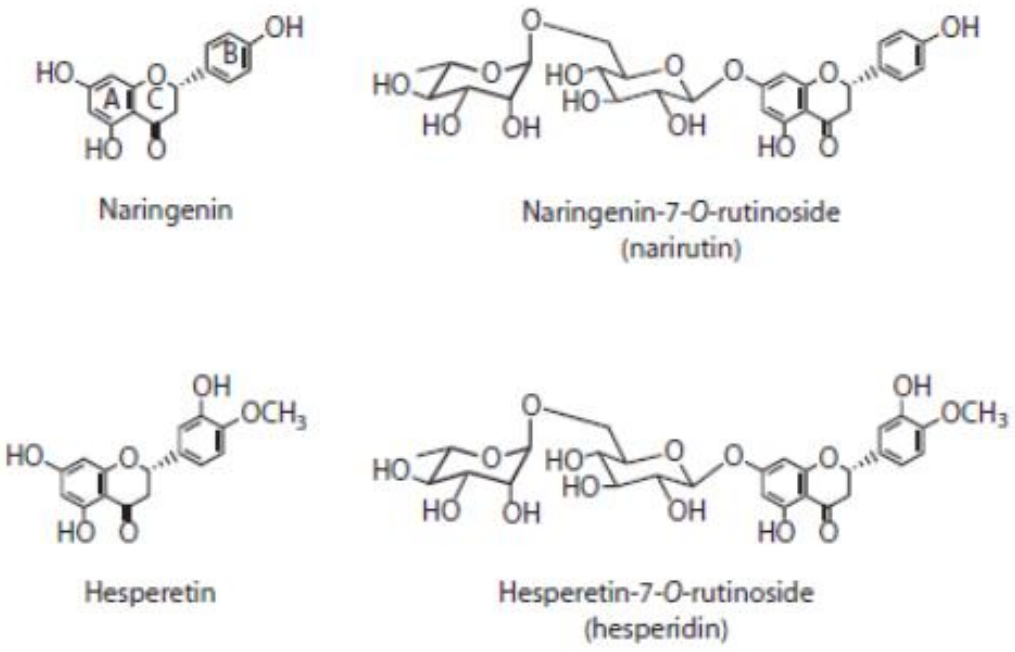


Figure 1-4 Structures of hesperetin and naringenin glycosides
(Wei et al., 2004).

1.3 Phenolic acids

Phenolic acids (Figure 1-5 A), including benzoic acid derivatives and cinnamic acid derivatives, are present in plants, such as *p*-coumaric acid (4-hydroxycinnamic acid), caffeic acid (3,4-dihydroxycinnamic acid), ferulic acid (4-hydroxy-3-methoxycinnamic acid), and sinapic acid (4-hydroxy-3,5-dimethoxycinnamic acid) and their esters with quinic and tartaric acids (chlorogenic, caftaric, etc.) (Manach et al., 2004), but are also metabolites produced in the body from polyphenols.

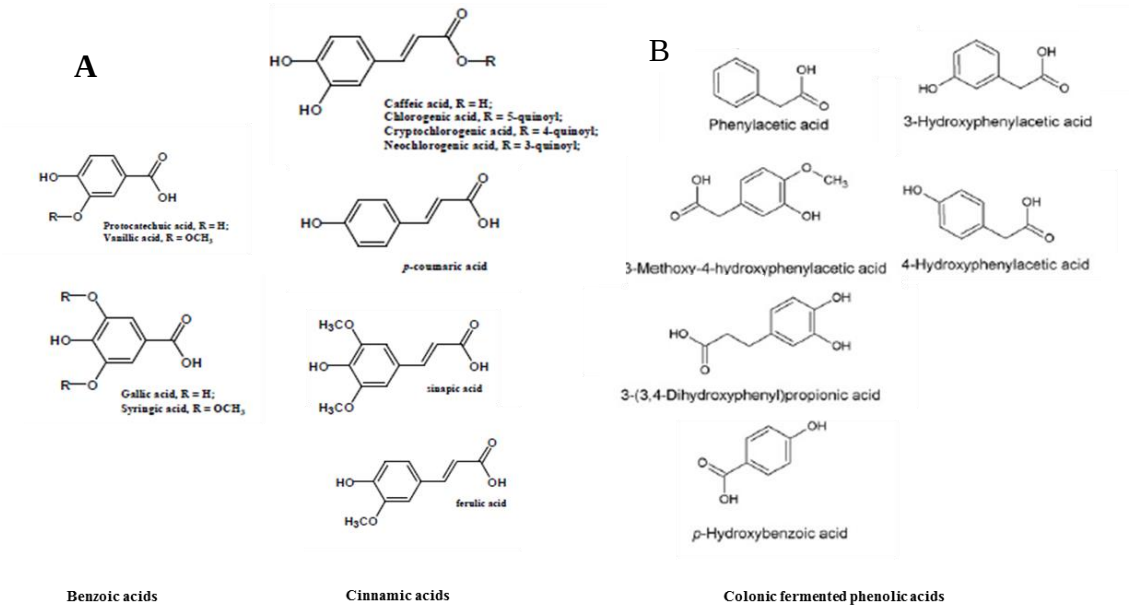


Figure 1-5 Phenolic acids in plants (A) and in biological fluids (B) derived from flavonoids by gut microbiota

(Manach et al., 2004, Crozier et al., 2010).

Phenolic acids identified in biological fluids (urine, plasma) as metabolites after ring fission of flavonoids by the gut microbiota, include phenylpropionic acids, phenylacetic acids, benzoic acids and hippuric acids (Figure 1-5 B) (Serra et al., 2011, Crozier et al., 2010, Lafay and Gil-Izquierdo, 2008, Tsao, 2010).

Colonic transformations of flavonoids into phenolic acids have been widely investigated. Most of these studies focused on *in vitro* incubation of specific flavonoids with human gut bacteria considering ring-cleavage, decarboxylation, demethylation, and dehydroxylation reactions (Selma et al., 2009, Aura, 2008, Laparra and Sanz, 2010, Rechner et al., 2002) (Table 1-2). For example, Selma et al showed that quercetin could be degraded into 2-(3,4-dihydroxyphenyl)acetic acid, 2-(3-hydroxyphenyl)acetic acid and 3,4-dihydroxybenzoic acid from the B ring, 3-(3,4-dihydroxyphenyl) propionic acid and 3-(3-hydroxyphenyl)propionic acid (Selma et al., 2009). Myricetin can be transformed into 2-(3,5-dihydroxyphenyl)acetic acid, 2-(3-hydroxyphenyl)acetic acid, and 2-(3,4,5-trihydroxyphenyl)acetic acid, and kaempferol was mainly degraded into 2-(4-hydroxyphenyl)acetic acid (Rechner et al., 2002). Phloroglucinol, 3-(3,4-dihydroxyphenyl)propionic acid and 3-(m-hydroxyphenyl)propionic acid were derived from naringin by human colonic microflora, while 3-hydroxyphenylacetic acid and 3-(3-hydroxyphenyl)-propionic acid was the major phenolic end products from rutin (Rechner et al., 2004). 2-(p-hydroxyphenyl)acetic acid, 2-(p-hydroxyphenyl)-propionic acid,

2-(m-hydroxyphenyl)acetic acid, 2-(m-hydroxyphenyl)propionic acid, 5-(m-hydroxyphenyl)valeric acid, and phenylpropionic acid were found by incubation of proanthocyanidins with human gut bacteria (Déprez et al., 2000). 2,4-dihydroxyphenylacetic acid, resorcinol and phlorogpucinol were found in fermentation of hesperidin by gut bacteria (Kim et al., 1998). (−)-Epicatechin and (+)-catechin could be degraded into 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, 5-phenyl-γ-valerolactone, and 3-phenylpropionic acid by human gut mictrobiota (Tzounis et al., 2008). Pyrogallol, 5-(3,4-dihydroxyphenyl)valeric acid, 5-(3-hydroxyphenyl)valeric acid, 3-(3,4-dihydroxyphenyl)propionic acid, 3-(3-hydroxyphenyl)propionic acid, 3-(3-methoxyphenyl)valeric acid, and 2,3-dihydroxyphenoxyl 3-(3′,4′-dihydroxyphenyl)propionic acid were derived from epicatechin by human intestinal bacteria (Meselhy et al., 1997). Caffeic acid was degraded into 3-hydroxyphenylpropionic acid and benzoic acid (Gonthier et al., 2006).

1.4 Metabolism of flavonoids

The bioavailability of flavonoids is an important factor determining their bioactivity and beneficial effects in cells and tissues (Silberberg et al., 2005). It is important to understand how the flavonoids are absorbed and metabolised in the gut and the form which reaches the systemic blood and tissues (Figure 1-5). The bioavailability of flavonoids has been investigated by measuring metabolites in plasma and urine, and flavonoid metabolites in tissues in animal models (Mullen et al., 2002, Suganuma et al., 1998, El Mohsen et al., 2006). Furthermore, the ranges from 11.7 % of peonidin-3, 5-*O*-diglucoside to 103.3 % of catechins and epicatechins in ileal fluid after ingesting a juice drink containing a diversity of dietary (poly)phenols derived from green tea, apples, grapes and citrus fruit by ileostomists (Borges et al., 2013), indicated that less anthocyanidins and more catechins could reach the colon.

Table 1-2 Phenolic metabolites produced from flavonoids

Flavonoids	Evidence	Parent Compound	Metabolites (phenolic acids) produced by colonic bacteria.
Flavonols	(Rechner et al., 2002)	Kaempferol	2-(4-Hydroxyphenyl)acetic acid
	(Selma et al., 2009)	Quercetin	2-(3,4-Dihydroxyphenyl)acetic acid, 2-(3-Hydroxyphenyl)acetic acid, 3,4-Dihydroxybenzoic acid, 3-(3,4-Dihydroxyphenyl) propionic acid 3-(3-Hydroxyphenyl)propionic acid
	(Rechner et al., 2002)	Myricetin	2-(3,5-Dihydroxyphenyl)acetic acid, 2-(3-Hydroxyphenyl)acetic acid, 2-(3,4,5-Trihydroxyphenyl)acetic acid
	(Rechner et al., 2004)	Rutin	3-Hydroxyphenylacetic acid 3-(3-Hydroxyphenyl)-propionic acid
Flavanones	(Kim et al., 1998)	Hesperidin	2,4-Dihydroxyphenylacetic acid, resorcinol Phlorogpucinol
	(Rechner et al., 2004)	Naringin	Phloroglucinol, 3-(3,4-Dihydroxyphenyl)propionic acid 3-(m-hydroxyphenyl)propionic acid
Anthocyanidins	(Déprez et al., 2000)	Pro anthocyanidin	2-(p-hydroxyphenyl)acetic acid, 2-(p-hydroxyphenyl)-propionic acid, 2-(m-hydroxyphenyl)acetic acid, 2-(m-hydroxyphenyl)propionic acid, 5-(m-hydroxyphenyl)valeric acid, phenylpropionic acid
	(Aura et al., 2005)	Cyanidin-3-glucoside	2,4,6-Trihydroxybenzaldehyde, 4-Hydroxybenzoic acid, 2,4,6-Trihydroxybenzoic acid, 4-Hydroxyphenylacetic acid 3,4-Dihydroxyphenylacetic acid
Flava-3-ol	(Tzounis et al., 2008)	(−)-Epicatechin (+)-Catechin	5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone, 5-Phenyl-γ-valerolactone, 3-Phenylpropionic acid
	(Meselhy et al., 1997)	Epicatechin	Pyrogallol, 5-(3,4-Dihydroxyphenyl)valeric acid, 5-(3-Hydroxyphenyl)valeric acid, 3-(3,4-Dihydroxyphenyl)propionic acid, 3-(3-Hydroxyphenyl)propionic acid, 3-(3-Methoxyphenyl)valeric acid, 2,3-Dihydroxyphenoxyl propionic acid, 3-(3′,4′-Dihydroxyphenyl)propionic acid
Phenolic acid	(Gonthier et al., 2006)	Caffeic acid	3-hydroxyphenylpropionic acid Benzoic acid

Flavonoid glycosides are cleaved off by acids or enzymes (mainly β-glucosidases) in the GI tract or by gut bacteria (Erlund et al., 2001b, Landete, 2012, Nmeth et al., 2003, Berrin et al., 2002, Day et al., 2003). Aglycones are then conjugated to glucuronides, sulfate esters and methyl esters by enzymes in the cells of the small intestine, colon and liver, prior to systemic circulation through blood stream in the body (Landete, 2012). Methylated, glucuronidated and sulphated metabolites are then eliminated via the kidney into urine or are excreted back into the gut via the biliary route. When excreted into bile, these metabolites flow into the small intestine and then reach the large intestine to be degraded into phenolic acids by the colon microflora, which are reabsorbed into blood and tissues (Spencer et al., 2004) .

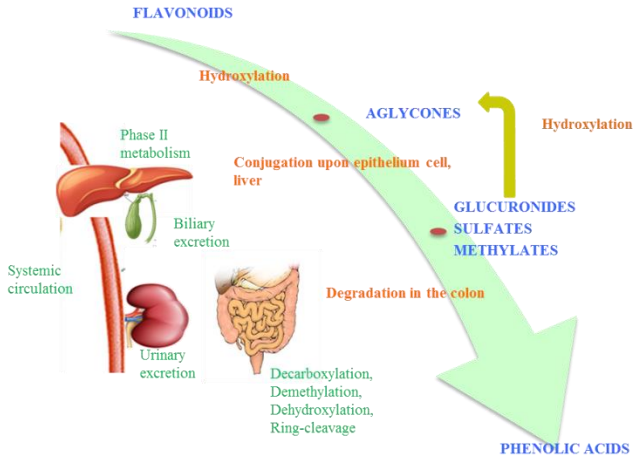


Figure 1-6 Formation of metabolites and conjugates of flavonoids in humans
Author's own figure based on published studies (Erlund et al., 2001b, Landete, 2012, Nmeth et al., 2003, Berrin et al., 2002, Day et al., 2003, Spencer et al., 2004)

1.4.1 Flavanone metabolites

Hesperidin is hydrolysed to release hesperetin by the colonic bacteria when it reaches the large intestine, and then hesperetin is conjugated into glucuronides or sulfates. The main hesperetin metabolites were glucuronidated and sulfoglucuronidated at the 7- and 3'-hydroxyl positions in urine collected from subjects after consumption of orange juices (Manach et al., 2003, Bredsdorff et al., 2010, Vallejo et al., 2010a). In a previous study, 5, 7-

O-diglucuronide and 3’, 7-*O*-diglucuronide were also identified in urine after orange juice consumption (Bredsdorff et al., 2010). However, sulphoglucuronides were less abundant or absent in plasma (Mullen et al., 2008a).

The types of metabolites produced from narirutin can be different in humans and animals. For example, naringenin glucuronides and sulphate metabolites were the main naringenin metabolites in human plasma and urine after consumption of orange juice (Vallejo et al., 2010b). whereas in rats the main metabolites after oral administration of narirutin (Abe et al., 1993) were naringenin 4′-glucuronide, naringenin-7, 4′-*O*-disulfate and naringenin-7-*O*-glucuronide in urinary and plasma, and the main biliary metabolites were naringenin-7-glucuronide, naringenin-7-sulfate 4′-glucuronide and naringenin-7-glucuronide 4′-sulfate. This may be due to differences in the microbiota and cellular metabolism.

Ring fission products are derived from hesperetin or metabolites by colonic bacteria. Roowi and colleagues reported 3-hydroxyphenylacetic acid, 3-hydroxyphenylhydracrylic acid, dihydroferulic acid, 3-methoxy-4-hydroxyphenylhydracrylic acid and 3-hydroxyhippuric acid in urine after orange juice consumption in humans (Roowi et al., 2009a).

1.4.2 Bioavailability of flavanones

The bioavailability of flavanones determines their health effects in the host. They are poorly absorbed from the intestine and rapidly eliminated into urine via kidney (Manach et al., 2004). As described in section 1.4, parent compounds can be degraded into aglycones and phenolic acids as they pass through the gut. Hesperidin is subject to both mucosal and gut bacterial metabolism and then its alycone (hesperetin) and catabolites (phenolic acids) can be absorbed in the body and excreted into urine. The fate of hesperidin in the gut has been studied in several human studies (Table 1-3). A study of ileostomists observed 30 % of hesperidin intake was recovered in 24 h ileal fluid after consumption of a polyphenol-rich juice (Borges et al., 2013). It is not clear how much phenolic acid absorption occurs in the small intestine.

Table 1-3 Recovery of hesperidin and metabolites after consumption of orange juice in humans

	Recovery	Reference
Hesperidin passing to the colon	30 %	(Borges et al., 2013)
Phenolic acids passing to the colon	--	--
Hesperidin excreted in urine	0.8 -16 %	(Pereira-Caro et al., 2014b, Brett et al., 2009, Bredsdorff et al., 2010, Erlund et al., 2001c, Silveira et al., 2014, Vallejo et al., 2010c, Tomas-Navarro et al., 2014, Manach et al., 2003)
Hesperidin and its catabolites (phenolic acids) in urine	37 – 88 %	(Pereira-Caro et al., 2014a, Roowi et al., 2009b)

There have been a number of studies focusing on the bioavailability of flavanones in orange juice, which ranged from 0.8 % ~ 16 % of ingested dose (Table 1-4). Pereira-Caro and colleagues reported the highest recovery of flavanones in urine at 16 % of flavanone intake after consumption of pulp enriched orange juice, where phenolic acids accounted for 88 % of flavanone dose in urine (Pereira-Caro et al., 2014b). Mullen et al found a lower urinary excretion of flavanone metabolites (7 % of hesperidin and narirutin intakes) than (Pereira-Caro et al., (2014b) , and characterised the excretion of five phenolic acids in urine after consumption of 250 mL orange juice (Mullen et al., 2008a, Roowi et al., 2009b). In addition, Brett and Bredsdorff reported 5.3 % and 7.0 % of flavanone intake excreted in urine after consumption of orange juice from concentrated and commercial orange juice respectively (Brett et al., 2009, Bredsdorff et al., 2010). In Erlund's study, 1.1 % of naringenin and 5.3 % of hesperetin were recovered in urine after ingestion of orange juice from concentrate (Erlund et al., 2001c). On the other hand, Silveira et al investigated urinary flavanone and metabolite after consumption of freshly-squeezed and commercially processed orange juices, They recovered respectively to 4.1 % and 3.8 % of hesperidin intake, 3.8 % and 8.7 % of naringenin intake in urine (Silveira et al., 2014). Vallejo et al estimated the bioavailability of five different beverages containing hesperidin and narirutin including two commercial orange juices and a pulp-enriched orange juice. The bioavailability of flavanones was 4.6 %, 1.4 % and 0.8 % (Vallejo et al., 2010c). In addition, Tomas-Navarro and colleagues reported various levels of bioavailability of flavanones after consumption of fresh hand-squeezed orange juice, high pressure homogenised orange juice and pasteurised orange juice ranging from 6.7 % - 11 % (Tomas-Navarro et al., 2014). Furthermore, Manach and colleagues

investigated two doses of orange juice (0.5 L and 1 L) and reported 4.6 % and 6.7 % of urinary recovery of flavanone intake in urine, but there was no significant difference and no doubling of flavanone bioavailability with the higher dose of orange juice (Manach et al., 2003). Thus with a wide range of estimates for bioavailability in the literature, it is important to determine the factors which affect this in humans.

Table 1-4 Bioavailability of flavanone (hesperidin and narirutin) after consumption of orange juice

Reference	No. of subjects	Orange Juice	Source	Consumption Volume (mL)	Intake of total flavanone (mg)	AUC (μg/L.h)	Percentage as urinary excretion of flavanone intake (%)
(Silveira et al., 2014)	24	Fresh-squeezed	Citrosuco, Matao, Brazil	786	41.4	255	1.6
		Commercial			250.2	848	0.9
(Vallejo et al., 2010c)	10	Commercial from concentrate	Spain	400	86.4	1218.5/375.7	4.6
		Commercial from concentrate			176.8	1532.9/485.6	1.4
		Pulp-enriched juice			247.2	695.9/206.5	0.8
(Mullen et al., 2008a)	8	Commercial	Somerfield's, Glasgow, U.K.	250	109.4	1959.8	7.0
(Erlund et al., 2001c)	8	Commercial from concentrate	Valio Ltd., Helsinki, Finland	8 mL/kg (400 ~ 760 ml)	41 mg/L of narigenin and 218 mg/L of hesperetin	3818	1.1 % of naringenin intake and 5.3 % of hesperetin intake
(Manach et al., 2003)	5	Commercial	France	500	270	1383	4.6
				1000	540	3156	6.7
(Brett et al., 2009)	20	Commercial from	UK	300 g	81.2	522	5.3

		concentrate					
(Bredsdorff et al., 2010)	16	Commercial	Nestlé, Thailand	--	0·83 mg/kg body weight	81.6 of naringenin	7.0 of naringenin
(Tomas-Navarro et al., 2014)		Fresh hand-squeezed juice			56.5	--	11.0
	18	High-pressure homogenized	Valencia, Spain	400	161.6	--	7.7
		Pasteurized			172.7	--	6.7
(Pereira-Caro et al., 2014b)	12	Pulp enriched	UK	250	230.3	--	16.0

1.5 Food matrix

The food matrix has been investigated for its effects on the metabolism and bioavailability of flavonoids. Foods containing proteins, lipids and polysaccharides might impact on the bioavailability and then the bioactivity of simultaneously consumed flavonoids through their interactions in the gastrointestinal tract. These effects must be considered when metabolism and bioavailability, and beneficial effects of flavonoids are studied.

1.5.1 Milk and milk products

Milk or milk products (yoghurt) are often added to polyphenol rich food (cocoa, tea, fruits, vegetables) or processed as commercial products (chocolate, fruit yoghurt). Flavonoids have the capacity for hydrophilic and hydrophobic interactions with proteins. The effect of milk or milk products has been wildly assessed for on interactions with flavonoids which affect their metabolism or bioavailability. In previous studies, tea polyphenols have been found bind to hydrophobic proline-rich proteins (α- and β-caseins) via both hydrophilic and hydrophobic interactions (Hasni et al., 2011, Yuksel et al., 2010).

Cocoa, as a flavonoid-rich food, has been linked to a range of beneficial effects, such as cardio-protective effects (Dower et al., 2015, Kris-Etherton and Keen, 2002), reduction of LDL cholesterol, elevation of HDL cholesterol and suppression of oxidized LDL (Baba et al., 2007), inhibition of platelet activation and function (Rein et al., 2000b, Rein et al., 2000a), enhancement of vascular function (Fisher et al., 2003, Rodriguez-Mateos et al., 2013). Regular consumption of cocoa powder and milk modulated the lipid profile and increased urinary polyphenol metabolites in subjects at high-risk of cardiovascular disease (Khan et al., 2012). However, Schroeter et al proposed that milk proteins and chocolate flavonoids interact to inhibit the absorption of epicatechin into the bloodstream (Schroeter et al., 2003). There was a minor effect on epicatechin-*O*-sulfate in plasma, reduced excretion of 4 flavan-3-ol metabolites in urine, and *O*-methyl-epicatechin-*O*-sulfate was not affected by milk in the cocoa. In some studies, milk had no effect on bioavailability and absorption of flavonoids. Roura et al showed that milk did not affect the bioavailability of epicatechin metabolite in terms of epicatechin-glucuronide in plasma (Roura et al., 2007a).

Milk has also been reported to change the metabolite profiles after intake of flavonoids. Roura and his colleagues reported that milk (250 ml) increased epicatechin sulfate when epicatechin glucuronide was still the main metabolite 0-6 h after intake, and milk influenced the sulfation position of epicatechin in terms of concentration of three isomers, but did not affect total epicatechin metabolites in urine after intake cocoa powder (40 g) containing 28.2 mg (-)-epicatechin, 25.5 mg procyanidin B2, 8.4 mg (+)-catechin, and 2 mg flavonols (Roura et al., 2008).

On the other hand, Neilson and colleagues conducted *in vivo* and *in vitro* studies. They found that the C_{max} of epicatechin metabolites was enhanced over 6 h after intake of catechin (C) and epicatechin (EC) from cocoa (approximately 9.44 mg C and 27.39 mg EC/serving (250 ml) in the form of sucrose milk protein (BSMP) and non-nutritive sweetener milk protein (BNMP) (9.46 mg C and 27.44 mg EC /serving) compared with that of a reference dark chocolate (CDK) (9.35 mg C and 27.13 mg EC)with high milk protein (CMP) (Neilson et al., 2009). They also found higher bioavailability of catechin from BSMP and BNMP than CDK, high sucrose and CMP in an *in vitro* gastric and small intestinal digestion model, while there was no difference in epicatechin (Neilson et al., 2009). This suggests that the food matrix has a different impact on metabolism and bioavailability of dietary polyphenols depending on their chemical structure. In addition, some of the effect of milk on cocoa flavonoids may be due to the dose of flavonoids ingested. It appears that at high dose (97.2 μmol EC and 29.0 μmol C) little effect is seen (Roura et al., 2007b) but effects are evident at low dose (45 μmol EC and C) (Mullen et al., 2009a). This may be due to saturation of the binding potential (Mullen et al., 2009a). Thus, it is important to consider the levels of flavonoids present in common foods.

There is a similar controversy with regards to the effect of milk on tea flavonoids. Consumption of black tea significantly increased concentrations of total phenols, catechins, and the flavonols quercetin and kaempferol with improvement of plasma antioxidant capacity within 80 min, but consumption of milk and tea did not affect these metabolites in the plasma (Kyle et al., 2007). In an *in vitro* digestion study, catechin recovery was 59 % for English black tea made with 5.6 % milk and was about 40 % for English and Indian black teas with 15-40% milk, but did not influence total catechin recovery after stimulated gastric and intestinal digestion (van der Burg-Koorevaar et al., 2011). Furthermore, polyphenol-protein complexes could be degraded during digestion. Blood catechin levels were not significantly

affected by the addition of milk (100 mL) to black tea (600 mL) (Van het Hof et al., 1998). Consumption of milk also did not change the plasma concentration-time curve of quercetin or kaempferol after drinking black tea by men (Hollman et al., 2001). In addition, plasma total catechin and antioxidant activity was not affected by addition of milk after consumption of black or green tea (Leenen et al., 2000).

However, defatted milk did not significantly affect plasma anthocyanins levels after the consumption of blackberry juices (Hassimotto et al., 2008).

For the isoflavones, milk decreased the urinary excretion of daidzein, genistein (parent compound) and increased the equol concentration (metabolites) in urine after tempeh (fermented beans) consumption in postmenopausal Malay women (Haron et al., 2011). However, urinary genistein recovery was higher after soya-milk consumption than textured vegetable protein (soyabean products) (Faughnan et al., 2004).

In an *in vitro* study, skimmed milk powder significantly increased degradation of isoflavone glycosides into isoflavone aglycones after 24 h incubation of soymilk with *Lactobacillus*, which was supposed due to growth of *Lactobacillus* stimulated by skim milk powder providing lactose and others nutrients (Pham and Shah, 2008).

Yoghurt, as a dairy product, was also investigated for its effect on bioavailability of flavonoids in orange juice (Mullen et al., 2008a). In this study, there was a significant delay in the initial appearance of flavanone metabolites, and reduced excretion of flavanone metabolites at 0-5 h after orange juice ingestion with yoghurt, but no significant difference in the excretion of flavanone metabolites (three hesperetin-O-glucuronide, two hesperetin-O-glucuronide-O-sulfates, a hesperetin-O-diglucuronide, a naringenin-O-diglucuronide, and naringenin-7-O-glucuronide and naringenin-4′-O-glucuronide) over the 24 h period after consuming orange juice without and with yoghurt (Mullen et al., 2008a). This may be attributed to a prolonged transit time to reach the large intestine after consumption of orange juice with yoghurt. However, full fat yoghurt reduced the excretion of the five phenolic acids equivalent to 37% of the ingested flavanones over 24h after orange juice consumption, compared with after drinking orange juice without full fat natural yoghurt (Roowi et al., 2009). Meanwhile, urinary excretion of 3-hydroxyphenylacetic acid, 3-hydroxyphenylhydracrylic acid, dihydroferulic acid, 3-methoxy-4-hydroxyphenylhydracrylic

acid and 3-hydroxyhippuric acid, which were derived from flavanone in orange juice by gut bacteria, reduced markedly from 62 ± 18 μmol to 6.7 ± 1.8 μmol after consumption of orange juice with yoghurt (Roowi et al., 2009a). In addition, soy consumption increased plasma phytoestrogen concentrations ($P < 0.0001$), but plasma phytoestrogen concentrations and the number of equol producers were not different between the soy protein and soy + probiotics diets in a 6-wk randomized, crossover design (20 women without a history of cancer and 20 women successfully treated for breast cancer)(Nettleton et al., 2004). The reasons for the impact of yoghurt on the phenolic acids in urine are unclear and are explored further in the studies described in this thesis.

1.5.2 Dietary fibre

Dietary fibre consists of non-starch polysaccharides, resistant oligosaccharides, resistant starch and lignin associated with the dietary fibre polysaccharides which include, hemicellulose, pectin, β-glucans, gums, classified as soluble dietary fibre and cellulose as insoluble dietary fibre (Gallaher and Schneeman, 2001, Gorinstein et al., 2001, Dhingra et al., 2012). Dietary fibre is considered to play an important role in human health and may interact with phytochemicals in food to influence the metabolism and beneficial effects of flavonoids in human. In an animal study, oatmeal (6.7 % (w/w)) increased anthocyanin rutinosides, but reduced anthocyanin glucosides in digesta, and decreased C_{max} of anthocyanins from 0.37 ± 0.07 μmol/L to 0.20 ± 0.05 μmol/L in plasma with delayed time from 0.25 h to 1 h, in addition to reduced maximum urinary excretion time of anthocyanins from 2 h to 3 h after consumption of blackcurrant powder containing 250 mg total anthocyanins per kg body weight of rats (Walton et al., 2009). A diet containing hydrolysed wheat, wheat, barley, maize and oats stabilised procyanidins in grape seed procyanidin extracts under GI digestion conditions using in vitro model, but reduced C_{max} of procyanidin trimer in plasma after feeding grape seed procyanidin extracts to rats (Serra et al., 2010). In addition, supplements of rice bran hemicellulose significantly reduced plasma daidzein in rats after feeding cellulose-daidzein diet for 30 days in rats (Tamura et al., 2009). Furthermore, phenolic metabolites have also been investigated. Gu et al reported that diet containing sorghum bran containing cellulose, catechin and epicatechin increased urinary excretion of catechin, 3'-*O*-methylcatechin in rats, 3-methoxy-4-hydroxyphenylacetic acid, 3-hydroxyphenylacetic acid, and 3-hydroxyphenylpropionic acid in rats after 50 days of consumption (Gu et al., 2007).

Different dietary fibre might have various effects on the bioavailability of flavonoids due to their different properties and fermentability. Nishijima and colleagues reported that co-ingestion of apple pectin (10 mg/ml) caused 2.5 times higher urinary excretion of quercetin and its metabolites (isorhamnetin & tamarixetin) after consumption of 250 ml drink containing quercetin(0.5 mg/ml), and high-methoxy pectin significantly enhanced the urinary excretion by 1.69 fold in humans (Nishijima et al., 2015). Saura-Calixto investigated in an *in vitro* study that dietary fibre from red grape subjected to digestion treatment containing 73.5 ± 0.8 g /100 g of total dietary fibre and 14.8 ± 0.2 g/100g proanthocyanidins produced more phenolic metabolites than non-digestible carob pod containing 15 ± 0.3 g/100g of total dietary fibre and 78.3 ± 1.8 g/100g proanthocyanidins based on same amount of non-extractable proanthocyanidins that are linked to cell wall constituents and not detected by the usual analytical procedures (Perez-Jimenez et al., 2013) in two different fibre containing products fermented by gut bacteria from rats (Saura-Calixto et al., 2010). Tamura et al also reported that concentrations of quercetin and isorhamnetin and ratios of isorhamnetin / quercetin were significantly higher in plasma after feeding a pectin-rutin diet in rats for 14 days than a cellulose-rutin diet (Tamura et al., 2007a), which suggested that dietary fibre can modify metabolism of flavonoids or gut physiological function to enhance bioavailability of flavonoids. Resistant starch acting as dietary fibre in the colon, improved urinary excretion of equol in ovariectomized mice feed formulated diet with daidzein (0.1%) and resistant starch (12%) for 6 weeks with increased proportion of *Bifidobacterium spp* in faecal bacteria (Tousen et al., 2011).

Other carbohydrates also affect bioavailability of flavonoids. Schramm and colleagues showed that sugar and bread meals increased plasma concentration of flavanols (epicatechin & catechin) to 140 % after consumption of cocoa providing 0.125 g/kg body weight of flavanol in subjects (Schramm et al., 2003).

1.5.3 Fat

Fat also influence the bioavailability and metabolism of flavonoids. A higher content of fat in flavonoid enriched food tended to enhance the bioavailability of flavonoids. Cream is high in fat. Mullen and colleagues reported that cream delayed T_{max} of pelargonidin glucuronide in plasma from 1.1 ± 0.4 to 2.4 ± 0.5 h after the consumption of strawberries containing 222 µmol of pelargonidin-3-O-glucoside, and there was a lower C_{max} (227 ± 35 nmol/L)

compared to that without cream (274 ± 24 nmol/L). Lower 0–2 h urinary excretion of total anthocyanins was found after intake of the strawberries with cream. The 24h overall excretion of anthocyanins was not significantly different after ingestion strawberries with cream or without cream, which delayed gastric emptying and extended mouth to caecum transit time (Mullen et al., 2008b). In an *in vitro* study, Ortega reported significantly higher procyanidins (monomers and dimers) after digestion of cocoa liquor containing ~50 % fat than cocoa powder containing ~15 % fat through GI digestion by using an in vitro digestion model, both of which contained similar amount of phenolic compositions (Ortega et al., 2009b). It indicated that higher fat reduced the loss of flavonoids under gastrointestinal digestion conditions. In animal studies, plasma quercetin metabolites were also enhanced after administration of quercetin (30 μmol/kg body weight) with a diet containing 17 % fat compared with 3% fat in pigs, whereas they were similar compared with meal containing 32 % fat (Lesser et al., 2004).

On the other hand, different types of fatty acids influenced the bioavailability of flavonoids. A diet rich in medium-chain fatty acids (162.8 fat/kg diet), not long chain fatty acids significantly enhanced the absorption of quercetin compared with the standard diet (16.7 g fat/kg diet), and delayed C_{max} of quercetin in pigs after supplementation of 30 mmol quercetin/kg body weight (Lesser et al., 2006). In an animal study, plasma quercetin metabolites were increased in rats after 2 weeks ingestion with diet contained soybean oil (> 4.6 % (w/w)), fish oil or beef (9.5 % lipid content) with 1.0 g of onion powder (Azuma et al., 2003).

1.6 Individual bioavailability- human factors

The bioavailability of flavonoids may depend on the ethnic origin, gender and age of the host. Ethnicity has been investigated in several previous studies. A higher maximum concentration in plasma and area under the plasma concentration-time curve of genistein and daidzein were found in Asians than in Caucasians (3.7 vs. 2.4 and 43.7 vs. 30.1 ng/ml per h per kg BW; 2.9 vs. 1.7 and 29.2 vs. 19.6 ng/ml per h per kg BW) after an acute intake of soya-based cheese (45.97 mg isoflavones), and 10 day daily consumption of soya-based cheese significantly increased these values in Caucasians, but not that of daidzein in Asians (Vergne et al., 2009). In an *in vitro* fermentation study, daidzein was more degraded by gut bacteria from Asians than Caucasians (0.25 mol/L ± 0.03 mol/L vs. 0.20 mol/L ± 0.03 mol/L in phases I, and 0.21

mol/L ± 0.02 mol/L vs. 0.12 mol/L ± 0.03 mol/L in phases II) after 24 h incubation of daidzein and genistein at a concentration of 100 mol/L in brain heart infusion culture medium (600 mol/L) (Zheng et al., 2003b). In another *in vitro* study, there was higher production of phenolic acids by gut bacteria from Indian participants than Caucasian subjects (9.4 ± 4.4 µg/ml vs. 4.1 ± 3.5 µg/ml) at 6 h of fermentation (Alkhaldy et al., 2012b). .

In terms of age, there was higher excretion of phenolic acids in older people 54-75 y than younger people 23-43 y (23.4 ± 31.7 vs. 227.4 ± 72.3µg/day after a high polyphenol diet (Alkhaldy et al., 2012b). In addition, Erlund et al reported that the 0-24 h area under the plasma concentration time curve of quercetin was higher in women than men (1946 µg·h/L vs. 678 µg·h/L) after intake of quercetin aglycone and rutin equivalent to 50 mg dose of quercetin (Erlund et al., 2001b). The rate of urinary excretion of daidzein and genistein was 39 % and 44 % higher respectively, and the total amount of urinary total isoflavonoids and metaboliteswas higher (32%) in children (3-12y old) than in adults over 12 h after intake of soya nuts containing 0·615 mg total isoflavones/kg (Halm et al., 2007).

Gender also affected the bioavailability of flavonoids. Quercetin in plasma was higher in women from 0 to 24 hours (1946 µg · h/L) than in men (678 µg · h/L) after ingestion of 50 mg quercetin aglycone and rutin, expressed as quercetin equivalents (Erlund et al., 2001a).

1.7 Other factors

Various other factors have been suggestd to affect the bioavailability and absorption of dietary flavonoids, such as food sources, dose, solubility and characteristics of flavonoids. Brett and colleagues revealed no different absorption and excretion of flavanone through intake of orange fruits and orange juice from concentrate (Brett et al., 2009). Charron et al. reported that plasma anthocyanin decreased with intake dose of total anthocyanins from 76 to 380 µmol through consumption of carrot juice from 50 to 250 mL, and C_{max} of nonacylated anthocyanins was 4 times higher than that of acylated anthocyanins so that acylated anthocyanins had lower bioavailability of acylated anthocyanins compared with non-acylated anthocyanins from purple carrots juices (Charron et al., 2009). Lower solubility of hesperidin in orange juice reduced urinary excretion of hesperetin metabolites and the C_{max} in plasma from humans after intakes of orange juices (Vallejo et al., 2010a). Krogholm showed that urinary excretion of naringenin and hesperetin (14.2 % and 22.6 % of intake) was higher than

quercetin (1.5 % of intake), C_{max} of naringenin (0.25 µM) was significantly higher than that of quercetin and hesperetin (0.15 µM and 0.18 µM), and T_{max} of quercetin and naringenin (3.6 ± 1.6 h) was earlier than that of hesperetin (4.9 ± 1.4 h) after consumption of a hesperetin, naringenin and quercetin mixed juice (Krogholm et al., 2010). In addition, urinary excretion of hesperidin metabolites was higher (12.0 %) than (epi)gallocatechin metabolites (6.2 %) after consumption of a flavonoids mixed juice containing green tea flavan-3-ols, grape seed and pomace procyanidins, apple dihydrochalcones, procyanidins and chlorogenic acid, citrus flavanones and grape anthocyanins (Borges et al., 2010).

1.8 Gut microbiota

There are up to 10^{14} total bacteria, more than 1000 different species in the human intestinal tract (Rajilić-Stojanović and de Vos, 2014). 99 % of the gut bacteria belongs to a few phyla, the main ones being *Bacteroidetes* and *Firmicutes*, whereas *Actinobacteria*, *Proteobacteria* and *Verrucomicrobia* are minor constituents (Lozupone et al., 2012, Eckburg et al., 2005, Guarner and Malagelada, 2003, Suau et al., 1999). The gut microbiota is individually different and modified over the whole life by diet, life-style, disease and antibiotic use, but its composition usually remains relatively stable (Nicholson et al., 2012, Moore and Moore, 1995).

The gut bacteria have been widely investigated for their interactions with food and flavonoids (Figure 1-7). Non-digestible carbohydrates, such as non-starch polysaccharides, resistant starch and oligosaccharides that are resistant to gastric acidity and enzymes (Dhingra et al., 2012), can be fermented into short chain fatty acids by gut microbiota (Crittenden et al., 2002) affecting the pH in the colon (Brøkner et al., 2012) which may affect the metabolism of flavonoids (Mansoorian et al., 2015).

Hawksworth detected β-glucosidase activity in *Bacteroides*, *Bifidobacteria*, and *Lactobacilli* in *in vitro* cultures (Hawksworth et al., 1971), these enzymes can degrade flavonoids into their aglycone. Rutin, hesperidin, naringin, poncirin and baicalin were degraded by gut bacteria isolated from human faeces with activity of α-rhamnosidase and β-glucosidase or endo-β-glucosidase (Kim et al., 1998). In addition, *Escherichia coli* HGH21 and a gram-positive strain HGH6 isolated from human faeces cleaved daidzin and genistin into daidzein and genistein (Hur et al., 2000). Gut bacteria are responsible for cleavage C-ring of

flavonoids. *Clostridium scindens* and *Eubacterium desmolans* degraded 20 ~ 25 % of naringenin during in vitro incubation at 37°C for from 4 h to 7 days (Winter et al., 1989). *Clostridium cocoides-Eubacterium rectale* group transformed (+)-catechin and (-)-epicatechin into 5-(3',4' -dihydroxyphenyl)-γ- valerolactone, 5-phenyl- γ -valerolactone and phenylpropionic acid by using batch-culture fermentation (Tzounis et al., 2008).

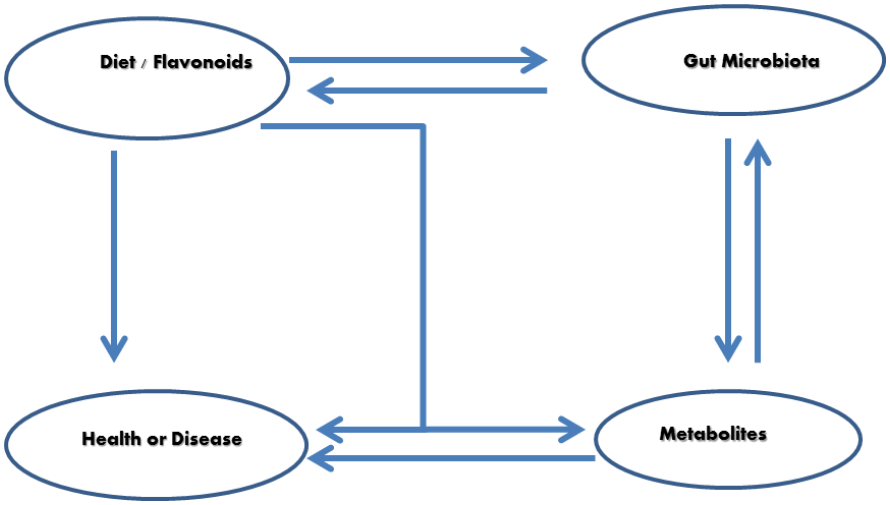


Figure 1-7 Interactions between diet / flavonoids, metabolites and health

Flavonoids and their metabolites may also modulate the gut microbial composition (Hervert-Hernández and Goñi, 2011). (+)-Catechin stimulated growth of *Clostridium coccoides–Eubacterium rectale* group, *Bifidobacterium spp.* and *Escherichia coli (E.coli)*, and inhibited growth of *C. histolyticum* group, while (−)-epicatechin only increased *C. coccoides–Eubacterium rectale* group through incubation of human gut bacteria with (−)-epicatechin or (+)-catechin (Tzounis et al., 2008). *Lactobacilli* and *Bifidobacteria* were increased in faeces of rats by feeding 1 mg of resveratrol/kg/day for 25 days, and this also reduced the colitis caused by 5% dextran sulfate sodium over the last 5 days, reducing systemic and colonic inflammatory markers and cytokines (Larrosa et al., 2009). *Bacteroides spp., Clostridium spp.*were inhibited by incubation with freshly brewed thick Yunnan Chinese tea containing epicatechin, catechin, 3-O-methylgallic acid, gallic acid and caffeic acid (*C. perfringens and C. difficile*), *Bifidobacterium* spp., *Lactobacillus* spp., *E. coli* and *Salmonella typhimurium,* but did not affect growth of *Clostridium spp., Bifidobacterium spp.* and *Lactobacillus sp.*by in vitro incubation with 28 common pathogenic, commensal and probiotic intestinal bacteria (Lee et al., 2006). Ellagitannins strongly inhibited the growth of *Staphylococcus bacteria* cultured aerobically at 37 °C on nutrient agar (Puupponen-Pimiä et al., 2005).

Fruits, vegetables, and their products containing polyphenols influence the composition of the colonic microbiota. A human intervention study revealed that *Enterococcus, Prevotella, Bacteroides, Bifidobacterium, Bacteroides uniformis, Eggerthella lenta,* and *Blautia coccoides–Eubacterium rectale* groups significantly increased in faeces of men after 4 week daily consumption of red wine (Queipo-Ortuño et al., 2012). In addition, 6 weeks daily consumption of a wild blueberry drink (25 g of wild blueberry powder in 250 mL of water) significantly increased *Bifidobacterium spp.* and *L. acidophilus* group in men faeces (Vendrame et al., 2011). There were several *in vitro* studies also showing the effects of polyphenol enriched fruits on proliferation of gut bacteria. Growth of *C. perfringens, C. ramosum, S. aureus,* and *C. clostridioforme* were inhibited, whereas *Bifidobacterium breve* and *Bifidobacterium infantis* were increased by incubation with commercial pomegranate peel extract (0.01% (v/v)) containing19% ellagitannins as punicalagins and punicalins, 4% free ellagic acid, and 77 % oligomers composed of 2-10 repeating units of gallic acid, ellagic acid, and glucose in different combinations in broth or /and agar (Bialonska et al., 2009). Purple and red vegetable and fruit juices (180 mL) at 1:2 to 1:16 dilutions and tea at 1:28 to 1:32 dilutions with single or double-strength trypticase soy broth inhibited growth of *S. epidermidis* and *K. pneumoniae* in 96-well microtiter plates with 20 mL innoculum (Lee et al., 2003). *Lactobacillus* spp. and *Bifidobacterium* spp. increased in faecal samples of women after 4 weeks consumption of a high cocoa flavanol drink every day (494 mg) compared with a low cocoa drink (29 mg) (Tzounis et al., 2011). The impact of other constituents of these fruits and vegetable juices on the bacteria should be considered to ensure the effects are due to the flavonoids alone.

1.9 Flavonoids and risk of disease

1.9.1 Mechanism of flavonoid bioactivity-antioxidant actions

Flavonoids are thought to play a role in protection against disease. Flavonoids as antioxidants can scavenge free radicals and chelate metal ions (Cook and Samman, 1996b). A catechol group in ring B determines the radical-scavenging capability of flavonoids, where electron donation is easier (Bors et al., 1990). A 3 hydroxyl group in the heterocyclic ring and polyhydroxylation of A and B aromatic rings of flavonoids enhance antioxidant and free radical scavenging functions (Sichel et al., 1991).

Dietary flavonoids have been shown to protect against oxidative stress and inflammation and have been suggested to reduce risk of chronic diseases to play an important role in human health (Tapiero et al., 2002) (Table 1-5). Naringin (0.03, 0.1, 0.3, and 1 mM) increased H_2O_2-induced cytotoxicity and inhibited chromatin condensation and DNA damage induced by H_2O_2 in mouse leukemia P388 cells (Kanno et al., 2003). (-)-epigallocatechin gallate, quercetin, rutin and hesperetin (1–50 mM) inhibited LDL oxidation, and inhibited HUVEC nuclear condensation and fragmentation induced by oxidized LDL in human endothelial cells (Jeong et al., 2005). In addition, epigallocatechin gallate and (+)-catechin, quercetin, myricetin, rutin, naringenin, hesperetin, luteolin and apigenin at 1–50 mM increased anti-apoptotic Bcl-2 expression, and reduced Bax expression and caspase-3 cleavage in human endothelial cells stimulated by oxidized LDL potentially against for atherosclerotic disease (Jeong et al., 2005). Apigenin at 20, 40 μM reduced LPS-induced expression of nitric oxide synthase, cyclooxygenase-2, expression of IL-1β, IL-2, IL-6, IL-8, and TNF-α, and AP-1 proteins (c-Jun, c-Fos, and JunB) in human lung A549 cells (Patil et al., 2015). Apigenin, luteolin and genistein (50 μM) reduced platelet aggregation induced by arachidonic acid and collagen related to platelet responses (Guerrero et al., 2005). Flavonoids also have shown inhibitory properties against viruses. Epicatechin (35 μM), epicatechin-gallete (50 μM), genistein (50 μM), naringenin (50 μM) and quercetin (50 μM) inhibited virus-induced cytopathic effect, and catechin, epigallocatechin, epigallocatechin gallate, naringenin, chrysin, baicalin, fisetin, myricetin, quercetin, and genistein at less than 100 μM moderately inhibited growth of HSV-1 virus (Lyu et al., 2005).

However, the studies above used parent compounds and aglycones which are not absorbed intact or when absorbed are at much lower concentrations. Thus much lower physiological concentrations are seen after ingestion of food and juices. For example, the C_{max} of hesperidin metabolites was 922 nM after the consumption of orange juice (Mullen et al., 2008a) and the C_{max} of total catechins, EGCG, ECG, EGC and EC were 461.8 ± 27.4 nM, 178.9 ± 12.7 nM, 147.3 ± 11.4 nM, 86.5 ± 5.1 nM, 48.2 ± 3.8 nM after the consumption of green tea (James et al., 2015) Futhermore daidzein aglycones in plasma were 7.7 ± 1.7 nM at 1.5 h and 14.5 ± 3.3 nM at 3.0 h, and genistein was 6.0 ± 4.2 nM at 1.5 h and 5.2 ± 3.4 nM at 3.0 h after consumption of sautéed onion and tofu (Nakamura et al., 2014). Thus the data generated may not be relevant to the physiological effects of dietary flavonoids.

Therefore the majority of flavonoids are not absorbed intact as the parent compound but are hydrolysed and conjugated, or catabolised into smaller molecular compounds, such as phenolic acids, and then these metabolites are distributed to organs and circulated through blood stream, finally eliminated into urine. The bioactive properties of these metabolites need to be demonstrated at physiological levels. There are few studies to investigate this. Spencer et al showed that epicatechin-5-O-β-D- and epicatechin-7-O-β-D-glucuronides did not inhibit peroxide-induced loss in neuronal or fibroblast viability, and caspase-3 activation in both cells, in contrast with 3′-O-methyl epicatechin and epicatechin (Spencer et al., 2001). However, epicatechin and 3′-O-methyl epicatechin have been shown to elicit cytoprotective effects in fibroblasts(Spencer et al., 2001). Concentrations of 26 phenolic compounds (benzoic acid, salicylic acid, 4-hydroxybenzoic acid, protocatechuic acid, gentisic acid, gallic acid, syringic acid, cinnamic acid, caffeic acid, sinapic acid, dihydrocaffeic acid, phloretic acid, 5-methoxysalicylic acid, vanillic acid, p-coumaric acid, m-coumaric acid, ferulic acid, 4-hydroxyhippuric acid, 3-hydroxyhippuric acid, 3-hydroxyphenylacetyl glycine, 3-hydroxyphenylpropionyl glycine, 4-hydroxyphenylpropionyl glycine, hippuric acid, pyrogallol, catechol, resorcinol) impacted neither platelet aggregation nor activation at the concentration less than 50 μmol/L (Ostertag et al., 2011). However, these studies have still not investigated their effects at the physiological conditions. Thus, it is essential to explore profile and content of flavonoids metabolites, as well as factors affecting concentrations of metabolites derived from polyphenols in our diet to provide insight for experimental and clinical studies.

Table 1-5 Mechanisms of flavonoids in reducing risk of diseases

References	Flavonoids	Actions	Treatment	Results
(Jeong et al., 2005)	(-)-epigallocatechin gallate, quercetin, rutin and hesperetin (1–50 mM)	Antioxidation of LDL, and induced oxidative stress and oxidized LDL-triggered atherogenesis	Cu2+ induced human umbilical vein endothelial cells (HUVEC)	Inhibited LDL oxidation, increased anti-apoptotic Bcl-2 expression, and reduced Bax expression and caspase-3 cleavage stimulated by oxidized LDL.
(Kanno et al., 2003)	Naringin (0.03, 0.1, 0.3, and 1 mM)	Antioxidant and anti-apoptopic properties	H_2O_2-induced mouse leukemic P388 cells	Reduced H_2O_2-induced cytotoxicity and inhibited chromatin condensation and DNA damage
(Patil et al., 2015)	Apigenin (20, 40 μM)	Anti-inflammation	lipopolysaccharide (LPS)-induced human lung A549 cells	Inhibited nitric oxide synthase, cyclooxygenase-2, expression of IL-1β, IL-2, IL-6, IL-8, and TNF-α), and AP-1 proteins (c-Jun, c-Fos, and JunB).
(Guerrero et al., 2005)	Apigenin and genistein (50 μM)	Antiplatelet	Arachidonic acid and collagen-induced platelet	Reduced platelet aggregation and TxA(2) generation linked to platelet responses
(Lyu et al., 2005)	EC, ECG, galangin, and kaempferol	Antivirus	HSV-1	Inhibited

1.9.2 Epidemiological studies in consumption of flavonoid-rich food

The association between flavonoids and disease has been investigated in several epidemiological studies. These studies show positive or neutral associations between flavanone or flavanone-rich food and health benefits. The details are illustrated in Table 1-6.

Over the past decades, flavonoid intake has been linked to reduced risk of cardiovascular disease. Cassidy et al reported that anthocyanin and flavanones intake reduced the risk of hypertension in a 14 y follow-up cohort study (Cassidy et al., 2011). An intake of 80 g/day fruits was associated with 6–7 % lower risk of CVD and CHD mortality, and the highest intake of grapes and citrus reduced the risk of CVD and stroke compared with non-consumers (Lai et al., 2015). Ponzo et al. also showed an inverse association of intakes of flavanones, flavan-3-ols, anthocyanidins and flavanones with CVD risk (Ponzo et al., 2015). Mink et al also observed an inverse association between flavanone intake and CHD risk in the highest intake group compared with the lowest intake group (Mink et al., 2007b). Mursu et found that the highest intake group of flavanone and flavone had an inverse correlation with risk for CVD death after multivariate adjustment (Mursu et al., 2008b). Tavani et al found lower non-fatal acute myocardial infarction (AMI) in the highest intake group of anthocyanidins, compared with the lowest intake, and flavanone intake had similar effect after alcohol adjustment (Tavani et al., 2006). These studies suggested potential benefits of flavanone or flavanone rich flood, especially citrus fruits /juices intake in the risk of human cardiovascular diseases.

However, some studies found the association only with total flavonoids intake. The 18 % lower risk of fatal CVD was associated with the highest intake of total flavonoids in men and women (McCullough et al., 2012). The highest intake of flavonoids reduced 46 % CVD risk compared with the lowest intake of flavonoids (Tresserra-Rimbau et al., 2014). In addition, a lower risk of all-cause, CVD, and cancer mortality was found in the highest intake of total flavonoid intake(Ivey et al., 2015).

There are some studies revealing their effects in cancer risks. The lower incidence of ovarian cancer was found in the highest intake of flavanol and flavanone compared with in the lowest group (Cassidy et al., 2014). However, there are several studies showing no association between flavanone intake and the risk of cancers. For instance, the highest intake of total

flavonoid, flavonol and flava-3-ol reduced risk of the lung cancer, but no effect was seen for flavanone intake (Mursu et al., 2008a).

Risk of type-2-diabetes has inversely been associated with flavanone intake. Higher urinary excretion of hesperetin reduced the risk (Sun et al., 2015). Citrus fruits, especially orange juice, tended to decrease plasma concentration of IL-18 (proinflammatory cytokines related to type 2 diabetes incidence) in women (Landberg et al., 2011). Wedick et al also reported that the intake of flavones and flavanones slightly reduced type 2 diabetes risk (Wedick et al., 2012).

Flavanone intake also affected cognitive functions. In addition, women had greater healthy aging when the highest intake of flavones, flavanone, anthocyanin, and flavonol compared with the lowest intake (Samieri et al., 2014). Butchart et al. reported an association between a higher IQ and intake of flavones and flavanones at age 11 years, the effects of citrus fruit consumption on mini-mental state examination, verbal fluency and speed tests in older people, as well as association between flavanones and verbal fluency in later life (Butchart et al., 2011). However, flavanone intake was not found to reduce risk of Parkinsons Disease, but total flavonoid intake affected the incidence in men (Gao et al., 2012).

Diet, nutritional status, and lifestyle are comprehensive factors to affect incidence of chronic diseases and cancers (Edmands et al., 2015). Epidemiological studies have revealed inconsistent associations of flavanone intake and chronic diseases. The dietary habits can be very varied. For instance, the main flavonoid intakes are different across countries. Proanthocyanidins (48.2 %) are the main sources along with flavan-3-ol monomers (24.9 %) in non-Mediterranean (MED) countries, while proanthocyanidins (59.0 %) were the main flavonoids ingested in MED countries. even though there were no differences in total flavonoid intake between non-MED countries (373.7 mg/d) and MED countries (370.2 mg/d). Tea (25.7 %) and fruits (32.8 %) were main sources of flavonoids in non-MED regions, while fruits (55.1 %), wines (16.7 %) and tea (6.8 %) in MED regions.(Zamora-Ros et al., 2013). In this study, the variability in the detailed intakes of flavonoids and metabolites might potentially explain the variation in outcomes of health in clinical studies and epidemiology studies.

Table 1-6 Flavanone or orange juice intake and diseases on cohort studies

Diseases	Reference	Hypothesis	Methods	Results	Comments
CVD, CHD mortality	(Lai et al., 2015)	The intake of polyphenol in total fruit and fruit subgroup is linked to reduced CVD mortality	30,458 women (aged 35–69 years) were recruited and completed FFQ providing information about 600 diet, health and lifestyle variables. During the mean follow-up period of 16.7 years from 1995–1998, fatal CVD deaths were recorded, and survival was analysed by using participants free from history of CVD at baseline.	80 g/day intake of total fruit reduced 6–7 % risk of CVD and CHD mortality. The highest intake of grapes and citrus significantly lowered risk of CVD and stroke respectively compared with non-consumer.	The cohort study was only conducted in women. Polyphenol profiles should be introduced to evaluate association between flavanone intake and CVD mortality.
All-cause, CVD, cancer mortality	(Ivey et al., 2015)	Flavonoid intake is associated with lower risk of mortality from all causes	1063 women aged over 75 y were recruited and followed for 5 years. Two estimates of flavonoid intake (total $flavonoid_{USDA}$ and total $flavonoid_{PE}$) were used to assess flavonoid intake. The association between flavonoid risk and all-cause, cancer, and cardiovascular mortalities was assessed.	High intake of total flavonoid was associated with a lower risk of 5-y all-cause, cardiovascular disease and cancer mortality.	Only the intake of total flavonoids was used for assessment. There is no information about attribution of specific flavonoid subclasses to reduce mortality risk. The different outcomes based on two databases provided an insight for further epidemiological studies.

CVD mortality	(Tresserra-Rimbau et al., 2014)	The intake of total polyphenol was associated with reduced myocardial infarction, stroke or death from cardiovascular causes	7172 participants were followed for 4.3 year and a 137-item FFQ at baseline and yearly thereafter was used for assessment of flavonoid intake. Hazard ratios were estimated by using a time-dependent Cox proportional hazards regression with updated diet and covariates information.	There was a 46 % lower risk of CVD in the highest flavonoid intake group compared with the lowest flavonoid consumer. The intake of flavanols, lignans and hydroxybenzoic acids had the strongest inverse associations with CVD risk.	In this study, The CVD risk was not associated with flavanone intake.
CVD mortality	(Tresserra-Rimbau et al., 2013)	The intake of flavonoid was inversely associated with the cardiovascular (CV) risk	1,658 individuals were recruited between 2001 and 2003, were followed for 12 years. Anthropometric, laboratory measurements, medical history and the vital status were collected at baseline and during 2014. The Framingham risk score was used to evaluate CV risk.	The lowest intakes of total and all subclasses of flavonoids, but isoflavones, were linked to the 2014 CV risk score and increased the risk score from baseline. Flavanones, flavan-3-ols, anthocyanidins had an inverse correlation with incident CV events. Flavan-3-ols, anthocyanidins and flavanones were linked to reduce morality of all-cause.	Food-frequency questionnaire completed from baseline was prone to cause bias, due to whether participants have changed their diets or not during the 12 years.
CVD, CHD mortality	(Mink et al., 2007a)	Flavonoid intake was associated with reduced risk of cardiovascular disease.	34,489 postmenopausal women participated to complete FFQ and were followed over 16 years. Intakes of total flavonoids and flavonoid subclasses were	Anthocyanidins intake was significantly associated with CHD, CVD, and total mortality after adjustment for all of non-dietary confounders, and	7 subclasses of flavonoid were included to evaluate flavonoid intake. The observation was achieved from postmenopausal

			assessed. Proportional hazards rate ratios (RR) were calculated for assessment of CVD, CHD, stroke, and total mortality.	flavonones intake associated with CHD. Consumption of apples, pears, and red wine were associated with reduced mortality of CHD and CVD. Consumption of bran, strawberries, and chocolate associated with mortality of CVD, and consumption of grapefruit was associated with lower mortality of CVD.	women. Sampling could be enlarged for all of population to identify cardio protective effects of flavonoids intakes. Food source should be further studied because of larger source of flavanone intake from orange and orange juice.
Hypertension	(Cassidy et al., 2011)	Habitual flavonoid intake is related to hypertension incident	46,672 women and 23,043 men were followed for 14 years. Food-frequency questionnaires were collected every 4 years.	The highest anthocyanin intake reduced hypertension risk by 8 %. Flavone (apigenin), flava-3-ol (catechin) intakes reduced hypertension risk separately by 5% for all participants and by 6% for participants over 60 y.	Flavanone intake was not associated with lower risk of hypertension.
CVD, Stoke	(Mursu et al., 2007)	The intake of five flavonoid subclasses (flavonols, flavones, flavanones, flava-3-ols and anthocyanidins) is associated with reduction of ischaemic stroke and CVD mortality.	1950 eastern Finnish men without CHD and stroke aged 42-60 years were followed for 15.2 years. Age, examination years, BMI, systolic blood pressure, hypertension medication, serum HDL- and LDL-cholesterol, serum TAG,	A lower CVD risk was associated with the highest intake of flavonol and flavan-3-ol. A lower CVD mortality was found in the highest intake of flavanone and flavone after adjustment for multivariate.	This study excluded women, and onle 4d food record from baseline was used. The result might exist in bias.

			maximal oxygen uptake, smoking, family history of CVD, diabetes, alcohol intake, energy-adjusted intake of folate, vitamin E, total fat and saturated fat intake were assessed.		
Acute Myocardial Infarction	(Tavani et al., 2006)	Intake of flavonoids is associated with risk of acute myocardial infarction (AMI).	760 patients (580 men and 180 women) were interviewed between 1995 and 2003, and 682 patients (439 men and 243 women) were assessed as control group. Flavonoids intake was assesses based on the US flavonoid food composition database. Adjusted odds ratios (OR) and 95% confidence intervals (CI) were calculated by using multiple logistic regression models. Energy and alcohol intake, sociodemographic factors, tobaccos were included as confounders.	Highest intake of anthocyanidins and flavonols reduced AMI risk compared with the lowest quintile.	Higher intake of flavanones was only associated with the reduced risk of AMI after non-adjustment for alcohol intake. Absorption of flavonoids might affect its protective effects on chronic disease.
Epithelial Ovarian Cancer	(Cassidy et al., 2014)	Habitual flavonoid subclass intake impacts on risk of ovarian cancer	171,940 participants were followed for 16–22 y. Validated food-frequency questionnaires were collected every 4 y. The	723 cases of ovarian cancer were confirmed during 16-22 years. The highest group of flavonol and flavanone intakes	The result strongly revealed the association between flavanone intake and reduced risk of ovarian

			risk of ovarian cancer was estimated by using Cox proportional hazards models.	was associated with lower risk of ovarian cancer than those in the lowest group. Flavanone intake had a stronger association with serous invasive and poorly differentiated tumors compared with nonserous and less-aggressive tumors. There was no significantly association between intake of other flavonoids and the risk.	cancer. Other databases can be used to confirm these positive effects.
Cancers	(Mursu et al., 2008a)	High intake of flavonoids decreases the risk of lung, prostate, and colorectal cancer.	2590 middle-aged eastern Finnish men participated and were followed for 16.2 years. Flavonoid intake, maximal oxygen uptake, body weight, body mass index, and resting systolic blood pressure were measured. Blood sample was collected.	62 lung, 138 prostate, and 55 colorectal cancers were reported during follow-up period. Highest intake of total flavonoid, flavanols, and flava-3-ols reduced risk of lung cancer, compared to the lowest intake. There was no association between flavonoid intake and the risk of prostate and colorectal cancer.	Women were excluded in this study, and 4 days food recording might cause bias of flavonoid intake on humans.

Cognitive Function	(Samieri et al., 2014)	Flavonoid intake in midlife is relative to prevalence of healthy aging.	13,818 women in late 50s without chronic diseases were recruited in 1984-1986 and were followed for 15 y. Healthy aging was defined on survival to ≥ 70 y with no major chronic diseases or major impairments in cognitive or physical function or mental health. Intakes of flavonoid-rich foods were retrieved from 2 food-frequency questionnaires.	1517 women achieved healthy aging. Women in the highest quintile of intake of flavones, flavanone, anthocyanin, and flavonol at midlife had greater odds of healthy aging. Oranges, berries, onions, and apples were associated with increased odds of healthy aging.	The results confirmed benefits of favanone intakes on cognitive function. Case control study might provide stronger evidence to support this association.
Cognitive Function	(Butchart et al., 2011)	Flavonoid intake is related to better cognitive function in later life.	IQ of 1091 men and women born in 1936 was measured at age 11 years. Moray House Test, National Adult Reading Test, and verbal fluency were estimated, and FFQ was completed at age of 70 years.	Total fruit, citrus fruits, apple and tea intakes significantly associated with better scores in cognitive tests before adjustment for confounders. Better verbal fluency in later life was related to flavanone intake before adjustment.	There was an association between citrus fruits, flavanone intake and cognitive function, but assessment of flavanoid intake was conducted only at 11 and 70 years old. It needs more works to confirm relationship between flavonoid intake and cognitive function.

Parkinson Disease (Kuo et al.)	(Gao et al., 2012)	Higher intakes of total flavonoids and their subclasses reduce risk of Parkinson disease.	49,281 men and 80,336 women were recruited. Dietary intakes were assessed at baseline and every 4 years. Tea, berry fruits, apples, red wine, and orange / orange juice and flavonoids were assessed by food composition database and FFQ.	Total flavonoids in the highest intake reduced 40% PD risk in men after adjustment for confounders. There was no association in women. Anthocyanins intake and berries decreased PD risk.	The association between total flavonoid intake and PD risk was different in women and men, which might require to be considered in studies on flavonoid intake and diseases.
Type 2 Diabetes	(Sun et al., 2015)	Urinary excretion of flavonoid and phenolic acid metabolites is estimated to associate with T2D risk.	29,611 subjects (NHSII) aged 32– 52 y provided from 1996 to 2001, and 18,743 participants aged 53–80 from 2000 to 2002 were recruited and provided blood and urine. 1111 T2D cases were identified and randomly selected 1 control for each case based on age at urine sample collection, month of sample collection, first morning urine (yes or no), race (white or other races), and menopausal status and postmenopausal hormone use (NHSII only). Metabolites in urine were analysed.	Higher urinary excretion of hesperetin was linked to a lower T2D risk, not other polyphenols. Markers of flavanone intakes (naringenin and hesperetin) and flavonol intakes (quercetin and isorhamnetin) were significantly correlated with a lower T2D risk during the early follow-up period (~4.6 y). None of flavonoids was associated with T2D risk during later follow-up. Flavan-3-ols and ferulic acid were not linked to T2D risk in either period.	It was the first time to use flavonoid metabolites for evaluation of association with diseases risk. The measurement of metabolites applied hydrolysis that is prone to under evaluation of the association.

Diabetes	(Wedick et al., 2012)	Intakes of flavonoid are associated with reduced risk of type 2 diabetes.	A total of 70,359 women in 1984-2008, 89201 women in 1991-2007, and 41,334 men in 1986-2006 were included. FFQ was sent to report their dietary food- rich flavonoids. Diabetes cases were reported.	During follow-up, 12,611 participants were diagnosed with type 2 diabetes. Higher intakes of anthocyanins reduced risk of type-2 diabetes after adjustment for age, BMI, lifestyle and dietary factors. Consumption of flavonoids-rich fruits, blueberries, strawberries and apples/pears, was associated with the risk. Flavnone intake was also associated with the risk, but not after adjustment.	The association between flavanone intake and diabetes was not found. Misclassification of flavonoids or population relative to food habits caused underestimation.

1.9.3 Clinical studies of role of flavanones in disease

Intervention studies can explore the mechanisms by which flavanone and/or metabolites impact on disease, when epidemiologic studies reveal an association between citrus flavanone intake and disease risk (Table 1-7). Rangel-Huerta and colleagues showed that urinary 8-hydroxy-2'-deoxyguanosine, 8-iso-prostaglandin F2α, erythrocyte catalase, glutathione reductase activities, body mass index, waist circumference and leptin decreased while erythrocyte superoxide dismutase activity increased after 12 weeks daily consumption of high polyphenol orange juice (745 mg/d) compared with low polyphenol orange juice (299 mg / d) (Rangel-Huerta et al., 2015a). Constans et al observed a significant association between antioxidant effect and plasma hesperetin levels, and reactive oxygen species reduced after 4-weeks daily consumption of 200 mL blond orange juice, which also tended to reduce concentration of endothelial dysfunction biomarkers (sICAM) from 172.1 ± 11.7 $ngmL^{-1}$ to 168.3 ± 11.0 $ngmL^{-1}$, and increase plasma apoA-I from 1.2 to 1.6 g L^{-1} and apoB from 0.5 to 1.3 g L^{-1} in men with two cardiovascular risk factors (Constans et al., 2014). However, consumption oforange juice or a hesperidin supplement (both providing 320 mg hesperidin) did not affect endothelial function, blood pressure, arterial stiffness, cardiac autonomic function, platelet activation, and NADPH oxidase gene expression, while plamsa concentrations of metabolites increased including eight flavanone metabolites (hesperetin-glucuronide, naringenin-7-glucuronide, hesperetin-glucuronide, hesperetin-diglucuronide, hesperetin-diglucuronide, naringenin-glucuronide, hesperetin and naringenin) and 15 phenolic metabolites (hippuric acid, dihydroferulic acid, dihydroferulic acid–3-glucuronide, 4-hydroxyphenylacetic acid, vanillic acid, hydroxyhippuric acid, iso/ferulic acid–glucuronide, 3- hydroxyhippuric acid, isovanillic acid, 3-hydroxyphenylacetic acid, vanillic acid–glucuronide, isovanillic acid–glucuronide, iso/vanillic acid–glucuronide, 4-hydroxy-benzoic acid, benzoic acid–4 glucuronide) in participants aged 51–69 y (Schar et al., 2015).

Consumption of orange juice displayed anti inflammatory properties in humans. Dalgard et al. observed that C-reactive protein (CRP) was decreased by 11 % and fibrinogen by 3 % after 4 weeks consumption of juice containing 250mL orange juice and 250mL blackcurrant juice in patients with peripheral arterial disease (Dalgard et al., 2009).

Consumption of orange juice protected against inflammation induced by meals. Ghanim et al. showed that a high-fat, high-carbohydrate meal coupled with orange juice increased plasma

glucose concentration, endotoxin, inflammatory indexes (protein expression of NADPH oxidase subunit p47phox, phosphorylated and total p38 mitogen-activated protein kinase, suppressor of cytokine signaling-3, TLR2 and TLR4 messenger RNA (mRNA) and protein expression, mRNA expression of matrix metalloproteinase in circulating mononuclear cells) (Ghanim et al., 2010). In addition, serum antioxidant capacity significantly increased by around 50 µmol TE/L after consumption of both orange juice or hesperidin and naringenin with 28 g of Kellogg's Corn Flakes and 118 mL of 2% milk as a typical, high-carbohydrate breakfast in health subjects (Snyder et al., 2011).

However, immune function was not affected by consumption of orange juice or hepseridin. Perche et al found that polymorphonuclear neutrophil cells, B and T lymphocytes, NK cells and monocytes, ROS production by stimulated phorbol myristate-acetate, lytic activity of NK cells or cytokine production capacity of leukocytes were not affected in well-nourished humans after 4 weeks of daily consumption of orange juice containing 292 mg hesperidin or isocaloric control beverage with a capsule of hesperidin (292 mg) compared with isocaloric control beverage with placebo (starch) (Perche et al., 2014).

Consumption of orange juice may improve cognitive function. Kean et al observed that better global cognitive function and improved global performance and memory were found after 8 weeks daily consumption of flavanone-rich orange juice (305 mg) compared with low-flavanone (37 mg) orange-flavored in older adults (Kean et al., 2015b).

Furthermore, Milenkovic et al. found that 4 weeks consumption of orange juice containing 292 mg/500 mL of hesperidin up-regulated 1,706 genes and down-regulated 1,716, while 4 weeks consumption of control drink with 2 capsules of hesperidin (146 mg each) up-regulated 1,033 genes and down-regulated 786 genes in overweight healthy male blood leukocytes compared to 4 weeks of control drink and placebo (starch). Those genes which have been shown to be involved in processes of signal transduction, cell adhesion, immune response, cell proliferation, chemotaxis and lipid metabolism were mostly downregulated, while those which were upregulated were linked to the inflammatory response or the I-kappaB kinase/NFkappaB (Milenkovic et al., 2011).

Table 1-7 Orange juice / flavanone and disease risk on intervention studies

Diseases	Reference	Hypothesis	Methods	Results	Comments
Cardiovascular disease	(Schar et al., 2015)	Orange juice or a dose-matched hesperidin supplement affects cardiovascular risk biomarkers in men at moderate CVD risk.	16 participants aged 51-69 y consumed orange juice or a hesperidin supplement (320 mg) or control. Endothelial function, blood pressure, arterial stiffness, cardiac autonomic function, platelet activation, NADPH oxidase gene expression and plasma flavanone metabolites were assessed at baseline and 5 h postintake.	Cardiovascular risk biomarkers were not changed.	An acute, randomized, placebo-controlled crossover trial, measurements conducted only at baseline and 5 h post intake. Other biological parameters in biological samples might improve accuracy of benefits of flavanone or orange juice intake in cardiovascular diseases.
Oxidative stress	(Rangel-Huerta et al., 2015a)	The intake of high polyphenol orange juice affects antioxidant defence system, oxidative stress biomarkers, and clinical signs of metabolic syndrome.	100 nonsmoking overweight or obese subjects participated in the randomized, double-blind crossover study for two 12-wk periods with a 7-wk washout period. Enzymatic and nonenzymatic blood antioxidant defence systems, urinary and plasma oxidative stress biomarkers, and clinical signs of metabolic syndrome were assessed before and after interventions.	Urinary 8-hydroxy-2'-deoxyguanosine, 8-iso-prostaglandin F2α, erythrocyte catalase, and glutathione reductase activities decreased, as well as body mass index, waist circumference and leptin. The consumption of high polyphenol orange juice increased erythrocyte	Flavanone content in orange juice, or flavanone metabolites in biological samples were not analysed, which caused a misevaluation whether orange juice flavanone contributed to these benefits.

				superoxide dismutase (SOD) activity.	
Cardiovascular disease	(Constans et al.)	Blond orange juice containing flavanone affects antioxidant markers, cardiovascular biomarkers and endothelial function	25 male volunteers with LDL-cholesterol between 130 and 190 mg/L over 50 years were recruited in this randomized cross-over study. 200 mL of either blond orange juice or control beverage was consumed 3 times every day for 4 weeks. Endothelial function (flow mediated dilatation and plasma markers), oxidative status, lipid profile and inflammatory markers were analysed.	Improved antioxidant effect was linked to hesperetin plasma levels, while reactive oxygen species decreased. Endothelial dysfunction tended to decrease and plasma apoA-I concentration tended to increase.	The results supported effects of orange juice hesperetin on cardiovascular diseases in subjects with risk factors. Sampling can be extended to healthy humans and females for assessment of flavanone in CVD diseases in future.
Endothelial and Vessel Function	(Milenkovic et al., 2011)	Chronic consumption of orange juice influence the gene expression profile of leukocytes and hesperidin contribute to nutrigenomic effects of orange juice.	24 overweight men at age of 50-65 years were divided into three groups to consume control drink with placebo, control drink with hesperidin, or orange juice for 4 weeks in a randomized, controlled, crossover study. Fasting blood samples were collected after 4-week dietary intervention. Global gene expression profiles were estimated by	Leukocyte gene expression was affected after 4-week orange juice and hesperidin intakes, which are related to chemotaxis, adhesion, infiltration of circulating cells to vascular wall and lower lipid accumulation.	It was first to study effect of orange juice and hesperidin on human vascular function at the molecular level. It is a suggestive direction to understand exact effect of flavonoids on human diseases.

			human whole genome cDNA microarrays.		
Arterial Inflammation	(Dalgard et al., 2009)	Both orange and blackcurrant juice reduce levels of inflammatory markers in patients with CVD.	48 patients with peripheral arterial disease (PAD) participated in the block-randomised, 2×2 factorial crossover trial with two intervention periods of 4 weeks and one wash-out period of 4 weeks. 250mL orange and 250mL blackcurrant juice with vitamin E; juice with placebo-vitamin E; placebo with vitamin E; placebo with placebo- vitamin E were consumed for 4 weeks. Venous blood samples were collected, 3d apart, at baseline and at the end of each intervention period. C-reactive protein (CRP) and IL-6 concentrations were measured.	Juice reduced C-reactive protein (CRP) by 11% and fibrinogen by 3%.	This study supported effects of orange juice on patients. Healthy subjects can be considered to extend the effect of fruit juice in population.
Inflammation and Oxidant Stress	(Ghanim et al., 2010)	Orange juice affects high-fat, high-carbohydrate (HFHC) meal-induced inflammation and	30 healthy subjects divided into 3 groups consuming water, 300 kcal glucose or orange juice with a 900-kcal HFHC meal. Blood were collected before and 1, 3, 5 h after	Orange juice with meal significantly reduced protein expression of the NADPH oxidase subunit p47(phox), phosphorylated and total p38	Orange juice was evidenced to display its postprandial effects. Flavanone and their metabolites could provide more information about their anti-inflammation and

		oxidative stress and the expression of plasma endotoxin and Toll-like receptors.	intaking. Circulating mononuclear cells (MNCs) were isolated. ROS-generation was measured by chemiluminescence. Total RNA was isolated and real-time reverse transcriptase polymerase chain reaction was measured. Endotoxin, serum MMP-9, plasma-glucose and insulin concentration were measured.	mitogen-activated protein kinase, and suppressor of cytokine signaling-3; TLR2 and TLR4 messenger RNA (mRNA) and protein expression; mRNA expression of matrix metalloproteinase (MMP)-9 in MNCs; and plasma concentrations of endotoxin and MMP-9, and generation of reactive oxygen species	autoxidation.
Blood Oxidant Stress	(Snyder et al., 2011)	Orange juice flavonoids reduce postprandial oxidation.	16 male and female participants were divided into 4 groups: placebo (ascorbic acid) with sugar and sucrose; placebo (ascorbic acid and sugar mixture) with hesperidin; placebo (ascorbic acid and sugar mixture) with naringenin and luteolin; fresh-squeezed navel orange juice. Serum oxygen radical absorbance capacity (ORAC), total plasma phenolics (TP), and serum	ORAC and LO lag time significantly increased after consumption of orange juice and placebo with hesperidin, lutelin and naringenin.	This study confirmed serum antioxidant capacity of flavonoids in orange juice.

			lipoprotein oxidation (LO) were measured before and 1, 2, and 3 h after intervention.		
Cognitive benefits	(Kean et al., 2015b)	Daily consumption of flavanone-rich orange juice benefits cognitive function in healthy older adults.	A crossover, double-blind, randomized trial was conducted. 37 healthy older adults daily consuming high-flavanone orange juice or a placebo for 8 wk. Cognitive function, mood, and blood pressure were assessed at baseline and after treatments.	Global cognitive function was significantly improved after 8-wk consumption of flavanone-rich juice compared with the low-flavanone control. Mood and blood pressure was not significantly affected.	The results supported benefits of flavanone rich orange juice in cognitive function. Flavanone metabolites might provide more accurate evdence.
Immune function	(Perche et al., 2014)	Orange juice and its major polyphenolic compound hesperidin affect immune cell functions.	24 healthy participants randomised for daily consumption of 500 mL orange juice, hesperidin, or placebo. Fasting bloods were taken at baseline and at the end of each intervention.Excreted cytokine, lytic activity of NK cells, and reactive oxygen species were assessed.	PMN, B and T lymphocytes, NK cells and monocytes, ROS production, lytic activity of NK cells or cytokine production capacity of leukocytes were not affected.	This study is inconsistent to other studies about orange juice intake in immunological function. Subjects (healthy or patients, male or female, active or inactive) or extended intervention period should be further studies to achieve the hypothesis.

1.9.4 Phenolic acids and diseases

In comparison with flavonoids, phenolic acids as metabolites from flavonoids have been little studied for their benefits in humans. The health effects of phenolic metabolites are mostly attributed to experimental studies linked to several chronic diseases by searching PubMed targeting specific phenolic acids and 'disease' or 'cancer', there were eight relevant papers (Table 1-8). 4-hydroxybenzoic acid, isovanillic acid, isovanillic acid -glucuronide, vanillic acid-glucuronide, protocatechuic acid-3-sulfate, and benzoic acid sulfate significantly reduced secretion of pro-inflammatory cytokine TNF-α, and IL-1β secretion was inhibited by 4-hydroxybenzoic acid at physiological concentrations in human THP-1 monocytic cells stimulated by lipopolysaccharide (Gesso et al., 2015). 3, 4-dihydroxybenzoic acid (protocatechuic acid) increased caspase-3 and caspase-8 activities, and decreased intercellular adhesion and concentration of interleukin IL-6 and IL-8 in human breast cancer, lung cancer, HepG2, cervix HeLa, and prostate cancer cells (Yin et al., 2009). Kampa and his colleagues also found anti-proliferation activity of 4-hydroxybenzoic acid in T47D human breast cancer cells (Kampa et al., 2004). It also increased activity of peroxisome proliferator–activated receptor-γ that controls glucose and lipid metabolism, and adiponectin and GLUT4 levels in human omental adipocytes (Scazzocchio et al., 2011). In addition, protocatechuic acid reduced IL-6 protein levels in ox LDL-stimulated and CD40L-stimulated vascular endothelial cells, and protocatechuic acid-4-sulfate decreased CD40L-stimulated vascular endothelial cells (Amin et al., 2015). Similarly, the anthocyanin and its metabolites reduced production of VCAM-1 protein, 3-O-methylgallic acid and 3, 4-dihydroxyphenylacetic acid respectively at the concentration of 260 μM and > 400 μM significantly inhibited proliferation of HCT116 colon cancer cells (Henning et al., 2013). 3, 4-dihydroxyphenylacetic acid also attenuated proliferation of prostate cancer cells (LNCaP) at135 μM and colon cancer cells (HCT116) at 90 μM, and had more inhibitory effect on colon cancer cells compared with normal intestinal epithelial cells (IEC6) (Gao et al., 2006b). 3, 4-dihydroxyphenylacetic acid and 4-hydroxyl-phenylacetic acid at 2 mM have been shown to have anti-platelet aggregation activity in cancer cells (Kim et al., 1998). 3-hydroxybenzoic acid and 3-(3´-hydroxyphenyl) propionic acid inhibited aggregation of β-amyloid peptides into neurotoxic β-amyloid, linked to Alzheimer's disease neuropathogenesis (Wang et al., 2015).

Table 1-8 Phenolic acids and diseases and cancers

Related disease	Sources	Phenolic acids	Results
Alzheimers Disease	(Wang et al., 2015)	3-hydroxybenzoic acid 3-(3´-hydroxyphenyl)propionic acid	The aggregation of β -amyloid peptides into neurotoxicamyloid was decreased in *in vitro* incubation of Aβ1–42 peptides (25 μM)
Vascular Disease	(Amin et al., 2015)	Protocatechuic acid	IL-6 protein levels in oxLDL stimulated vascular endothelial cells was reduced
		Protocatechuic acid-4-sulfate	IL-6 protein production was reduced In CD40L-stimulated cells
Diabetes	(Scazzocchio et al., 2011)	Protocatechuic acid	Increased adipocyte glucose uptake and GLUT4 membrane translocation, nuclear PPARγ activity, adiponectin and GLUT4 expressions, and prevented against pathology associated with insulin resistant.
Monocytic Leukemia	(Gesso et al., 2015)	Isovanillic acid, Isovanillic acid-glucuronide, Vanillic acid-glucuronide, Protocatechuic acid-3-sulfate, Benzoic acid-sulfate, 4-Hydroxybenzoic acid Protocatechuic acid	TNF-β secretion of human monocytic cells (THP-1) stimulated by LPS was reduced.
		4-Hydroxybenzoic acid	IL-1β secretion of human monocytic cells

			(THP-1) stimulated by LPS was reduced.
Cancers	(Yin et al., 2009)	Protocatechuic acid	Human breast cancer MCF7 cell, lung cancer A549 cell, HepG2 cell, cervix HeLa cell, and prostate cancer LNCaP cell, breast MCF10A, lung IMR-90, and prostate PrEC cells viability, mitochondrial membrane potential, and Na^{+}-K^{+}-ATPase activities were reduced, while lactate dehydrogenase leakage, caspase-3 activity (2-8 μmol/L), DNA fragmentation in cancer cell were increased. Cell adhesion, vascular endothelial growth factor (IL-6 and IL-8) was inhibited at 2-8 μmol/L.
	(Kampa et al., 2004)	Caffeic acid, ferulic acid, protocatechuic acid, 3, 4-dihydroxyphenylacetic acid, sinapic acid and syringic acid	Growth of breast cancer cells was inhibited.
		3, 4-dihydroxyphenylacetic acid	Nitric oxide synthase of breast cancer cells was reduced.
	(Gao et al., 2006a)	3, 4-dihydroxyphenylacetic acid	Proliferation of the prostate and colon cancer cells were inhibited, and colon cancer cells were inhibited more than normal intestinal epithelial cells.
	(Kim et al., 1998)	3, 4-dihydroxyphenylacetic acid, 4-hydroxylphenylacetic acid	Inhibited platelet aggregation of human stomach cancer cells (SNU-1), human colon cancers (SNU-C4), mouse

		lymphoid neoplasma cells (P-388D1), mouse lymphocytic leukemia cells (L-1210), human hepatoblastoma cells (HepG2), human lung cancer cells (A549) and Macacus' rhesus monkey kidney cells (MA-104), quercetin and ponciretin were more effective on cytotoxicity for tumor cell lines.
	2, 4, 6-trihydroxybenzaldehyde	Cytotoxic activities on human stomach cancer cells (SNU-1), human colon cancers (SNU-C4), mouse lymphoid neoplasma cells (P-388D1), mouse lymphocytic leukemia cells (L-1210), human hepatoblastoma cells (HepG2), human lung cancer cells (A549) and Macacus' rhesus monkey kidney cells (MA-104)

1.10 Flavonoids, health effects and exercise

Flavonoids have been widely studied to explore their beneficial effects in athletes and sports. Endurance capacity and $VO_{2\ max}$ were increased during a cycling test by 13.2 % and 3.9 % in healthy untrained volunteers supplementing quercetin (500 mg) twice daily for 7 days (Davis et al., 2010). The increase in F_2-isoprostanes induced by exercise was significantly attenuated in athletes after supplementing 120 mg resveratrol and 225 mg quercetin for 6 days and 240 mg resveratrol and 450 mg quercetin on day 7 (McAnulty et al., 2013). This indicated that flavonoid supplements can reduce lipid peroxidation induced by exercise. In addition, blood lactate concentration decreased by 27 % in overweight, middle-aged women conducting 1 h aerobic training 3 times a week for 3 months and consumed 500 mL/d of orange juice compared with control group who did not drink orange juice, which revealed that consumption of orange juice was potential to attenuate muscle fatigue and enhance exercise performance (Aptekmann and Cesar, 2010). Furthermore, the inhibitory effects of flavonoid intake on muscle force loss, muscle damage and oxidative stress biomarkers (creatine phosphokinase , lactate dehydrogenase and malondialdehyde) induced by exercise, was confirmed in mice by feeding catechin for 8 weeks (Haramizu et al., 2011).

However, the effect of physical activity on the bioavailability and absorption of dietary flavonoids has been rarely investigated. In a cross sectional study, it was found that 24 h urinary excretion of naringenin, eriodictyol, and hesperetin metabolites were 92 %, 88 %, and 53 % higher in very well trained athletes than in sedentary individuals after ingestion of aronia-citrus juice (a mixture of citrus juice (95 %) with 5 % of *Aronia. Melanocarpa* juice containing 86.1 mg of flavanone per 400 ml) (Medina et al., 2012).

Gut bacteria is associated with metabolism of polyphenols in the colon. The gut microbiota could be modified by frequent physical activity, which also influences bowel movements (Zheng et al., 2003a, Holdstock et al., 1970). Clark et al reported that there were 48 bacterial taxa in significantly greater abundance in athletes than in high BMI (> 28) controls excluding *Bacteroidetes*, and 40 taxa excluding *Lactobacillaceae*, *Bacteroides* and *Lactobacillus* than lower BMI (< 25) controls by measuring 16S rRNA of fresh stool samples (Clarke et al., 2014). An animal study revealed different TGGE profiles of gut bacteria 16S rRNA from 5 of 7 male rats which accessed voluntary wheel-running exercise for 5 weeks compared with 7 male rats without exercise for 5 weeks, where another two rats subjected to the exercise had

different TGGE profiles from others (Matsumoto et al., 2008). If the bacteria change with exercise then the flavanone metabolism in the gut may also change.

Intensive exercise has also been illustrated to prolong gut transit time that might affects the timing and amount of dietary flavonoids reaching the colon for bacterial degradation and further absorption(Brouns and Beckers, 1993, Spencer and Crozier, 2012). In addition, many gastrointestinal hormone related to motility, metabolism and absorption functions have been reported to change by increased physical activity (O'connor et al., 1995).

Absorption of flavonoids involves epithelial brush border membrane transporters, such as Na-dependent GLUT-1, and UDP-glucuronosyl transferase (Gee et al., 1998, Henry-Vitrac et al., 2006). However, Piatkowski et al reported that 8-week treadmill running did not affect UDP-glucuronosyl transferase, beta-glucuronidase, and glutathione-S-transferase in young and middle-aged male rats (Piatkowski et al., 1993).

In addition, physiological functions of gastrointestinal intestine tract have been linked to absorption or/and rate of drug and flavonoids, such as gastric emptying and gut transit time (Clements et al., 1978, Mullen et al., 2008a, Mullen et al., 2008b, Mullen et al., 2009a). In addition, marathon runners had faster gastric emptying than sedentary subjects (67.7 min vs. 85.3 min) measured by recording stomach radioactivity of an isotope marker (human serum albumin), which was injected into a raw egg for making an omelette (Carrio et al., 1989). Koffler and his colleagues found that whole bowel transit time was significantly shortened from 41 ± 11 h to 20 ± 7 h in 7 healthy sedentary males through 13-wk (3 $d \cdot wk^{-1}$) body strength training program by using keiser K-300 variable resistance machines, whereas mouth to cecum transit time was not influenced (1.83 ± 0.32 hr vs. 1.40 ± 0.25 hr) (Koffler et al., 1992).

Gastrointestinal hormones might be related to intestinal physiological functions. Peptide YY delayed gut transit time from 67 ± 4 mins to 94 ± 7 mins and 192 ± 9 mins, and prolonged gastric emptying from 37 ± 8 mins to 63 ±10 mins and 130 ±12 mins when subjects received low dose of infusion at 0.4 pmol/kg/min dose (50 ml), and high dose infusion at 1.1 pmol/kg/min (50 ml) (Savage et al., 1987). Naslund and his colleagues supposed that peptide YY was responsible for intestinal motility and glucagon-like peptide -1 dominated early gastric emptying (Naslund et al., 1998). The gastrointestinal hormones (vasoactive intestinal

polypeptide, gastrin, secretin, pancreatic polypeptide, neurokinin A, pancreastatin, and glucagon-like peptide 1) were significantly increased after a 30 min race in 26 marathon runners (O'connor et al., 1995).

In addition, metabolites are circulated through blood stream in the body. However, blood flow required for exercising muscle and lung is diverted away from the GI tract and tissues (Duncker and Bache, 2008, Flamm et al., 1990, Khazaeinia et al., 2000). Cycling decreased blood flow in the spleen, kidney and liver by 46 %, 24 % and 18 % whereas blood flow increased in active muscle (leg) by 50 % in humans (Flamm et al., 1990). In comparison with resting, renal blood flow was decreased by 53.4 %, 17.5 % and 21.1 % respectively immediately, 30 min and 60 min after cycling in six males (Suzuki et al., 1996).

However, forearm blood flow was 64 % higher in athletes than sedentary people by using capacitance plethysmography (Ebeling et al., 1993). There was a marginally significantly lower hepatic blood flow (15.6 mL/min/kg vs. 18.2 mL/min/kg) in participants undertaking 16 weeks exercise (40 min treadmill walking, stairclimbing, or combination three times per week) compared with subjects without exercise (Pollock et al., 1995). In addition, biliary excretion of endogenous bile acids and reduced and oxidized glutathione were increased respectively by 190 % and 173 % respectively in female rats with 6 weeks running exercise compared with sedentary rats (Watkins et al., 1994). Therefore, physical activity modified gut physiological function, gut bacteria and circulatory system that potentially influence bioavailability of flavanone so that induce variable benefits in humans by the intake of dietary flavonoids.

1.11 Aim of thesis

Based on the review of the literature above, it is clear that flavanones and flavanone-rich food constitute an integral part of the diet and may enhance human health. However, many factors could cause inconsistent effects, such as food sources, food matrix, age, ethnicity and physical activity. On the other hand, although the bioactive properties of flavanone have been reported in several studies flavanone bioavailability is low in humans. The impact of metabolites and phenolic acids produced in the body may be more important. Flavanone metabolites are more complex than the ingested parent flavanone, and possible synergistic or antagonistic effects can not be ignored. Thus in this thesis, factors affecting the bioavailablity

and metabolism of orange juice flavanones, including food source, food matrix and physical exercise, were considered to investigated in order to enhance their beneficial effects in humans.

The following hypotheses were to be tested:

1 The release and stability of of flavanones in orange juice and their degradation by gut bacteria in the gastrointestinal tract are influenced by the source and formulation of the juice.

2 Full fat natural yoghurt affects the stability and gut bacterial metabolism of flavanone in orange juices in *in vitro* models of the gastrointestinal tract

3 Physical exercise affects the bioavailability and metabolism of orange juice flavanones in healthy sedentary humans

To investigate these hypotheses, the following studies were conducted:

1 Smooth orange juice and orange juice 'with bits' from six different brands were analysed to assess the variability in flavanone content between brands and the impact of inclusion of pulp 'bits'(chapter 3)

2 The metabolism of flavanones was investigated in smooth orange juice and orange juice 'with bits' using stimulated GI digestion and fermentation models (chapter 3)

3 Full fat natural yoghurt (no live bacteria) was incubated with smooth orange juice and orange juice 'with bits' to investigate any differences in metabolism of orange juice flavanone in an in vitro model of the upper GI tract and colonic fermentation model (chapter 4)

4 To investigate the mechanism by which yoghurt may influence flavanone metabolism, the fate of orange juice flavanones in models of the upper GI tract were compared in the presence of Full fat natural yoghurt (no live bacteria), probiotic yoghurt (live bacteria) and fruit yoghurt (no live bacteria but additional polyphenols from the fruit) (chapter 4). Raftiline and glucose were used to investigate the effect of inclusion of different types of fermentable non digestible carbohydrates (dietary fibre) on colonic metabolism of hesperidin by gut bacteria (chapter 5)

5 A 4 week physical exercise intervention study was conducted to assess bioavailability and metabolism of orange juice flavanone in previously healthy sedentary humans (chapter 6)

CHAPTER 2 *General Methods*

2.1 Introduction

As discussed in chapter 1, it is essential to understand the metabolism and bioavailability of dietary flavonoids which may determine thebeneficial effects of dietary polyphenols in the host. Thus, it is important to consider factors that may affect bioavailability of orange juice flavanone by interactions between dietary flavonoids (flavanone) and the food matrix, and physical activity which m act through physiological, metabolic and absorptive processes. This chapter covers the common methods used throughout the whole thesis. More specific methods will be described in the individual chapters.

Advanced analytical equipment, such as HPLC-PDA, HPLC-MS and GC-MS were used in this thesis to measure polyphenols and their metabolites. The total phenol content and antioxidant capacity of orange juices and biological samples (plasma, urine,) were also measured using colorimetric assays. Hesperidin, narirutin, total phenol, total flavonoid content, and antioxidant capacity were measured in a range of orange juices. Flavanone metabolites, phenolic acids, total phenols and antioxidant capacity, pH were measured in the in vitro models of the GI tract. In the human studies, flavanone metabolites in plasma and or urine were measured. These methods are detailed below (Figure 2-1).

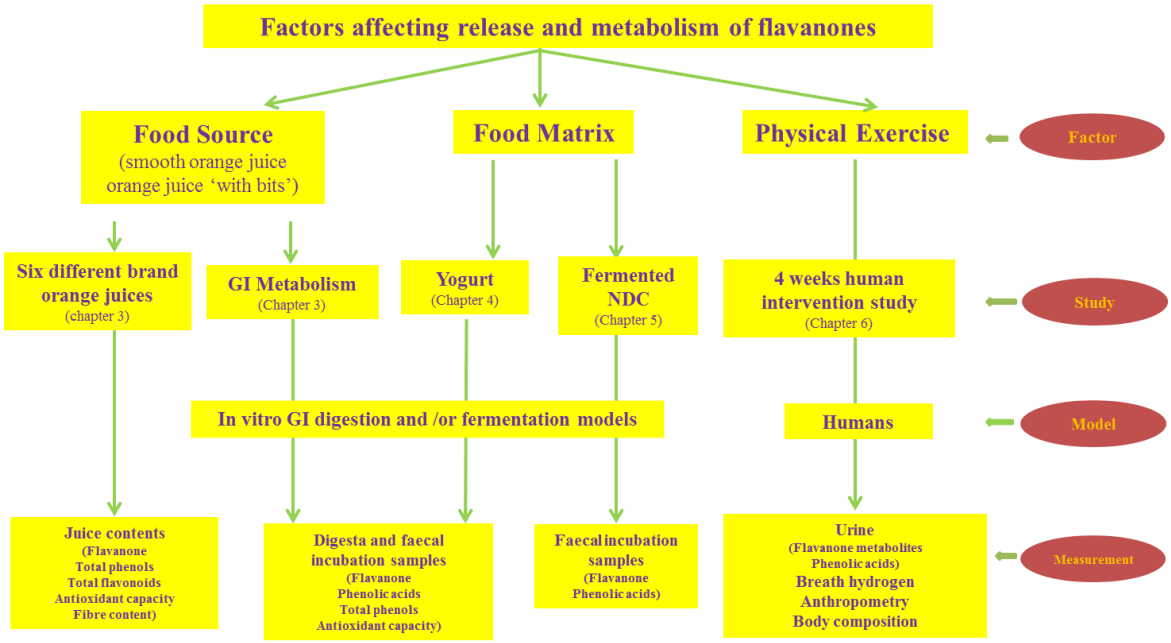


Figure 2-1 Flow of studies in this thesis and the factors studied

The factors affecting bioavailability of orange juice flavonoids were tested in different models, including food source, food matrix, people and physical exercise, the final boxes are the analysis done on samples in this thesis. NDC: Non-digestible carbohydrates.

2.2 Orange Juices

The orange juices used in this thesis were purchased from local supermarkets in Glasgow: Tropicana Company, Leicester, U.K; Innocent, Innocent Ltd, London, U.K; Sainsbury's own brand orange juice, Sainsbury's Supermarkets Ltd, London, U.K; Tesco own brand orange juice, Tesco Stores Ltd., U.K; Morrison's own brand orange juice, Wm Morrison Supermarkets PLC, Bradford, U.K; Waitrose own brand orange juice, U.K. These companies all produce smooth orange juice and a corresponding orange juice ''with bits''. The term ''with bits'' is used by these manufacturers to describe the inclusion of pulp in the juice. The actual content of the 'bits' (pulp) in orange juices was not clear. However orange pulp has been reported to be 9.8 % of proteins, 2.4 % of lipids, 2.7 % of ash contents, 9.3 % of total carbohydrates and 74.9 % of total dietary fibre content 54.8 % (insoluble dietary fiber and 20.1 % soluble dietary fibre) (Larrea et al., 2005). We had the dietary fibre content of the juices used in this thesis measured by the AOAC method (Prosky et al., 1988) (The analysis of dietary fibre was conducted by Prof Kofi E Aidoo at School of Health and Life Science, Glasgow Caledonian University) There was much less fibre than that reported above (see

chapter 3). The juices used were 100 % squeezed, not from concentrate. Tropicana orange juices were purchased in April of 2013, Sainsbury's, Tesco and Innocent orange juices were purchased in August of 2013, Waitrose and Morrison's orange juices were obtained in March of 2014.

2.3 *In vitro* gastrointestinal digestion and fermentation model

The model to simulate gastric and small intestinal digestion was adapted from Gil-Izquierdo with some modifications (time and orange juice volume shown below) (Gil-Izquierdo et al., 2003). This in vitro procedure consisted of two sequential steps: an initial gastric phase: pepsin / HCl (pH 2) for 1 h and a small intestinal phase: pancreatin enzymes/bile salts pH 7 for 6 h. Orange juice (40 mL) was used for gastric and small intestinal digestion, and then colonic fermentation based a proportion of 250 mL orange juice reaching colon consumed by subjects in Mullen's study (Mullen et al., 2008a).

2.3.1 Gastric phase

The sample was incubated for 1 hour at 37 °C in a shaking water bath at 100 stokes/min with pepsin (from porcine gastric mucosa, EC 3.4.23.1, Sigma, Steinheim, Germany). For the gastric phase digestion, orange juice was adjusted to pH 2 by adding concentrated HCl (5 M), and pepsin added (0.32 % w/v) in 0.1 M HCl in cap-sealed bottles, and then incubated in a 37 °C shaking water bath at 100 stokes/min for 1 h.

2.3.2 Small intestinal phase

After the gastric phase, the orange juice digesta was neutralized with $NaHCO_3$ before adding pancreatin (4 g/l; from porcine pancreas, Sigma, UK) and bile extract (25 g/l; containing a mixture of sodium cholate and sodium deoxycholate, Sigma, UK) in 0.1 M $NaHCO_3$. Digestion was performed at 37 °C for 6 h in a shaking water bath at 100 stokes/min. During the intestinal digestion, cellulose dialysis tubing (molecular weight cut-off 0.1-0.5 kDa, Sigma) was used to allow carbohydrates, fatty acids and peptides to pass through the dialysis

membrane into a 5L beaker filled with 5 L of distilled water. At the end of the small intestinal digestion with cellulose dialysis tubing, digestas were collected and subjected to freeze drying to remove water for colonic fermentation by using Freeze Dryer (Edwards Micro Modulyo.)

For evaluation of stability/ release of hesperidin and narirutin in orange juice during this stage, the digesta was not incubated with the dialysis membrane in order to avoid loss of flavanones released (Bermúdez-Soto et al., 2007). Aliquots (1.5 mL) were taken before and after the pepsin digestion, 2 h, 4 h and 6 h during the pancreatin / bile acids digestion, and quickly stored at -80 °C until analysis.

2.3.3 Colonic phase

After collection of digesta from the dialysis membrane tubing, digesta was freeze-dried by using Edwards Micro Modulyo Freeze Dryer. The residues were stored at - 80 °C until colonic fermentation.

2.3.3.1 Faecal sample collection

Faecal samples were collected from Caucasian volunteers who had a normal diet, were non-smokers, had no digestive diseases, no food allergies, and had not received antibiotics for at least 6 months before the study. The volunteers followed a low-polyphenol diets avoiding fruits, vegetables, tea, coffee, wine and wholemeal foods for 2 days before providing a faecal sample (Appendix-3). The faecal sample was collected in a plastic box containing an AnaeroGen sacket (Oxioid, Basingstoke, Hampshire, UK) to generate anaerobic conditions, and processed within 1-2 h of passage.

2.3.3.2 *In vitro* fermentation with human colonic bacteria

The fermentation medium was prepared as described by Jaganath (Jaganath et al., 2009) . In brief, 2 g of tryptone was mixed in 400 mL of distilled water and 100 µl of micromineral solution consisting of 13.2 g of $CaCl_2 \cdot 2H_2O$, 10 g of $MnCl_2 \cdot 4H_2O$, 1 g of $CoCl_2 \cdot 6H_2O$, 8 g of $FeCl_3 \cdot 6H_2O$, and distilled water up to 100 ml, 200 ml of buffer solution (2 g of NH_4HCO_3,

17.5 g of $NaHCO_3$, and distilled water up to 200 ml), 200 ml of macromineral solution (2.85 g of Na_2HPO_4, 3.1 g of KH_2PO_4, 0.3 g of $MgSO_4 \cdot 7H_2O$, and distilled water up to 200 ml), and 1 mL of 1 % (w/v) of resazurin solution (a redox indicator). The medium was adjusted to pH 7 using 6 M HCl, boiled, and allowed to cool under oxygen-free nitrogen (OFN) to remove oxygen. Reducing solution (312 mg of cysteine hydrochloride, 2 mL of 1 M NaOH, 312 mg of $Na_2S \cdot 9H_2O$, and distilled water up to 50 ml) was added at 0.5 ml / 10 ml of medium after which the solution was purged with OFN until anaerobic conditions were obtained.

Fresh faeces were homogenized with sodium phosphate buffer (pH = 7) to obtain 32 % faecal slurry. Faecal slurries were then strained through a nylon mesh to remove particulate materials. Strained slurry (5 ml) was added to the mixture of fermentation medium and reducing solution. Each batch culture consisted of slurry mixture (50 ml) with digested orange juice powder, or hesperidin, or hesperidin plus 0.5 g glucose, or hesperidin plus raftilne. No substrates were added for the controls. The amount of hesperidin (10 µg) was based on the levels of orange juice consumed by subjects in chapter 3.

The 50 ml fermentation bottles were sealed air-tight with polyethylene caps and flushed with OFN before incubation in a 37 °C shaking water bath at 60 stokes/min and protected from the light by a metal cover on the top of the water bath. The fermentation bottles were incubated for 24 h in duplicate. Aliquots of the fermentation fluid (8 ml) were collected at 0, 2, 4, 6, 24 h, and stored at -80 °C until analysis. pH was measured at 0, 2, 4, 6, 24 h by an auto calibrated portable digital pH meter model (Hanna pH20instruments, USA).

2.4 Analysis of flavanones in orange juice

Orange juice (1 mL) was mixed with 1 ml of methanol for 10 min by using an Ultraturrax homogenizer (Fisher Scientific, UK). The mixture was centrifuged at 4500 g at 4 °C for 10 min. The pellet was extracted with 0.5 mL of methanol for 10 min, and then centrifuged at 4500 g at 4 °C for 10 min in duplicate. The extracts were centrifugally evaporated under vacuum at room temperature using a Thermo Speedvac concentrator. The residue was resuspended with 25 µl of methanol and made up to 1 mL with 0.1 % formic acid for analysis.

2.4.1 Determination of total phenolic compounds

Total phenolic compounds were determined by using the Folin-Ciocalteau method (Singleton and Rossi, 1965). The above extracts (20 µl) were added and mixed with 100 µl of Folin-Ciocalteu's reagent (1:10) and 70 µl of distilled water in 96-well plates in triplicate. After 5 min, 70 µl of sodium carbonate solution was added. The samples were incubated for 2 h and then read at 765 nm by using a Multiskan spectrum plate reader (Thermo Fisher Scientific Inc., Stafford ST, UK). Gallic acid was used for the standard curve ranging from 50 to 800 µg / ml. All results were expressed as grammes gallic acid (g GAE) / ml orange juice.

2.4.2 Determination of total flavonoids

Total flavonoids were determined by using the spectrophotometric method based on the production of complex compounds of flavonoids with aluminium chloride (Chang et al., 2002). The extracts (100 µl) were added into 96 well plates. Aluminum chloride solution (10 %, 60 µl) and potassium acetate (30 µl), distilled H_2O (95 µl) were then added. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm. Total flavonoids were expressed as mg/ml quercetin equivalent (QE). Total flavonoid content was calculated with respect to a quercetin standard curve ranging from 50 to 200 µg/ mL.

2.4.3 Determination of antioxidant capacity (FRAP)

Antioxidant capacity was determined by FRAP assay in a redox-linked colourimetric reaction. The method is based on the reduction of ferric (Fe^{3+}) to ferrous (Fe^{2+}) ion at low pH. This causes the formation of a blue colored ferrous-tripyridyltriazine (Fe^{2+}-TPTZ) complex, which absorbs at 593 nm. Orange juice (25 µl) was incubated with 225 µl FRAP reagent for 4 min in 96-well plates in triplicate. Samples were read at 593 nm by using the Thermo Multiskan spectrum plate reader (Thermo Fisher Scientific Inc., Stafford ST, UK). Aqueous solutions of known Fe^{2+} concentration (0.2 to 1 mmol/l; $FeSO_4 \cdot 7\ H_2O$) were used as the standard curve and all results are expressed in mmol Fe^{2+} equivalents (TE) per litre (mM).

2.5 Analysis of digesta, faecal samples or urine

2.5.1 Urine

In this thesis, urine was directly injected into HPLC-MS after centrifugation at 13,000 rpm at 4 °C for 10 min. Supernatants were injected into HPLC-MS for analysis.

2.5.2 Extraction of digested and faecal samples for flavanone and total phenols analysis

The polyphenols were extracted by using methanol or ethyl acetate (Mullen et al., 2008a, Mullen et al., 2009a). Samples were thawed and homogenized with a Disruptor Genie (Scientific Industries, UK). Sample (1 mL) and methanol (1 mL) containing 1% formic acid were added to the glass tube and vortexed for 10 min at room temperature. The mixtures were centrifuged at 13,000 rpm at 4 °C for 10 min. Supernatants were collected, and pellet was mixed with 0.5 ml of methanol and vortexed for 10 min and centrifuged at 13,000 rpm at 4 °C for 10 min in duplicates. Supernatants were combined and homogenized, and aliquot (1.5 mL) was centrifugally evaporated under vacuum at room temperature using a Thermo Speedvac concentrator. Residues were then resuspended with 25 µl of methanol and 475 µl of 0.1% formic acid, and centrifuged at 13,000 rpm at 4 °C for 5 min until analysis by HPLC-PDA.

2.5.3 Extraction of digesta and faecal samples for phenolic acids analysis

Phenolic acid extraction, derivatisation and analysis were performed by using the method from Combet (Combet et al., 2011). Each faecal sample and calibration standard (0.5 ml) were mixed with 30 µl of 2, 4, 5-trimethoxycinnamic acid (TMCA) as an internal standard and 60 µl of aqueous 1M HCl. Mixtures were vortexed and then placed at 4 °C for 10 min. Anhydrous ethyl acetate (1.5 mL) was added to mixtures for 30 s vortex, and then centrifuged at 2700 g at 4 °C for 10 min. The upper organic layer was transferred to an amber glass vial in a 37 °C aluminium block and dried under a gentle flow of nitrogen. The residue solutions

were extracted with 1.5 ml of anhydrous ethyl acetate, and upper layers were transferred to the amber vial and dried under a gentle flow of nitrogen. Dichloromethane (DCM) (200 µl) was added to rinse walls of the vials and dried under a slight stream of nitrogen. Derivatization reagent (50 µl) [N, o-Bis (Trimethylsilyl) trifluoroacetamide (BSTFA) + 10% trimethylchlorosilane (TMCS)] was added to the amber vials for derivatisation. The headspace was flushed with a gentle flow of nitrogen before sealing. Samples were vortexed each 30 min during derivatisation at 70 °C for 4 h. And then anhydrous hexane (350 µl) was added to the vials before injection to GC-MS.

2.5.4 Analysis of digesta and faecal samples for total phenols and antioxidant capacity

Total phenols of the extracts were measured by using the Folin-Ciocalteau method mentioned in 2.4.1. Antioxidant capacity was measured by the FRAP assay mentioned in 2.4.3.

2.6 Identification and quantification of flavanones in orange juice by HPLC-PDA

Samples were injected onto a Thermo Surveyor HPLC system comprising an HPLC pump, PDA detector scanning from 250 to 700 nm, and an autosampler cooled to 4 °C (Thermo Finnigan, San Jose, CA, USA). Separation was carried out using a 250 × 4.6 mm i.d. 4 µm Synergi Max-RP column (Phenomenex, Macclesfield, UK) and eluted from 5 to 50 % methanol in 0.1% formic acid within 60 min at a flow rate of 1 ml/min and maintained at 40 °C. Hesperidin, narirutin, hesperetin and narigenin were identified based on retention times of individual authentic standards. Peak area versus concentration was obtained by linear regression analysis of concentration at range of 0.1 ng / µl to 300 ng / µl at 290 nm.

2.7 Identification and quantification of flavanone metabolites by using LC-MS

HPLC-PDA-MS^2 was used to identify and quantify hesperetin and naringenin metabolites in biological samples. Urine was analysed on a surveyor HPLC - PDA - MS^2 composed of an

HPLC pump, a PDA detector, scanning from 200 to 800 nm, and auto-sampler cooled to 4 °C (Thermo Fisher Scientific). Reverse-phase separations were carried out by using a 250 × 4.6 mm i.d. 4 µm Synergi Max-RP column (Phenomenex, Macclesfield, UK) maintained at 40 °C, and eluted from 25 % - 80 % methanol in 0.1 % aqueous formic acid over 60 min at a flow rate of 1ml/min. After passing through the flow cell of the photodiode array detector, the column elute was split, and 0.3 ml/min elute was directed to a LCQ Deca XP ion trap mass spectrometer fitted with an electrospray interface operating in negative ionization mode for hesperetin and narigenin metabolites. The capillary temperature was 325 °C, sheath gas was 40 units, auxiliary gas was 20 units, and source voltage was 6 kv. Identifications were based on full-scan, data-dependent MS scanning from 100 to 800 *m/z*, MS-MS fragmentation, and comparisons of the retention time and absorption λ_{max} with available standards. Hesperetin and narigenin metabolites were quantified based on chromatographic peak areas acquired at 290 nm and expressed relative to standards of hesperetin-7-*O*-rutinoside and naringenin-7-*O*-rutinoside dissolved in methanol at concentrations ranging from 0.1 to 100 ng/µl. Hesperetin and naringenin metabolites were quantified as hesperetin-7-O-glucuronide and naringenin-7-O-glucuronide equivalents. A linear response was obtained for all the standard curves when a linear regression analysis was $R^2 > 0.999$. Values were expressed in µM as mean values ± SE. Peak identifications were based on full-scan, data dependent MS scanning from 100 to 800 m/z, MS-MS fragmentation, and comparisons of the retention time and absorption λ_{max} with available standards. Limits of detection ranged from 0.1 to 1.0 ng, limits of quantification ranged from 1.0 to 5.0 ng, and the precision of the assay (as the coefficient of intraassay variation, which ranged from 8.7 % to 9.0 %) were considered acceptable allowing the quantification of flavonoids and their metabolites.

2.8 Identification and quantification of phenolic acids by GC-MS

GC-MS was performed to give high sensitivity and specificity for analysis of phenolic acids. Derivatized phenolic acids were analysed using a Trace DSQ single quadruple GC-MS, equipped with an AI 300 autosampler (Thermo Finnigan Ltd, Hempstead, Hertfordshire, UK). Samples were injected in the split mode with a 25:1 ratio. The injector temperature was maintained at 220 °C. The mass spectrometer was used in the positive ionization mode with

the ion source and transfer line set at 180 °C and 310 °C, respectively. Separations were carried out 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). Helium was the carrier gas with a flow rate of 1.2 ml min^{-1}. The column temperature was initially set at 40 °C and raised to 160 °C at 20 °C min^{-1}, 200 °C at 1.5 °C min^{-1} and 250 °C at 10 °C min^{-1} to a final temperature of 300 °C at 40 °C min^{-1}, held for 5 min. Data acquisition was performed in full scan mode (*m/z* 50 - 470) with ionization energy of 70 eV, and analysis was carried out by using Xcalibur software version 2.0 (Thermo Fisher Scientific UK,, Hempstead, Hertfordshire, UK). Phenolic acids were identified based on the mass spectra and retention time of authentic standards analysed under identical conditions. When standards were not commercially available, identification was achieved through the integrated National Institute of Standards and mass spectral library 2008 (Scientific Instruments Services Inc., Ringoes, NJ, USA), with a confidence of 70 % or above. Calibration curves of the ratio between the target ion of the interested analyte and of the internal standard (m/z 279) at concentrations ranging from 3-40 mg ml^{-1} were computed (R^2 >0.95).

Standards: 3-hydroxyphenylacetic acid, 4-hydroxyphenylacetic acid, 4-hydroxyphenylpropionic acid, 3, 4-dihydroxyphenylacetic acid, 3-(3, 4-dihydroxyphenyl) propionic acid, 4-hydroxy-3-methoxy-phenylpropionic acid (dihydrofeulic acid) were purchased from Sigma-Aldrich Co Ltd (Poole, Dorset, UK) or Alfa Aesar (Karlsruhe, Germany). The internal standard was 2, 3, 5-trimethylcinamic acid.

2.9 Data analysis

Analyses of orange juices in chapter 3 were carried out in triplicate, and data was presented as mean values with standard deviation. All data was assumed to be normally distributed and independent t-test or ANOVA were employed to test for differences between smooth orange juice and orange juice with bits, and significant differences were defined as $p < 0.05$.

For orange juice flavanones and or phenolic in digestive fluids and faecal samples, data are presented as mean values with standard error. The unit used for the upper GI digestion was based on total amount of flavanone or total phenols in the digestive fluids expressed as

mg/250ml, and used for the fermeantion was based on concentration of flavanone or total phenols in faecal sample expressed as μM. All data were assumed to be normally distributed; t-tests or ANOVA tests with post hoc Tukey-HSD was used to determine significant differences defined as $p < 0.05$.

Chapter 3 *Metabolism of orange juice flavanone from different sources in the in vitro gastrointestinal tract*

3.1 Introduction

Flavonoids and flavonoids enriched foods, have been reported to have antibacterial (Lee et al., 2003), anti-inflammatory (Coelho et al., 2013), and to inhibit lipid peroxidation (Cesar et al., 2010, Constans et al., 2014) and reduce the risk of chronic diseases (Bahorun et al., 2012) including type-2-diabetes (Jacques et al., 2013), Parkinson disease (Gao et al., 2012) and some cancers (Zamora-Ros et al., 2014). Consumption of orange juice or flavanones modulated inflammatory markers (Coelho et al., 2013), enhanced cognitive function (Kean et al., 2015a), reduced diastolic blood pressure (Rangel-Huerta et al., 2015b) and improved postprandial microvascular endothelial reactivity (Morand et al., 2011), and increased antioxidant capacity (Constans et al., 2014, Vinson et al., 2002), inhibited bone loss and decreased serum and hepatic lipids (Chiba et al., 2003) in humans.

According to the Phenol-Explorer database, orange juice (150 ml) contains 90 mg of flavanones (Neveu et al., 2010). Hesperidin constitutes 90 % of the flavanones in orange juice, followed by narirutin (Manach et al., 2003, Abad-García et al., 2012). In the juice, they are mainly present as glycosides (Rangel-Huerta et al., 2015b). However, flavanone metabolites and phenolic metabolites can be absorbed into the circulatory system after deglycosylation and degradation of the parent compounds by intestinal enzymes or colonic bacteria (Manach et al., 2005, Crozier et al., 2010, Pereira-Caro et al., 2014a, Roowi et al., 2009b).

A human study observed ~ 30 % of hesperidin intake was recovered in ileal fluid 0–24 h after consumption of a polyphenol-rich juice by ileostomists (Borges et al., 2013). In an *in vitro* study, 63 % of hesperidin was transformed to the corresponding chalcone during *in vitro* simulated pancreatin-bile digestion (Gil-Izquierdo et al., 2001). Aglycones are conjugated into glucuronides, sulfates and sulfoglucuronides by the intestine epithelium cells and in the liver, or degraded to phenolic acids by gut bacteria in the colon (Kim et al., 1998, Khan and Dangles, 2014, Roowi et al., 2009a). In addition, the plasma concentration of flavonoids has been highly studied. The maximum concentrations of hesperetin metabolites were 0.9 – 2.2 µmol/L after consumption of orange juice or hesperidin (Mullen et al., 2008a, Manach et al., 2003, Erlund et al., 2001c). Conjugated metabolites with a glucuronide, sulfate, or methoxy moiety have been detected and characterised in the stomach, intestine, liver, heart, kidney,

spleen, pancreas, prostate and bladder by using animal models (Suganuma et al., 1998, Chang et al., 2000, Kim et al., 2000).

Bacteroides, bifidobacteria, and lactobacilli have the highest enzymatic activity of *β*-glucosidase, *α*-rhamnosidase and *β*-glucuronidase (Steer et al., 2003, Turner, 2008). Clostridium, Eubacterium genera, and beneficial generes bifidobacteria and lactobacilli are involved in metabolism of flavonoids via ring-cleavage, decarboxylation, demethylation, and dehydroxylation reactions in the human intestinal tract (Selma et al., 2009, Aura, 2008). *Clostridium* genus, *Clostridium cocoides* and *Eubacterium rectaleare* are responsible for C-ring cleavage (Winter et al., 1989, Tzounis et al., 2008). It has been thought that fibre enriched products may modify the composition and content of gut microbiota, in turn impacting on the metabolism of flavonoids. In an *in vitro* study, hydroxyphenylacetic acid, hydroxyphenylvaleric acid and two isomers of hydroxyphenylpropionic acid were produced at a higher amount by fermentation with extractable proanthocyanidins than with red grapes which contained dietary fibre (Saura-Calixto et al., 2010). In addition the polyphenol rich foods may affect the growth of the bacteria. Cherry, pomegranate, raspberry, and strawberry inhibited growth of *Staphylococcus epidermis*, *Klebsiella pneumonia*, *Staphylococcus spp.*, *Salmonella spp.*, *Helicobacter pylori*, and *Bacillus cereus* (Lee et al., 2003, Puupponen‐Pimiä et al., 2005). What is more, pomegranate peel extract inhibited growth of *C. perfringens*, *C. ramosum*, *S. aureus*, and *Clostridium clostridioforme*, but significantly increased number of *Bifidobacterium breve* and *Bifidobacterium infantis* (Bialonska et al., 2009).

As discussed in section 1.4.2, the bioavailability of flavanones varied considerably after consumption of orange juice ranging from 0.8 ~ 16 % in humans after consumption of orange juice (Pereira-Caro et al., 2014b, Brett et al., 2009, Bredsdorff et al., 2010, Erlund et al., 2001c, Silveira et al., 2014, Vallejo et al., 2010c, Tomas-Navarro et al., 2014, Manach et al., 2003). This may be related to the difference in the content of the fruit or to changes made in processing of the juice. One example is a difference in the inclusion of pulp between smooth orange juice and orange juice with pulp or ‘bits’ as they are called in UK market, depends on product processing. The orange juice, may include segment membranes and other connective tissues, and pulp derived from peel by squeezing (Grigelmo-Miguel and Martín-Belloso,

1998). The pulp may contain pectins, cellulose, hemicellulose and lignin as well as small amounts of protein, fat and ash (Grigelmo-Miguel and Martín-Belloso, 1998).

The amount of cellular material is variable by differences in the techniques used in orange juice processing. Processing of orange juice can reduce the insoluble dietary fibre content by 39.1 % from 58.8 % to 32.3 % and increased soluble dietary fibre by 80 % from 20.1 % to 37.9 % in orange pulp (Larrea et al., 2005). In addition, pulp and orange juice cloud may include non-cell wall proteins (50 %), and cell wall fragments (Brat et al., 2003). Flavanones can be bound to fibre solid particles by hydrophilic interaction (Manthey and Grohmann, 1996). The bioavailability of flavanones depends on their digestive stability and the release of flavonoids from the food matrix as they pass through the intestine tract (Rubió et al., 2014). Various processing of orange juice affected flavanone under the gastrointestinal pH, temperature, and enzyme and chemical conditions (Gil-Izquierdo et al., 2003). As a result, the interactions of flavanones and these particles in orange juice from different sources might affect the metabolism of flavanone in the gastrointestinal tract to render varying levels of bioavailability of flavanones in studies. In addition, orange juice of different types might have various actions on gut bacteria to modify metabolism of flavanones in the colon. To our knowledge, there have been no *in vitro* studies to investigate mechanisms by which orange juice from different sources affect the release and metabolism of flavanones via digestion and fermentation in the gastrointestinal tract. Therefore, the aim of this chapter was to examine flavanone metabolism by using an *in vitro* GI model to determine the likely bioavailability from different sources in humans (Figure 3-1).

In this chapter, smooth orange juice and orange juice with ‘bits’ from 6 brands in the UK were measured in terms of flavanone content, dietary fibre, total phenols, total flavonoids, and antioxidant capacity to compare physical characteristics. The stability and release of flavanone and degradation of flavanone were assessed by using stimulated gastrointestinal digestion and fermentation to investigate metabolism of flavanone in smooth orange juice and orange juice ‘with bits’. In addition, total phenol and antioxidant capacity of digesta and faecal incubations were also evaluated.

3.1.1 Hypothesis

To test the hypothesis that the release and stability of of flavanones in orange juice and their degradation by gut bacteria in the gastrointestinal tract are influenced by the source and formulation of the juice, for example the inclusion of pulp

1) What is the variability of flavanone content in 6 different brands of smooth orange juice and orange juice 'with bits?'

2) Does the presence of 'bits'affect the flavanone content in the original juice, release of flavanones in a model simulating the upper GI tract or the production of phenolic acids in a faecal bacteria incubation model?

3.2 Materials and methods

3.2.1 Chemicals

Hesperidin and narirutin were purchased from Extrasynthese (Genay, France). HPLC grade methanol was obtained from Rathburn Chemicals (Walkerburn, Borders, UK). Formic acid was purchased from Riedel-deHaen (Seelze, Germany). Hydrochloric acid, sodium bicarbonate, pepsin (from porcine stomach mucosa), pancreatin (from porcine pancrease), bile acids (from porcine); tryptone, 2,4,5 – trimethoxycinnamic acid, anhydrous ethyl acetate, dichloromethane (DCM), N-methyl-N- (trimethylsily) trifluoracetateamide, *N*, *O*-bis (Trimethylsilyl) trifluoroaceta mide (BSTFA) + 10 % trimethylchlorosilane (TMCS),prydine. $CaCl_2 \cdot 2H_2O$, $MnCl_2 \cdot 4H_2O$, NH_4HCO_3, $NaHCO_3$, Na_2HPO_4, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, cysteine hydrochloride, NaOH, $Na_2S \cdot 9H_2O$, and resazurin solution were purchased from Sigma-Aldrich Co Ltd (Poole, Dorset, UK). Ethyl acetate and dichloromethane (DCM) were purchased from Rathburn Chemicals Ltd (Walkerburn, Peeblesshire, UK).

3.2.2 Orange juice analysis

Four popular supermarkets were visited to obtain six brands of orange juice. They supply smooth orange juice and orange juice 'with bits' (ie including pulp), freshly squeezed, not from concentrate. Appendix 1 shows ingredients labelled in the bolttles. The juices were Tropicana orange juices, Sainsbury's, Tesco, Innocent, Waitrose and Morrison's orange juices obtained between April of 2013 and March of 2014.

The content of dietary fibre in each juice was analysed by using AOAC method (Prosky et al., 1988) conducted by the School of Health and Life Science, Glasgow Caledonian University. Total phenols, total flavonoids and antioxidant capacity were measured as described in section 2.4.2, 2.4.2 and 2.4.3.

3.2.3 *In vitro* gastrointestinal digestion and fermentation models

3.2.3.1 Orange juices for *in vitro* GI digestion and fermentation

Two types of 100 % pure squeezed orange juice (smooth orange juice and orange juice 'with bits') used were from Tropicana Company purchased at Tesco Express, Dumbarton Road, Glasgow, UK) for the gastrointestinal digestion and fermentation.

3.2.3.2 *In vitro* gastrointestinal digestion

The model to simulate gastric and small intestinal digestion was adapted from Gil-Izquierdo (Gil-Izquierdo et al., 2003). 40 ml of orange juice was chosen based on the orange juice consumption by humans in chapter 3. *In vitro* gastrointestinal digestion was conducted as described in section 2.3.1.

Smooth orange juice and orange juice with bits had similar hesperidin content (37.3 mg/100 ml vs. 40.5 mg/100 ml) and narirutin (4.1 mg/100 ml and 4.9 mg/100 ml), which did not confirm results from previous studies reporting higher amounts of hesperidin and narirutin in pulp enriched orange juice (Table 3-3). This may have been different for just this one brand of juice so we purchased examples of all juices which had both smooth orange juice

and orange juice ‘with bits’ in local supermarkets. Six brands had smooth orange juice and orange juice ‘with bits’ (Appendix 1), these juices were then compared to investigate the variation in flavanones between smooth orange juice and orange juice with bits (Table 3-3).

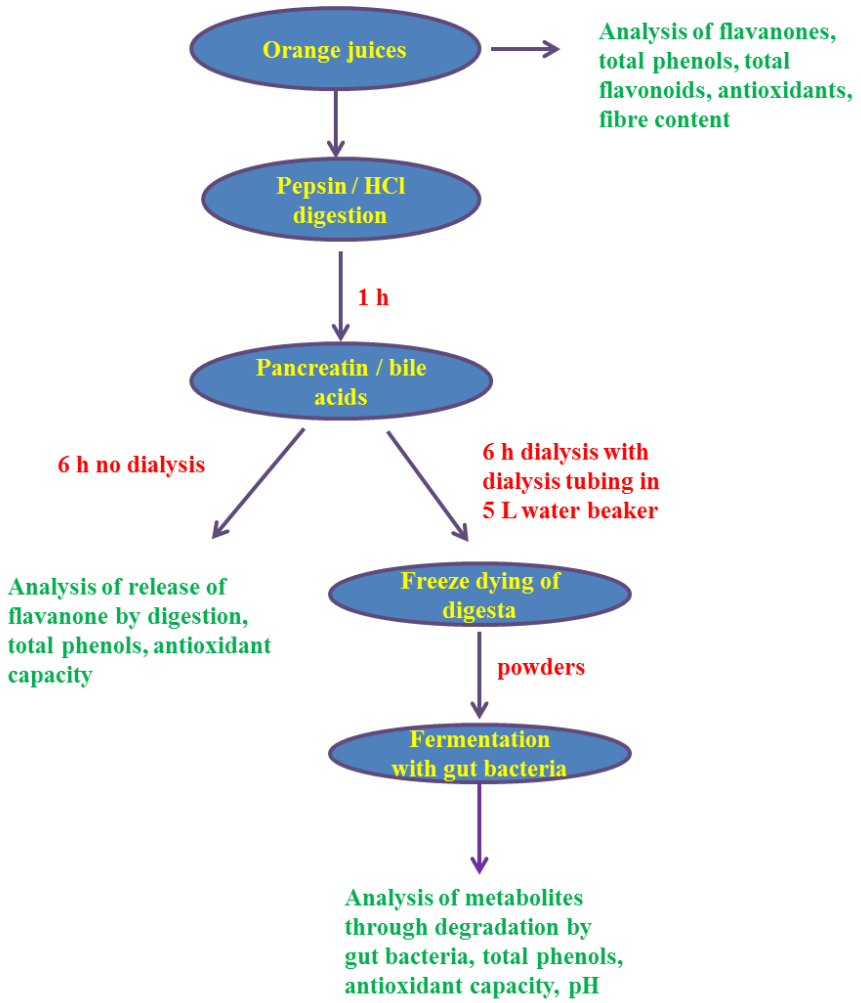


Figure 3-1 Details of the different stages of the simulated gastrointestinal tract model

3.2.3.3 Faecal sample collection

Six healthy Caucasian volunteers (Table 3-1) who participated in this study had a normal diet, were non-smokers, had no digestive diseases, no food allergies, and had not received antibiotics for at least 6 months before the study. The volunteers followed a low-polyphenol diet avoiding fruits, vegetables, tea, coffee, wine and wholemeal foods for 2 days (details in appendix 2) before providing a faecal sample according to information sheet (appendix 2). Each faecal sample was collected by subjects in a special plastic box containing an AnaeroGen sachet (Oxoid, Hampshire, UK) to generate anaerobic conditions. Faecal samples

were processed when brought to the lab. The food has been recorded in dietary record (Appendix 4).

Table 3-1 Body characteristics of participants

	Gender	Age(years)	Height (cm)	Weight (kg)	BMI
1	F	22	172	73	24.7
2	M	21	181	84	25.6
3	F	27	161	52	20.1
4	F	21	168	57	20.2
5	F	23	164	60	22.3
6	M	26	185	76	22.2
Mean ± SD		23 ± 2.6	171 ± 9.5	67 ± 12.5	22.5 ± 2.3

Values are mean ± SD, M: male, F: female, BMI: body mass index.

3.2.3.4 *In vitro* fermentation with human colonic bacteria

The fermentation was conducted as described in section 2.3.3.

3.2.4 Extraction of hesperidin and narirutin in orange juices

Orange juice (1 ml) was extracted as described in section 2.9.1.

3.2.5 Extraction of hesperidin, narirutin, hesperetin, and naringenin after in vitro digestion and fermentation

Samples were thawed and homogenized with a Disruptor Genie (Scientific Industries, UK). Extraction was the same as orange juice described in 2.9.1.

3.2.6 Analysis with HPLC-PDA

Hesperidin, narirutin, hesperetin and naringenin were analysed by using HPLC-PDA as described in section 2.10.

3.2.7 Extraction and derivatization of phenolic acids in faecal slurries

Extraction of phenolic acids was done by the methods published by Combet et al.(Combet et al., 2011), as described in section 2.5.3.

3.2.8 Analysis of phenolic acids by GC-MS

GC-MS analysis was explained in details in chapter 2.12.

3.2.9 Total phenol measurement for orange juice, digested and faecal samples

Total phenols were measured by using the Folin–Ciocalteau method. See section 2.6 for more details.

3.2.10 Total flavonoids measurement for orange juice

Total flavonoids were measured by using the spectrophotometric method based on the production of complex compounds of flavonoids with aluminium chloride. See section 2.7 for more details.

3.2.11 FRAP assay for orange juice, digesta and faecal samples

Antioxidant capacity was determined by using FRAP assay in a redox-linked colorimetric reaction. See chapter 2.8 for more details.

3.3.12 Release of NEPP by hydrolysis with driselase and pectinase / cellulase

Enzymatic hydrolysis was conducted by previous studies with some modification (Kang et al., 2008, Fry et al., 2001, Shirakawa et al., 2007, Mandalari et al., 2006, Wilkins et al., 2007). Orange juice (5 ml) was centrifuged at 4500 g for 10 min at 4 °C. Supernatants were

extracted with or without methanol (2 ml) in 0.1 % formic acid and centrifuged at 4500 g for 10 min at 4 °C in duplicate. Supernatants were collected in the 10 ml tubes. Extracted pellets and non-extracted pellets were incubated individually with 2 %, 1 %, or 0.1 % of pectinase / cellulase (pH 4.4) at 37 °C or driselase at 45 °C (pH 5) for 2, 4, 6, 20, and 24 h.

Aliquots (0.5 ml) were extracted with ethyl acetate (1 ml), and the extracts were centrifugally evaporated under vacuum at room temperature using a Thermo Speedvac concentrator. The residue was dissolved with 25 µl of methanol and made up to 1 mL with HPLC-grade water containing 0.1 % formic acid for analysis. All of experiments were carried out in triplicate.Total phenols were measured by using the Folin–Ciocalteau method. See section 2.6 for more details.

3.2.13 Statistical analyses

All data analysis was conducted with SPSS software version 22. Analyses of orange juices were carried out in triplicate, and data was presented as mean values with standard deviation. , and was assumed to be normally distributed and independent t-test or ANOVA were employed to test for differences between smooth orange juice and orange juice with bits, and significant differences were defined as $p < 0.05$.

For orange juice flavanones and or phenolic in digestive fluids and faecal samples, data are presented as mean values with standard error. The unit used for the upper GI digestion was based on total amount of flavanone or total phenols in the digestive fluids expressed as mg/250ml, and used for the fermentation was based on concentration of flavanone or total phenols in faecal sample expressed as µM. All data were assumed to be normally distributed, t-tests or ANOVA tests with post hoc Tukey-HSD was used to determine significant differences between smooth orange juice and orange juice with bits ($P < 0.05$).

3.3 Results

3.3.1 Analysis of orange juices

Total phenols, total flavonoids, antioxidant capacity (FRAP), hesperidin and narirutin were varied by 26.6 %, 36.8 %, 31.6 %, 158.7 % and 72.1 % (Table 3-2). According to type of orange juice, OJ bits had higher total phenolic content compared with smooth OJ (225.9 ± 7.5 µg GAE/ ml vs. 208.4 ± 6.1 µg GAE / ml, $p < 0.05$) (Table 3-2). However, total flavonoids were similar between OJ and OJ bits (81.4 ± 3.8 vs. 93.0 ± 12.1 µg QAE/ml, $p = 0.058$). There was no significant different antioxidant capacity between OJ and OJ bits. Hesperidin and narirutin were 32.9 ± 10.3 mg / 100 ml and 5.1 ± 2.4 mg / 100 ml in OJ bits, while 30.0 ± 9.1 mg / 100 ml and 3.9 ± 1.4 mg / 100 ml in OJ respectively. Hesperidin was correlated with total flavonoids in orange juice (Pearson correlation coefficient = 0.67). In addition, the antioxidant capacity of orange juice (FRAP) was correlated with hesperidin (Pearson correlation coefficient = 0.86).

Table 3-2 Total phenolic content (µg GAE/ml), total flavonoids (µg QE/ml), antioxidant capacity (mM Fe^{2+}), narirutin (mg/100ml), hesperidin (mg/100ml) and total flavanone (mg/100ml) in orange juices

Brand	Type	Total Phenols	Total flavonoids	FRAP φ	Narirutin	Hesperidin φ
Tropicana	Smooth OJ	216.5 ± 12.0 ^{dghik}	76.8 ± 0.5^{dghijkl}	5.9 ± 0.3 ^{dfl}	4.2 ± 0.2 ^{ckl}	37.3 ± 1.8^{cdefgijkl}
	OJ bits	228.4 ± 5.9 ^{cdghijk}	86.7 ± 17.1^{dghijkl}	6.4 ± 0.4 ^{d}	4.9 ± 0.3 ^{cjkl}	40.5 ± 1.6^{acghijkl}
Sainsbury's	Smooth OJ	213.5 ± 2.7^{bdfghik}	84.9 ± 13.6^{dghijkl}	6.0 ± 0.1 ^{dfl}	2.3 ± 0.1^{abjhijk}	34.5 ± 0.5^{adefghijkl}
	OJ bits	252.3 ± 6.6 ^{abcefghijkl}	100.2 ± 15.0^{abcefghijkl}	7.4 ± 0.4 ^{abceghijk}	3.7 ± 0.3^{ijk}	40 ± 1.5^{acghijkl}
Tesco	Smooth OJ	220.9 ± 1.9^{dghijl}	79.2 ± 3.3^{dghijkl}	6.3 ± 0.5 ^{d}	3.7 ± 0.1^{ijk}	38 ± 0.8^{acghijkl}
	OJ bits	226.1 ± 5.9^{cdghijl}	78.9 ± 7.7^{dghijkl}	6.1 ± 0.6 ^{acghij}	4.1 ± 0.1^{cjkl}	41.9 ± 1.5^{acgijkl}
Innocent	Smooth OJ	197.2 ± 9.1^{abcdefkl}	82.5 ± 5.2^{abcdefijl}	4.6 ± 0.3 ^{dfl}	4.4 ± 0.2^{cjkl}	30 ± 1.2^{abcdefkijkl}
	OJ bits	199.8 ± 9.6 ^{abcdefkl}	81.9 ± 3.5^{abcdef}	5.0 ± 0.2 ^{dfl}	5.3 ± 0.1^{cdefkl}	37.4 ± 1.9^{bcdegijkl}
Morrison's	Smooth OJ	207.8 ± 3.2 ^{bdefjkl}	86.4 ± 1.2^{abcdefg}	5.2 ± 0.1 ^{dfl}	7.8 ± 0.1^{bcdefghkl}	21.5 ± 1.0^{abcdefgkl}
	OJ bits	224.0 ± 7.7 ^{dghijkl}	101.1 ± 7.0^{abcdefg}	5.3 ± 0.1 ^{dfl}	9.6 ± 0.9^{abcdefghijl}	21 ± 1.7^{abcdefgkl}
Waitrose	Smooth OJ	194.5 ± 8.2 ^{abcdefjk}	78.6 ± 12.9^{abcdef}	4.9 ± 0.3 ^{d}	2.6 ± 0.1^{abcghijk}	17.8 ± 0.2^{abedefgkil}
	OJ bits	224.6 ± 9.1^{dghijk}	108.9 ± 14.6 ^{abcdefg}	5.2 ± 0.1 ^{acghij}	2.8 ± 0.4^{ahhijk}	18.5 ± 1.3^{abedefgkl}
Mean	Smooth OJ	208.4 ± 10.7	81.4 ± 3.8	5.5 ± 0.7	3.9 ± 1.4	20.0 ± 9.1
	OJ bits	225.9 ± 16.7 *	93.0 ± 12.1	5.9 ± 0.9	5.1 ± 2.4	32.9 ± 10.3

Data expressed as mean values ± SEM (n = 3). Repeated measures ANOVA test with LSD post hoc was performed to determine difference within orange juice, different letters were expressed significantly different within orange juice ($p < 0.05$), independent t-test was preformed to determine difference from Smooth OJ, * Significantly different from smooth OJ, $p < 0.05$, Pearson's correlation analysis was performed for each column, φ significantly correlated with hesperidin (correlation coefficient = 0.86).

The fibre content in the orange juices ranged from 0.1 g / 100 ml to 0.3 g / 100 ml. There was no difference between smooth orange juice and orange juice 'with bits' (Table 3-3).

Table 3-3 Total dietary fibre (g/100 ml) in orange juices

	OJ	OJ bits
Tropicana	0.1	0.1
Sainsbury's	0.1	0.3
Tesco	0.1	0.2
Innocent	0.1	0.2
Morrison's	0.1	0.2
Waitrose	0.3	0.2
Mean	0.1 ± 0.1	0.2 ± 0.1

Data expressed as mean values, Mean value was calculated based on six orange juice expressed as mean values ± SD (n=6), OJ: smooth orange juice, OJ bits: orange juice 'with bits'. Fibre measured by AOAC method (Prosky et al., 1988) by the School of Health and Life Science, Glasgow Caledonian University.

3.3.2 Stability and release of hesperidin and narirutin during in vitro gastrointestinal digestion

In the gastric phase, hesperidin was higher in orange juice 'with bits' than smooth orange juice ($p < 0.05$), while there was no differences of narirutin (Table 3-4). Hesperidin was more stable in smooth orange juice than orange juice 'with bits' ($p < 0.014$), while narirutin was similar between the two orange juices (Table 3-4).

Table 3-4 Contents of hesperidin and narirutin (mg/250 ml) in smooth orange juice and orange juice 'with bits' in the gastric phase

	0 h		1 h	
	Hesperidin	Narirutin	Hesperidin	Narirutin
OJ	25.8 ± 2.6	1.7 ± 0.2	28.6 ± 1.3	1.8 ± 0.0
Recovery (%)	--	--	112.6± 0.1	103.7± 0.1
OJ bits	33.1 ± 0.6 *	1.7 ± 0.0	22.0 ± 0.6 *	1.6 ± 0.1
Recovery (%)	--	--	66.6 ±0.0	97.4 ±0.0

Values were expressed as mean values ± SE (n=3), Calculation was based on total amount of hesperidin or narituitn from 250 ml of orange juice, OJ: smooth orange juice, OJ bits: orange juice 'with bits', * significantly different from OJ, $p < 0.05$ (paired t-test).

After the small intestinal digestion, hesperidin and narirutin were similar between smooth orange juice and orange juice 'with bits' (Table 3-5). However, there was more hesperidin recovery from smooth orange juice than orange juice 'with bits' (90.1 ± 6.8 % vs. 67.9 ± 3.2%, $p = 0.062$).

Table 3-5 Contents of hesperidin and narirutin (mg/250 ml) in smooth orange juice and orange juice 'with bits' under small intestinal condition

	2 h		4 h		6 h	
	Hesperidin	Narirutin	Hesperidin	Narirutin	Hesperidin	Narirutin
OJ	20.5 ± 1.6	1.1 ± 0.1	22.2 ± 1.1	1.1 ± 0.1	22.9 ± 0.6	1.0 ± 0.1
Recovery (%)	81.9 ± 12.0	61.6 ± 5.1	87.4 ± 5.5	62.8 ± 4.2	90.1 ± 6.8	57.7 ± 2.8
OJ bits	22.0 ± 0.6	1.0 ± 0.1	25.0 ± 1.3	1.0 ± 0.1	22.5 ± 1.3	1.0 ± 0.1
Recovery (%)	66.6 ± 2.7	60.6 ± 2.2	75.5 ± 3.1	61.5 ± 0.9	67.9 ± 3.2	57.1 ± 3.0

Values were expressed as mean values ± SE (n=3), Calculation was based on total amount of hesperidin or narituitn from 250 ml of orange juice, OJ: smooth orange juice, OJ bits: orange juice 'with bits' * significantly different from OJ, $p < 0.05$ (paired t-test).

Narirutin had a greater decrease when transferred from gastric condition to small intestinal condition from 1.8 ± 0.0 mg / 250 mL to 1.1 ± 0.1 mg / 250 mL in smooth orange juice ($p < 0.02$) and from 1.6 ± 0.1 mg / 250 mL to 1.0 ± 0.1 mg / 250 mL in orange juice 'with bits' ($p < 0.01$). However, total flavanone (hesperidin + narirutin) was was not significantly

difference between orange juice ‘with bits’ and smooth orange juice before and after the upper GI digestion (Table 3-6).

Table 3-6 Contents of total flavanone (hesperidin + narirutin) (mg/250 ml) in smooth orange juice and orange juice ‘with bits’ during the GI digestion

	0 h	1 h	2 h	4 h	6 h
OJ	27.5 ± 2.7	30.3 ± 1.2	21.6 ± 1.6	23.3 ± 1.1	23.9 ± 0.9
OJ bits	34.8 ± 0.6	23.7 ± 0.6 *	23.1 ± 0.6	26.1 ± 1.4	23.5 ± 1.4

Values were expressed as mean values ± SE (n=3), Calculation was based on total amount of hesperidin or narituitn from 250 ml of orange juice, OJ: smooth orange juice, OJ bits: orange juice ‘with bits’, Gastric digestion for 1 h, small intestinal digestion for 6 h from 1 h to 7 h, * significantly different from OJ, $p < 0.05$ (paired t-test).

3.3.3 Metabolism of orange juice flavanone in the large intestine

3.3.3.1 Degradation of hesperidin and narirutin in the large intestine

Hesperidin and narirutin were analysed by HPLC-PDA to monitor degradation of flavanones by gut bacteria (Table 3-7). There was a significant decrease in total flavanone by 56.6 %in orange juice with bits after 24 h fermentation by gut bacteria ($p < 0.02$). The gut bacteria transformed 65.7 % of narirutin into naringenin or phenolic acids from 3.5 ± 0.6 µM to1.2 ± 0.3 µM ($p < 0.001$) during fermentation with orange juice ‘with bits’ . Hesperidin was significantly higher at 2 h of fermentation with orange juice ‘with bits’ than smooth orange juice (89.6 ± 9.5 µM vs. 77.1 ± 8.9 µM, $p < 0.05$).

Table 3-7 Concentration of hesperidin and narirutin (μM) during *in vitro* fermentation of smooth orange juice and orange juice 'with bits' by human gut bacteria

	Time	0 h		2 h		4 h		6 h		24 h	
	Flavanone	Narirutin	Hesperidin	Narirutin	Hesperidin	Narirutin	Hesperidin	Narirutin	Hesperidin	Narirutin	Hesperidin
OJ	μM	2.7 ± 0.6	70.0 ± 13.3	2.7 ± 0.6	77.1 ± 8.9	2.5 ± 0.7	72.8 ± 8.4	2.0 ± 0.7	63.0 ± 13.8	1.4 ± 0.3	44.4 ± 12.6
	Recovery (%)	--	--	100	112	80.6	105.7	64.5	91.4	45.2	64.4
OJ bits	μM	3.5 ± 0.6	97.9 ± 10.1	2.8 ± 0.6	89.6 ± 9.5 *	2.4 ± 0.6	79.5 ± 14.8	1.8 ± 0.5	68.6 ± 18.7	1.2 ± 0.3	42.5 ± 15.9
	Recovery (%)	--	--	80.0	91.5	68.6	81.2	51.4	70.1	34.3	43.4

Data expressed as mean values ± SE (n=6). OJ: smooth orange juice, OJ bits: orange juice 'with bits', ANOVA tests with post hoc Tukey-HSD were performed to determine significant difference from OJ, * significantly different from OJ, $P < 0.05$.

Table 3-8 Concentrations of hesperetin and naringenin (μM) during 24 h fermentation with smooth orange juice and orange juice 'with bits' by human gut bacteria

Sources		0h		2h		4h		6h		24h	
		Naringenin	Hesperetin	Naringenin	Hesperetin	Naringenin	Hesperetin	Naringenin	Hesperetin	Naringenin	Hesperetin
OJ	Concentration	0.7 ± 0.4	0.4 ± 0.3	2.0 ± 0.7	1.4 ± 0.6	2.7 ± 0.9	2.7 ± 1.2	3.0 ± 1.2	2.9 ± 1.2	2.7 ± 1.0	4.6 ± 1.9
OJ bits	Concentration	0.7 ± 0.4	0.4 ± 0.3	1.8 ± 0.8	1.6 ± 0.8	2.2 ± 0.8	2.4 ± 1.1	2.2 ± 0.7	2.7 ± 0.9	2.4 ± 0.7	3.4 ± 1.4

Data expressed as mean values ± SE (n=6). OJ: smooth orange juice, OJ bits: orange juice 'with bits', ANOVA tests with post hoc Tukey-HSD were performed to determine significant difference from OJ, $P < 0.05$.

The aglycones, hesperetin and naringenin were produced by fermentation with gut bacteria. There were 4.6 ± 1.9 µM of hesperetin and 2.7 ± 1.0 µM of naringenin after 24 h fermentation with smooth orange juice, while 3.4 ± 1.4 µM of hesperetin and 2.4 ± 0.7 µM of naringenin were produced by fermentation with orange juice 'with bits' (Table 3-8).

Total flavanone (hesperidin, narirutin, hesperetin and naringenin) was decreasing over 24 h of fermentation with human gut bacteria (Figure 3-2). Flavanones were more degraded into smaller molecular compounds in orange juice 'with bits' by gut bacteria than smooth orange juice ($P < 0.001$). At the end of fermentation by gut bacteria, 52 % of flavanone was degraded into smaller molecular compounds from orange juice 'with bits', in comparison with 28 % of flavanone from smooth orange juice (0 h compared with 24 h).

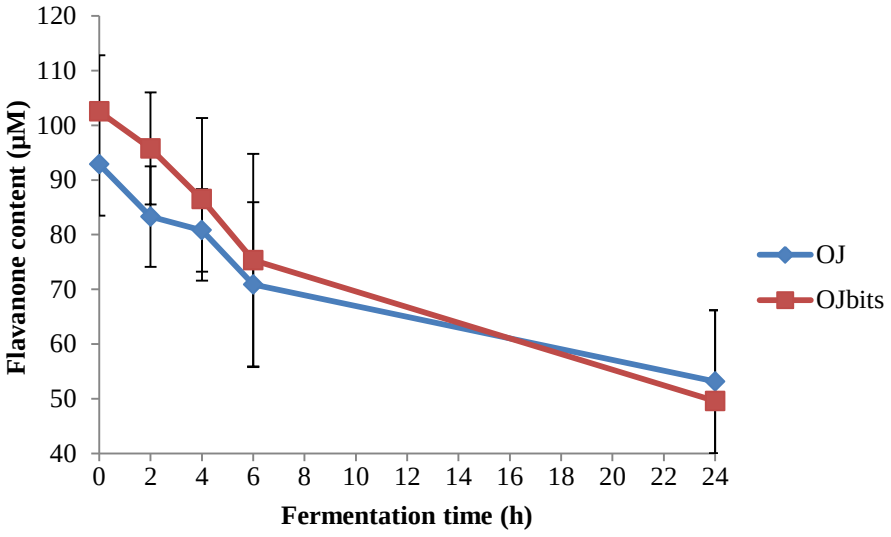


Figure 3-2 Degradation of total flavanone (hesperidin, narirutin, hesperetin and naringenin) in smooth orange juice and orange juice 'with bits' by human gut bacteria

Values are expressed as mean values ± SE (n=6). OJ: smooth orange juice, OJ bits: orange juice 'with bits', ANOVA tests with Tukey-HSD post hoc were performed to determine significant difference from OJ, $P < 0.05$.

3.3.3.2 Production of phenolic acids in the large intestine

There were 7 phenolic acids found during the fermentation of orange juices by human colonic bacteria. They were 3-(phenyl)propionic acid, 3-(phenyl)lactic acid, 4-hydroxybenzoic acid,

4-hydroxyphenylacetic acid, 3-(4'-hydrophenyl)propionic acid, dihydroferulic acid, and 3,4-dihydroxyphenylpropionic acid were during the fermentation of orange juices by gut bacteria (Figure 3-4). The five phenolic acids (4-hydroxybenzoic acid, 4-hydroxyphenylacetic acid, 3-(4'-hydrophenyl) propionic acid, dihydroferulic acid, and 3, 4-dihydroxyphenylpropionic acid) that assumed derived from orange juice flavanone were summed to give total phenolic content. Production of phenolic acids were similar between smooth orange juice and orange juice 'with bits' after 24 h of colonic fermentation (39.0 ± 12.5 µM and 42.3 ± 12.7 µM) (Figure 3-3).

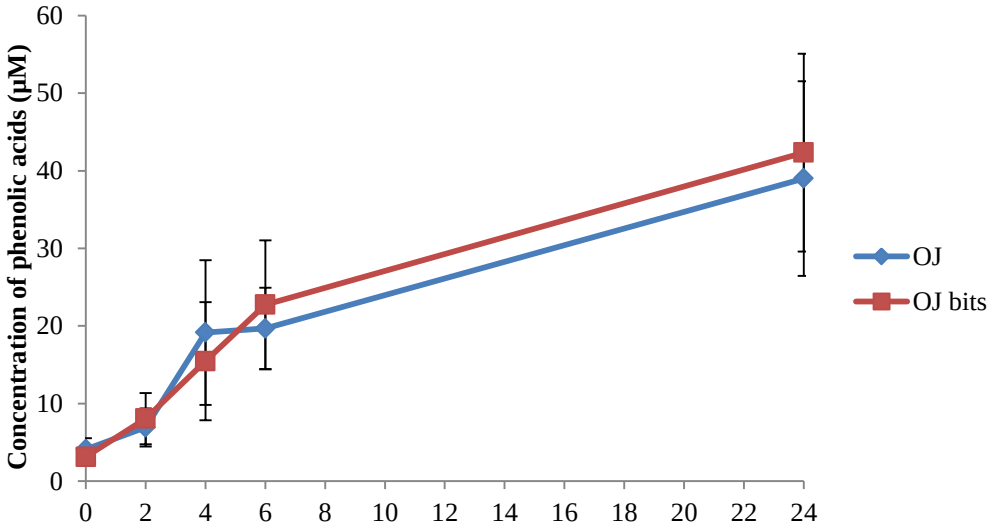


Figure 3-3 Change in concentration of phenolic acids (µM) during 24 h fermentation of orange juices by gut bacteria

Data expressed as mean values ± SE (n=6). Calculation was based on 7 phenolic acids, Blue line: smooth orange juice, Red line: orange juice 'with bits'. ANOVA tests with post hoc Tukey-HSD were performed to determine significant difference from OJ, $P < 0.05$.

3-(phenyl)propionic acid, 3-(phenyl)lactic acid, 4-hydroxybenzoic acid, 4-hydroxyphenylacetic acid, 3-(4'-hydrophenyl)propionic acid, dihydroferulic acid, and 3,4-dihydroxyphenylpropionic acid were produced during the fermentation of orange juices by gut bacteria (Figure 3-4). 4-dihydroferulic acid and 3-(3, 4-dihydroxyphenyl) propionic acid were significantly higher during fermentation of smooth orange juice than orange juice 'with bits' ($P < 0.05$). In addition, 3, 4-dihydroxyphenylpropionic acid was significantly highly

produced from orange juice ‘with bits’ by gut bacteria than smooth orange juice (8.6 ± 2.8 µM vs.3.4 ± 1.6 µM, $p \leq 0.05$).

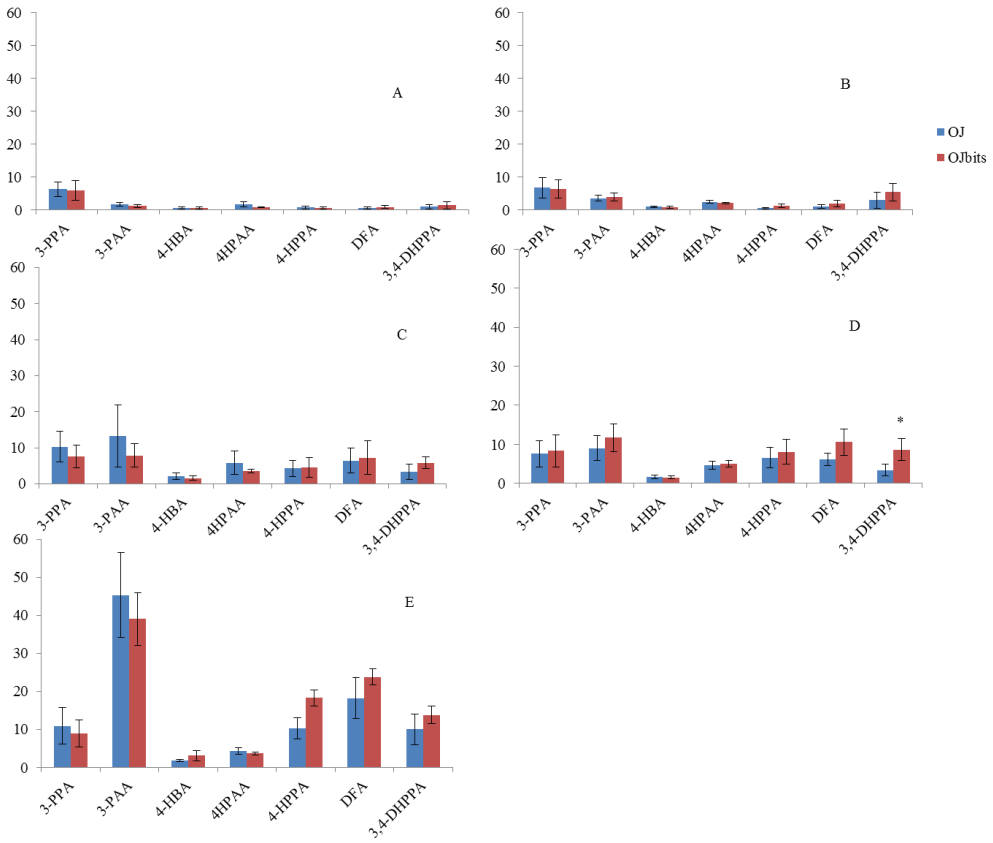


Figure 3-4 Production of phenolic acids (µM) at 0 h (A), 2 h (B), 4 h(C), 6 h(D) and 24 h(E) of the fermentation by human gut bacteria

Expressed as mean value in µM ± SE (n=6), OJ: smooth orange juice, OJ bits: orange juice ‘with bits’, 3-PPA: 3-(phenyl)propionic acid, 3-PAA: 3-(phenyl)acetic acid, 4-HBA: 4-hydroxybenzoic acid,4-HPAA: 4-hydroxyphenylacetic acid, 4-HPPA: 4-hydroxyphenylpropionic acid, 4 - HPPA: 3-(4'-hydrophenyl)propionic acid, DFA: dihydroferulic acid, 3,4-DHPPA: 3,4-dihydroxyphenylpropionic acid. ANOVA tests with post hoc Tukey-HSD were performed to determine significant difference from OJ, $P < 0.05$.

At the beginning of fermentation, 3-(phenyl) propionic acid was the main phenolic acid (6.3 ± 2.3 µM and 6.0 ± 3.0 µM). The total phenolic acid were 12.9 ± 3.1µM and 11.7 ± 3.5 µM in the incubation of smooth orange juice and orange juice ‘with bits’ respectively.

During the period of 0 h to 2 h fermentation, 3-(3,4-dihydroxyphenyl)propionic acid increased by 3 times and 4 times respectively from smooth orange juice and orange juice

‘with bits’ (from 1.0 µM to 3.0 µM and from 1.4 µM to 5.5 µM). 3-(phenyl) propionic acid did not change (6.8 ± 3.2 µM and 6.5 ± 2.8 µM). The sum of 7 main phenolic acids was 18.6 ± 4.1 µM vs. 22.3 ± 5.4 µM) by fermentation of orange juice ‘with bits’ than smooth orange juice. From 2 h to 4 h, 3-pheylacetic acid increased to 13.3 ± 8.7 µM and 7.9 ± 3.2 µM, accounting for 29 % and 21 % of total seven phenolic acids respectively. Dihydroferulic acid and 3-(4'-hydrophenyl) propionic acid increased by 6 ~ 7 times through the fermentation of smooth orange juice and by 3.5 ~ 3.7 times through the fermentation of orange juice ‘with bits’. There was 45.8 ± 20.7 µM of total phenolic acids produced from smooth orange juice, while 38.4 ± 12.1 µM were produced from orange juice ‘with bits’.

After 6 h fermentation, higher phenolic acids were found in the incubation of orange juice ‘with bits’ compared with smooth orange juice (53.7 ± 14.0 µM vs. 38.8 ± 11.1 µM, $p < 0.05$). In addition, 3-(3, 4-dihydrophenyl)propionic acid was significantly higher during fermentation with orange juice ‘with bits’ than smooth orange juice (8.6 ± 2.8 µM vs. 3.4 ± 1.6 µM, $p < 0.05$).

At the end of 24 h fermentation, total phenolic acids increased to 100.9 ± 25.2 µM and 110.7 ± 20.5 µM in the incubation of smooth orange juice and orange juice ‘with bits’ respectively. 3-phenylacetic acid (45.3 ± 11.2 µM, $p < 0.05$) was the highest phenolic acid compared with 4-hydroxybenzoic acid (1.9 ± 0.3 µM), 4-hydroxyphenylacetic acid (4.4 ± 0.9 µM), 3-(4’-hydrophenyl)propionic acid (10.3 ± 2.8 µM), dihydroferulic acid (18.2 ± 5.4 µM), 3-(3,4-dihydroxyphenyl)propionic acid (10.0 ± 4.0 µM) in the incubation of smooth orange juice. In terms of orange juice ‘with bits’, 3-phenylacetic acid (39.0 ± 6.9 µM, $p < 0.05$) was the highest phenolic acid compared with 4-hydroxybenzoic acid (3.1 ± 1.3 µM), 4-hydroxyphenylacetic acid (3.7 ± 0.4 µM), 3-(3,4-dihydroxyphenyl)propionic acid (13.8 ± 2.3 µM).

3.3.4 Total phenolic contents and antioxidant capacity of orange juice in GI tract

3.3.4.1 Upper GI digestion

Total phenolic contents (Table 3-9) and antioxidant capacity (Figure 3-5) were analyzed by Folin-Ciocalteau method and FRAP assay. Total phenols and antioxidant capacity of smooth orange juice and orange juice 'with bits' were similar during the upper GI digestion. The increase of total phenolic content was by 10.8 % and by 4.8 % respectively in smooth orange juice and orange juice 'with bits' than after the gastric digestion, while decreased to 104.1 ± 13.5 mg GAE/250 ml and 112.3 ± 0.6 mg GAE/250 ml after the GI digestion (Table 3-9).

Table 3-9 Total phenols of orange juices (mg GAE/ 250 ml) during the upper gastrointestinal digestion

	Gastric phase		Small intestinal phase		
	0 h	1 h	3 h	5 h	7 h
OJ	150 ± 6.5	166.3 ± 3.3	108.8 ± 0.1	118.6 ± 0.6	104.1 ± 13.5
Recovery (%)	--	110.8	72.5	79.1	69.4
OJ bits	175.8 ± 0.6	184.3 ± 2.7 *	144.7 ± 5.4 *	143.1 ± 3.1 *	112.3 ± 0.6
Recovery (%)	--	104.8	82.3	81.4	63.8

Values are expressed as mean ± SE (n=3). OJ: smooth orange juice, OJ bits: orange juice 'with bits'. Calculation based on 250 ml of orange juice Gastric digestion for 1 h, small intestinal digestion for 6 h from 1 h to 7 h, ANOVA tests with post hoc Tukey-HSD were performed to determine time was significantly different from 0 h, * , $p < 0.05$.

However, antioxidant capacity of smooth orange juice and orange juice 'with bits' decreased by 56.1 % and 41.6 % respectively after the upper GI digestion (Figure 3-5). The antioxidant capacity of smooth orange juice and orange juice 'with bits' was significantly different during the upper GI digestion at 0 h (1.1 ± 0.0 mM Fe^{2+} vs. 0.7 ± 0.0 mM Fe^{2+}, $p = 0.004$), 1 h (1.0 ±0.0 mM Fe^{2+} vs. 0.7 ± 0.0 mM Fe^{2+}, $p = 0.004$), 3 h (0.2 ± 0.0 mM Fe^{2+} vs. 0.2 ± 0.0 mM Fe^{2+}, $p = 0.001$), 5 h (0.2 ± 0.0 mM Fe^{2+} vs. 0.2 ± 0.0 mM Fe^{2+}, $p = 0.04$).

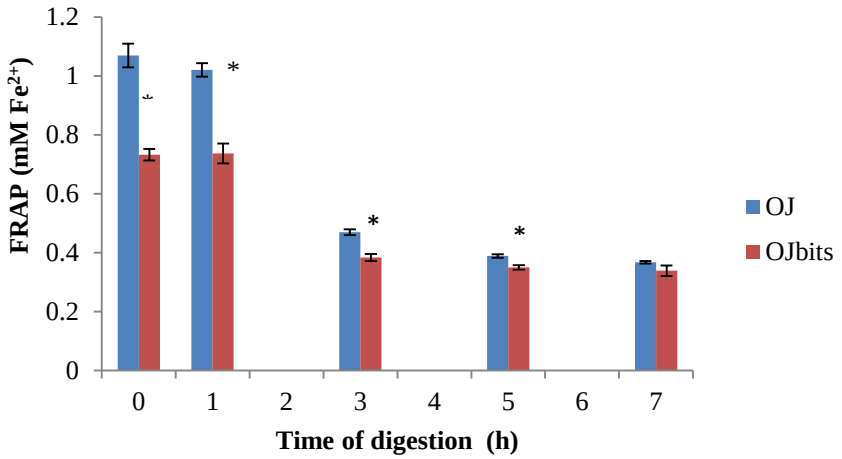


Figure 3-5 Antioxidant capacity of orange juices during the GI digestion

Values are expressed as mean ± SE (n=3). OJ: smooth orange juice, OJ bits: orange juice 'with bits', Gastric digestion for 1 h, small intestinal digestion for 6 h from 1 h to 7 h, ANOVA tests with post hoc Tukey-HSD were performed to determine significant difference from OJ, * $p < 0.05$, ** $P < 0.001$.

3.3.4.2 Colonic fermentation

Total phenolic content (Table 3-10) and antioxidant capacity (Figure 3-6) were similar between orange juices 'with bits' and smooth orange juice during fermentation. Total phenols decreased to 4.5 mM GAE/ ml after 24 h fermentation with smooth orange juice, while it increased to 4.9 mM GAE/ ml with orange juice 'with bits'. The antioxidant capacity decreased from 0.8 mM Fe^{2+} to 0.7 mM Fe^{2+} in the incubation with smooth orange juice, and from 0.8 mM Fe^{2+} to 0.7 mM Fe^{2+} in the incubation with with orange juice 'with bits'.

Table 3-10 Total phenols (mM GAE/ml) in incubation with orange juice digesta during 24 h fermentation with gut bacteria

		0h	2h	4h	6h	24h
OJ		5.3 ± 0.2	5.1 ± 0.4	4.9 ± 0.7	5.2 ± 0.4	4.5 ± 0.8
	Recovery (%)	100	96.7	92.2	98.9	84.4
OJ bits		4.6 ± 0.7	5.6 ± 0.2	5.0 ± 0.8	5.5 ± 0.4	4.9 ± 1.0
	Recovery (%)	100	121.5	107.6	119	105.1

Data expressed as mean values ± SE (n=6). OJ: smooth orange juice, OJ bits: orange juice 'with bits', ANOVA tests with post hoc Tukey-HSD were performed to determine significant difference from OJ, * $p < 0.05$.

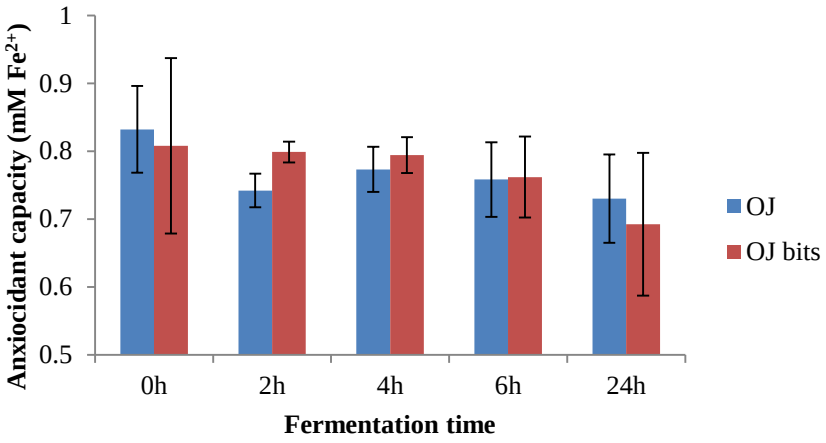


Figure 3-6 Antioxidant capacity during 24 h fermentation with gut bacteria

Data expressed as mean values ± SE (n=6). OJ: smooth orange juice, OJ bits: orange juice 'with bits', ANOVA tests with post hoc Tukey-HSD were performed to determine significant difference from OJ, * $p < 0.05$.

3.3.5 Change of faecal pH during in vitro fermentation

pH did not change during fermentation with hesperidin or no substrates, while smooth orange juice and orange juice 'with bits' significantly decreased pH to 4.5 after 24 h fermentation ($p < 0.05$) (Figure 3-7). In addition, glucose significantly decreased pH from 7.2 to 5.1.

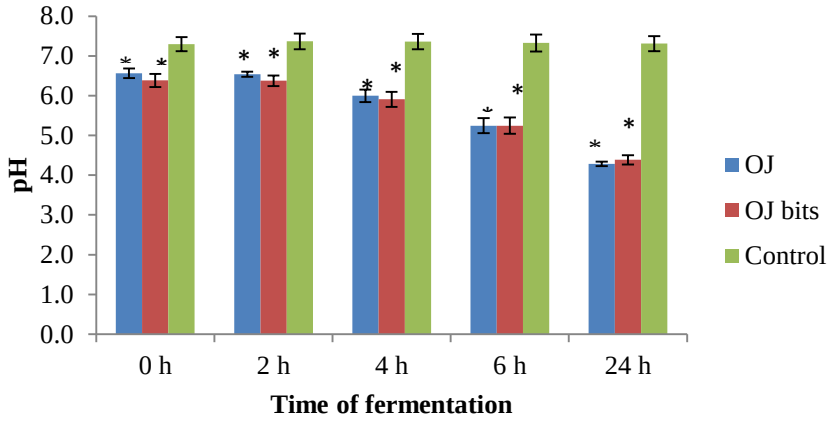


Figure 3-7 pH in faecal samples during fermentation with human gut bacteria

OJ: smooth orange juice, OJ bits: orange juice 'with bits', one way ANOVA with post hoc Tukey-HSD to determine significant differences, * significantly different from control, $P < 0.05$, $ significantly different from Hes, $P < 0.05$.

3.3.6 NEPP in orange juice with bits

Higher polyphenols could be recovered in orange juice by using hydrolysis of extracted pellets with a mixture of pectinase and cellulase. There was 81.6 % of total phenol content extractable in orange juice.

3.4 Discussion

In this chapter, the variation in the content of a range of orange juices were determined and the impact of inclusion of 'bits' was tested in *in vitro* the upper GI tract and colon models. Total phenols were significantly higher in orange juice with bits compared with smooth orange juice (225.9 ± 16.7 µg GAE/ ml vs. 208.4 ± 10.7 µg GAE/ ml, $p < 0.05$), but hesperidin, narirutin, antioxidant capacity, total flavonoids were not different between smooth orange juice and orange juice with bits. In addition, the dietary fibre content ranged from 0.1 g / 100 ml to 0.3 g / 100 ml in the orange juices, but there was no difference between smooth orange juice and orange juice 'with bits' (Table 3-2). Thus, dietary fibre content was not a factor which affected the content of hesperidin and narirutin and degradation of phenolic acids during incubation in models simulating GI digestion and fermentation between smooth orange juice and orange juice with bits.

This may differ from other orange juices with different processing and formulation but as other studies have not characterised the content of their juices in terms of dietary fibre and moreover sometimes have not given sufficient details of the juice to identify its origin it is not possible to explore the potential differences further. In addition, NEPP was detected about 3 mg GAE/ml in extracted orange juice residues by using a mixture of pectinase and cellulase, or driselase.

However, hesperidin was higher in orange juice 'with bits' compared with smooth orange juice when they were subjected to incubation with pepsin/HCl (33.1 ± 1.0 mg / 250 ml vs. 25.8 ± 4.5 mg/ 250 ml hesperidin). This release of hesperidin may be due to non-extractable polyphenols. Non-extractable polyphenols that are those linked to cell wall constituents (polysaccharides and protein) (Arranz et al., 2010, Hasni et al., 2011, Yuksel et al., 2010) which prevents extraction and detection by the usual analytical procedures (Perez-Jimenez et

al., 2013). Thus the acidic gastric conditions in the *in vitro* model released extra hesperidin from orange juice 'with bits'. However, hesperidin was higher in smooth orange juice compared with orange juice 'with bits' after simulating gastric digestion (28.6 ± 2.2 mg / 250 ml vs. 22.0 ± 1.0 mg/ 250 ml). Narirutin had similar amount between both of orange juices during the gastric digestion. Thus, the release / stability of hesperidin were determined by orange juice sources, but not narirutin in stomach.

After the upper GI digestion, hesperidin and narirutin was reduced by 32.1 % and 42.9 % in orange juice 'with bits', which was similar to one study showing 70 % of hesperidin passed to the colon after a polyphenol-rich drink by ileostomists (Borges et al., 2013). However, 9.9 % and 42.3 % were respectively not recovered in smooth orange juice, but no difference between juices. There was similar amounts of hesperidin and narirutin in the smooth orange juice and orange juice 'with bits' after the upper GI digestion at 22 mg and 1.0 mg /250 ml respectively even though there was a higher hesperidin in orange juice with bits. Thus, the different orange juice source might not affect the amount of hesperidin and nairutin passing to the colon.

On the other hand, total phenolic contents reduced by 47 % in fruit beverages in models mimicking gastrointestinal digestion (Cilla et al., 2009). In this study, total phenols also decreased in both of orange juice by 30.6 % and 36.2 % respectively in smooth orange juice and orange juice 'with bits' during in vitro digestion, However, total phenols was higher in orange juice 'with bits' than smooth orange juice during the gastrointestinal digestion, except at the end of the digestion. The antioxidant capacity decreased by 43.9 % and 47.6 % between smooth orange juice and orange juice 'with bits' after 2 h of small intestinal digestion under the mild alkaline conditions of the small intestine. In terms of antioxidant capacity (FRAP), smooth orange juice and orange juice 'with bits' decreased respectively by 56.1 % and 41.6 % after the upper GI digestion. However, total phenols and antioxidant capacity did not change during fermentation with gut bacteria. Therefore, orange juice sources might mainly influence total phenolic content and antioxidant capacity of orange juice during the upper GI digestion.

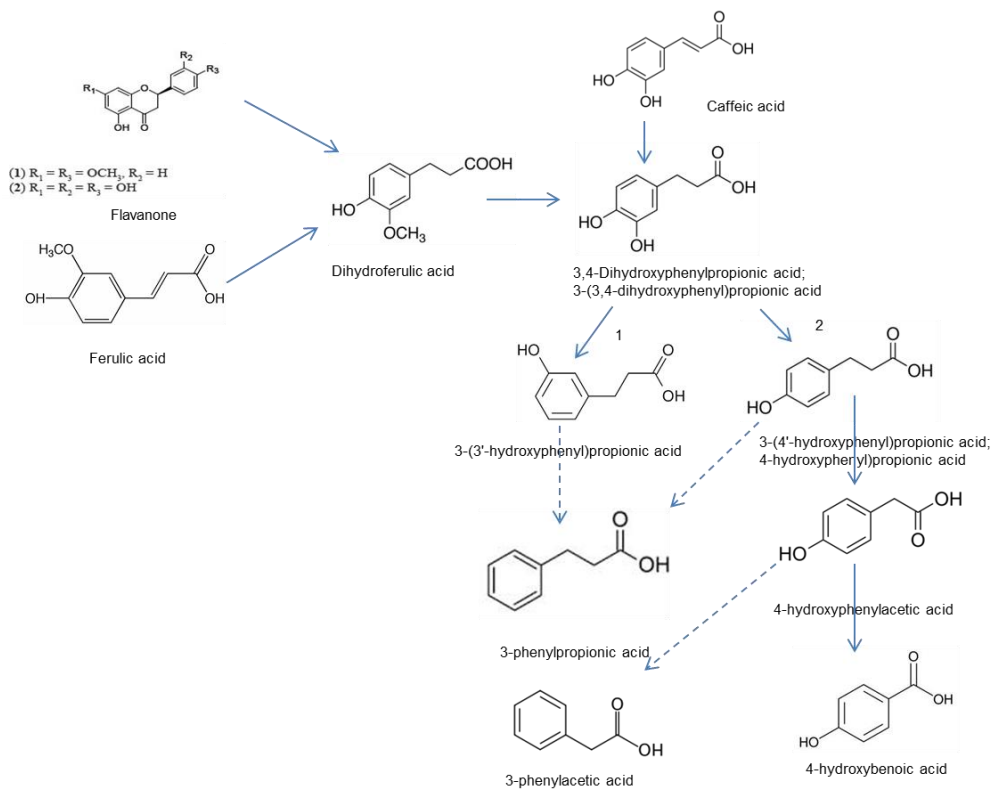


Figure 3-8 the proposed catabolic pathways of orange juice flavanone degradation by human colonic microbiota

Based on previous studies (Duda-Chodak et al., 2015, Rechner et al., 2004, Wang et al., 2015, Jaganath et al., 2009).

In our study, acidic gastric conditions reduced hesperidin to a lower level in orange juice 'with bits' and released higher amounts of hesperidin in incubation with orange juice 'with bits' during fermentation by gut bacteria. Other studies focusing on the stability of flavonoids by using *in vitro* upper GI digestion, orange juice flavanones were transformed into chalcones by 70 % during gastrointestinal digestion (Gil-Izquierdo et al., 2003) and hand squeezed, pasteurized and industrial proceed orange juice had varied flavanone during simulating gastrointestinal digestion (Gil-Izquierdo et al., 2003). In human studies, Tomas-Navarro and colleagues reported various levels of bioavailability of flavanones after consumption of fresh hand-squeezed orange juice, high pressure homogenised orange juice and pasteurised orange juice ranging from 6.7 % - 11 % (Tomas-Navarro et al., 2014). On the other hand, insoluble and soluble dietary fibre were changed by 39.06 % and 80 % respectively by processing in orange pulp (Larrea et al., 2005). Furthermore, dietary fibre was low and similar between

smooth orange juice and orange juice with bits in this study. Thus, this suggests that this difference in stability of polyphenols during digestion may not require high fibre contents.

In addition, the narirutin content significantly decreased when passing from the acidic condition of the stomach model to the alkaline condition of the small intestinal tract, but hesperidin was not affected. This was similar with a study reporting the effects of gastrointestinal digestion on flavonoids. The decreased polyphenols, flavonoids and especially anthocyanins were caused by the transition from the acidic gastric to the mild alkaline intestinal environment (Tagliazucchi et al., 2010). In addition, Rodríguez and colleagues found no change of hesperidin and quercetin, but reduction of naringenin, caffeic acid, ferulic acid, p-coumaric acid, and 4-hydroxybenzoic acid, as well as an increase in chlorogenic acid, sinapic acid, rutin and (+)-catechin in a blended fruit juice containing orange, kiwi, pineapple and mango by the gastric digestion process (Rodríguez-Roque et al., 2014). Low stability of narirutin during the gastrointestinal digestion could be attributed to the higher solubility of narirutin (Gil-Izquierdo et al., 2001). However, hesperidin is more abundant in 'bits' particles by its hydrophobic characteristics (Vallejo et al., 2010c). Thus, the chemical structures of flavanones potentially determine their stability /release in the gastrointestinal tract, so that food sources might influence flavanone in the gastrointestinal tract.

Hesperidin was higher in the faecal incubations with orange juice 'with bits' compared with smooth orange juice at 2 h fermentation (89.6 ± 9.5 mg vs. 77.1 ± 8.9 mg, $p \leq 0.05$). In addition, production of phenolic acids was higher at 6 h fermentation with orange juice 'with bits' compared with smooth orange juice (53.7 ± 14.0 μM vs. 38.8 ± 11.1 μM, $p \leq 0.04$). However, hesperidin and narirutin, hesperetin and naringenin were similar between incubated smooth orange juice and orange juice 'with bits' after 24 h fermentation. Furthermore, pH was decreased to 4.5 in faecal samples after 24 h fermentation with both of orange juices. Thus, the presence of 'bits' affected the initial rate of changes in hesperidin content and production of phenolic acids, but not metabolism of flavanone over 24 h of fermentation.

Hesperidin and narirutin are metabolised depending on their concentration and structure, and the composition of the human gut microflora (Rechner et al., 2004). β-rhamnosidases and β-glucosidases of colonic bacteria are responsible for cleavage of the rutinoside moiety (Aura et

al., 2002a). In this study, narirutin was degraded by 43 % from smooth orange juice and orange juice 'with bits', while hesperidin was transformed by 19 % and 32 % respectively, which was in accordance with higher urinary excretion of narirutin intake after consumption of orange juice by humans (Mullen et al., 2008a). Hesperidin and narirutin require hydrolysed into hesperetin and naringenin with relevant enzymes, such as with activity of α-rhamnosidase and β-glucosidase, or catabolised to phenolic acids by gut bacteria prior to absorption through intestinal epithelium cells (Nielsen et al., 2006b, Brand et al., 2008, Manach et al., 2005). Hesperidin and narirutin in smooth orange juice were reduced to 42.5 ± 15.9 μM and 1.2 ± 0.3 μM from 97.9 ± 10.1 μM and 3.5 ± 0.6 μM after 24 h fermentation with human gut bacteria. However, their aglycones, hesperetin and naringenin were not significantly different between smooth orange juice and orange juice 'with bits' during the fermentation (p = 0.75 and 0.52 respectively). After 6 h fermentation with gut bacteria, phenolic acids were higher in the incubation with orange juice 'with bits' than smooth orange juice. Phenolic acids were derived from aglycones by gut bacteria. The varied production of phenolic acids might reflect previous human studies that revealed various urinary excretion of flavanone dose after consumption of orange juice (Tomas-Navarro et al., 2014, Silveira et al., 2014, Vallejo et al., 2010c). Thus, orange juice sources might influence the metabolism of orange juice flavanones.

Gut bacteria, such as *Bifidobacterium spp.* and *Lactobacillus spp.* (Selma et al., 2009, Hidalgo et al., 2012) were affected by polyphenols. Tea polyphenols, (+)-catechin, and anthocyanins improved growth of *Bifidobacterium spp.* and *Lactobacillus spp.*(Lee et al., 2006, Tzounis et al., 2008, Hidalgo et al., 2012). The different degree of flavanone degradation depending on sources provided various concentration of metabolites that might influence composition of gut bacteria during the fermentation. Thus, orange juice sources might affect the metabolism of flavanones relevant to absorption and elimination.

In conclusion, this chapter illustrated the metabolism of flavanones in smooth orange juice and orange juice 'with bits' using simulating gastrointestinal digestion and colonic fermentation models. The GI stability / release of orange juice flavanones, especially hesperidin were affected by the types of orange juice. Production of phenolic acids was significantly different between orange juice sources. The derived aglycones were similar in incubation with both of orange juices during fermentation by gut bacteria. These results

might be a reason for the variability of bioavailability ranging from 0.8 to 16 % after consumption of orange juice in humans through gut metabolism. Thus, it is necessary to consider in detail the properties of the tested food in future clinical studies and when measuring consumption habits in epidemiological studies. In addition it is essential that full details of the juices are provided in papers which evaluate health outcomes of flavonoids intake from our diet.

Chapter 4 *Effect of yoghurt on the metabolism of orange juice flavanones in an in vitro model of the GI tract*

4.1 Introduction

Polyphenol-rich food is often ingested with food containing polysaccharides, lipids and proteins, which may influence the bioavailability of polyphenols by interacting with them directly or by influencing the processes of digestion and absorption. Polyphenols have hydrophilic and hydrophobic qualities that interact with food ingredients (Roura et al., 2007a, Mullen et al., 2009b, Ortega et al., 2009a).

Polyphenols have been reported to interact with milk proteins, particularly proline-rich proteins with hydrophobic characteristics (α- and β-caseins) (Hasni et al., 2011, Yuksel et al., 2010). (+) Catechin in green tea was able to interact with β-casein to form complexes visualised by using fluorescent probe binding method and isothermal titration calorimetry analysis (Yuksel et al., 2010). Dairy matrices (milk, yoghurt and cheese) increased the amount of total phenols from green tea and antioxidant activity at the end of intestinal digestion in an *in vitro* digestion model (Lamothe et al., 2014).

Yoghurt is a key source of milk proteins that is often mixed with fruits. There may be direct binding of flavonoids to these proteins. Caseins, 80 % of milk proteins, are aggregated to form a network during the fermentation of milk, which have a strong affinity for hydroxyl groups (Tamime and Robinson, 1999, Yuksel et al., 2010). The majority of anthocyanins (84.73%) were bound to proteins when pomegranate juice was added in 15 % reconstituted skimmed milk made from milk powder to a final concentration of 40 % (v/v) within one day and 90.06 % bound anthocyanins after 28 days, revealing the high affinity of anthocyanins for milk proteins (Trigueros et al., 2014).

In addition, yoghurt also contains lactic acid bacteria. These bacteria include the starter cultures, for example *Streptococcus thermophilus* and *Lactobacillus delbrueckii spp. bulgaricus*. which ferment the milk to produce yoghurt (Ng et al., 2011). In a human study, *Streptococcus thermophilus* and *Lactobacillus Delbrueckii* survived in the gastrointestinal tract of subjects after 12 days consumption of fresh yoghurt (Mater et al., 2005). Some yoghurt contains live probiotic bacteria, such as Bifidobacterium and Lactobacilli (Hotel and Córdoba, 2001, Fuller, 2012, Rybka and Kailasapathy, 1995), which can survive in the human gut after consumption of yoghurt containing probiotics (Gorbach, 2000).

Bifidobacteria and lactobacilli have the highest activity of the β-glucosidases and sulfatases responsible for glycoside hydrolysis and subsequent aglycone cleavage (Steer et al., 2003, Turner, 2008). Thus, probiotics in yoghurt might affect polyphenols as they pass through the gastrointestinal tract and are degraded by gut bacteria in the colon.

Physiological pH, nutrients from food, pepsin, pancreatin and bile salts, can influence probiotics in the gastrointestinal tract (Huang and Adams, 2004, Krasaekoopt et al., 2003, Gregurek, 1999). In an *in vitro* study, passion fruit or mixed berry improved survival of *L. acidophilus (*LAFTI L10) by, but not *Lactobacillus acidophilus* and *Bifidobacterium animalis spp.* Lactis during storage (Kailasapathy et al., 2008). In addition, green tea improved the survival of *B. infantis* by 5.05% and *B. breve* by 7.38% after simulated gastrointestinal digestion (Vodnar and Socaciu, 2012). Therefore, yoghurt bacteria might be affected by these flavonoid-rich products, and in turn, affect metabolism of flavonoids in GI tract.

In a previous study in Glasgow, Mullen et al reported that full fat natural yoghurt reduced the urinary excretion of flavanone metabolites from 3.3 μM to1.5 μM at 0-5 h after ingestion of orange juice, but did not affect the 0-24 h urinary excretion, maximal plasma concentration and T_{max} of two hesperetin-O-glucuronides (Mullen et al., 2008a). In addition, Roowi et al reported that full fat natural yoghurt also greatly reduced from 62 ± 18 μmol to 6.7 ± 1.8 μmol in the urinary excretion of five phenolic acids (3-hydroxyphenylacetic acid, 3-hydroxyphenylhydracrylic acid, dihydroferulic acid, 3-methoxy-4-hydroxyphenylhydracrylic acid and 3-hydroxyhippuric acid) (Roowi et al., 2009a). This study suggested that full fat natural yoghurt might supress the degradative activity of gut bacteria to break down flavanones into phenolic acids. However the mechanism is not clear. It is possible that delivery of polyphenols to the colon may have been affected or absorption from the gut in the colon. There was no effect of full fat natural yoghurt on gastric emptying or small bowel transit time.

Flavanone metabolites and phenolic metabolites are absorbed into the circulatory system after deglycosylation and degradation by intestinal enzymes or colonic bacteria (Manach et al., 2005, Crozier et al., 2010, Pereira-Caro et al., 2014a, Roowi et al., 2009b). However, the mechanism by which yoghurt affects the production of flavanone metabolites and phenolic acids in the GI tract has not been explored. In addition, orange juice source (smooth orange

juice and orange juice ‘with bits’) affected metabolism of flavanone in GI tract, where there was lower hesperidin after gastric digestion and higher 3,4 –dihydroxyphenylpropionic acid at 6 h of fermentation from orange juice ‘with bits’ (Chapter 3). Thus, variable interactions of full fat natural yoghurt with smooth orange juice / orange juice ‘with bits’ might affect metabolism of flavanone in the GI tract. Moreover, various yoghurts containing probiotics or fruits might influence metabolism of orange juice flavanone in GI tract based on their ingredients (probiotics or extra polyphenols). The studies in this chapter were designed to elucidate the action of yoghurt/flavanone interactions using the GI model described in chapter 3 by exploring the effects of natural full fat yoghurt which contained only starter culture organisms in *in vitro* models of the upper and lower gut. The starter culture organisms should not survive in the model. The effects were compared with yoghurt containing probiotic bacteria which should survive in the model. In addition fruit containing yoghurt (live bacteria but extra polyphenols) were studied see any additional effects of the fruit. Thus the impact of yoghurt alone was tested in case this inhibited the phenolic acid production, probiotic bacteria may affect what phenolics enter the colon as they may metabolise the flavanones in the upper gut models as well as affect phenolic acid production in the colonic model. The fruit yoghurts may behave the same as the normal full fat yoghurt but the extra polyphenols from the fruit may interact with the gut bacteria and change total output.

4.1.1 Hypothesis

These studies aimed to test the hypothesis that yoghurt as part of the food matrix influences the metabolism of flavanones in an *in vitro* model of the upper and lower gut and to explore the mechanisms for any interactions.

The following research questions were investigated:

1) Does full fat natural yoghurt affect the stability / release of orange juice flavanones in the upper gut model which may decrease delivery to the colon?

2) Was the reduced urinary excretion of phenolic acids seen in the *in vivo* study (Roowi et al., 2009b) with full fat yoghurt due to decreased production in the colon?

3) Does full fat natural yoghurt have different actions between orange juice ‘with bits’ and orange juice ‘no bits’?

4) Do probiotics containing behave differently, can the probiotic bacteria degrade the flavanones in the upper gut models and or phenolic acid release in the colonic model resulting in reduced delivery of polyphenols to the colon?

5) Does probiotic fruit yogurt behave the same as non probiotic full fat yoghurt, or does the extra fruit affect phenolic acids production?

4.2 Subjects and methods

4.2.1 Chemicals and materials

HPLC-grade methanol was obtained from Rathburn Chemicals (Walkerburn, UK). Formic acid was purchased from Riedel-deHaen (Seelze, Germany). Hydrochloric acid, sodium bicarbonate, pepsin (from porcine stomach mucosa), pancreatin (from porcine pancrease), bile acids (from porcine); tryptone, 2,4,5 – trimethoxycinnamic, anhydrous ethyl acetate, dichloromethane (DCM), N-methyl-N- (trimethylsily) trifluoracetateamide, prydine. $CaCl_2.2H_2O$, $MnCl_2.4H_2O$, NH_4HCO_3, $NaHCO_3$, Na_2HPO_4, KH_2PO_4, $MgSO_4.7H_2O$, cysteine hydrochloride, NaOH, $Na_2S \cdot 9\ H_2O$ and resazurin solution were purchased from Sigma-Aldrich Co Ltd (Poole, Dorset, UK). Hesperetin-7-*O*-rutinoside, narigenin-7-*O*-rutinoside, 3-hydroxyphenylacetic acid, 4-hydroxyphenylacetic acid , 4-hydroxyphenylpropionic acid, 3, 4-dihydroxyphenylacetic acid, 3-(3, 4-dihydroxyphenyl) propionic acid, 4-hydroxy-3-methoxy-phenylpropionic acid (dihydrofeulic acid), 3-hydroxybenzoic acid, vanillic acid, 3-hydroxy-4-methoxyphenyl propionic acid, 3-methoxy-4-hydroxyphenyl propionic acid, p-coumaric acid and ferulic acid were purchased from Extrasynthese, Sigma-Aldrich Co Ltd (Poole, Dorset, UK) or Alfa Aesar (Karlsruhe, Germany).

4.2.2 Orange juices and yoghurt

Two types of 100 % squeezed orange juice (Tropicana, not from concentrate, smooth orange juice (OJ) and orange juice 'with bits' (OJ bits)) were purchased from local supermarkets.

Full fat natural yoghurt was made by the researcher from whole milk (Yeo Valley whole milk) and fermented by yoghurt cultures (*Streptococcus thermophiles* and *Lactococcus bulgaricus*) (Cheesemakingshop, Prospect Lane, Solihull, UK) in the lab with the following recipe: 1L of milk was heated and then quickly cooled down to 45 °C. A sachet of yoghurt starter (1 g) was dissolved with a cup of the heated milk, and then added to the rest of the milk. The flask was sealed and allowed milk to ferment for 7 hours. And then, full fat natural yoghurt was kept in fridge for next day use.

Probiotic containing natural full fat yoghurt was purchased from local supermarkets; Yeo Valley Natural Yoghurt containing *Bifidobacterium (no species given), Lactobacillus acidophilus,* and *Streptococcus thermophilus*.

Strawberry yoghurt was purchased from local supermarket; Yeo Valley Strawberry yoghurt containing organic whole milk (86%), organic Strawberry Purée (5%), and live cultures: *Bifidobacterium, Lactobacillus acidophilus,* and *Streptococcus thermophilus*.

4.2.3 Simulating GI digestion and fermentation of orange juice and full fat yoghurt with only starter culture

The experiments conducted in this chapter are illustrated in Figure 4-1.Orange juice (40 ml) and 25 ml of full fat natural yoghurt (25 ml) was chosen based on the consumption of orange juice (250 ml) and full fat natural yoghurt (150 ml) in previous study (Mullen et al., 2008a).

4.2.3.1 *In vitro* gastrointestinal digestion

The model to simulate gastric and small intestinal digestion was adapted from Gil-Izquierdo (Gil-Izquierdo et al., 2003). *In vitro* gastrointestinal digestion was conducted as described in section 2.3.1.

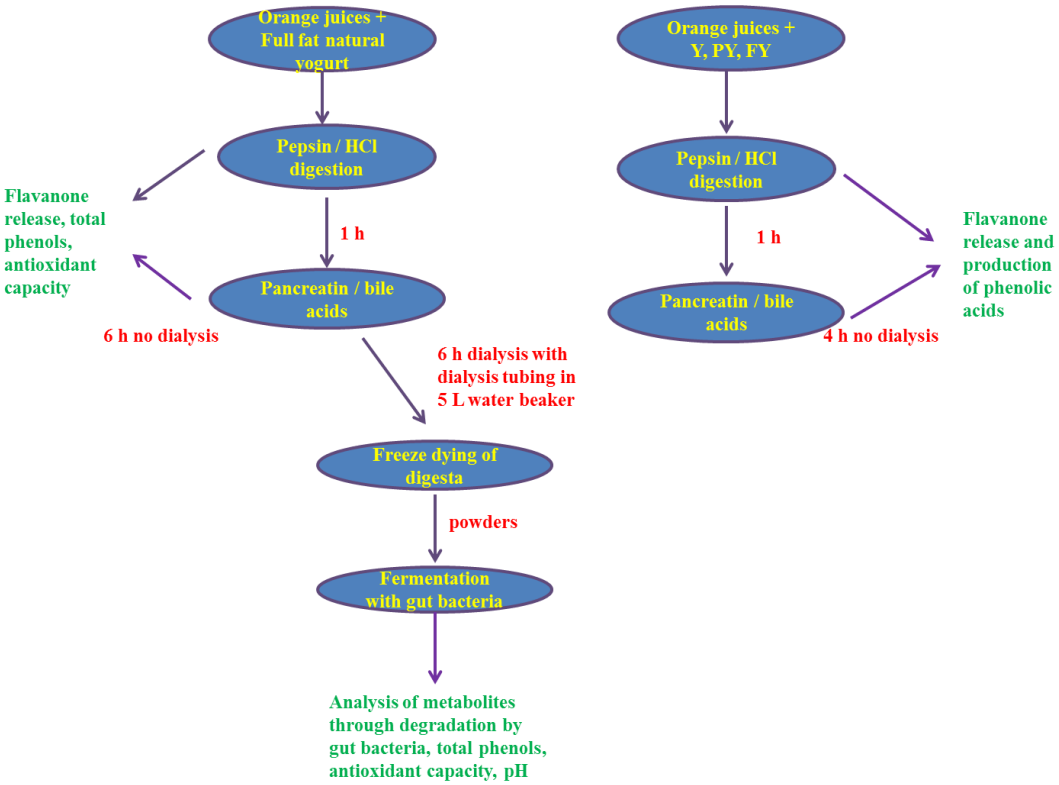


Figure 4-1 Details of the different stages of the simulating gastrointestinal model

Y: full fat natural yoghurt, PY: probiotic containing yoghurt, FY, probiotic containing yoghurt with strawberry Purée (5 %)

4.2.3.2 Faecal sample collection

Six healthy Caucasian volunteers (same in Chapter 3) participated in this study and had a normal diet, were non-smokers, had no digestive diseases, no food allergies, and had not received antibiotics for at least 6 months before the study. Before providing faeces, participants agreed to take part in this study, and signed consent form. The volunteers followed a low-polyphenol diet avoiding fruits, vegetables, tea, coffee, wine and wholemeal foods for 2 days (details in Appendix 3) before providing a faecal sample according to information sheet (Appendix 2). Each faecal sample was collected by subjects in a special plastic box containing an AnaeroGen sacket (Oxioid, Hampshire, UK) to generate anaerobic conditions. Faecal samples were processed when brought to the lab. The food was recorded in a dietary record (Appendix 4).

4.2.3.3 *In vitro* fermentation with human colonic bacteria

After in vitro gastrointestinal digestion, digesta was freeze-dried by using Edwards Micro Modulyo Freeze Dryer. The residues were stored at - 80 °C until colonic fermentation. The fermentation was conducted as described in section 2.3.3.

4.2.4 Analysis of flavanone and phenolic acids in digested and faecal samples

Samples (1 ml) were extracted as described in section 2.9.1. The extracts were analysed by using HPLC-PDA as described in section 2.10. Phenolic acids were extracted as described in section 2.6. The extracts were analysed by using GC-MS as described in chapter 2.8.

4.2.5 Analysis of total phenol and antioxidant capacity in digested and faecal samples

Total phenols in digested and faecal samples were measured by using the Folin–Ciocalteau method. See section 2.4.1 for more details. Antioxidant capacity was determined by using FRAP assay in a redox-linked colorimetric reaction. See chapter 2.4.3 for more details.

4.2.6 Survival test of yoghurt starter culture bacteria

After gastric digestion of full fat natural yoghurt, digesta (1 ml) was diluted with sodium citrate (0.1 M) ranging from 1:10 to 1:1000 and poured in MRS agar plate. The plates were incubated 2 days at 37°C.

4.2.7 Statistical analysis

All data were presented as mean values with standard error. The unit used for the upper GI digestion was based on total amount of flavanone or total phenols in the digestive fluids expressed as mg/250ml, and used for the fermentation was based on concentration of flavanone or total phenols in faecal sample expressed as µM. All data were assumed to be normally distributed, t-tests or ANOVA tests with Tukey-HSD was used to determine significant differences between orange juice with yogurt and orange juice without yogurt ($P < 0.05$). The analysis was conducted with SPSS software version 22.

4.3 Results

4.3.1 The effect of full fat natural yoghurt (Y) on GI metabolism of orange juice flavanone

4.3.1.1 Hesperidin and narirutin in orange juices mixed with full fat natural yoghurt

Hesperidin was higher in OJ + Y compared with OJ (40.5 ± 4.5 mg vs. 25.8 ± 4.5 mg, $p < 0.001$) and OJ bits + Y compared with OJ bits (41.3 ± 2.1 mg vs. 33.1 ± 1.0 mg, $p < 0.05$) at the beginning of the GI digestion (Table 4-1). Narirutin was higher in OJ + Y compared with OJ at beginning of GI digestion (1.8 ± 0.3 mg vs. 1.7 ± 0.3 mg, $p < 0.001$). However, there was no difference of hesperidin and narirutin between OJ +Y and OJ bits + Y, even though hesperidin was higher in OJ bits than OJ (33.1 ± 1.0 mg vs. 25.8 ± 4.5 mg, $p < 0.05$).

Table 4-1 Content of hesperidin and narirutin (mg/250ml) in orange juices mixed with and without full fat natural yoghurt at initial gastric digestion

	Hesperidin	Narirutin
OJ	25.8 ± 2.6	1.7 ± 0.2
OJ + Y	40.5 ± 2.6 **	1.8 ± 0.2
OJ bits	33.1 ± 0.6 £	1.7 ± 0.1
OJ bits + Y	41.3 ± 1.2 *	1.8 ± 0.1

Data expressed as mean values ± SE (n=3). Samples were collected when pepsin/HCl was added to individual inbubation. OJ: smooth orange juice, OJ+Y: smooth orange juice with full fat natural yoghurt, OJ bits: orange juice 'with bits', OJ bits +Y: orange juice 'with bits' with full fat natural yoghurt, * significantly different from OJ or OJ bits, $P < 0.05$ (pair t-test), ** significantly different from OJ or OJ bits, $P < 0.001$ (pair t-test), £ significantly different from OJ, $p < 0.05$ (independent t-test).

4.3.1.2 Hesperidin and narirutin in orange juices mixed with full fat natural yoghurt during in vitro GI digestion

After gastric digestion, hesperidin was higher in OJ + Y (41.6 ± 2.0 mg/250 ml vs. 28.6 ± 1.6 mg/250 ml, p = 0.002) and OJ bits + Y (38.5 ± 1.9 mg/250 ml vs. 22.0 ± 0.7 mg/250 ml, p = 0.012) compared with OJ and OJ bits after the gastric digestion (Table 4-2). After the small intestinal digestion, hesperidin was higher in OJ bits + Y (47.9 ± 7.2 mg/250 ml vs. 22.5 ± 1.7 mg/250 ml, p = 0.002) compared with OJ bits (p < 0.004), while hesperidin was higher in OJ + Y compared with OJ (34.9 ± 1.1 mg/250 ml vs. 22.9 ± 1.4 mg/250 ml, p = 0.031). Furthermore, narirutin was about 1.0 ± 0.1 mg / 250 ml in orange juices after the upper GI digestion.

Table 4-2 Content of hesperidin and narirutin (mg/250ml) in digestive fluids during the upper GI digestion *in vitro*

	Narirutin		Hesperidin		Total Flavanone	
	After gastric digestion	After intestinal digestion	After gastric digestion	After intestinal digestion	After gastric digestion	After intestinal digestion
OJ	1.8 ± 0.0	1.0 ± 0.1	28.6 ± 1.6	22.9 ± 1.0	30.3 ± 1.6	23.9 ± 1.1
OJ +Y	1.8 ± 0.1	1.5 ± 0.1 *	41.6 ± 2.0 *	34.9 ± 1.1*	43.4 ± 2.1 *	36.4 ± 1.2*
OJ bits	1.6 ± 0.1	1.0 ± 0.1	22.0 ± 0.7	22.5 ± 1.7	23.7 ± 0.7	23.5 ± 1.7
OJ bits +Y	1.6 ± 0.1	1.6 ± 0.1	38.5 ± 1.9 *	47.9 ± 7.2 *	40.2 ± 1.9 *	49.6 ± 7.3 *

Data expressed as mean values ± SE (n=3), samples were collected when pepsin/HCl was added to each inbubation. Calculation was based on total amount of hesperidin or narirutin from 250 ml of orange juice, OJ: smooth orange juice, OJ+Y: smooth orange juice with full fat natural yoghurt, OJ bits: orange juice 'with bits', OJ bits +Y: orange juice 'with bits' with full fat natural yoghurt, ANOVA tests with Tukey-HSD test was used to determine significant differences * significantly different from OJ or OJ bits, P < 0.05..

4.3.1.3 Hesperidin and narirutin in orange juices mixed with full fat natural yoghurt during *in vitro* fermentation by human gut bacteria

Compared with OJ, hesperidin was higher in OJ + Y after 6 h fermentation with gut bacteria (63.0 ± 13.8 μM vs. 54.2 ± 11.9 μM, p ≤ 0.03) (Table 4-3). However, hesperidin and narirutin were higher in OJ bits than OJ bits + Y (97.9 ± 10.1 μM vs. 73.2 ± 11.8 μM, p ≤ 0.01; 3.5 ±

0.6 µM vs. 2.6 ± 0.5 µM, $p < 0.05$) at 0 h fermentation (Table 4-4). However, narirutin, hesperidin, hesperetin and naringenin were not significantly different between OJ and OJ +Y, or OJ bits and OJ bits + Y after 24 h colonic fermentation with gut bacteria. Total flavanone was 53.1 ± 13.1 µM and 55.1 ± 11.8 µM in the incubation with OJ and OJ +Y, 49.6 ± 16.6 µM and 59.2 ± 15.2 µM in the incubation of OJ bits and OJ bits + Y respectively.

Table 4-3 Concentration of hesperidin, narirutin, hesperetin and naringenin (μM) from smooth orange juice during fermentation by human colonic bacteria in the presence of full fat natural yoghurt

	0 h		2 h		4 h		6 h		24 h	
	OJ	OJ +Y	OJ	OJ +Y	OJ	OJ +Y	OJ	OJ +Y	OJ	OJ +Y
Narirutin	2.7 ± 0.6	3.6 ± 0.5	2.7 ± 0.6	3.1 ± 0.5	2.5 ± 0.7	2.0 ± 0.4	2.0 ± 0.7	2.5 ± 0.6	1.4 ± 0.3	1.2 ± 0.3
Hesperidin	70.0 ± 13.3	79.2 ± 11.1	77.1 ± 8.9	87.4 ± 5.9	72.8 ± 8.4	64.1 ± 6.9	63.0 ± 13.8	54.2 ± 11.9 *	44.4 ± 12.6	47.4 ± 12.0
Naringenin	0.7 ± 0.4	0.4 ± 0.2	2.0 ± 0.7	1.7 ± 0.6	2.7 ± 0.9	2.3 ± 0.9	3.0 ± 1.2	1.5 ± 0.4	2.7 ± 1.0	2.7 ± 0.6
Hesperetin	0.4 ± 0.3	0.2 ± 0.1	1.4 ± 0.6	1.1 ± 0.4	2.7 ± 1.2	2.3 ± 1.1	2.9 ± 1.2	1.1 ± 0.3	4.6 ± 1.9	3.8 ± 1.4
Total	73.8 ± 14.1	83.4 ±11.4	83.3 ± 9.2	93.3 ± 6.6	80.8 ± 7.6	70.7 ± 7.8	70.9 ± 15.1	59.3 ± 11.7 *	53.1 ± 13.1	55.1 ± 11.8

Data expressed as mean values ± SE (n=6), OJ: smooth orange juice, OJ +Y: smooth orange juice with full fat natural yoghurt, ANOVA tests with Tukey-HSD test was used to determine significant differences * significantly different from OJ, $P < 0.05$.

Table 4-4 Concentration of hesperidin, narirutin, hesperetin and naringenin (μM) from orange juice 'with bits' during fermentation by human colonic bacteria in the presence of full fat natural yoghurt

	0 h		2 h		4 h		6 h		24 h	
	OJ bits	OJ bits+ Y	OJ bits	OJ bits+ Y	OJ bits	OJ bits+ Y	OJ bits	OJ bits+ Y	OJ bits	OJ bits+ Y
Narirutin	3.5 ± 0.6	2.6 ± 0.5 *	2.8 ± 0.6	2.8 ± 0.5	2.4 ± 0.6	2.2 ± 0.3	1.8 ± 0.5	1.9 ± 0.5	1.2 ± 0.3	1.4 ± 0.5
Hesperidin	97.9 ± 10.1	73.2 ± 11.8 *	89.6 ± 9.5	72.8 ± 15.3	79.5 ± 14.8	78.2 ± 11.2	68.6 ± 18.7	70.1 ± 16.0	42.5 ± 15.9	52.9 ± 14.5
Naringenin	0.7 ± 0.4	0.4 ± 0.3	1.8 ± 0.8	1.0 ± 0.3	2.2 ± 0.8	2.3 ± 0.8	2.2 ± 0.7	1.6 ± 0.6	2.4 ± 0.7	1.7 ± 0.7
Hesperetin	0.4 ± 0.3	0.2 ± 0.2	1.6 ± 0.8	0.6 ± 0.3	2.4 ± 1.1	2.1 ± 0.8	2.7 ± 0.9	1.9 ± 0.5	3.4 ± 1.4	3.1 ± 1.0
Total	102.5 ± 10.3	76.4 ± 12.3 *	95.8 ± 10.2	77.2 ± 15.9	86.5 ± 14.9	84.8 ± 11.9	75.3 ± 19.4	75.6 ± 16.9	49.6 ± 16.6	59.2 ± 15.2

Data expressed as mean values ± SE (n=6), OJ bits: orange juice 'with bits', OJ bits +Y: orange juice 'with bits' with full fat natural yoghurt, ANOVA tests with Tukey-HSD test was used to determine significant differences, * significantly different from OJ bits, $P < 0.05$.

4.3.1.4 Phenolic acids derived from orange juices mixed with full fat natural yoghurt after *in vitro* fermentation

The five phenolic acids (4-hydroxybenzoic acid, 4-hydroxyphenylacetic acid, 3-4'-hydrophenylpropionic acid, dihydroferulic acid, and 3, 4-dihydroxyphenylpropionic acid) that are assumed to be derived from orange juice flavanones were summed to give total phenolic content. There was no significantly different production of phenolic acids in the incubations of orange juices with or without yoghurt after 24 h fermentation (Table 4-5). In addition, the yoghurt did not affect the amount of each phenolic acid during the fermentation of smooth orange juice and orange juice 'with bits'.

Table 4-5 Concentration of five phenolic acids (μM) derived from orange juice during fermentation by human colonic bacteria in the presence of full fat natural yoghurt

	0 h	2 h	4 h	6 h	24 h
OJ	4.1 ± 1.4	6.9 ± 2.5	19.2 ± 9.3	19.7 ± 5.3	39.0 ± 12.5
OJ +Y	3.1 ± 1.0	8.1 ± 3.3	15.5 ± 7.6	22.7 ± 8.3	42.3 ± 12.7
OJ bits	4.7 ± 1.6	10.7 ± 3.6	20.1 ± 10.4	26.1 ± 8.5	37.4 ± 10.3
OJ bits + Y	4.9 ± 1.8	15.8 ± 8.6	17.3 ± 9.8	21.3 ± 9.1	42.2 ± 12.0

Data expressed as mean values ± SE (n=6), OJ: smooth orange juice, OJ+Y: smooth orange juice with full fat natural yoghurt, OJ bits: orange juice 'with bits', OJ bits +Y: orange juice 'with bits' with full fat natural yoghurt, ANOVA tests with Tukey-HSD test was used to determine significant differences, * significantly different from OJ or OJ bits, $P < 0.05$.

4.3.2 The GI metabolism of hesperidin and narirutin from different orange juice in the presence of full fat natural yoghurt

4.3.2.1 The stability / release of hesperidin and narirutin during the upper GI digestion

There was no significant difference in hesperidin and narirutin between OJ + Y and OJ bits + Y during the upper GI digestion. Hesperidin decreased from 40.5 ± 2.6 to 34.9 ± 0.9 mg/ 250ml in OJ +Y, and ranged from 41.3 ± 2.1 to 47.9 ± 10.0 mg/ 250ml in OJ bits +Y. Narirutin decreased from 1.8 ± 0.3 to 1.5 ± 0.1 mg/ 250ml ($p < 0.02$) in OJ + Y. Total flavanone decreased to 36.4 ± 0.9 mg/ 250ml (13.9 % of loss) in OJ + Y, but increased to 49.6 ± 5.9 mg/ 250 ml (115.1 % of recovery) in OJ bits +Y.

Table 4-6 Contents of hesperidin and narirutin (mg/250ml) in orange juices in the presence of full fat natural yoghurt during the upper GI digestion

	0 h		1 h		3 h		5 h		7 h	
	Hesperidin	Narirutin	Hesperidin	Narirutin	Hesperidin	Narirutin	Hesperidin	Narirutin	Hesperidin	Narirutin
OJ+Y	40.5 ± 2.6	1.8 ± 0.2	41.6 ± 1.6	1.8 ± 0.1	35.2 ± 1.7	1.6 ± 0.1	29.0 ± 2.3	1.4 ± 0.1	34.9 ± 0.9	1.5 ± 0.1
Recovery (%)	--	--	103.5 ± 5.9	100.4 ± 10.9	87.3 ± 4.6	90.6 ± 9.0	73.1 ± 11.2	76.3 ± 11.3	87.4 ± 8.3	81.7 ± 12.1
OJ bits +Y	41.3 ± 1.2	1.8 ± 0.1	38.5 ± 1.5	1.6 ± 0.1	30.4 ± 2.4	1.4 ± 0.1	32.0 ± 0.5	1.4 ± 0.1	47.9 ± 5.8	1.6 ± 0.1
Recovery (%)	--	--	94.1 ± 6.4	91.5 ± 4.2	74.2 ± 7.3	79.2 ± 8.1	78.0 ± 3.0	79.7 ± 5.3	117.2 ± 16.1	91.0 ± 10.5

Data expressed as mean values ± SE (n=3). Calculation was based on total amount of hesperidin or narirutin from 250 ml of orange juice, OJ+Y: smooth orange juice with full fat natural yoghurt, OJ bits + Y: orange juice 'with bits' with full fat natural yoghurt, gastric digestion for 1 h, small intestinal digestion for 6 h from 1 h to 7 h, ANOVA tests with Tukey-HSD was used to determine significant differences, * significantly different from OJ + Y, $P < 0.05$.

Table 4-7 Concentration of hesperidin and narirutin (μM) from orange juices during colonic fermentation by human gut bacteria in the presence of full fat natural yoghurt

	0 h		2 h		4 h		6 h		24 h	
	Hesperidin	Narirutin	Hesperidin	Narirutin	Hesperidin	Narirutin	Hesperidin	Narirutin	Hesperidin	Narirutin
OJ+Y	79.2 ± 11.1	3.6 ± 0.5	87.4 ± 5.9	3.1 ± 0.5	64.1 ± 6.9	2.0 ± 0.4	54.2 ± 11.9	2.5 ± 0.6	47.4 ± 12.0	1.2 ± 0.3
OJ bits +Y	73.2 ± 11.8	2.6 ± 0.5	72.8 ± 15.3	2.8 ± 0.5	78.2 ± 11.2	2.2 ± 0.3	70.1 ± 16.0	1.9 ± 0.5	52.9 ± 14.5	1.4 ± 0.5

Data expressed as mean values ± SE (n=6), OJ+Y: smooth orange juice with full fat natural yoghurt, OJ bits + Y: orange juice 'with bits' with full fat natural yoghurt. ANOVA tests with Tukey-HSD test was used to determine significant differences, * significantly different from OJ + Y, $P < 0.05$.

4.3.2.2 Degradation of hesperidin and narirutin during in *vitro* fermentation

4.3.2.2.1 Hesperidin and narirutin, and their aglycones (hesperetin and naringenin) in the incubation with orange juices in the presence of full fat natural yoghurt

Hesperidin and narirutin were similar between OJ and OJ bits in the presence of full fat natural yoghurt during fermentation by gut bacteria (Table 4-7). There was 44 ± 11 % of hesperidin degraded by colonic bacteria in OJ, while 39 ± 15% was transformed from OJ bits in the presence of full fat natural yoghurt, but there was no significantly different degradation of hesperidin between smooth orange juice and orange juice with bits in the presence of the yoghurt. Narirutin was degraded by 67.6 ± 4.9 % and 46.5 ± 15.4 % after 24 h fermentation of OJ +Y and OJ bits +Y.

Hesperetin and naringenin, aglycones of hesperidin and narirutin, were produced in similar amounts between OJ and OJ bits in the presence of full fat natural yoghurt (Table 4-8). There was 22.5 % of flavanone including hesperidin, narirutin, hesperetin and naringenin reduced by 27.8 ± 13.6 % in OJ bits + Y, and 34.4 ± 9.6 % in OJ + Y after 24 h fermentation (Table 4-9).

Table 4-8 Concentration of hesperetin and naringenin (μM) produced from orange juices during colonic fermentation by human gut bacteria in the presence of full fat natural yoghurt

	0h		2h		4h		6h		24h	
	Hesperetin	Naringenin	Hesperetin	Naringenin	Hesperetin	Naringenin	Hesperetin	Naringenin	Hesperetin	Naringenin
OJ + Y	0.2 ± 0.2	0.4 ± 0.2	1.1 ± 0.4	1.7 ± 0.6	2.3 ± 1.1	2.3 ± 0.9	1.1 ± 0.4	1.5 ± 0.4	3.8 ± 1.4	2.7 ± 0.6
OJ bits + Y	0.2 ± 0.2	0.4 ± 0.3	0.6 ± 0.3	1.0 ± 0.3	2.1 ± 0.8	2.3 ± 0.8	1.9 ± 0.5	1.6 ± 0.7	3.1 ± 1.0	1.8 ± 0.7

Data expressed as mean values ± SE (n=6), OJ+Y: smooth orange juice with full fat natural yoghurt, OJ bits + Y: orange juice 'with bits' with full fat natural yoghurt, ANOVA tests with Tukey-HSD test was used to determine significant differences, * significantly different from OJ + Y, $P < 0.05$.

Table 4-9 Concentration of total flavanone (μM) from orange juice during fermentation by human colonic bacteria in the presence of full fat natural yoghurt

	0 h	2 h	4 h	6 h	24 h
OJ + Y	83.4 ± 12.0	93.3 ± 7.4	70.7 ± 9.3	59.3 ± 13.3	55.2 ± 14.4
Recovery (%)	--	111.9	84.8	71.1	66.2
OJ bits +Y	76.4 ± 12.8	77.1 ± 16.4	84.8 ± 13.1	75.6 ± 17.5	59.2 ± 16.6
Recovery (%)	--	100.9	111	99	77.5

Data expressed as mean values ± SE (n=6), Total flavanone includes hesperidin, narirutin, hesperetin and naringenin, OJ+Y: smooth orange juice with full fat natural yoghurt, OJ bits + Y: orange juice 'with bits' with full fat natural yoghurt. , ANOVA tests with Tukey-HSD test was used to determine significant differences, * significantly different from OJ + Y, $P < 0.05$

4.3.2.2.2 Production of phenolic acids in the incubation with orange juices in the presence of full fat natural yoghurt

Phenolic acids were produced through the activity of gut bacteria over 24 h of fermentation (Figure 4-2). After 24 h fermentation, five phenolic acids increased to 107.4 ± 37.1 µM and 110.8 ± 36.6 µM respectively in the incubation with OJ + Y and OJ bits + Y.

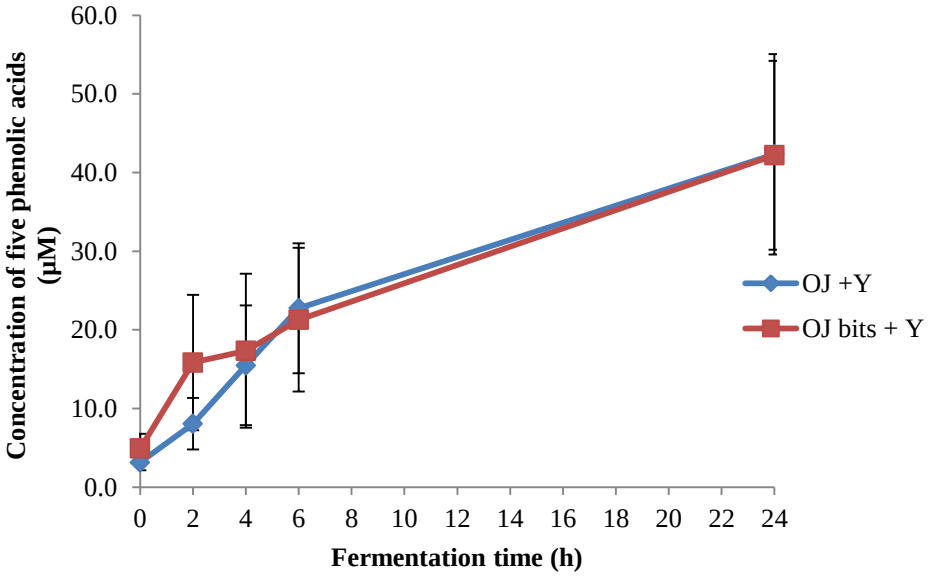


Figure 4-2 Production of five phenolic acids (µM) by human gut bacteria in incubation of smooth orange juice and orange juice 'with bits' in the presence of full fat natural yoghurt
Values expressed as mean values ± SE (n=6), OJ+Y: smooth orange juice with full fat natural yoghurt, OJ bits + Y: orange juice 'with bits' with full fat natural yoghurt. ANOVA tests with Tukey-HSD test was used to determine significant differences

As shown in Figure 4-3, 3-phenylpropionic acid was main phenolic acid at the beginning of the fermentation of OJ and OJ bits (6.1 ± 2.9 µM and 7.7 ± 3.0 µM), followed by 3-(3, 4-dihydroxyphenyl) propionic acid (2.4 ± 1.9 µM and 3.4 ± 2.9 µM) and 3-phenylacetic acid (2.5 ± 0.5 µM and 3.1 ± 0.6 µM) at 0 h of fermentation. At first 2 h, 3-phenylpropionic acid increased to 12.3 ± 4.7 µM in fermentation of OJ bits +Y ($p < 0.05$). 3-phenylacetic acid increased to 9.1 ± 2.3 µM in OJ + Y ($p < 0.03$). From 2 h to 4 h of fermentation, 3-(phenyl) acetic acid was produced to 21.9 ± 11.8 µM and 21.5 ± 10.2 µM respectively in the incubation of OJ + Y and OJ bits + Y. 4-hydrophenylpropionic acid significantly increased to 8.9 ± 2.1 µM ($p < 0.02$) and 5.9 ± 2.9 µM ($p < 0.02$) in incubation with OJ+Y and OJ bits + Y at 6 h of fermentation. 3, 4-dihydroxyphenyl) propionic acid was higher in incubation of OJ + Y compared with OJ bits +Y (7.4 ± 1.9 µM vs. 4.6 ± 1.2 µM, $P < 0.02$). Dihydroferulic

acid was significantly increased from 3.8 ± 2.8 µM to 6.5 ± 2.9 µM during the period of 4 h to 6 h. Among the phenolic acids, 3-phenylpropionic acid and 3-phenylacetic acid had higher amount (11.1 ± 4.0 µM & 12.3 ± 4.1 µM; 21.9 ± 9.9 µM & 19.1 ± 7.9 µM) separately in the fermentation with OJ+Y and OJ bits + Y.

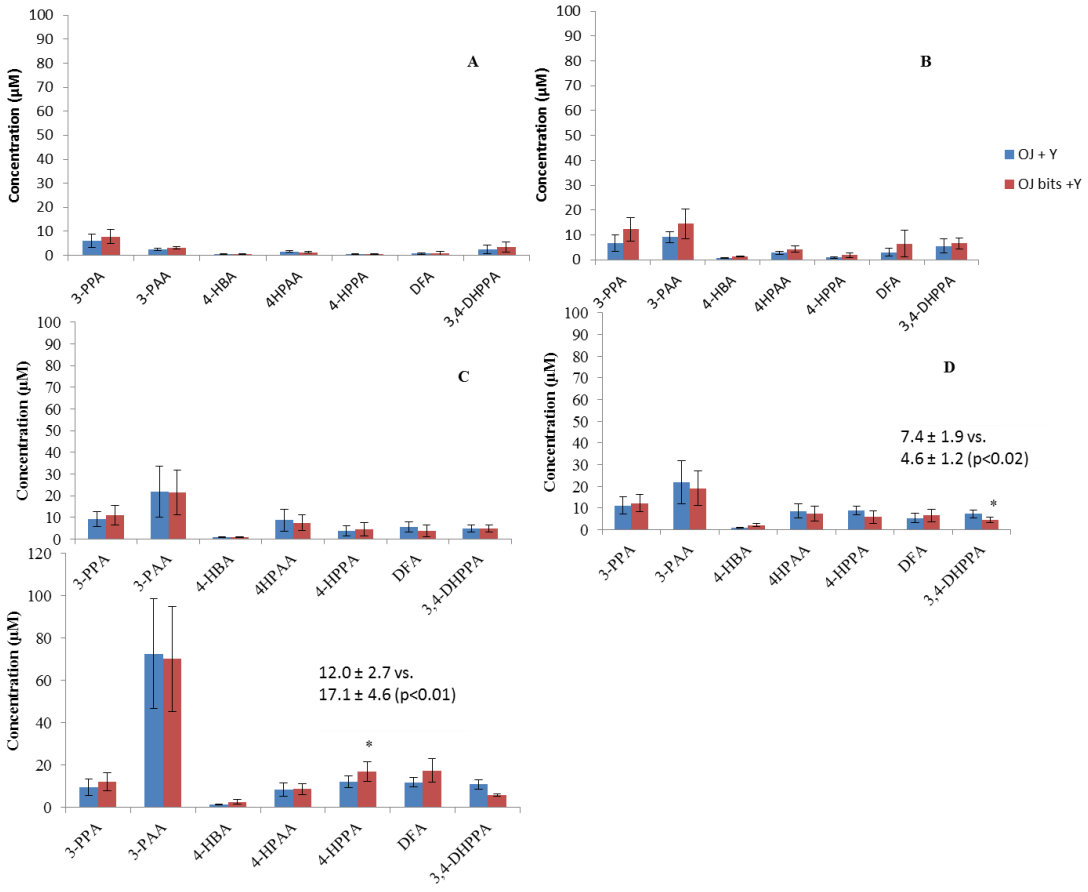


Figure 4-3 Production of phenolic acids by gut bacteria at 0 h (A), 2 h (B), 4 h(C), 6 h(D) and 24 h(E) of fermentation of smooth orange juice and orange juice 'with bits' in the presence of full fat natural yoghurt

Expressed as mean value in µM ± SE (n=6), quantified using the available standard. 3-PPA: 3-(phenyl)propionic acid, 3-PAA: 3-(phenyl)acetic acid, 4-HBA: 4-hydroxybenzoic acid,4-HPAA: 4-hydroxyphenylacetic acid, 4-HPPA: 4-hydroxyphenylpropionic acid, 3-HPPA: 3-3'-hydroxyphenylpropionic acid, 4 - HPPA: 3-(4'-hydrophenyl)propionic acid, DFA: Dihydroferulic acid, 3,4-DHPPA: 3,4-dihydroxyphenylpropionic acid, OJ+Y: smooth orange juice with full fat natural yoghurt, OJ bits + Y: orange juice 'with bits' with full fat natural yoghurt. ANOVA tests with Tukey-HSD test was used to determine significant differences, * 3, 4-DHPPA Significantly different from 6 h, $P < 0.05$.

4.3.2.3 Total phenolic contents and antioxidant capacity in orange juices in the presence of full fat natural yoghurt during *in vitro* GI digestion and fermentation

Total phenolic contents was analysed by Folin-Ciocalteau method (Figure 4-4). Total phenolic content of OJ + Y kept stable during pepsin/HCl digestion about 484.7 ± 16.0 mg GAE / 250ml, and then decreased to 339.4 ± 1.5 mg GAE / 250 ml after pancreatin/ bile acids digestion. The total phenols for the OJ bits + Y was stable during the gastrointestinal digestion from 367.9 ± 83.4 mg GAE / 250ml to 352.4 ± 12.0 mg GAE /250 ml. Total phenol was higher in OJ + Y digesta than OJ bits + Y digesta after the pepsin / HCl digestion.

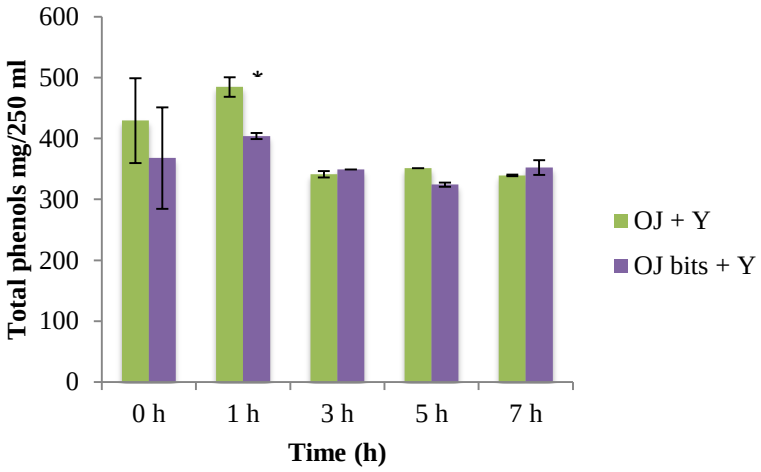


Figure 4-4 Total phenols of orange juice (mg/250ml GAE) during *in vitro* gastrointestinal digestion in the presence of full fat natural yoghurt

Data expressed as mean values ± SE (n=3), total phenol was based on 40 ml of orange juice, OJ+Y: smooth orange juice with full fat natural yoghurt, OJ bits + Y: orange juice 'with bits' with full fat natural yoghurt, gastric digestion for 1 h, small intestinal digestion for 6 h from 1 h to 7 h, ANOVA tests with Tukey-HSD test was used to determine significant differences, * Significantly different from OJ + Y, $P < 0.05$.

Antioxidant capacity were respectively analyzed by FRAP assay (Figure 4-5). There was no significant difference in terms of antioxidant capacity, which was 0.6 mM Fe^{2+} during the upper GI digestion with OJ + Y or OJ bits + Y.

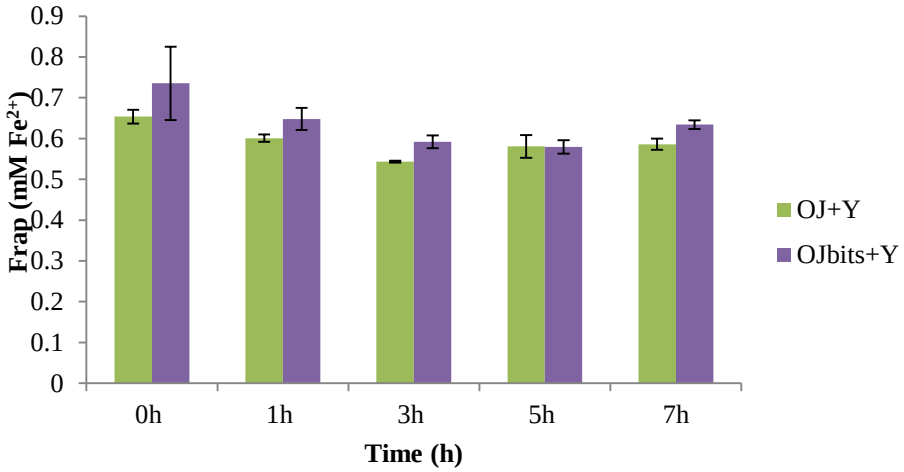


Figure 4-5 Antioxidant capacity of orange juice (mM Fe^{2+}) during in *vitro* gastrointestinal digestion in the presence of full fat natural yoghurt

Data expressed as mean values ± SE (n=3). OJ+Y: smooth orange juice with full fat natural yoghurt, OJ bits + Y: orange juice 'with bits' with full fat natural yoghurt, Gastric digestion for 1 h, small intestinal digestion for 6 h from 1 h to 7 h. ANOVA tests with Tukey-HSD test was used to determine significant differences, * Significantly different from OJ + Y, $P < 0.05$.

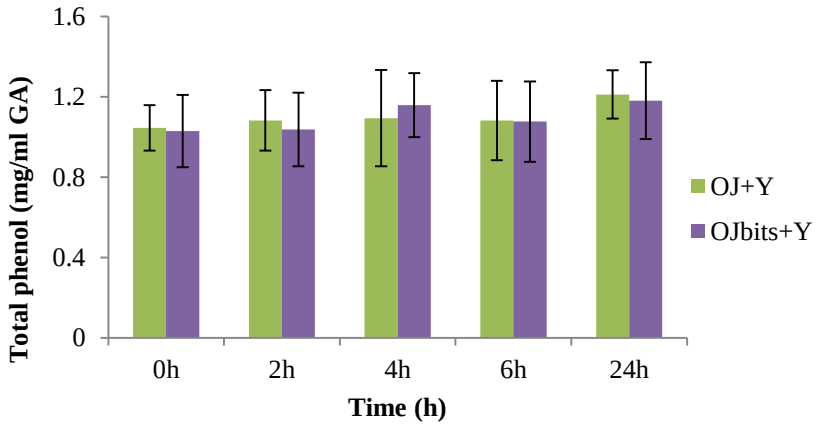


Figure 4-6 Total phenols in the incubation with orange juices by gut bacteria in the presence of full fat natural yoghurt

Data expressed as mean values ± SE (n=6). OJ+Y: smooth orange juice with full fat natural yoghurt, OJ bits + Y: orange juice 'with bits' with full fat natural yoghurt, ANOVA tests with Tukey-HSD test was used to determine significant differences, * significantly different from OJ + Y, $P < 0.05$.

Total phenolic content of faecal water is shown in Figure 4-6. Total phenolic content remained stable during fermentation with OJ + Y and OJ bits +Y from 1.1 ± 0.1 mg/ml GAE to 1.2 ± 0.1 GAE mg/ml and 1.0 ± 0.2 to 1.2 ± 0.2 GAE mg/ml. Compared with fermentation of OJ and OJ bits, total phenolic contents were similar between in the incubation of OJ +Y and OJ bits + Y (1.2 ± 0.2 vs. 1.2 ± 0.2 GAE mg/ml).

In terms of antioxidant capacity, faecal samples were 0.8 m MFe^{2+} during fermentation of OJ + Y and OJ bits + Y with gut bacteria over 24 h (Figure 4-7).

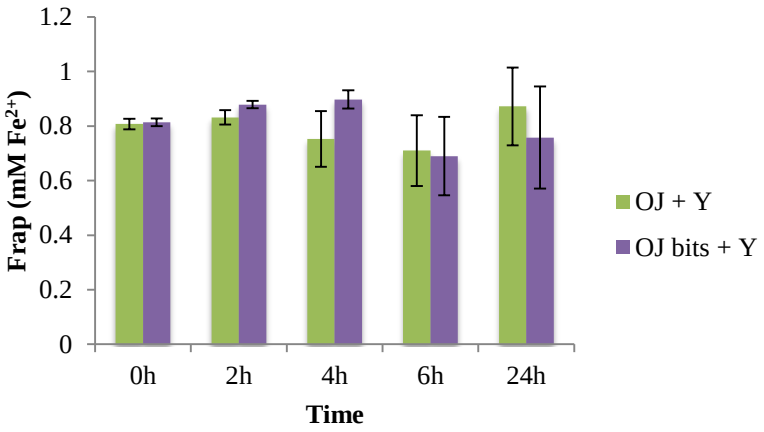


Figure 4-7 Antioxidant capacity in the incubation with orange juices by gut bacteria in the presence of full fat natural yoghurt

Data expressed as mean values ± SE (n=6). OJ+Y: smooth orange juice with full fat natural yoghurt, OJ bits + Y: orange juice 'with bits' with full fat natural yoghurt, ANOVA tests with Tukey-HSD test was used to determine significant differences, * significantly different from OJ + Y, $P < 0.05$.

4.3.2.4 pH in incubation with orange juices in the presence of full fat natural yoghurt during *in vitro* fermentation

pH was significantly lower in the incubation of orange juices in the presence of full fat natural yogurt (4.2 ~ 4.5) compared with control (7.3) after 24 h colonic fermentation ($p < 0.05$), but there was no difference between OJ / OJ +Y, OJ bits / OJ bits + Y, OJ + Y / OJ bits + Y Compared with fermentation with OJ or OJ bits, full fat natural yoghurt did not affect the pH (Figure 4-8).

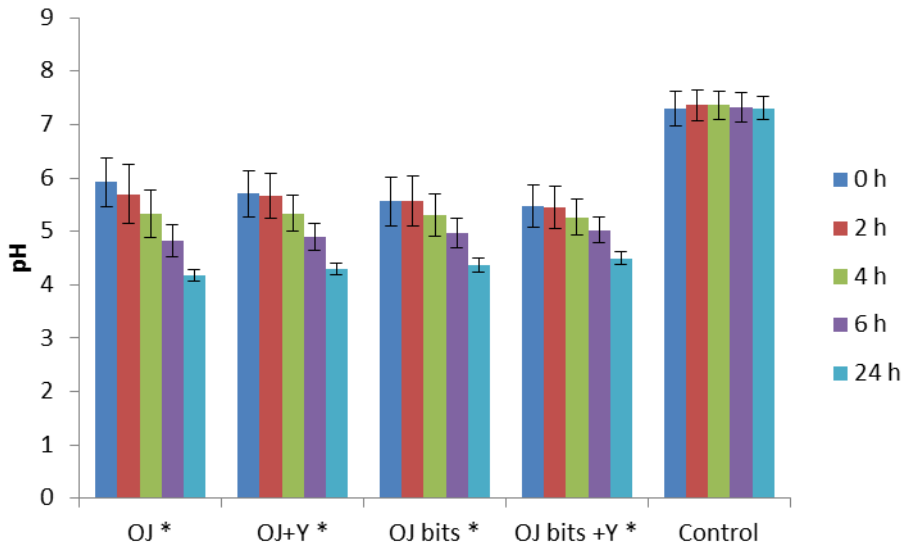


Figure 4-8 pH during fermentation with colonic bacteria

OJ: smooth orange juice, OJ bits: orange juice 'with bits', OJ+Y: smooth orange juice adding yoghurt, OJ bits + Y: orange juice 'with bits' adding yoghurt, ANOVA tests with Tukey-HSD test was used to determine significant differences,* significantly different from control, $p < 0.05$.

4.3.3 Probiotics containing yoghurt and strawberry purée (5 %) and probiotics containing yoghurt on flavanone and phenolic acids in orange juices during the upper GI digestion

4.3.3.1 The effect of yoghurt containing probiotics (PY) on hesperidin and narirutin during the upper GI digestion

As shown in Table 4-11, hesperidin was significantly different in OJ + PY than OJ during the upper GI digestion ($p < 0.05$). Hesperidin and narirutin were similar between OJ + PY and OJ before the upper GI digestion (4.9 ± 0.1 mM vs. 4.5 ± 0.2 mM, 10.0 ± 0.6 mM vs. 10.4 ± 0.1 mM) (Table 4-10). After gastric digestion, hesperidin was lower in OJ + PY than OJ (8.2 ± 0.3 mM vs. 10.2 ± 0.2 mM, $p < 0.05$) after the gastric digestion, and was lower in OJ + FY (9.3 ± 1.7 mM vs. 11.1 ± 0.4 mM, $p < 0.05$) after the upper GI digestion.

Table 4-10 Effect of probiotic yoghurt (PY), yoghurt containing fruits (FY) on flavanones in smooth orange juice during simulating upper GI digestion

	0 h		After gastric digestion		After small intestinal digestion	
	Narirutin	Hesperidin	Narirutin	Hesperidin	Narirutin	Hesperidin
OJ + PY $^{\&}$	4.9 ± 0.1	10.0 ± 0.6	3.6 ± 0.1	8.2 ± 0.3 *	3.5 ± 0.3	14.7 ± 0.8
OJ + FY $^{*\&€}$	3.2 ± 0.3 $^{*\$}$	9.2 ± 1.0	2.6 ± 0.5	8.4 ± 1.4	2.7 ± 0.3	9.3 ± 1.7 *
OJ	4.5 ± 0.2	10.4 ± 0.1	3.6 ± 0.2	10.2 ± 0.2	2.8 ± 0.3	11.1 ± 0.4
OJ + Y $^{*\&}$	4.4 ± 0.2	9.3 ± 0.2	3.0 ± 0.2	8.1 ± 0.3	3.4 ± 0.4	12.4 ± 1.7

Values are means ± SE (mM) (n = 3). OJ+Y: smooth orange juice with full fat natural yoghurt, OJ + Y: smooth orange juice with full fat natural yoghurt, OJ + PY: smooth orange juice with probiotic yoghurt, OJ + FY: smooth orange juice with strawberry yoghurt, OJ: smooth orange juice. ANOVA tests with Tukey-HSD test was used to determine significant differences,* significantly different amount of narirutin from OJ, $p < 0.05$, $^{\&}$ significantly different amount of hesperidin from OJ, $p < 0.05$, $^{€}$ significantly different amount of narirutin from OJ + Y, ^{ƒ} significantly different amount of hesperidin from OJ+Y, $^{£}$ significantly different from OJ + Y, $p < 0.05$, $^{\$}$ significantly different from OJ + PY, $p < 0.05$.

Narirutin was higher in OJ bits + PY than OJ bits (3.5 ± 0.1 mM vs. 2.3 ± 0.1 mM, $p < 0.05$), after the upper GI digestion (Table 4-11).

Table 4-11 Effect of full fat natural yoghurt (Y), probiotic containing yoghurt (PY), yoghurt containing fruits (FY) on flavanone in orange juice 'with bits' during gastrointestinal digestion

	0 h		After gastric digestion		After small intestinal digestion	
	Narirutin	Hesperidin	Narirutin	Hesperidin	Narirutin	Hesperidin
OJ bits + PY *	3.9 ± 0.2	10.1 ± 0.8	3.5 ± 0.3	10.7 ± 0.4 $^{£}$	2.4 ± 0.1	13.7 ± 1.6
OJ bits + FY *	3.5 ± 0.2	10.2 ± 0.1	3.3 ± 0.1 *	10.2 ± 0.1 $^{£}$	3.5 ± 0.1 $^{*£}$	14.7 ± 0.5
OJ bits	4.3 ± 0.2	9.6 ± 0.4	2.0 ± 0.1	7.7 ± 0.5	2.3 ± 0.1	11.1 ± 0.2
OJ bits + Y *	3.7 ± 0.3	9.4 ± 0.5	2.9 ± 0.2	8.8 ± 0.3	2.9 ± 0.2	14.3 ± 0.8

Values are means ± SE (mM) (n = 3).OJ bits + Y: orange juice 'with bits' with full fat natural yoghurt; OJ bits + Y: orange juice 'with bits' with probiotic yoghurt; OJ bits: orange juice 'with bits', OJ bits + FY: orange juice 'with bits' with strawberry, OJ bits + Y: orange juice 'with bits' with full fat natural yoghurt; ANOVA tests with Tukey-HSD test was used to determine significant differences, ,* significantly different amount of narirutin from OJ bits, $p < 0.05$, $^{£}$ Significantly different from OJ bits + Y, $p < 0.05$, $^{\$}$ significantly different from OJ bits + PY, $p < 0.05$.

4.3.3.2 The effect of strawberry purée (5 %) and probiotic containing yoghurt (FY) on hesperidin and narirutin during the upper GI digestion

Narirutin was lower in OJ + FY than OJ (3.2 ± 0.3 mM vs. 4.5 ± 0.2 mM, $p < 0.05$), while hesperidin was similar between OJ + FY and OJ before the upper GI digestion (Table 4- 11). Hesperidin and narirutin was similar between OJ bits + FY and OJ bits (Table 4-12). Narirutin was higher in OJ bits + FY than OJ bits after the gastric digestion (3.3 ± 0.1 mM vs. 2.0 ± 0.1, $p < 0.05$) (Table 4-12). There were similar amount of narirutin and hesperidin at 2.6 ± 0.5 mM and 8.4 ± 1.4 mM in OJ + FY, and 3.6 ± 0.2 mM and 10.2 ± 0.2 mM after the gastric digestion. Hesperidin was significantly lower in OJ + FY compared with OJ after small intestinal digestion (9.3 ± 1.7 mM vs. 11.1 ± 0.4 mM, $P < 0.05$), while narirutin was similar. However, narirutin was higher in OJ bits + FY than OJ bits (3.5 ± 0.1 mM vs. 2.3 ± 0.1 mM, $p < 0.05$), while hesperidin was similar between OJ bits + FY and OJ bits.

Furthermore, PY and FY did not affect hesperidin and narirutin during the upper GI digestion compared with smooth orange juice with full fat natural yogurt (Table 4-10). Hesperidin was higher in OJ bits + PY (10.7 ± 0.4 mM) and OJ bits + FY (10.2 ± 0.1 mM) compared with OJ bits + Y (8.2 ± 0.3 mM) ($P < 0.05$) after the gastric digestion, even though it was similar in OJ bits + PY, OJ bits + FY and OJ bits + Y after the small intestinal digestion (Table 4-12). After the small intestinal digestion, narirutin was higher in OJ bits + FY compared with OJ bits + Y (3.5 ± 0.1 mM vs. 2.9 ± 0.2 mM, $P < 0.05$), but was similar between OJ bits + PY (2.4 ± 0.1mM) and OJ bits + Y (2.9 ± 0.2 mM) (Table 4 -12).

4.3.3.3 Phenolic acids

There were eight phenolic acids were detected in digesta during the upper GI digestion. They were 3-hydroxybenzoic acid, vanillic acid, 3,4-dihydroxyphenylacetic acid, 3-hydroxy-4-methoxyphenyl propionic acid, 3-methoxy-4-hydroxyphenyl propionic acid, 3,4-dihydroxyphenylpropionic acid, p-coumaric acid and ferulic acid. 3, 4-dihydroxyphenylacetic acid, 3-hydroxy-4-methoxyphenyl propionic acid, 3-methoxy-4-hydroxyphenyl propionic acid, 3, 4-dihydroxyphenylpropionic acid were assumed to be derived from orange juice flavanone. The others were already present in the OJ. Four phenolic acids was higher at 6.3 ± 0.7 mM in OJ + FY compared with OJ + PY, at 6.2 ± 3.2 mM in OJ bits + Y, compared with OJ bits + PY and OJ bits + FY after the upper GI digestion ($p < 0.05$) (Table 4-12).

Table 4-12 Effect of Y, PY and FY on sum of phenolic acids (mM) in smooth orange juice and orange juice 'with bits' during the upper GI digestion

	OJ		OJ bits	
	Before	After	Before	After
Y	0.0 ± 0.0	3.4 ± 0.7 ↑	0.3 ± 0.2	6.2 ± 3.2 ↑
PY	0.0 ± 0.0	2.2 ± 1.2* ↑	0.2 ± 0.2	2.3 ± 0.8$^{\&}$ ↑
FY	0.5 ± 0.0	6.3 ± 0.7↑	0.2 ± 0.1	1.8 ± 1.4$^{\& \$}$ ↑

Values are means ± SE (n = 3).The sum of phenolic acids was based on 8 phenolic acids, Y:full fat natural yoghurt, PY: probiotic containing yoghurt, FY: strawberry purée (5 %) and probiotic containing yoghurt, OJ: smooth orange juice, OJ bits: orange juice 'with bits', nd, not detected, ANOVA tests with LSD test was used to determine significant differences, * significantly different from OJ + FY, $p < 0.05$; $^{\&}$ significantly different from OJ bits + Y, $p < 0.05$, ↑ increase after the upper GI digestion.

4.3.3.3.1 The effect of full fat natural yoghurt on phenolic acids

Phenolic acids were significantly higher in OJ bits + Y compared with OJ bits during the upper GI digestion (0.0 ± 0.0 mM vs. 0.5 ± 0.0 mM at 1 h, 1.0 ± 0.2 mM vs. 6.8 ± 1.3 mM at 2 h and 2.8 ± 0.5 mM vs. 6.1 ± 2.0 mM, $P < 0.05$) (Table 4-13). There were similar amount of phenolic acids between OJ and OJ + Y. Phenolic acids were significantly higher during small intestinal digestion compared with gastric digestion. Moreover, there were more phenolic acids from the digestion of OJ bits with full fat natural yoghurt.

Table 4-13 Effect of full fat natural yoghurt (Y) on phenolic acids (mM) in smooth orange juice and orange juice 'with bits' during the upper GI digestion

	0 h	1 h	2 h	4 h
OJ	nd	nd	4.2 ± 2.7 $^{\#£}$	1.8 ± 0.4 $^{\#£}$
OJ + Y	nd	nd	2.9 ± 0.8 $^{\#£}$	2.8 ± 0.5 $^{\#£}$
OJ bits	nd	nd	1.0 ± 0.2 $^{\#£}$	0.6 ± 0.0 $^{\#£}$
OJ bits + Y *	0.2 ± 0.2	0.5 ± 0.0	6.8 ± 1.3 $^{\#£}$	6.1 ± 2.0$^{* \#£}$

Values are means ± SE (n = 3). sum of phenolic acids was based on 8 phenolic acids, OJ+Y: smooth orange juice adding full fat natural yoghurt, OJ bits + Y: orange juice 'with bits' adding full fat natural yoghurt; OJ + Y: smooth orange juice adding full fat natural yoghurt, OJ bits + Y: orange juice 'with bits' adding full fat natural yoghurt; OJ : smooth orange juice, OJ bits + W: orange juice 'with bits', nd, not detected, univariate Analysis of Variance test with post hoc LSD was used to determine significant differences, $^{\#}$ different from at 0h, $P < 0.05$; $^{£}$ different from sum of phenolic acids at 1 h, $P < 0.05$; $^{\wedge}$ different from sum of phenolic acids at 2 h, $P < 0.05$, $^{\&}$ significantly different from at sum of phenolic acids 2 h, $P < 0.05$, * significantly different from OJbits, $p < 0.05$.

4.3.3.3.2 The effect of probiotic containing yoghurt on phenolic acid production

There were similar amount of phenolic acids during the digestion with orange juices with yoghurt containing probiotics (Table 4-14). Phenolic acids were increased after small intestinal digestion.

Table 4-14 Effect of probiotics containing yoghurt (PY) on phenolic acids (mM) in smooth orange juice and orange juice 'with bits' during the upper GI digestion

	0 h	1 h	2 h	4 h
OJ	nd	nd	4.2 ± 2.7 $^{\#£}$	1.8 ± 0.4 $^{\#£}$
OJ + PY	nd	nd	4.2 ± 1.6 $^{\#£}$	1.8 ± 0.9 $^{\#£}$
OJ bits	nd	nd	1.0 ± 0.2 $^{\#£}$	0.6 ± 0.0 $^{\#£}$
OJ bits + PY	0.2 ± 0.1	0.4 ± 0.1	5.0 ± 1.0 $^{\#£}$	2.2 ± 0.6 $^{\#£\wedge}$

Values are means ± SE (mM) (n = 3). OJ + PY: smooth orange juice with probiotic yoghurt, OJ bits + PY: orange juice 'with bits' with probiotic yoghurt; OJ: smooth orange juice, OJ bits: orange juice 'with bits', , univariate Analysis of variance test with LSD was used to determine significant differences, $^{\#}$ significantly different from sum of phenolic acids at 0 h, P < 0.05; $^{£}$ significantly different from at sum of phenolic acids 1 h, P < 0.05; $^{\wedge}$ significantly different from at sum of phenolic acids 2 h, P < 0.05. * Significantly different from OJbits, p < 0.05.

Table 4-15 Effect of strawberry purée (5 %) and probiotic containing yoghurt (FY) on sum of phenolic acids (mM) in smooth orange juice and orange juice 'with bits' during the upper GI digestion

	0 h	1 h	2 h	4 h
OJ	0.0 ± 0.0	0.0 ± 0.0	4.2 ± 2.7 $^{\#£}$	1.8 ± 0.4 $^{\#£}$
OJ + FY	0.4 ± 0.0 $^{\eta\beta}$	1.1 ± 0.6 $^{\eta\beta}$	5.2 ± 1.3 $^{\#£\beta}$	7.0 ± 0.6 $^{\alpha\#£\beta}$
OJ bits	0.0 ± 0.0	0.0 ± 0.0	1.0 ± 0.2 $^{\#£}$	0.6 ± 0.0 $^{\#£}$
OJ bits + FY	0.2 ± 0.1	0.3 ± 0.1	4.3 ± 1.4 $^{\#£}$	1.9 ± 0.3 $^{\#£}$
FY	0.1 ± 0.1	0.2 ± 0.1	5.1 ± 3.1 $^{\#£}$	1.6 ± 0.7 $^{\#£}$

Values are means ± SE (mM) (n = 3), OJ + FY: smooth orange juice with strawberry yoghurt, OJ bits + FY: orange juice 'with bits' with strawberry yoghurt; OJ: smooth orange juice, OJ bits: orange juice 'with bits', nd, not detected, univariate Analysis of Variance test with post host hoc LSD was used to determine significant differences, * significantly different from OJ + FY, P < 0.05; $^{\#}$ significantly different from sum of phenolic acids at 0 h, P < 0.05; $^{£}$ different from sum of phenolic acids at 1 h, P < 0.05; ^ different from sum of phenolic acids at 2 h, P < 0.05. $^{\eta}$ significantly different from OJ, p < 0.05, $^{\beta}$ significantly different from FY, p < 0.05, $^{\alpha}$ significantly different from OJ bits + FY, p < 0.05.

4.3.3.3.3 The effect of strawberry purée (5 %) and probiotic containing yoghurt on phenolic acids

Phenolic acids were significantly higher in OJ + FY than OJ or FY after the upper GI digestion (Table 4-15).

4.4 Discussion

It had previously been shown that yoghurt fed to humans with orange juice had limited effects on their metabolism and bioavailability in the upper GI tract but decreased phenolic acid excretion in urine substantially. In this chapter the mechanisms for this effect were explored *in vitro* by investigating the effect of full fat yogurt on the metabolism of the flavanones from orange juice (and orange juice with bits) on their own and in the presence of full fat yoghurt, probiotic containing yoghurt and fruit and probiotic contained yoghurt in models of the upper and lower GI tract, Full fat natural yoghurt was made by using yoghurt starter culture bacteria (*Streptococcus thermophilus* and *Lactococcus bulgaricus*). In this study, there no bacteria survived after simulating upper GI digestion. Hesperidin was increased by 1.5 fold in OJ and 1.8 fold in OJ bits after the gastric digestion and by 1.4 fold in OJ bits after the small intestinal digestion in the presence of full fat natural yoghurt. In addition, full fat natural yoghurt increased four phenolic acids from 0.6 ± 0.0 mM to 6.1 ± 2.0 Mm during the upper GI digestion with OJ bits. However, the yoghurt did not affect individual phenolic acid during the fermentation of smooth orange juice and orange juice with bits. During 24 h fermentation, full fat natural yoghurt did not affect hesperetin and naringenin in the incubation batches, as well as production of phenolic acids from OJ and OJ bits. Furthermore, full fat natural yoghurt increased total phenolic contents from 104.1 ± 13.5 mg GAE/250 ml to 339.4 ± 1.5 mg GAE/250 ml in OJ and from 112.3 ± 0.6 mg GAE to 352.4 ± 12.0 mg GAE in OJ bits after the upper GI digestion ($p < 0.05$). Full fat natural yoghurt did not affect the antioxidant capacity in smooth orange juice and orange juice ‘with bits’ during the gastrointestinal digestion and did not change antioxidant capacity in faecal samples during *in vitro* fermentation. Thus this does not explain the results of the human study, where phenolic acids in urine were substantially decreased (Roowi et al., 2009b).

On the other hand, stability / release of hesperidin and narirutin and production of hesperetin and naringenin were similar in smooth orange juice and orange juice ‘with bits’ during the GI

digestion and fermentation. There was 44 ± 11 % and 39 ± 15 % of hesperidin degradation by colonic bacteria respectively in OJ and OJ bits in the presence of full fat natural yogurt after 24 h fermentation. Five phenolic acids were 107.4 ± 37.1 μM and 110.8 ± 36.6 μM respectively in the incubation with OJ + Y and OJ bits + Y.

Hesperidin was higher in OJ + Y and OJ bits + Y compared with OJ bits after the small intestinal digestion. The results was consistent with a previous study (Rodríguez-Roque et al., 2014) that investigated increased release of many phenolic substances around 5 % and 93 % during *in vitro* gastrointestinal digestion after adding milk to a blended fruit juice (orange, kiwi, pineapple and mango). As we know, polyphenols have hydrophobic activity that are able to bind to carbohydrates and protein (Siebert et al., 1996, Seshadri and Dhanaraj, 1988). In addition, interactions between milk protein and polyphenols are responsible for polyphenol release during small intestinal digestion (Moser et al., 2014, Neilson et al., 2007). On the other hand, stability of orange juice flavanones partially depends on precipitation of transformed chalcones into the cloud under physiological conditions of the intestine(Gil-Izquierdo et al., 2003). The reaction between flavanone and yoghurt might inhibit formation of chalcone to increase flavanone recovery in the upper GI tract. Thus, yoghurt may have interactions with orange juice to promote hesperidin release during upper GI digestion.

Hesperidin was lower in OJ + Y and OJ bits + Y compared with OJ and OJ bits respectively at 6 h and 0 h during the fermentation with gut bacteria. Polysaccharide was reported to promote formation of complexes with whey proteins and caseins (Tromp et al., 2004). Dietary fibre was low and similar amounts between smooth orange juice and orange juice 'with bits'. However, insoluble fibre and soluble fibre in orange pulp varied depending on extrusion processing (Larrea et al., 2005). The "bits" in the orange juice are likely to be responsible for actions of full fat natural yoghurt on flavanone stability /release in the GI tract. The results confirmed different interaction of yoghurt with orange juices during the colonic fermentation.

Yoghurt did not affect the amount of each phenolic acid during the fermentation of smooth orange juice and orange juice with bits. However, Roowi et al reported that full fat natural yogurt reduced urinary excretion of five phenolic acids including 3-hydroxyphenylacetic acid, 3-hydroxyphenylhydracrylic acid, dihydroferulic acid, 3-methoxy-4-hydroxyphenylhydracrylic acid and 3-hydroxyhippuric acid from 62 ± 18 μmol to 6.7 ± 1.8

μmol (Roowi et al., 2009a), which was inconsistent with our study(Roowi et al., 2009b). Our results did not confirm those of the *in vivo* study, which might be due to physiological functions in humans after consumption of orange juice and yoghurt, or types of yoghurt. Even though probiotic yoghurt and strawberry purée (5 %) and probiotics yoghurt had similar effects with full fat natural yoghurt on hesperidin and narirutin during the upper GI digestion in terms of hesperidin and narirutin, full fat yoghurt significantly increased four phenolic acids from 0.6 ± 0.0 mM to 6.1 ± 2.0 mM. This is not due to bacteria in the yoghurt, but may be a property of other yoghurt components which survive digestion in the upper gut. This needs to be explored further. The lack of extra effects of probiotic yoghurts apart from some release of phenolic acids in the upper gut model means that it is unlikely that the reduction in phenolic acids seen *in vivo* was due to any probiotics in the yoghurt used.

Phenolic acids were significantly produced during small intestinal digestion in smooth orange juice and orange juice 'with bits' with or without the presence of yogurts, which revealed that the alkaline condition of small intestine could stimulate production of phenolic acids. In this study, strawberry purée (5 %) containing yoghurt significantly increased four phenolic acids (3, 4-dihydroxyphenylacetic acid, 3-hydroxy-4-methoxyphenyl propionic acid, 3-methoxy-4-hydroxyphenyl propionic acid, 3, 4-dihydroxyphenylpropionic acid) in OJ during the upper GI digestion compared with full fat natural yoghurt and probiotic containing yoghurt, while it did not affect the increase of these phenolic acids in OJ bits compared with probiotic containing yoghurt. It indicated that different types of yoghurt have different effects on phenolic acids, even yoghurt with *Bifidobacterium spp* and *Lactobacillus acidophilus* that might enhance production of phenolic acids derived from flavanones during the upper GI digestion.

In conclusion, hesperidin was higher in smooth orange juice and orange juice 'with bits' in the presence of full fat natural yoghurt after the upper GI digestion. During *in vitro* fermentation with gut bacteria, lower hesperidin was found at 6 h incubation with smooth orange juice, and at 0 h fermentation with orange juice with bits. However, full fat natural yoghurt did not affect degradation of flavanone and production of phenolic acids after 24 h fermentation. In Mullen's human study, 24 h urinary excretion of flavanone was also not affected, but the 0-5 h excretion of flavanone metabolites in urine was reduced by the consumption of full fat natural yoghurt and orange juice. The temporary reduction might relate to formation of the polysaccharide-protein-flavanone complexes, which needs to be

verified in future, and could be broken down by gut bacteria to release flavanone in the colon. In terms of phenolic acids, the reduction of urinary excretion was not found in this *in vitro* study. During the upper digestion phase, strawberry containing yoghurt increased four phenolic acids, while full fat natural yoghurt increased phenolic acids in orange juice with bits, which also could not explain the reduced phenolic acids in urine. Even though starter culture did not survive in the upper GI model, the probiotic bacteria may have survived but still had no effect in the phenolic acids and metabolism of flavanones in the colon. In general, the food matrix (yoghurt) influenced flavanone and phenolic acids in the GI tract so that potentially affect absorption of aglycone and /or phenolic acids, but this needs to be confirmed *in vitro* and *in vivo* in future.

CHAPTER 5 *Effect of raftiline (non-digestible carbohydrate) and glucose on metabolism of hesperidin by gut bacteria in vitro*

5.1 Introduction

As the main site of flavanone metabolism is in the human colon and by the colonic microbiota, any component of food which influences the gut bacteria has the potential to modify the bacterial metabolism of the flavanones and the production of phenolic acids.

Dietary fibre consists of non-starch polysaccharides, resistant oligosaccharides, resistant starch and lignin associated with the dietary fibre polysaccharides which include cellulose, hemicellulose, pectin, β-glucans, gums, and lignin, classified as soluble dietary fibre and insoluble dietary fibre (Gallaher and Schneeman, 2001, Gorinstein et al., 2001, Dhingra et al., 2012).

In this chapter the impact of the non-digestible carbohydrate raftiline (fructo-oligosaccharides) and also glucose were used as models for fermentable fibres to test the effect of increased carbohydrate fermentation on flavanone metabolism in *in vitro* models of the large intestine. Raftiline is a low molecular weight component of inulin that is rapidly fermented by the colonic bacteria producing short chain fatty acids, such as propionate, acetate and butyrate and reducing colonic pH (Ramnani et al., 2012, Pascoal et al., 2013) it also selectively stimulates the growth of lactic acid bacteria (Ramchandran and Shah, 2010). Many fibres contain glucose (eg cellulose and beta glucan) and while raftiline is a selective substrate most bacteria can ferment glucose so comparing the two gives more information about the mechanism by which fermentation may affect bacterial flavanone degradation.

There are several mechanisms by which different dietary fibres may affect the bioavailability of flavanones. Various cellular components in fruits and vegetables and other products can act as a physical barrier to the release of nutrients (bioaccessibility) during digestion in the GI tract (Jeffery et al., 2012, Saura-Calixto, 2010). Therefore, the physical state of bioactive compounds in the plant cell determines their release and subsequent absorption. For example, β-carotene was approximately 3 times higher in plasma 9.5 h after consumption of papayas (13.0 mg lycopene and 1.3 mg β-carotene) than carrots (13.0 mg lycopene & 1·6 mg β-carotene and tomatoes (13.0 mg lycopene & 2.3 mg β-carotene), and 2.6 fold plasma lycopene than after the tomatoes in healthy humans (Schweiggert et al., 2014). The maximal concentrations of quercetin in plasma were higher at 0.7 μM at 0.7 h after consumption of fried onions containing 225 ± 43 μmol quercetin than apple (apple sauce and apple peel)

providing 325 ± 7 μmol quercetin (0.3 μM at 2.5 h), and urinary excretion of quercetin was also higher (1.4 % vs. 0.4 %) of intake dose and in humans (Hollman et al., 1997).

On the other hand, non-extractable polyphenols (NEPP) are linked to cell wall constituents (polysaccharides and protein) or can bind with food matrix components by hydrophobic interactions (Arranz et al., 2010, Hasni et al., 2011, Yuksel et al., 2010). These polyphenols are not released by the usual extraction process during analysis for food polyphenols (Perez-Jimenez et al., 2013), but may be released in the gut and have physiological effects. The NEPP content ranged from 210 mg to 880 mg per 100 g in dried fruits and cereals (Arranz et al., 2010) and the true ingestion of polyphenols can be underestimated if they are not considered. NEPP which are attached by ester bonds to polysaccharides in foods can be released by the action of esterase in both the small and large intestine (Saura-Calixto, 2010). In chapters 3 and 4, we found that the amount of hesperidin increased during the small intestinal digestion phase of the model with orange juice suggesting there are some NEPP in the juice. Thus, dietary fibre probably influences flavonoids available for metabolism and absorption, however there was very little fibre in these juices so it may be small amounts of cell wall and other structural tissues that are important rather than a high fibre content.

The bulking and hydrophobic binding properties of dietary fibre may impact on the metabolism and bioavailability of flavonoids. A wheat fibre supplement reduced 24 h plasma genistein by 55 %, and total urinary genistein by 20 %, but not daidzein after intake of soy in 7 healthy women (Tew et al., 1996). In a study in rats, plasma daidzein was significantly lower after feeding cellulose-daidzein diet with added rice bran hemicellulose for 30 days compared with the same diet without dietary fibre in rats (Tamura et al., 2009). A carbohydrate-rich food containing starch (hydrolysed wheat, wheat, barley, maize and oats) and malt extract (2 g/kg of body weight) reduced C_{max} of procyanidin trimer in plasma after feeding grape seed procyanidin extracts (1 g / kg of body weight) to rats (Serra et al., 2010). Oatmeal consumption delayed maximum urinary excretion of anthocyanins after blackcurrant ingestion in rats (Walton et al., 2009).

Some dietary fibres are able to increase viscosity of intestinal contents to slow gastric emptying and small intestinal absorption (Dikeman et al., 2006). Many soluble dietary fibres are viscous (Dikeman and Fahey Jr, 2006). Rice brans, soy hulls, and wood cellulose have the lowest viscosities, whereas guar gum, psyllium, and xanthan gum had the highest viscosities,

regardless of concentration under gastric and small intestinal conditions (Dikeman et al., 2006) Psyllium (3.6 g) significantly delayed gastric emptying by 10 % and 20 % at 3 h and 6 h after a meal (40 g carbohydrates, 23 g lipids, 21 g proteins) in healthy humans (Bergmann, Chassany et al. 1992). High-methoxy pectin was more viscous than low methoxy pectin (Yoo, Fishman et al. 2006). Nishijima and colleagues found that apple highly methyoxyl esterified pectin increased 2.3 times urinary excretion of quercetin and its metabolites compared with non-methoxy pectin, while there was no difference between non-methoxy pectin and low-methoxy pectin after consumption of quercetin drink in humans (Nishijima et al., 2015) .

On the other hand, the major mechanism by which dietary fibre impacts on the metabolism and absorption of flavonoids is likely to be in the colon by influencing the metabolism or composition of the gut microbiota with resultant changes in pH, bacterial metabolic capability, gut mucosal mass (Cummings et al., 1987), and colonic motility (Squires et al., 1992). Fermentation of carbohydrates in the colon produces short chain fatty acids, these reduce pH which can be as low as 5.5 in the proximal colon when highly fermentable non digestible carbohydrates are ingested (Cummings et al., 1987, Cummings and Bingham, 1986, Hijova and Chmelarova, 2007).

The gut microflora plays an essential role in the metabolism and bioavailability of flavonoids. They deconjugated the parent compound and release the aglycone and then produce metabolites such as phenolic acids which may be responsible for the health effects of high flavonoid ingestion (Selma et al., 2009). Bacteroides, bifidobacteria, and lactobacilli have the highest activity of the β-glucosidases and sulfatases responsible for glycoside hydrolysis and subsequent aglycone cleavage (Steer et al., 2003, Turner, 2008). Dietary fibre may affect the bioavailability of flavonoids through changing colonic microbiota composition. Plasma quercetin and isorhamnetin concentrations were significantly higher in rats after 14 d feeding pectin-rutin diet (2.5 ± 1.3 and 6.7 ± 4.4 vs. 0.9 ± 0.3 and 0.6 ± 0.3) with an increased number of faecal bacteria (9.7 ± 0.4 vs. 10.8 ± 0.2 expressed as log10 of the number of viable total bacteria per gram wet weight of faeces) including Bacteroidaceae, Eubacteria, Clostridia, Lactobacilli, Enterobacteria, and Streptococci compared with a cellulose-rutin diet (Tamura et al., 2007b).

It is well established that some non-digestible carbohydrates such as inulin (which includes raftiline) increase the proportion of Bifidobacteria and Lactobacilli in the colon and when present in the diet alongside flavonoids there may be some effects on phenolic acid production due to changes in the bacterial populations and also the chemical environment in terms of pH. *Bifidobacterium longum, Bifidobacterium pseudocatenulatum* and *Bifidobacterium adolescentis* were increased after intake of inulin-type fructans prebiotics for 3 months by obese women (Salazar et al., 2015). Moreover, inulin-type fructans stimulate the growth of Bifidobacteria, Lactobacilli and other lactic acid bacteria (Campbell et al., 1997, Ito et al., 2011). Raftiline (inulin) as a fructo-oligosaccharide (soluble fibre) which is not viscous, is well established as a prebiotic that increases the growth of Bifidobacteria (Clark et al., 2012, Gibson et al., 1995).

Citrus fruits contain dietary fibre (pectin, cellulose,etc.) (Figuerola et al., 2005), with properties of water and oil holding capacity and colonic fermentability (Grigelmo-Miguel and Martín-Belloso, 1998), and the amount varies depending on the processing of the orange juice (Figuerola et al., 2005). Even dietary fibre was low in orange juices, the physiological characteristics of dietary fibre might influence metabolism of orange juice by modifing if sufficient fibre is present. In this chapter, raftiline and glucose were used to test the effects of fermentation on the metabolism of hesperidin by human gut bacteria in the *in vitro* model.

5.1.1 Hypothesis

To test the hypothesis that the presence of raftiline or glucose changes degradation of hesperidin in the large intestinal tract

Do Fermentable carbohydrates affect degradation of hesperidin into aglycone and production of phenolic acids using in vitro fermentation model with human gut bacteria?

5.2 Materials and methods

5.2.1 Chemicals

Hesperidin, formic acid, hydrochloric acid, 2, 4, 5–trimethoxycinnamic acid, anhydrous ethyl acetate, dichloromethane (DCM), N-methyl-N- (trimethylsily) trifluoracetateamide, prydine.

CaCl2.2H2O, MnCl2.4H2O, NH_4HCO_3, $NaHCO_3$, Na2HPO4, KH_2PO4, $MgSO_4.7H_2O$, cysteine hydrochloride, NaOH, $Na_2S{\cdot}9H_2O$ and resazurin solution were purchased from Sigma-Aldrich Co Ltd (Poole, UK). Raftiline was purchased from Siber Hegner Ltd (Beckenham, UK).

3-phenylpropionic acid, 3-hydroxyphenylacetic acid, 4-hydroxybenzoic acid, 4-HPAA: 4-hydroxyphenylacetic acid, 3-hydroxyphenylpropionic acid, 3, 4-dihydroxyphenyl propionic acid were purchased from Sigma-Aldrich Co Ltd (Poole, Dorset, UK) or Alfa Aesar (Karlsruhe, Germany). N, o-bis (Trimethylsilyl) trifluoroacetamide (BSTFA) + 10 % trimethylchlorosilane (TMCS), 2, 4, 5– trimethoxycinnamic acid and HPLC grade methanol were obtained from Fisher Scientific (Loughborough, UK).

5.2.2 *In vitro* fermentation

The fermentation was conducted as described in section 2.3.3. The amount of hesperidin (10 µg) with or without glucose (0.5 g) (Jaganath et al., 2009) for incubation was based on the levels of orange juice consumption by subjects in previous study (250 mL) for investigation of effect of glucose on metabolism of hesperidin (n=6).

Raftiline (1 g) was added to fermentation medium (50 ml) based on fibre consumption (6 g) in 300 ml of colonic content (Khossousi et al., 2008) for investigation of dietary fibre on metabolism of hesperidin.

5.2.2.1 Faecal sample collection from participants

For investigation in metabolism of hesperidin in the presence of dietary fibre (Raftiline), ten healthy Caucasian volunteers 19 - 33 y with BMI 22.5 ± 3.0 (Table 5-1) who participated in this study followed a 3 days low-polyphenol diet avoiding fruits, vegetables, tea, coffee, wine, food high in polyphenols (Appendix 3) before providing a faecal sample on the 4^{th} day. The faecal sample was collected in a special plastic box containing an AnaeroGen sacket (Oxioid, Hampshire, UK) to generate anaerobic conditions (done by Dr Bahareh and Areej). I analysed samples from 7 participants as the other samples were missing.

Table 5-1 Body characteristics of participants

	Gender	Age(years)	Height (cm)	Weight	BMI
1	M	22	175.5	84	19.7
2	F	19	161	47.9	27.4
3	F	21	158	63.9	18.4
4	F	21	156	54	25.6
5	F	26	174	57.2	22.2
6	F	22	160	50.5	18.9
7	F	25	161	54.3	20.9
8	M	23	183	77.8	23.2
9	M	33	175	76	24.8
10	M	23	176	72.2	23.3
Mean ± SD		23 ± 3.8	152 ± 53.7	63.8 ± 12.8	22± 2.9

Values are mean ± SD, M: male, F: female, BMI: body mass index

For investigation of metabolism of hesperidin in the presence of glucose, six different healthy Caucasian volunteers (Chapter 3) who participated in this study had a normal diet, were non-smokers, had no digestive diseases, no food allergies, and had not received antibiotics for at least 6 months before the study. The volunteers followed a low-polyphenol diet avoiding fruits, vegetables, tea, coffee, wine and wholemeal foods for 2 days (details in Appendix 3) before providing a faecal sample according to information sheet (Appendix 2). Each faecal sample was collected by subjects in a special plastic box containing an AnaeroGen sacket (Oxioid, Hampshire, UK) to generate anaerobic conditions. Faecal samples were processed when brought to the lab. The food was recorded in a dietary record (Appendix 4).

5.2.3 Extraction of hesperidin, narirutin, hesperetin, and naringenin after *in vitro* digestion and fermentation

Samples were thawed and homogenized with a Disruptor Genie (Scientific Industries, UK). Extraction was the same as orange juice described in 2.9.1.

5.2.4 Analysis of faecal samples by using HPLC-PDA

Hesperidin, narirutin, hesperetin and naringenin were analysed by using HPLC-PDA as described in section 2.10.

5.2.5 Extraction and derivatization of phenolic acids in faecal slurries

Extraction of phenolic acids was done by the methods published by Fry et al (Fry et al., 2001), as described in section 2.9.2.

5.2.6 Analysis of phenolic acids in faecal samples by using GC-MS

GC-MS analysis was illustrated in chapter 2.12. Phenolic acids including 3-hydroxyphenylacetic acid, 4-hydroxyphenylacetic acid, 4-hydroxyphenylpropionic acid, 3, 4-dihydroxyphenylacetic acid, 3- (3, 4-dihydroxyphenyl) propionic acid, 4-hydroxy-3-methoxyphenylpropionic acid (dihydrofeulic acid), 3-(3'-hydroxyphenyl) propionic acid, 3-4'-hydroxyphenyl propionic acid were identified according to mass spectra and retention times obtained from authentic standards analysed under identical conditions. When standards were not commercially available, identification was achieved through the integrated NIST mass spectral library 2008 (Scientific Instruments Services Inc., Ringoes, NJ, USA), with a confidence of 70 % or above. Calibration curves of the ratio between the target ions of the internal standard (2, 4, 5–trimethoxycinnamic acid, m/z 279) were computed, with concentrations ranging from 3- 40 mg ml^{-1} ($R^2 > 0.95$). Values for phenolic acids in the faecal slurries were expressed in µM as mean values ± S.E (n = 6 or 7).

5.2.7 Statistical analysis

Analysis was conducted in duplicate and data are presented as mean values ± SE (n=6 or 7). Where appropriate, data was subjected to statistical analysis using paired t-test or one way ANOVA of the results to determine significant differences ($P < 0.05$) with SPSS software version 22.

5.3 Results

5.3.1 Effect of raftiline on metabolism of hesperidin

5.3.1.1 Degradation of hesperidin and production of hesperetin by human colonic bacteria in the presence of raftiline

Hesperidin was hydrolysed into hesperetin by gut bacteria during fermentation (Table 5-2). The amount of hesperidin decreased from 50.9 μM to 30.2 μM by activity of gut bacteria – after 6 h of fermentation in the absence of raftiline. There was 35.5 μM of hesperidin present in the incubation with raftiline higher than that without raftiline at 6 h, even though they were not significantly different. From 6 h to 24 h fermentation, hesperidin still kept higher concentration in the fermentation with raftiline (35.2 μM) than that without raftiline (27.6 μM). What is more, hesperetin was also higher in the fermentation with raftline during 24 h of fermentation of hesperidin hanging from 0.3 μM to 0.7 μM. There was about 0.2 μM of hesperetin in the absence of hesperidin during 24 h of fermentation of hesperidin by gut bacteria. In total, 46 % of hesperidin was degraded over 24 h of fermentation by gut bacteria.

Table 5-2 Concentrations of hesperidin and hesperetin (μM) during 24 h fermentation by gut bacteria

	Treatment	0 h	2 h	4 h	6 h	24 h
Hesperetin	No Raf	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
	Raf	0.3 ± 0.2	0.7 ± 0.2	0.6 ± 0.2	0.3 ± 0.1	0.4 ± 0.2
Hesperidin	No Raf	50.9 ± 5.1	44.3 ± 5.2	39.3 ± 3.6	30.2 ± 3.6	27.6 ± 6.1
	Raf	54.2 ± 3.3	40.3 ± 6.4	40.5 ± 6.9	35.5 ± 5.6	35.2 ± 4.1

Values are means ± SEMs (n = 7). No Raf: no adding raftiline, Raf: adding raftiline, * Different from fermentation without raftiline, P <0.05 (paried t-test).

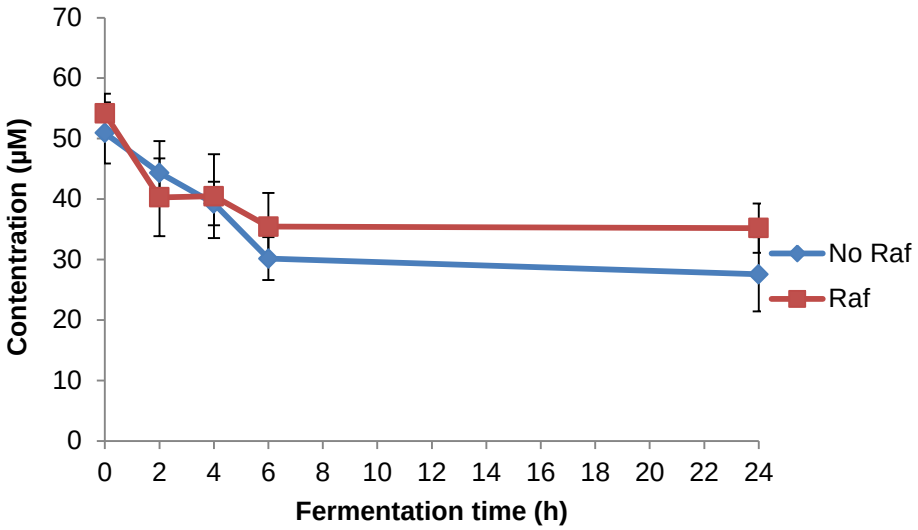


Figure 5-1 Effect of raftiline on total flavanone (hesperetin + hesperidin) (μM) during 24 h fermentation of hesperidin by gut bacteria

Values are means ± SEM (n=7), No Raf: no adding raftiline, Raf: adding raftiline, * Different from fermentation without raftiline, P <0.05 (paried t-test).

5.3.1.2 Production of phenolic acids by human colonic bacteria in the presence of raftiline

There were six phenolic acids present in the batches of fermentation through cleavage of hesperetin by gut bacteria. They are 3-phenylpropionic acid, 3-hydroxyphenylacetic acid, 4-hydroxybenzoic acid, 4-hydroxyphenylacetic acid, 3-hydroxyphenylpropionic acid, and 3, 4-dihydroxyphenyl propionic acid (Table 5-3). The sum of 6 phenolic acids was increasing during 24 h of fermentation of hesperidin by gut bacteria (Figure 5-2). The sum of 6 phenolic acids was significantly increased with or without raftiline by gut bacteria at the end of fermentation (220.3 μM, $p< 0.05$; 126.6 μM, $p < 0.05$). Raftiline significantly decreased production of phenolic acids from 220.3 μM to 126.6 μM over 24 h of fermenting hesperidin by gut bacteria ($p < 0.05$).

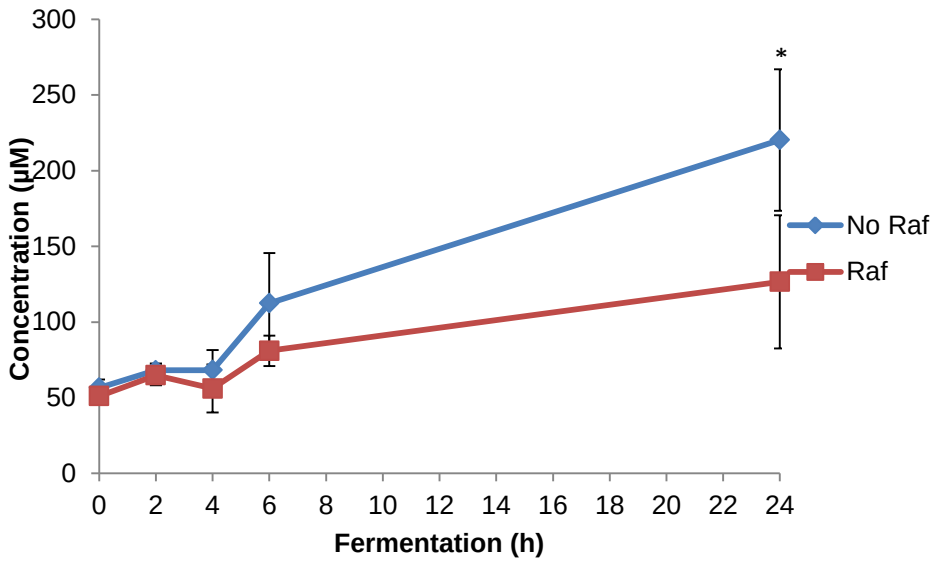


Figure 5-2 Concentration of phenolic acids over 24 h of fermentation with hesperidin or hesperidin and raftiline by gut bacteria

Values are means ± SE (µM) (n = 7), No Raf: no added raftiline; Raf: added raftiline, * Different from fermentation without raftiline, P <0.05

Among the phenolic acids, 3-phenylacetic acid, 4-hydroxyphenylacetic acid and 3-hydroxyphenylpropionic acid were main products from hesperidin degradation over 24 h in the absence of raftiline by gut bacteria (22.5 µM, 43.2 µM and 102.1 µM, $p < 0.05$). In addition, 4-hydroxybenzoic acid was significantly increased by adding raftiline (0 µM vs. 0.6 µM at 0 h, 0.2 µM vs. 1.0 µM at 2 h, 0.2 µM vs. 0.9 µM at 4 h, 0.4 µM vs. 1.3 µM at 6 h, $p <0.05$). The appearance of 4-hydroxyphenylacetic acid was also inhibited by raftiline to 20.5 µM from 43.2 µM (without raftiline) at the end of fermentation by gut bacteria ($p < 0.05$). 3-phenylpropionic acid accounted for about 37 % and 47 % of phenolic acids at 0 h of fermentation with hesperidin with or without raftiline respectively.

Table 5-3 Production of phenolic acids during 24 h fermentation of hesperidin with or without raftiline by human gut bacteria

Time	Treatment	3-PPA	3-HPAA	4-HBA	4-HPAA	3-HPPA	3,4-DHPPA	Total
0h	No Raf	21.0 ± 0.9	0.1 ± 0.1 [£]	0.6 ± 0.2 [£]	15.6 ± 3.7	16.4 ± 2.8	2.8 ± 1.8 [£]	56.6 ± 5.4
	Raf	24.2 ± 1.5	0.2 ± 0.2 [£]	nd [*£]	10.2 ± 3.7 [£]	13.4 ± 3.5 [£]	3.1 ± 2.0 [£]	51.1 ± 4.0
2h	No Raf	23.0 ± 2.0	0.4 ± 0.2 [£]	1.0 ± 0.3 [£]	18.3 ± 5.3	19.7 ± 0.7	5.7 ± 2.0 [£]	68.1 ± 4.5
	Raf	24.9 ± 1.6	4.2 ± 2.6 [£]	0.2 ± 0.1 [*£]	12.3 ± 3.5 [£]	19.0 ± 0.6	4.1 ± 2.0 [£]	64.8 ± 6.6
4h	No Raf	20.6 ± 3.7	0.7 ± 0.4 [£]	0.9 ± 0.3 [£]	19.0 ± 6.4	16.9 ± 2.9	10.1 ± 4.5 [£]	68.2 ± 13.2
	Raf	19.6 ± 5.6	3.5 ± 2.5 [£]	0.2 ± 0.1 [*]	11.9 ± 3.3	13.5 ± 3.5	7.5 ± 4.6	56.1 ± 15.8
6h	No Raf	26.7 ± 3.2	0.6 ± 0.2	1.3 ± 0.3	20.2 ± 3.2	22.1 ± 1.0	41.4 ± 32.2	112.4 ± 33.3
	Raf	27.1 ± 1.9	7.7 ± 3.8 [£]	0.4 ± 0.1 [*£]	19.1 ± 3.7	19.5 ± 0.9	8.3 ± 5.3 [£]	81.0 ± 10.0
24h	No Raf	22.5 ± 4.4 [&]	8.1 ± 7.6	1.7 ± 0.6	43.2 ± 9.8 [&]	102.1 ± 41.9 [&]	39.5 ± 33.3	220.3 ± 46.8 [&]
	Raf	27.2 ± 2.5	2.3 ± 0.9	0.7 ± 0.3	20.5 ± 6.5 [*]	34.7 ± 17.7	41.5 ± 10.0	126.6 ± 44.1 [*&]

Values are means ± SE (µM) (n = 7). 3-PPA: 3-phenylpropionic acid; 3-HPAA: 3-hydroxyphenylacetic acid; 4-HBA: 4-hydroxybenzoic acid; 4-HPAA: 4-hydroxyphenylacetic acid; 3-HPPA: 3-hydroxyphenylpropionic acid; 3, 4-DHPPA: 3, 4-dihydroxyphenyl propionic acid; No Raf: no added raftiline; Raf: added raftiline, ANOVA tests with LSD test was used to determine significant differences, * Different from fermentation without raftiline, $P < 0.05$; [&] Different from fermentation at 0 h, $P < 0.05$; [£] Different from 3-PPA, $P < 0.05$;

The first metabolite broken down from hesperetin by gut bacteria was 3, 4-dihydroxyphenyl propionic acid (Figure 5-4). The gut bacteria were significantly active in pathway 1 ($p < 0.05$), regardless of addition of raftiline. However, raftiline significantly impacted amount of phenolic acids in pathway 2 at the end of fermentation (21.3 µM vs. 43.9 µM, $p < 0.05$).

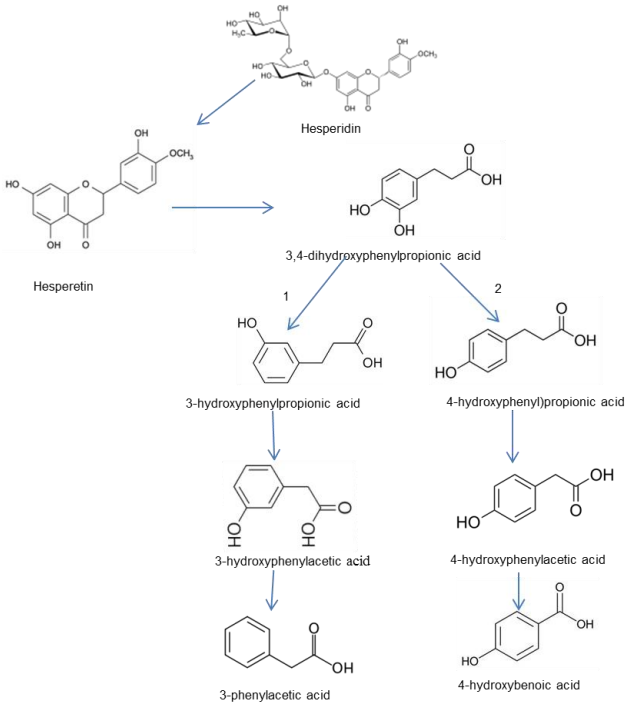


Figure 5-3 Proposed pathway of degradation of hesperidin to phenolic acids

Table 5-4 Phenolic acids produced in pathway1 and pathway 2 over 24h of fermentation of hesperidin and hesperidin with raftiline by human gut bacteria

	No Raf		Raf	
Time	Pathway 1	Pathway 2	Pathway 1	Pathway 2
0 h	37.5 ± 2.8 $^{\&}$	16.3 ± 3.8	37.8 ± 3.5 $^{\&}$	10.2 ± 3.8 *
2 h	43.1 ± 1.8 $^{\&}$	19.3 ± 5.2	48.1 ± 3.5 $^{\&}$	12.6 ± 3.5
4 h	38.2 ± 6.6$^{\&}$	19.9 ± 5.6	36.5 ± 9.8 $^{\&}$	12.1 ± 3.3
6 h	49.4 ± 3.2$^{\&}$	21.5 ± 3.1	53.2 ± 4.4 $^{\&}$	19.5 ± 3.6
24 h	136.9 ± 31.3$^{\&}$	43.9 ± 10.3	63.9 ± 19.9 $^{\&}$	21.2 ± 6.3 *

Values are means ± SE (µM) (n = 7). No Raf: no added raftiline; Raf: added raftiline. * Different from fermentation without raftiline, P <0.05; $^{\&}$: Different from pathway 2 (paired t-test).

5.3.2 Effect of glucose on metabolism of hesperidin

5.3.2.1 Degradation of hesperidin and production of hesperetin by human colonic bacteria in the presence of glucose

Hesperidin was rapidly degraded by colonic bacteria in the presence of glucose (Table 5-6 and Figure 5-5). There was 78.1 µM and 64.8 µM of detected hesperidin and 0.7 µM and 0.2 µM of hesperetin production at initial fermentation. There was 87.6 % and 84.1 % of hesperidin degraded by gut bacteria after 24 h fermentation of hesperidin and hesperidin with glucose respectively. Glucose induced rapid decrease of hesperidin over 2 to 4 h of fermentation (22.0 ± 2.5 µM vs. 9.2 ± 6.2 µM, p = 0.073). At the end of fermentation, there was about 10 µM of flavanone (hesperidin + hesperetin) that was not degraded.

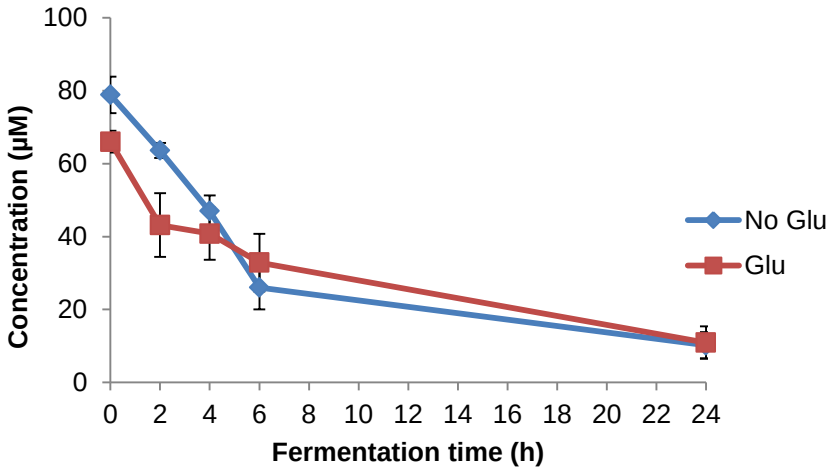


Figure 5-4 Effect of glucose on hesperetin and hesperidin) (µM) during 24 h of fermentation
Values are means ± SE (n = 6); No Glu: no adding glucose, Glu: adding glucose, ANOVA tests with LSD test was used to determine significant differences, * significantly different from No Glu.

Table 5-5 Effect of glucose on production of hesperetin (μM) and degradation of hesperidin (μM) by gut bacteria

	Treatment	0 h	2 h	4 h	6 h	24 h
Hesperidin	No Glu	78.1 ± 4.8	60.9 ± 2.6	39.0 ± 4.1	20.6 ± 5.3	5.7±1.3
	Glu	65.9 ±3.3	40.3 ±10.0	31.0 ± 9.7	22.8 ± 9.6	10.3 ± 4.4
Hesperetin	No Glu	0.7 ± 0.4	2.7 ± 0.8	8.0 ± 1.6	5.4 ± 2.0	4.5 ± 3.4
	Glu	0.2 ± 0.1	2.9 ± 0.8	9.8 ± 2.1	10.1 ± 3.9	0.6 ± 0.5

Data expressed as mean values ± SE (n=6). No Glu: no adding glucose, Glu: adding glucose
ANOVA tests with LSD post hoc was used to determine significant differences, * significantly different from No Glu.

6.3.2.2 Production of phenolic acids by human colonic bacteria in the presence of glucose

The sum of total phenolic acids were 280.3 ± 90.8 μM and 226.1 ± 41.4 μM respectively by 24 h fermentation of hesperidin and hesperidin with glucose respectively (p = 0.4) (Figure 5-5). 3-(phenyl) propionic acid, 3-phenyllactic acid, 4-hydroxybenzoic acid, 4-hydroxyphenylacetic acid, 3-hydroxyphenyl) propionic acid, 4-hydrophenyl propionic acid, dihydroferulic acid, and 3, 4-dihydroxyphenyl) propionic acid were found (Figure 5-6).

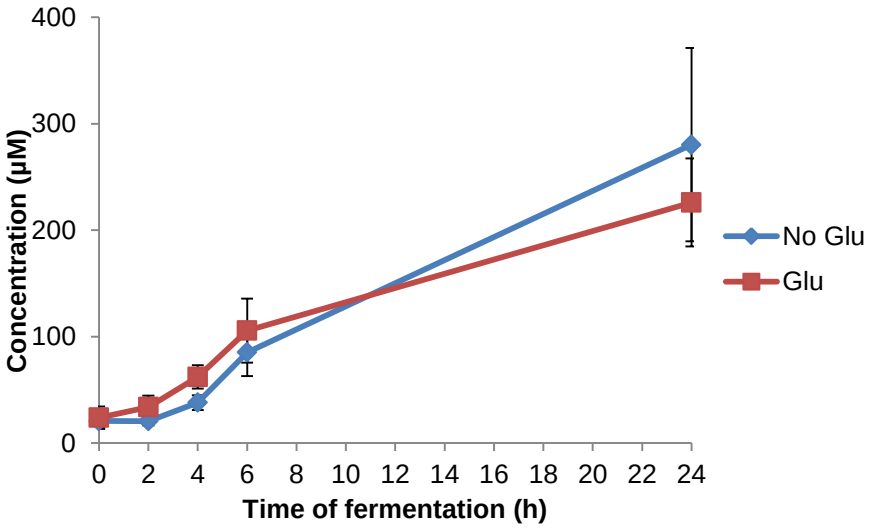


Figure 5-5 Concentration of phenolic acids (μM) after 24 h fermentation with hesperidin or hesperidin and glucose by gut bacteria

Values are means ± SE (n = 6), No Glu: no adding glucose, Glu: adding glucose, * significantly different from No Glu, P < 0.05 (paired t-test)

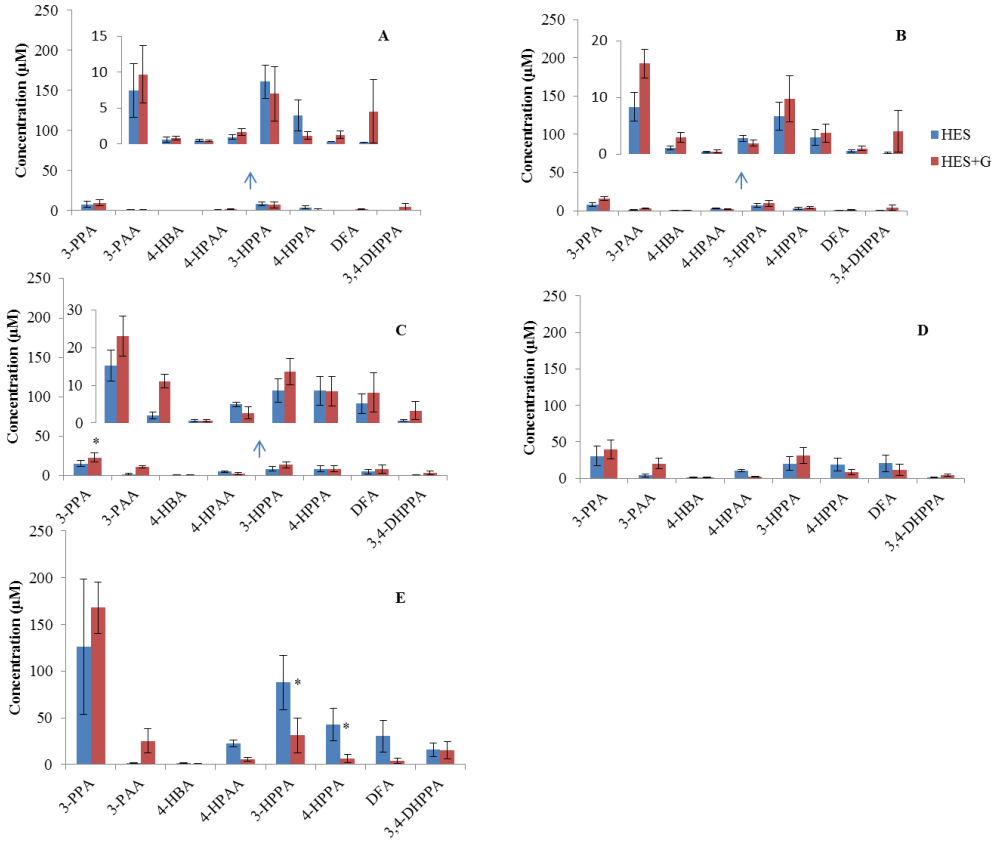


Figure 5-6 Impact of glucose on phenolic acids at 0 h (A), 2 h (B), 4h (C), 6 h (D) and 24 h (E) of hesperidin fermentation with human gut bacteria

Expressed as mean value in μM ± SE (n=6), blue bar: not adding glucose, red bar: adding glucose, 3-PPA: 3-(phenyl)propionic acid, 3-PAA: 3-(phenyl)acetic acid, 4-HBA: 4-hydroxybenzoic acid, 4-HPAA: 4-hydroxyphenylacetic acid, 3-HPPA: 3-(3'-hydroxyphenyl)propionic acid, 4-HPPA): 3-(4'-hydrophenyl)propionic acid, DFA: Dihydroferulic acid, 3,4-DHPPA: 3,4-dihydroxyphenylpropionic acid, 3-(3,4-DHPPA): 3-(3,4-dihydroxyphenyl)propionic acid, HES: hesperidin, HES+G: hesperidin plus glucose, ANOVA tests with LSD test was used to determine significant differences, * significantly different from No Glu.* Significantly different from HES fermentation, $P < 0.05$.

At the beginning, 3-phenylacetic acid, 3-hydroxyphenylpropionic acid, and 4-hydrophenyl propionic acid were rapidly produced respectively at 7.5 μM, 8.7 μM and 4.0 μM. In addition, 3, 4-dihydroxyphenylpropionic acid was present in the incubation of hesperidin plus glucose (4.1 μM). From 0 h to 2 h, the addition of glucose significantly increased 4-hydroxyphenyl)propionic acid and dihydroferulic acid (3.0 ± 1.4 μM vs. 3.7 ± 1.6 μM, $p < 0.03$; 0.5 ± 0.2 μM vs. 1.1 ± 0.4 μM, $p \leq 0.05$). From 2 h to 4 h, glucose significantly increased production of phenolic acids from 38.0 ± 6.8 μM compared with 62.1 ± 11.0 μM ($p < 0.02$). Addition of glucose significantly increased 3-phenyl acetic acid from 15.3 ± 4.1 μM to 23.1 ± 5.4 μM ($p < 0.01$). The phenolic acids was increased to 85.3 ± 22.4 μM and 105.7 ±

30.0 μM by 6 h fermentation of hesperidin and hesperidin plus glucose. Addition of glucose significantly increased 3-phenylacetic acid from 30.6 ± 13.6 μM to 39.5 ± 12.8 μM ($p \leq 0.04$). However, addition of glucose decreased production of 3'-dihydroxyphenylpropionic acid from 88.0 ± 29.1 μM to 31.3 ± 18.5 μM after 24 h of fermentation ($p \leq 0.04$).

5.4 Discussion

The human gut microbiota has the ability to degrade hesperidin into its aglycone and smaller molecular weight compounds (phenolic acids). Two different fermentable carbohydrates were tested for their effects on these processes. Raftiline is selectively used by lactic acid bacteria and glucose is used by a wider range of colonic bacteria, so by comparing their effects the mechanism for the effects of increased fermentation can be considered. Glucose changed the rate of hesperidin degradation over 2-4 h in the faecal incubations, while raftiline did not affect the metabolism of hesperidin during 24 h fermentation. However, raftiline significantly decreased production of phenolic acids from 220.3 μM to 126.6 μM after 24 h fermentation of hesperidin by gut bacteria ($p < 0.05$). However, glucose did not affect the production of phenolic acids after 24 h of fermentation, but increased production of phenolic acids from 38.0 ± 6.8 μM to 62.1 ± 11.0 μM at 4 h fermentation ($p < 0.02$) suggesting a change in the rate of metabolism rather than an increase in total phenolic acid production.

There were 46 % and 87.6 % of hesperidin degraded after 24 h fermentation with gut bacteria, but there was no significant difference after adding raftiline or glucose. Fructo-oligosaccharides stimulated the growth of Bifidobacterium *in vivo* and *in vitro* (Bouhnik et al., 1997, Steer et al., 2003(Yang, 2013 #618). In addition, raftline stimulates the growth of lactic acid bacteria (Ramchandran and Shah, 2010). However, degradation of hesperidin was not increased during the fermentation with raftiline through stimulation of gut bacteria *in vitro*.

Hesperetin was similar deglycosided from hesperidin by gut bacteria during the fermentation with or without glucose. Jaganath et al detected phenolic acids ranging from 33 μmol to 39 μmol at 24 h during fermentation of rutin without / with glucose by gut bacteria from 3 participants (Jaganath et al., 2009), showing limited effect of glucose in production of phenolic acids, which was similar with our results. It is well known that glycosylated polyphenols can be hydrolysed to aglycones by β –glucosidases (Dabek et al., 2008, Laparra

and Sanz, 2010). There are several studies describing the ability of β-glucosidase and β-rhamnosidase of gut bacteria to hydrolyse polyphenols *in vitro* and *in vivo* (Aura et al., 2002b, Bokkenheuser et al., 1987, Schneider et al., 1999). The gut microbiota can degrade flavonoids, and transform aglycones and conjugated metabolites into phenolic acids (Hooper et al., 2002, Wilson and Nicholson, 2009, Dueñas et al., 2015). The phenolic acids were higher at 4 h in the presence of glucose. This might indicate that gut bacteria can rapidly use glucose as energy source and increase degradation of hesperidin and the flux to phenolic acids rather than decreasing production of hesperidin.

However, the inhibitory property of raftiline on the production of phenolic acids was found during the fermentation. There was 72 %, 59 %, 53 %, and 76 % reduction of 3-hydroxyphenylacetic acid, 4-hydroxybenzoic acid, 4-hydroxyphenylacetic acid and 3-hydroxyphenylpropionic acid in the presence of raftiline, which was similar with *in vitro* incubation of rutin with dietary fibre, raftiline reduced total phenolic acids, 3-hydroxyphenylacetic acid and 4-hydroxyphenylacetic acid at the end of 24 h fermentation with rutin (Mansoorian et al., 2015). The major fermentation products in our study were 3,4-dihydroxyphenyl propionic acid, 3-hydroxyphenylpropionic acid, 4-hydroxyphenylacetic acid, which were similar to metabolites reported from the fermentation of rutin, naringin, quercetin, naringenin, and procyanidins (Rechner et al., 2002, Serra et al., 2012, Serra et al., 2011). Thus, raftiline may have inhibited these gut bacteria and reduced transformation of hesperidin into phenolic acids.

The production of phenolic acids were 3 fold higher in pathway 1 compared with pathway 2 during the fermentation with or without raftiline. However, raftiline significantly decreased production of phenolic acids in proposed pathway 2. Flavonoids are dehydrolysed, demethylated, decarboxylated to metabolites through the action of dehydroxylase, demethylase or decarboxylase by Ruminococcus, Eggertheilla, Bifidobacterium, Clostridium, Bacterioides, Eubacterium, Streptococcus and Lactobacillus bacteria (Clavel et al., 2005, Keppler and Humpf, 2005, Schneider et al., 1999, Blaut and Clavel, 2007, Kim et al., 1998). This implies that raftiline influenced pathway 2 related bacteria.

As we know, the level of flavonoid metabolites produced in the colon has been linked to their potential health effects. Hesperidin and /or hesperetin were reported to reduce cardiovascular disease related adiponectin expression (Liu et al., 2008), inflammation biomarker (IL-6)

(Choi and Lee, 2010), and carcinoid tumors cell proliferation. In addition, 3,4-dihydroxyphenylacetic acid was observed to have anti-proliferative activity in prostate and colon cancer cells *in vitro*, and protected against pancreatic β-cells dysfunction that represents a transition from pre-diabetes to diabetes (Gao et al., 2006a, Carrasco-Pozo et al., 2015). The phenolic acids, 4-hydroxybenzoic and 4-hydroxyphenylacetic protected against pathogenic bacteria in the gut (Si et al., 2006, Lee et al., 2006, Cueva et al., 2010). It indicated that inhibitory effect of raftiline on production of phenolic acids may affect properties of polyphenols.

In conclusion, in this study, the rate and extent of hesperidin degradation was affected by fermentable carbohydrates that might be due to modification of gut bacteria or their relevent enzymes. Raftiline had an inhibitory effect on degradation of hesperidin probably through selective modification of gut bacteria. Production of phenolic acids was more sensitive to fermentable carbohydrate than hesperidin or hesperetin. Thus, dietary fibre or other aspects of the food matrix should be considered when investigating bioavailability or bioactivity of flavonoids through modification of metabolites, especially phenolic acids. Although not studied in this thesis the flavonoids or their derived products (aglycones and phenolic acids) may influence the composition of gut bacteria that should be explored in future. In addition, the effect of dietary fibre on colonic metabolism of hesperidin or other flavonoids should be considered when the benefits of flavonoids or dietary fibre in humans are investigated *in vitro* or *in vivo*.

CHAPTER 6 *Impact of a four week exercise intervention on bioavailability of flavanone from orange juice in previously sedentary women*

6.1 Introduction

As we discussed before, flavanone bioavailability is very low in humans (section 1.4.2). Factors affecting bioavailability have been increasingly explored by researchers in order to improve health effects of dietary flavonoids in humans. The bioavailability was enhanced from 7 % to 47 % by using α-rhamnosidase to hydrolyse flavanone in orange juice (Bredsdorff et al., 2010). The fortified juice with orange flavonoid extract had the highest urinary excretion of flavanone metabolites in comparison with commercial orange juice and flavonoid contained drinks (Vallejo et al., 2010a). Moreover, full fat natural yoghurt reduced maximal concentration (C_{max}) of flavanone metabolites in plasma and production of five phenolic acids in urine, and increased the time to achieve maximal concentration (T_{max}) of metabolites in plasma, but did not modify 24 h urinary excretion of flavanone metabolites after orange juice intake (Roowi et al., 2009b, Mullen et al., 2008a).

There is limited evidence which suggest that the bioavailability of flavonoids can be favourably modified by participation in exercise. As described in chapter 1, 24 h urinary excretion of naringenin, eriodictyol, and hesperetin metabolites were 92 %, 88 %, and 53 % higher in very well trained athletes than in sedentary individuals after ingestion of aronia-citrus juice (Medina et al., 2012). However, this study has several limitations. Firstly, it focused on cross sectional populations with different lifestyles (active and sedentary). Secondly, both of the groups consumed different doses of aronia-citrus juice (200 ml for atheletes /400 ml for non-trained subjects) with different one week diet (a diet without polyphenol for non-trained group, another one diet for triathletes included the plant-foods containing flavanones with a balanced carbohydrates, proteins, fats, vitamins and microelements), even though flavanone dose was similar. Thirdly, the athlete group kept their active lifestyle during this one week juice consumption. Additionally, urine samples were hydrolysed with β-glucuronidase for analysis. The one week diet before urine collection was not same between two groups. Therefore the study does not allow a conclusion that exercise can directly enhance bioavailability of flavanone in those who are not professionally trained as athletes.

Flavanone metabolites and phenolic metabolites can be absorbed into the circulatory system or excreted into urine after deglycosylation, degradation and conjugation of flavanone by intestinal enzymes or colonic bacteria. The deglycosylation of dietary flavonoids depends on

β-glucosidase activity from human small intestine mucosa (Németh et al., 2003). Absorption of flavonoids involves epithelial brush border membrane transporters, such as Na-dependent GLUT-1, and UDP-glucuronosyl transferase (Gee et al., 1998, Henry-Vitrac et al., 2006). In addition, physiological functions of gastrointestinal intestine tract have been linked to absorption or/and rate of drug and flavonoids, such as gastric emptying and gut transit time (Clements et al., 1978, Mullen et al., 2008a, Mullen et al., 2008b, Mullen et al., 2009a). It is well known that flavonoid metabolites can be distributed into tissues through the blood stream in the host. Gastrointestinal hormones might impact on intestinal functions. Peptide YY delayed gut transit time from 67 ± 4 mins to 94 ± 7 mins and 192 ± 9 mins, and prolonged gastric emptying from 37 ± 8 mins to 63 ±10 mins and 130 ±12 mins when subjects received low dose of infusion at 0.4 pmol/kg/min dose (50 ml), and high dose infusion at 1.1 pmol/kg/min (50 ml) (Savage et al., 1987). Naslund and his colleagues supposed that peptide YY was responsible for intestinal motility and glucagon-like peptide -1 dominated early gastric emptying (Naslund et al., 1998).

Colonic microbiota have enormous catalytic and hydrolytic ability to degrade flavonoids and involving demethylation, dehydroxylation and decarboxylation activities and thus play an important role in metabolism of flavonoids (Aura, 2008). The activities of β-glycosidases and β-glucuronidases are the main enzymes to degrade polysaccharides and xenobiotics by gut bacteria (Laparra and Sanz, 2010). Clostridium, Butyrivibrio and Bacteroides species have enzyme activities to hydrolyse glycosides and ring diffusion of flavonoids in the colon in relation to their α-rhamnosidase, β-glycosidase and β-glucuronidase (Winter et al., 1989, Kim et al., 1998, Selma et al., 2009, Minamida et al., 2006, Tzounis et al., 2008, Laparra and Sanz, 2010, Rechner et al., 2002).

Clark et al reported more diversity of gut bacteria in athletes compared with sedentary humans (Clarke et al., 2014). However, higher quantities of calories, protein, fat, carbohydrates, sugar and saturated fat per day were consumed by athletes than the sedentary population, among which protein intake in athletes was positively associated with microbial diversity (Clarke et al., 2014). However, it is not clear if it is the effect of physical activity or diet that changes the composition of the gut bacteria, even though an animal study revealed different TGGE profiles of gut bacteria 16S rRNA from 5 of 7 male rats subjected to wheel-running exercise for 5 weeks (Matsumoto et al., 2008). If the bacteria change with exercise then the flavanone metabolism in the gut may also change.

Exercise modifies physiological functions as described in chapter 1. Chronic exercise can be expected to modify flavanone metabolism by several other mechanisms, including physiological functions of gastrointestinal tract and circulatory system (Ebeling et al., 1993, Pollock et al., 1995, Watkins et al., 1994, Carrio et al., 1989, Koffler et al., 1992, Piatkowski et al., 1993).

Due to lack of studies on effects of physical activity on dietary flavonoids in a normal population, a crossover controlled study was carried out to investigate whether a 4 weeks moderate intensity exercise programme physical activity affects the bioavailability of flavanones and the colonic catabolism of flavanone after orange juice in humans. Based on present evidence, it has been hypothesised that metabolism of flavonoids can be impacted by physical activity in sedentary humans.

6.1.1 Hypothesis

To test the hypothesis that 4 weeks moderate intensity exercise increases bioavailability and metabolism of orange juice flavanone in previously sedentary females

Does a 4 weeks moderate physical intensity physical exercise affect bioavailability of flavanone and production f phenolic acids after the consumption of orange juice ‘with bits’?

6.2 Subjects and study design

6.2.1 Study design

Twenty sedentary females were recruited by advertisement in a Glasgow community website, by Student voice (a student community social website) (Appendix 6), posters and leaflets in the area of Glasgow city. Inclusion criteria for this study were Caucasian females, sedentary for at least 6 weeks, 18-45 years old, non-smoker, not taking any medication, non-gastrointestinal disease, non-vegetarian, non-pregnant, BMI < 30. The study was explained to all of potential participants. Potential participants went through a screening process. They were asked to fill physical activity readiness questionnaire and health screening questionnaire (Appendix 10). If they were willing to take part in this study, the consent form was signed before the intervention study. MVLS Ethics Committee at University of Glasgow reviewed

and approved this study (No. 200140020), and participants provided implied consent by returning their questionnaires.

In this study, healthy Caucasian women, aged 18-45 years, had sedentary lifestyle (less than 1h physical activity per week and a sedentary job) for at least 6 weeks, had no digestive problems, diabetes, thyroid problems, blood disorders, kidney and liver problems, convulsion and epilepsy, non-smokers, not pregnant, had a regular menstrual cycle, non any medications or nutritional supplements before 6 months, and those who said 'no' to all of PRQ were recruited (Appendix 11).

Fifteen participants needed to undergo 4 weeks of moderate intensive exercise including running, cycling or combination of both, 4 sessions every week. Participants needed to complete experimental tests before and after 4 weeks of intervention. Prior to experimental tests, participants followed a well-designed list of polyphenol-free foods for 2 days (appendix 8) and the food was same for all of participants during the experimental day. The low polyphenol diet was strict in order to obtain an ideal baseline that excluded fruit, vegetables, legumes, whole cereal, wine, tea, cocoa, coffee, and so on, which was recorded in food inventory and participants repeated the same diet for 2 days before the second experimental test. The two days low polyphenol food was recorded in food inventory (Appendix 7). In addition, for the 2 days, the participants were restricted from high fibre containing food to minimize non-orange juice fibre induced H_2 exhalation from any remaining fermentable carbohydrates in the colon (Bertram et al., 2014, Brummer et al., 1985).

Participants attended the metabolic investigation room between 0800 and 0900 h after a 12 h fast and brought an overnight urine sample. Body composition (weight, BMI, body fat), was measured using TBF-310GS body composition analyzer (Tanita), and height and waist circumference were also measured using standard protocols (Marfell-Jones et al., 2012). Blood pressure and heart pulse was measured by using a portable blood pressure monitor. After body composition measurement, a venous cannula was introduced into an antecubital vein. After an interval of 10 min, a baseline blood sample (9 ml) was obtained into an EDTA tube. The first breath hydrogen value was tested by a breath hydrogen monitor (Bedfont Scientific Ltd., Kent, UK). Immediately, subjects were provided a cup of orange juice 'with bits' (500 mL). Blood samples were obtained at 1, 2, 3, 4, 5, 6, 7, 8 h after the juice intake. Expiratory breath hydrogen levels were monitored every 30 minutes at following 8 hours

after the orange juice intake. For breath hydrogen test, they were asked to maximally inhale and hold the inhalation for 15 s and then expire into the monitor. The time of first sustained rise of breath hydrogen was taken as the mouth to caecum transit time. They were allowed to drink water. Lunch included a white roll, butter and ham, a package of ready salted crisps (carbohydrates, 39.88 g; fat, 18.15 g; protein, 17.44 g; energy, 388.5 kcal) after 4 h consumption of orange juice and at the end of the experimental test. The dinner was of polyphenol-free food and was recorded. The cannula was removed after 8 h blood sample collection and the participants left the laboratory to sleep at home, and then came back in the next morning for the fasting blood sampling. Urine samples were collected before orange juice intake and 0-2, 2-5, 5-8, 8-10, 10-20, 20-24 h after juice consumption.

Exercise training consisted of 4 weeks' sessions of moderate intensity exercise (cycling, running or combination of both). Each exercise session lasted for 40 minutes in week 1, 50 minutes in week 2, 60 minutes in week 3 and 4. The exercise programme was set at an individually pre-determined work rate and achieved 70-80 % of their predicted maximal heart rate. Exercise sessions could be separated into smaller sessions if required by the participants, but finished in the same day. The training sessions took place at the Exercise and Energy Balance Laboratory (New Lister Building, the Glasgow Royal Infirmary), or Stevenson Building (Glasgow University).

6.2.2 Orange juice and chemicals

Orange juice was purchased in the local supermarket, freshly squeezed, not from concentration. Orange juice was homogenized in a large clean container, and separated to 500 ml, and stored in freezer until consumption (flavanone kept stable during the whole study). Aliquots of orange juice were used to analyze flavanone content.

HPLC grade methanol was purchased from Fisher Scientific (Loughborough, UK). Hesperetin-7-O-rutinoside, narigenin-7-O-rutinoside, 3-hydroxyphenylacetic acid , 4-hydroxyphenylacetic acid , 4-hydroxyphenylpropionic acid, 3, 4-dihydroxyphenylacetic acid, 3-(3, 4-dihydroxyphenyl) propionic acid, 4-hydroxy-3-methoxy-phenylpropionic acid were purchased from Sigma-Aldrich Co Ltd (Poole, Dorset, UK) or Alfa Aesar (Karlsruhe, Germany). Formic acid, N-methyl-N-(trimethylsilyl) trifluoroacetamide, pyridine, 2, 4 ,5 - trimethoxycinnamic acid, 4-hydroxybenzoic acid, ferulic acid, 4'-hydroxyphenylacetic acid,

3’-methoxy-4’-hydroxyphenylacetic acid, hippuric acid, 3-(3’-methoxy-4’-hydroxyphenyl)propionic acid, and 3-(3’-hydroxy-4’-methoxyphenyl)propionic acid were purchased from Sigma-Aldrich Co Ltd (Poole, UK). 4’-hydroxyhippuric acid was obtained from Bachem (UK) Ltd. Hesperetin-7-*O*-rutinoside and narigenin-7-*O*-rutinoside were acquired from Extrasyntheses (Genay Cedex, France). 3-(3’-Hydroxyphenyl) hydracrylic acid, hesperetin-7-*O*-glucuronide, naringenin-4’-*O*-glucuronide, and naringenin-7-*O*-glucuronide were purchased from Toronto Research Chemicals Inc. Ethyl acetate and dichloromethane (DCM) were purchased from Rathburn Chemicals Ltd (Walkerburn, Peeblesshire, UK).

6.2.3 Extraction of flavonoids in orange juice and phenolic acids in urine

Orange juice (1 ml) was extracted as mentioned in section 2.9.1. Extraction of phenolic acids was done by the methods, as described in section 2.9.2.

6.2.4 Identification and quantification of flavonoids in orange juice with HPLC- PDA-MS

Orange juice was analyzed by HPLC-PDA-MS^2 described in section 2.11. The extracted samples were injected in duplicate.

6.2.5 Identification of flavanone metabolites in urine by HPLC- PDA-MS

Urine samples were centrifuged at 13,000 rpm at 4 °C for 10 min. Supernatants (100 µl) were analyzed by using HPLC–PDA-MS^2 as described in section 2.11.

Quantification of hesperetin and narigenin metabolites was respectively based on chromatographic peak areas acquired at 290 nm and expressed against standards of hesperetin-7-*O*-glucuronide and naringenin-7-*O*-glucuronide equivalents with limit of detection at 0.1ng. The standards were dissolved in methanol at concentrations ranging from

0.1 to 100 ng. A linear response was obtained for all the standard curves when a linear regression analysis was $R^2 > 0.999$.

6.2.6 Identification and quantification of phenolic acids by GC-MS

GC-MS analysis was illustrated in section 2.12. Phenolic acids (3-hydroxybenzoic acid, 3'-hydrophenylacetic acid, 4-hydroxybenzoic acid, 4'-hydrophenylacetic acid, vanillic acid, 3-methoxy-4-hydroxyphenylacetic acid, 3, 4-dihydroxybenzoic acid, 3, 4-dihydroxyphenylacetic acid, 3-hydoxy-4-methoxphenylhydracrylic acid, hippuric acid, 3-hydoxyphenylhydracrylic acid, 3-(3-methoxy-4-hydroxyphenyl) propionic acid, 3-(3,4-dihydroxyphenyl) propionic acid, 3'-hydroxyhippuric acid, 4'-hydroxyhippuric acid were identified according to mass spectra and retention times obtained from authentic standards analysed under identical conditions. When standards were not commercially available, identification was achieved through the integrated NIST mass spectral library 2008 (Scientific Instruments Services Inc., Ringoes, NJ, USA), with a confidence of 70 % or above. Calibration curves of the ratio between the target ion of the internal standard (2, 4, 5-trimethoxycinnamic acid, m/z 279) were achieved with concentrations ranging from 3- 60 ng/µl ($R^2 > 0.95$).

6.2.7 Sample size and statistical analysis

The sample size was based on pilot data (Malkova et al unpublished data) obtained in a previous study on the impact of detraining on urinary excretion of flavanone metabolites. Assuming that the intervention will induce an effect size of ~0.5 SD units (i.e. it is half as effective), 10 subjects were needed to complete the study to achieve a significant difference before and after the intervention with 85% power at $p < 0.05$. All data were presented as mean values with standard error. All data were assumed to be normally distributed and was subjected to statistical analysis using a paired t-test, independent t-test and ANOVA tests with LSD post-hoc test. Statistical significance was set at a p-value < 0.05 for two-tailed comparisons.

6.2.8 Hypothesis

Four weeks moderate intensity physical exercise programme affects bioavailability of flavanone metabolites and phenolic acids

6.3 Results

6.3.1 Pre intervention characteristics of subjects by groups

Age, height, weight, BMI, WC (waist circumference) and body fat were not different between exercise and control groups prior to the intervention study (Table 6-1).

Table 6-1 Anthropometric characteristics and body composition of healthy females before exercise and habitual life intervention

Age (years)	22.4 ± 1.3
Height (cm)	167.9 ± 6.4
Weight (kg)	64.5 ±8.6
BMI (Kg/m^2)	22.9 ± 2.2
Waist circumference (cm)	79.9 ± 9.0
Body fat (%)	28.0 ± 6.3
SBP (mm Hg)	119.1 ± 8.6
DBP (mm Hg)	77.0 ± 11.7
Heart rate (bpm)	77.2 ± 8.1

Values are expressed as means ± SE (n = 15). SBP: systolic blood pressure, DBP, diastolic blood pressure

6.3.2 Identification and quantification of flavanones in orange juice

The orange juice (500 ml) consumed by participants contained 1.1 mmol of hesperidin and 0.1 mmol of narirutin respectively. There were no detectable aglycones in orange juice (Table 6-2).

Table 6-2 Quantities of hesperidin and narirutin in 500 ml orange juice 'with bits'

Flavanone	Concentration (mmol)
Narirutin	0.1 ± 0.01
Hesperidin	1.1 ± 0.1

Values are means ± SD (mmol / 500 mL) (n=3).

6.3.3 Effect of exercise intervention on body composition and anthropometric characteristics

As shown in Table 6-3, height, weight, BMI, waist circumference, body fat, SBP, DBP, and pulse were similar before and after 4 weeks of moderate intensive exercise programme within exercise group.

Table 6-3 Anthropometric characteristics in healthy females before and after 4 weeks intensive exercise

	Exercise	
	Before	After
Age (years)	24.7 ± 6.6	24.7 ± 6.6
Height (cm)	166.8 ± 6.0	165.9 ± 6.1
Weight (Friesenecker et al.)	64.5 ± 7.7	63.6 ± 7.0
BMI (Kg/m^2)	23.2 ± 2.6	23.2 ± 2.7
Waist circumference (cm)	80.8 ± 8.4	78.2 ± 6.9
Body fat (%)	29.3 ± 6.3	28.6 ± 6.6
SBP (mm Hg)	116.1 ± 8.1	114.6 ± 8.5
DBP (mm Hg)	74.6 ± 9.3	69.6 ± 9.2
Pulse	78.0 ± 7.9	70.0 ± 10.2

Values are expressed as means ± SD (n= 15), ANOVA test was performed to determine intervention was significantly different from baseline. * Significantly different from baseline (before), $p < 0.05$.

6.3.4 Effect of 4 weeks intensive exercise intervention on mouth to caecum transit time (MCTT) in sedentary healthy females

We measured MCTT by the breath hydrogen technique. We assumed that the orange juice contained sufficient fibre to be fermented in the colon. This was before we had the results on the actual fibre content. In reality this was too low to produce large changes in breath hydrogen. We could have used a small dose of lactulose to supplement the substrate available for hydrogen production (Mullen et al., 2008a, Bertram et al., 2014). Thus may however, change the transit time by the osmotic effect of the lactulose in the small intestine stimulating peristalsis. Even though the hydrogen value was lower than desired and disappeared quickly when reaching the first time rise (Figure 6-1), the result was still valuable. Data from two subjects were excluded because the monitor malfunctioned or the subject had not complied with the proper diet before the test. The breath hydrogen was measured for 8 h, so data from those transit times > 8 h was considered 8 h for calculation. In addition, mouth to caecum transit time was delayed through 4 weeks intensive physical activities in females (3.0 ± 0.2 h vs. 4.5 ± 0.7 h, $p \leq 0.056$) (Table 6-4).

Table 6-4 First time sustained rise of breath hydrogen in sedentary females before and after 4 weeks exercise or habitual life intervention

	Gut transit time (h)
Before	3.0 ± 0.2
After	4.5 ± 0.7 *

Values are means ± SE (n = 15). Paired t-test analysis was performed to determine intervention was significantly different from baseline. * Significantly different from baseline (before) at P ≤ 0.056.

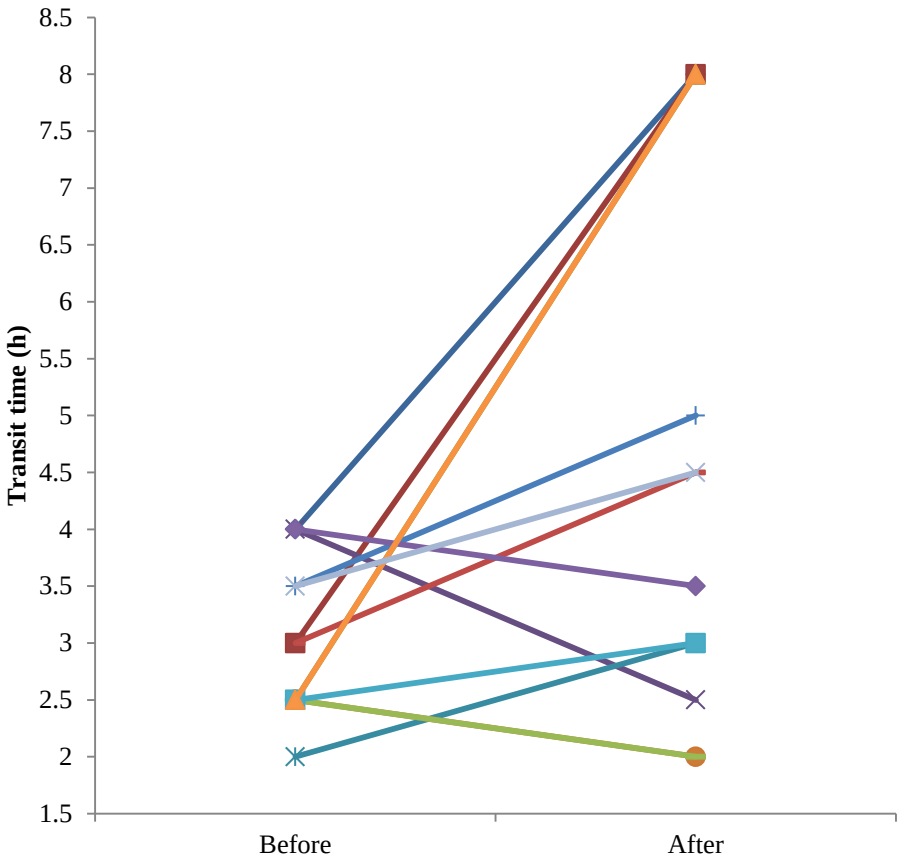


Figure 6-1 Individual gut transit time after intake of 500 ml of orange juice 'with bits' in females before and after 4 weeks exercise intervention

Values are the first time rise of breath hydrogen measured by breath hydrogen monitor (h) (n = 13). Paired t-test analysis was performed to determine. * Significantly different from baseline (before) at P ≤ 0.056.

6.3.5 Effect of 4 weeks intensive exercise intervention on urinary excretion of flavanone metabolites in sedentary humans

The excretion of flavanones over 0-2, 2-5, 5-8, 8-10, 10-20, and 20-24 h urine after orange juice consumption are shown in Table 6-5. Quantities of metabolites (µM) were based on total amount in urine. The flavanone metabolites included hesperetin sulfoglucoside, naringenin glucuronide (2 isomers), hesperetin diglucuronide, naringenin diglucuronide, hesperetin sulfoglucuronide, hesperetin glucuronide (3 isomers), and hesperetin sulphate detected in urine after intake of orange juice 'with bits'. The hesperetin and naringenin metabolites in total accounted for 67.3 % and 32.7 % of the total intake before 4 weeks intensive physical exercise in females after the consumption of orange juice 'with bits'. After the 4 weeks exercise intervention, 65.7 % were hesperetin metabolites in urine. Naringenin -

O- glucuronide (2 isomers), hesperetin -*O*- sulfoglucoside, hesperetin -*O*- glucuronide were the main metabolites in urine. Naringenin-diglucuronide was the lowest metabolite after orange juice intake (Table 6-5).

The urinary excretion of ten flavanone metabolites was 96.4 ± 10.6 µmol in total after intake of 500 mL orange juice 'with bits' by sedentary females, which reduced to 87.0 ± 12.5 µmol after 4 weeks of exercise training, but the reduction was not significant. However, one isomer of hesperidin glucuronide was significantly decreased from 10.4 ± 2.9 µmol to 6.2 ± 1.7 µmol after 4 weeks of exercise training ($p < 0.02$). In addition, the urinary excretion of flavanone metabolites (hesperetin metabolites and naringenin metabolites) was significantly reduced after 4 weeks intensive physical activities in females over 10-20 h after orange juice intake (21.4 ± 5.5 µmol vs. 18.8 ± 4.8 µmol, $p \leq 0.008$).

Naringenin metabolites expressed as a percentage of orange juice flavanone intake was higher than hesperidin metabolites in urine over 24 h after juice intake before and after 4 weeks intensive physical activities (Before: 6.0 ± 1.0 % vs. 23.2 ± 4.0 %; After: 5.3 ± 1.0 % vs. 21.9 ± 3.0 %) (Table 6-6). However, there was no significantly different excretion of flavanone metabolites in urine after 4 weeks intensive physical activities (before: 7.8 ± 0.9 %, after: 7.1 ± 1.0 % exercise). During the period of 24 h urine collection, the largest amount of flavanone metabolites were excreted over 5-8 h after orange juice intake, accounting for 29.4 % and 37.1 % of total urinary excretion in females respectively before and after 4 weeks physical activities.

Table 6-5 Quantities (μmol) of flavanone metabolites excreted in urine over 24 h after consumption of 500 ml orange juice by sedentary females before and after 4 weeks of moderate intensive exercise programme

	0-2 h		2-5 h		5-8 h		8-10 h		10-20 h		24 h		Total excreted	
	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
Hesperetin sulfoglucoside	1.0±0.4	1.0±0.4	3.5±1.3	2.0±0.5	3.7±1.2	2.6±1.2	1.8±0.7	1.5±0.6	2.5±1.1	1.3±0.4	0.1±0.0	0.1±0.0	12.5±3.9	8.5±1.9
Naringenin glucuronide	1.3±0.4	1.0±0.5	2.2±0.8	2.3±0.4	3.8±1.0	2.1±0.9	4.4±1.6	3.7±0.8	2.5±0.9	5.2±1.4	1.0±0.4	0.7±0.2	14.5±2.6	14.8±2.4
Naringenin glucuronide	1.8±0.6	2.2±0.9	2.6±0.9	2.4±0.6	5.2±2.3	4.5±2.0	3.1±1.5	1.0±0.3	1.9±0.5	3.5±1.0	0.9±0.4	0.1±0.0	14.7±4.5	14.4±2.9
Hespereretin diglucuronide	0.6±0.4	0.1±0.0	0.7±0.4	0.2±0.1	0.7±0.5	1.4±1.0	0.7±0.4	0.4±0.2	1.5±0.8	1.0±0.5	0.2±0.1	0.2±0.1	4.3±1.6	3.1±1.3
Naringenin diglucuronide	nd	nd	0.3±0.2	0.2±0.1	0.4±0.2	0.8±0.4	0.5±0.3	0.2±0.1	1.0±0.6	0.3±0.2	nd	0.1±0.0	2.3±0.9	1.6±0.8
Hesperetin sulfoglucuronide	0.5±0.3	0.1±0.1	1.8±0.6	1.1±0.4	1.3±0.4	1.3±0.3	0.7±0.3	0.3±0.2	0.9±0.6	0.5±0.3	nd	nd	5.1±1.0	3.3±0.9
Hesperetin glucuronide	0.6±0.3	0.3±0.1	3.6±0.8	2.0±0.8*	3.0±1.2	2.4±0.8	1.2±0.6	0.5±0.3	2.1±1.0	0.7±0.4	0.1±0.0	0.3±0.3	10.4±2.9	6.2±1.7*
Hesperetin glucuronide	0.3±0.2	0.1±0.0	3.9±0.8	3.7±1.2	4.3±1.4	4.4±1.4	3.1±1.9	1.7±0.8	4.7±1.6	2.5±1.3*	0.2±0.1	0.2±0.1	16.3±4.3	12.4±3.5
Hesperetin glucuronide	nd	0.2±0.1	2.7±1.3	2.6±1.3	2.7±1.8	8.4±4.0	0.5±0.3	2.4±1.5	3.2±3.1	2.3±2.2	nd	0.3±0.2	9.1±4.2	16.1±7.5
Hesperetin sulfate	nd	nd	1.7±0.9	0.3±0.2	1.3±0.6	0.8±0.3	0.1±0.1	0.5±0.3	0.6±0.5	1.3±0.9	nd	nd	3.7±1.5	3.0±1.3
Hesperetin metabolites	2.8±1.1	5.1±1.1	18.8±2.9	16.9±3.0	18.8±4.0	32.3±5.8	8.4±3.1	12.6±2.8	15.9±5.1	18.8±4.8*	0.5±0.1	1.60.7	64.9±10.5	57.2±11.5
Naringenin metabolites	3.1±0.7	1.8±0.5	5.1±1.2	12.2±3.0	9.4±2.5	25.0±5.0	7.4±2.4	7.6±2.4	5.5±1.3	9.8±4.0	1.4±0.5	0.9±0.6	31.5±5.4	29.8±4.1
Flavanone metabolites	5.9±1.5	5.1±1.1	23.9±3.0	16.9±3.0	28.3±4.2	32.3±5.8	15.8±3.9	12.6±2.8	21.4±5.5	18.8±4.8*	1.9±0.5	1.6±0.7	96.4±10.6	87.0±12.5

Values are means ± SE (n=15). Univariate analysis of variance test with post hoc LSD was used to determine significant differences * significantly different from baseline at $P \leq 0.05$.

Table 6-6 Urinary excretion of hesperetin, narigenin, and flavanone metabolites as a percentage of orange juice hesperidin, narirutin, and flavanone intakes over 24 h by sedentary females before and after 4 weeks of moderate intensity intensive exercise programme

Urinary excreted metabolites	0-2 h		2-5 h		5-8 h		8-10 h		10-20 h		24 h		Total	
	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
Hesperetin metabolites	0.2±0.1	0.1±0.0	1.8±0.3	1.0±0.3	1.8±0.4	2.4±0.4	0.8±0.3	0.7±0.2	1.5±0.5	1.0±0.4	0.0±0.0	0.1±0.1	6.0±1.0	5.3±1.0
Naringenin metabolites	2.3±0.5	2.4±0.7	3.7±0.9	3.5±0.5	6.9±1.8	5.4±1.6	5.4±1.7	3.6±0.7	4.0±0.9	6.6±1.6	1.1±0.4	0.5±0.2	23.2±4.0	21.9±3.0
Flavanone metabolites	0.5±0.1	0.4±0.1	1.9±0.2	1.4±0.2*	2.3±0.3	2.6±0.5	1.3±0.3	1.0±0.2	1.7±0.4	1.5±0.4	0.2±0.0	0.1±0.1	7.8±0.9	7.1±1.0

Values are means ± SEMs (n=15). Univariate analysis of variance test with LSD was used to determine significant differences * significantly different from baseline ($P \leq 0.05$).

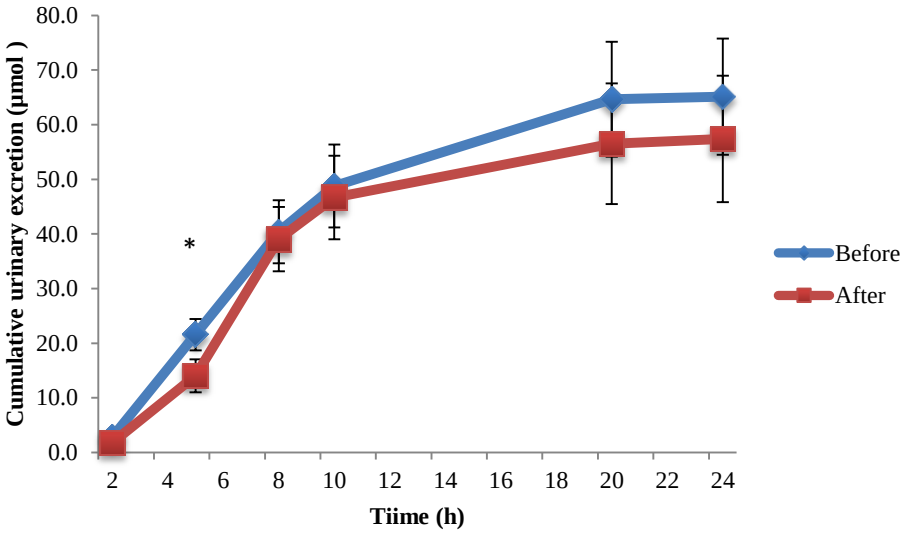


Figure 6-2 Effect of 4 weeks moderate intensive exercise on 24 h cumulative urinary excretion of hesperetin metabolites (µmol) after 500ml of orange juice intake in sedentary females

Values are means ± SE (n= 15). ANOVA with post host hoc HSD was used to determine significant differences * significantly different after 4 weeks intensive physical activities in exercise group, P ≤ 0.05.

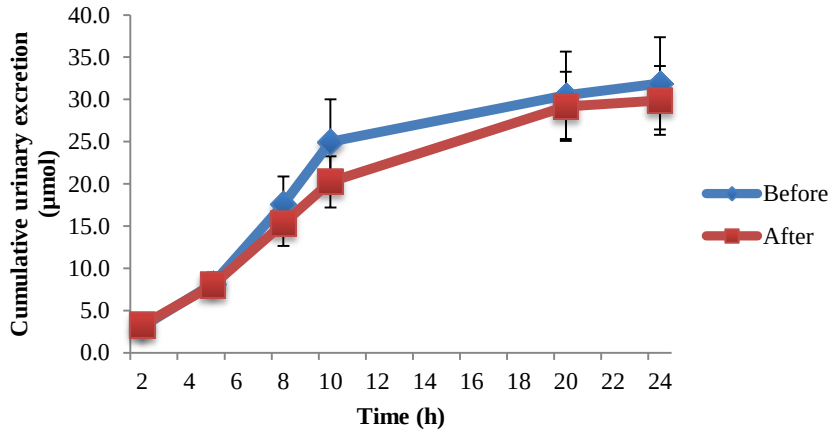


Figure 6-3 Effect of 4 weeks moderate intensity exercise programme on cumulative urinary excretion of naringenin metabolites (µmol) over 24 h after 500ml of orange juice intake in sedentary females

Values are means ± SE (n= 15). ANOVA with post hoc HSD was used to determine significant differences * significantly different after 4 weeks of exercise programme P ≤ 0.05.

Hesperidin metabolites were significantly reduced from 21.6 ± 2.8 µmol to 14.0 ± 3.0 µmol in urine over 2-5 h after 500 mL of orange juice with bits after 4 weeks moderate physical

activities in females ($p < 0.03$) (Figure 6-2). In terms of flavanone metabolites as cumulative urinary excretion following orange juice intake, 4 weeks of moderate intensity exercise significantly decreased 0-5 h cumulative excretion in urine (29.7 ± 2.9 µmol vs. 22.0 ± 2.9 µmol, $p < 0.02$) (Figure 6-4).

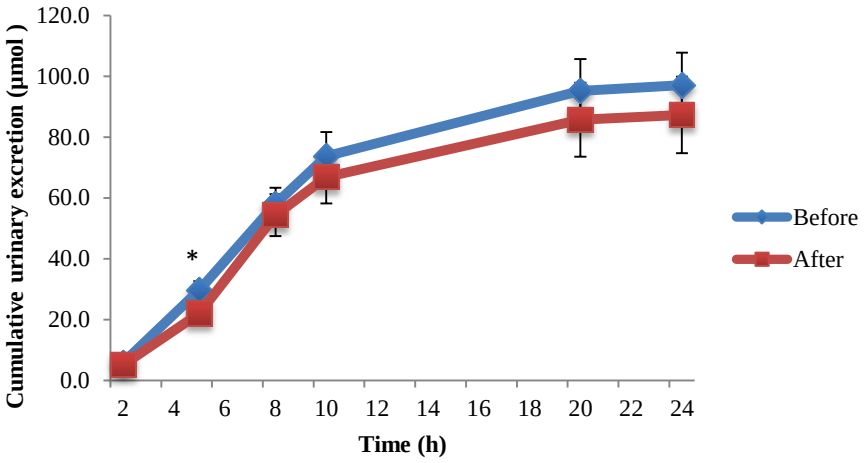


Figure 6-4 Effect of 4 weeks moderate intensity exercise programme on cumulative urinary excretion of total flavanone metabolites (µmol) over 24 h after 500ml of orange juice intake in sedentary females

Values are means ± SE (n= 15). ANOVA with post hoc HSD was used to determine significant difference, * significantly different after 4 weeks of exercise programme $p \leq 0.05$.

Table 6-7 Urinary excretions (µmol) of phenolic acids before and after 4 weeks of moderate intensity exercise programmme over 24 h after intake of 500 ml orange juice 'with bits'

	0-2 h		2-5 h		5-8 h		8-10 h		10-20 h		20-24 h		Total	
	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
3-HBA	0.0 ± 0.03	0.1 ± 0.1	0.4 ± 0.4	0.5 ± 0.5	0.5 ± 0.5	0.3 ± 0.3	0.3 ± 0.2	0.1 ± 0.1	0.5 ± 0.4	0.3 ± 0.3	0.1 ± 0.1	0.1 ± 0.1	1.8 ± 1.5	1.4 ± 1.3
3-HPAA	1.7 ± 0.6	1.2 ± 0.6	1.9 ± 1.1	1.2 ± 0.6	2.0 ± 0.8	1.0 ± 0.5	1.5 ± 0.6	0.6 ± 0.2 *	3.9 ± 1.1	1.4 ± 0.5	0.8 ± 0.3	0.7 ± 0.4	11.8 ± 3.4	6.0 ± 2.3
4-HBA	14.9 ± 3.5	13.2 ± 3.8	14.8 ± 3.7	14.9 ± 4.3	13.7 ± 3.2	11.8 ± 2.9	2.7 ± 1.1	3.0 ± 1.3	7.4 ± 1.9	7.0 ± 3.6	3.5 ± 2.4	0.6 ± 0.2	56.8 ± 11.5	50.5± 12.3
4-HPAA	13.1 ± 1.8	15.5 ± 2.1	12.1 ± 1.6	13.3 ± 2.2	15.3 ± 2.5	19.2 ± 2.2	9.4 ± 2.2	14.9 ± 4.6	27.6 ± 5.1	27.7 ± 3.6	6.4 ± 1.7	3.5 ± 0.6	83.5 ± 10.4	94.0 ± 7.7
VA	4.1 ± 1.8	3.2 ± 2.2	3.7 ± 1.5	4.1 ± 1.7	4.7 ± 2.4	3.0 ± 1.6	1.5 ± 0.7	1.1 ± 0.8	3.9 ± 2.0	1.0 ± 0.4	1.0 ± 0.7	0.9 ± 0.5	18.9 ± 8.2	13.3 ± 4.6
3-m-4-HPAA	8.9 ± 1.4	9.1 ± 1.6	9.4 ± 1.4	10.3 ± 1.9	9.2 ± 1.7	9.9 ± 1.9 &	4.0 ± 0.9	4.3 ± 0.9	8.5 ± 1.5	8.5 ± 1.1	2.2 ± 0.6	1.4 ± 0.2 &	42.0 ± 5.2	43.4 ± 6.0
3.4-DHBA	2.1 ± 1.2	0.8 ± 0.6	2.7 ± 1.6	0.8 ± 0.6	2.0 ± 1.2	1.2 ± 0.8	1.2 ± 0.7	0.5 ± 0.4	3.9 ± 1.9	0.6 ± 0.5	0.4 ± 0.2	0.1 ± 0.0	12.3 ± 5.4	3.9 ± 2.3
3,4-DHPAA	1.7 ± 0.7	1.9 ± 0.8	2.1 ± 0.9	3.6 ± 1.7	1.6 ± 0.6	5.6 ± 2.6	1.0 ± 0.5	1.3 ± 0.5	1.3 ± 0.5	2.8 ± 1.1	0.3 ± 0.2	0.9 ± 0.3	8.0 ± 2.8	16.0 ± 5.9
3-h-4-MPHA	7.4 ± 1.4	13.6 ± 4.4	8.9 ± 1.8	11.1 ± 2.4	9.7 ± 1.4	17.3 ± 7.7	5.8 ± 1.6	7.1±1.9	15.0 ± 3.7	14.5 ± 2.8	4.6 ± 1.6	3.0 ± 0.8	51.2 ± 8.8	66.5± 14.6
HA	120.0 ± 28.0	186.2 ± 86.2	114.1 ± 30.8	106.4 ± 35.4	203.9 ± 39.5	199.0 ± 45.6	148.8 ± 34.7	153.3 ± 41.1	373.1 ± 118.8	384.4 ± 59.1	77.8 ± 13.3	85.5 ± 19.7	1032.7 ± 229.6	1109.2 ± 225.5
3-HPHA	4.9 ± 1.6	3.2 ± 1.0	4.3 ± 1.5	4.8 ± 1.9	7.8 ± 3.0	10.3 ± 4.3	2.8 ± 1.1	2.8 ± 1.0	6.5 ± 1.7	5.0 ± 1.3	1.6 ± 0.4	1.1 ± 0.4	27.7 ± 6.3	27.1 ± 7.0
3-m-4-HPPA	1.1 ± 0.5	4.0 ± 1.4	2.8 ± 1.0	3.8 ± 1.6	6.0 ± 1.4	6.3 ± 1.4	2.0 ± 0.7	1.9 ± 0.6	2.5 ± 1.1	3.3 ± 1.0	1.1 ± 0.7	0.8 ± 0.2	15.4 ± 3.8	20.1 ± 5.3
3,4-DHPPA	0.6 ± 0.5	0.6 ± 0.5	0.6 ± 0.4	1.7 ± 0.8	0.7 ± 0.4	0.8 ± 0.4	0.4 ± 0.3	0.7 ± 0.4	0.7 ± 0.4	0.6 ± 0.4	0.3 ± 0.2	0.4 ± 0.3	3.4 ± 1.8	4.7 ± 2.3
3-HHA	7.1 ± 1.8	6.8 ± 1.7	7.1 ± 1.5	6.8 ± 2.0	9.9 ± 1.9	9.9 ± 2.3	7.1 ± 2.0	7.1 ± 2.3	20.7 ± 5.6	25.3 ± 9.3	5.9 ± 2.2	3.5 ± 1.0	57.3 ± 11.8	59.1± 12.1
4-HHA	17.4 ± 1.9	13.0 ± 2.3	0.4 ± 0.4	0.5 ± 0.5	22.9 ± 2.9	23.4 ± 3.2	0.3 ± 0.2	0.1 ± 0.1	27.3 ± 5.5	28.2 ± 7.4	9.0 ± 2.6	4.3 ± 0.8	108.0±11.2	99.8± 14.1

Values are expressed as means ± SE (n = 15). 3-HBA: 3'-hydroxybenzoic acid, 3-HPAA: 3'-hydrophenylacetic acid, 4-HBA: 4'-hydroxybenzoic acid, 4-HPAA: 4'-hydrophenylacetic acid, VA: vanillic acid, 3-m-4-HPAA: 3-methoxy-4-hydroxyphenylacetic acid, 3.4-DHBA: 3, 4-dihydroxybenzoic acid, 3,4-DHPAA: 3, 4-dihydroxyphenylacetic acid, 3-h-4-MPHA: 3-hydoxy-4-methoxphenylhydracrylic acid, HA: hippuric acid, 3-HPHA: 3'-hydoxyphenylhydracrylic acid, 3-m-4-HPPA: 3-(3-methoxy-4-hydroxyphenyl) propionic acid, 3,4-DHPPA: 3-(3', 4'-dihydroxyphenyl) propionic acid, 3-HHA: 3'-hydroxyhippuric acid, 4-HHA: 4'-hydroxyhippuric acid, univariate analysis of variance test with post hoc LSD was used to determine significant differences * significantly different from baseline at $p \leq 0.05$.

6.3.6 Effect of 4 weeks moderate intensity exercise on urinary excretion of phenolic acids in sedentary humans

There were 15 phenolic acids detected in urine samples, including 3'-hydroxybenzoic acid, 3'-hydrophenylacetic acid, 4'-hydroxybenzoic acid, 4'-hydrophenylacetic acid, vanillic acid, 3-methoxy-4-hydroxyphenylacetic acid, 3, 4-dihydroxybenzoic acid, 3, 4-dihydroxyphenylacetic acid, 3-hydoxy-4-methoxphenylhydracrylic acid, hippuric acid, 3'-hydoxyphenylhydracrylic acid, 3-(3-methoxy-4-hydroxyphenyl) propionic acid, 3-(3', 4'-dihydroxyphenyl) propionic acid, 3'-hydroxyhippuric acid, 4'-hydroxyhippuric acid (Table 6-7). The baseline level of urinary phenolic acids in urine collected overnight prior to consumption of orange juice 'with bits', was similar before and after 4 weeks physical activities intervention in exercise group (82.9 ± 15.5 µmol vs. 81.8 ± 15.8 µmol, p = 0.87).

Cumulative urinary excretion of phenolic acids without hippuric acid was not affected through 4 weeks moderate intensity exercise (498.0 ± 37.2 µmol vs. 505.8 ± 40.8 µmol) (Figure 6-5). During the period of 8-24 h after orange juice intake, cumulative urinary excretion of 3'- hydroxyl phenylacetic acid was significantly reduced after 4 weeks moderate intensity exercise (7.2 ± 2.5 µmol vs. 3.9 ± 1.8 µmol, p ≤ 0.01) (Figure 6-6).

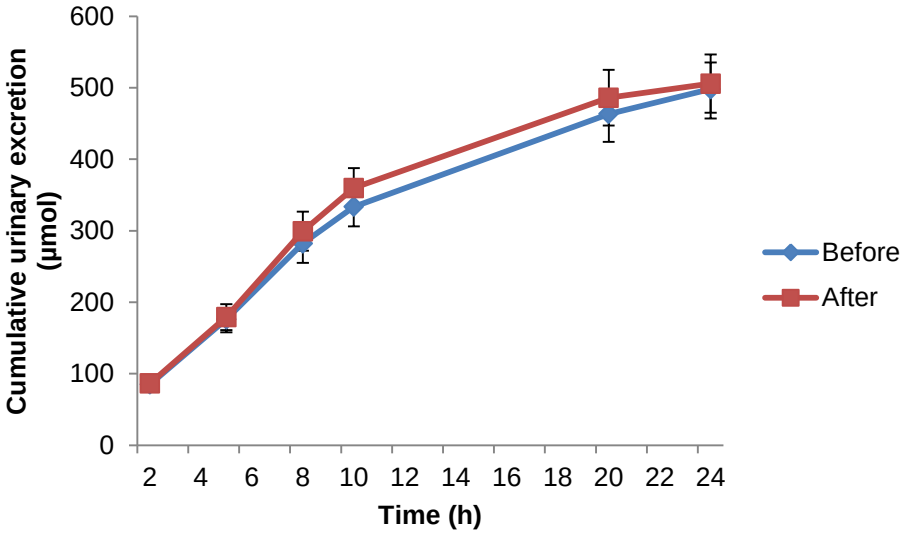


Figure 6-5 Urinary excretions (µmol) of phenolic acids without hippuric acid during 24 h after intake of orange juice before and after 4 weeks moderate intensity exercise.

Values are means ± SE (n = 15), ANOVA with post host hoc HSD was used to determine significant differences, * significantly different after 4 weeks of exercise programme, P ≤ 0.05.

For excreted phenolic acids over 0-2, 2-5, 5-8, 8-10, 10-20, and 20-24 h urine, there was no significant difference before and after 4 weeks moderate intensity physical exercise (Figure 6-6). The production of 3'-hydrophenylacetic acid reduced after 4 weeks of exercise programme over 8-10 h after orange juice intake (1.5 ± 0.6 µmol vs. 0.6 ± 0.2 µmol, $p < 0.05$) (Figure 6-7).

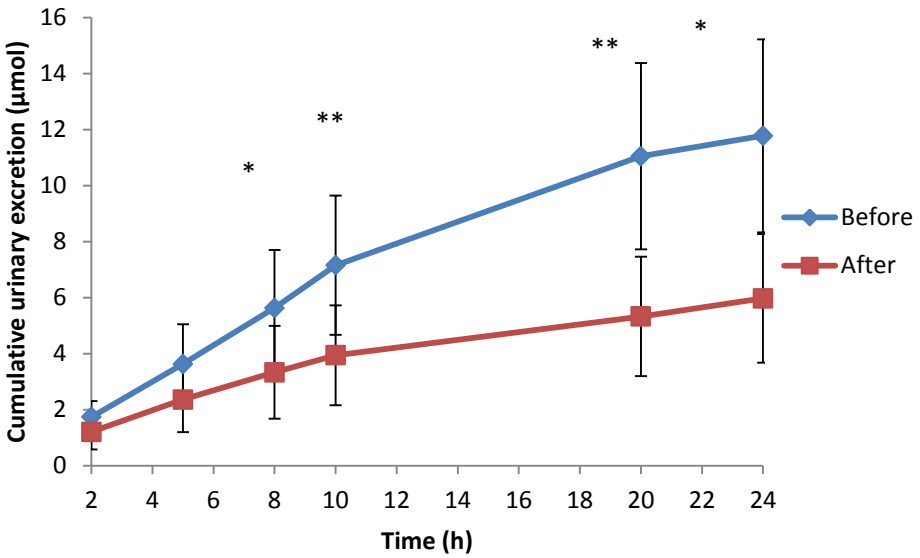


Figure 6-6 Cumulative urinary excretions of 3'- hydroxylphenylacetic acid (µmol), over 24 h after 500 ml of orange juice 'with bits' before and after 4 weeks of exercise

Values are means ± SE (n = 15). ANOVA with post host hoc HSD was used to determine significant differences * significantly different after 4 weeks of exercise programme, P < 0.05, ** significantly different after 4 weeks

6.3.7 Effect of 4 week moderate intensity exercise intervention on urinary excretion of total metabolites in sedentary females

The total metabolites (flavanone metabolites and phenolic acids) were 594.4 ± 40.3 µmol and 592.8 ± 46.0 µmol before and after 4 weeks physical exercise in urine over 24 h after consumption of orange juice 'with bits' (Table 6-8). Exercise intervention had no effect on total flavanone and phenolic metabolite excretion and the metabolites were mainly excreted during the period of 5 - 8 h (Before, 134.3 ± 7.3µmol; after, 152.4 ± 15.9 µmol) and 10 - 20 h (Before, 151.1 ± 23.6 µmol; After, 145.1 ± 26.4 µmol) after orange juice consumption (Table 6-8).

Table 6-8 Urinary excretion of total metabolites (µmol) over 24 h after intake of orange juice before and after 4 week moderate intensity exercise programme in previously sedentary females

	0-2 h	2-5 h	5-8 h	8-10 h	10-20 h	20-24 h
Before	92.5 ± 6.0	118.9 ± 13.9	136.4 ± 6.0 ƒ¥	53.7 ± 10.9 *£	164.2 ± 33.1 ƒ¥	46.9 ± 14.3 *£
After	96.6 ± 11.0	109.6 ± 15.0	152.7 ± 22.0 ƒ¥	64.1 ± 16.8 *£	160.4 ± 36.8 ƒ¥	22.3 ± 5.3 *£

Values are means ± SE (n = 10).ANOVA tests with post hoc LSD analysis were performed to determine different, * significantly different from 5 - 8 h of urinary excretion, $p < 0.05$; ƒ significantly different from 8-10 h of urinary excretion, $p < 0.05$; £ significantly different from 10-20 h of urinary excretion, $p < 0.05$; ¥ significantly different from 20 - 24 h of urinary excretion, $p < 0.05$.

3-hydroxyphenylacetic acid, 3-hydroxyphenylhydracrylic acid, dihydroferulic acid, 3-methoxy- 4-hydroxyphenylhydracrylic acid, 3-(3-hydroxy-4- methoxyphenyl)hydracrylic acid, 3-(3-hydroxy-4-methoxyphenyl)propionic acid, 3-(4-hydroxy-3-methoxyphenyl)propionic acid, 3-methoxy-4-hydroxyphenylacetic acid, 3-hydroxyhippuric acid, and 4-hydroxyhippuric acid has been considered as main phenolic acids produced from flavanone in urine after consumption of orange juice (Roowi et al., 2009b, Pereira-Caro et al., 2014a). Thus, 7 phenolic acids including 3-hydroxyphenylacetic acid, 3-methoxy-4-hydroxyphenylacetic acid, 3-hydroxy-4- methoxyphenyl)hydracrylic acid, 3-hydroxyphenylhydracrylic acid, dihydroferulic acid, 3-hydroxyhippuric acid, and 4-hydroxyhippuric acid, were contributed to metabolites with hesperetin and naringinin metabolites, which was around 33 % of consumed flavanones in urine over 24 h after orange juice intake (Table 6-9).

6.4 Discussion

The main finding of this study is that 4 week moderate intensity training programme did not affect urinary excretion of total metabolites (flavanone metabolites and phenolic acids) over 24 h after intake of orange juice in sedentary females. At the same time it demonstrated that following a 4 week exercise programme urinary excretion of flavanone metabolites from orange juice was diminished in some fractions. Hesperetin metabolites significantly decreased in urine collected over 0-5 h after juice consumption. In addition, the urinary excretion of 3-hydroxyphenylacetic acids was significantly reduced over 24 h after consumption of orange juice. The MCTT was prolonged from 3.0 ± 0.2 h to 4.5 ± 0.7 h in females by 4 weeks moderate intensity exercise programme. Physical exercise did not affect

anthropometric characteristics in healthy sedentary females: weight, BMI, body fat, waist circumference, and SBP, DBP, or heart rate (bpm).

Table 6-9 Cumulative urinary excretion of metabolites as a percentage of intakes (%) over 24 h after intake of orange juice before and after 4 weeks moderate exercise in previously sedentary females

Time (h)	Before	After
0	--	--
0-2	4.4 ± 0.4	4.5 ± 0.5
0-5	10.8± 0.9	10.3± 1.1
0-8	18.6 ± 1.0	19.3 ± 2.0
0-10	22.7± 1.4	23.4± 2.3
0-20	31.3 ± 2.5	32.0± 3.2
0-24	33.8± 2.8	33.2± 3.3

Values are means ± SE (n = 15). ANOVA tests with post hoc LSD analysis were performed to determine different from before, $p < 0.05$.

Previous studies showed variable bioavailability of flavanone metabolites (1 ~ 16 %) as a percentage of flavanone intake, 1.4 – 33 % of narirutin intake and 0.7 -14 % of hesperidin intake from orange juice, citrus juice or pure compounds (Vallejo et al., 2010c, Mullen et al., 2008a, Manach et al., 2003, Bredsdorff et al., 2010, Perez-Jimenez et al., 2010, Ameer et al., 1996). Brett et al revealed no significant different urinary production of hesperetin and naringenin between orange fruit and juice (Brett et al., 2009). Vallejo et al found lowest urinary excretion of flavanone metabolites after consuming pulp enriched juice (1.3 %) compared with other four beverages (two commercial orange juice, flavonoid rich orange juice, and water containing orange flavanone extract) (Vallejo et al., 2010c). Our study had a similar bioavailability of flavanone that was 7.8 % and 7.1 % over 24 h after consumption of orange juice in females before and after 4 weeks moderate intensity exercise programme.

Several studies demonstrated that structure and solubility of flavonoids determine their bioavailability (Mullen et al., 2008a, Brett et al., 2009, Vallejo et al., 2010c). Narirutin is more soluble than hesperidin in orange juice (Vallejo et al., 2010c). It is consistent with our study that naringenin metabolites represented 23.2 % and 21.9 % of narirutin intake, significantly higher than that of hesperidin intake (Before: 6.0 %, after: 5.3 %) in females before and after 4 weeks physical exercise.

Previous studies showed phenolic acids to be the majority of metabolites in urine after orange juice intake (37 - 88 %) (Pereira-Caro et al., 2014a, Roowi et al., 2009b). Hippuric acid is the product of glycine conjugated with benzoic acid (Hutt and Caldwell, 1990), mainly from amino acids and plant phenolics derived from polyphenols by gut bacteria, and sodium benzoate (Sakai et al. 1995). In our study, urinary excretion of metabolites was ~ 33 % of flavanone intake before and after the 4 week exercise in females.

It is well known that flavanone linked to a rhamnose moiety (hesperidin and narirutin) need to reach the colon for hydrolysis and cleavage into aglycones and then be absorbed by passive diffusion through the epithelial cells into blood stream (Manach et al., 2004). In addition, absorption, metabolism and bioavailability depends on the solubility of flavonoids, the interaction with ingredients in diet, intestinal enzymes, transporters, and the metabolic activity of gut microbiota (Neilson and Ferruzzi, 2011, Rein et al., 2013). Thus, gut transit time might determine rate of flavanone metabolism by gut bacteria. In a previous study, mouth to cecum transit time increased in 9 subjects but decreased in 5 even though transit time was not significantly modified by physical activity or fitness (Bingham and Cummings, 1989). In our study, 4 weeks of participation in moderate intensity exercise programme prolonged mouth to caecum transit time from 3.0 ± 0.2 h to 4.5 ± 0.7 h in healthy previously sedentary females. Prolonged transit time found in this study was matched with lower excretion of flavanones in urine over 0-5 h after orange juice intake following 4 weeks of moderate intensity exercise training (Before, 29.7 ± 2.9 µmol, After, 22.0 ± 2.9 µmol; $p < 0.05$). Lower urinary excretion of hesperidin occurred over 0-5 h after the intake of orange juice 'with bits' in females. It could be that longer delivery of orange juice to the intestine tract after exercise training delayed degradation of flavanones by gut bacteria.

In addition, longer residence of flavanone in the upper GI tract tended to reduce bio accessibility of hesperidin from orange juice reaching to the colon (data not shown), which probably also reduced urinary excretion of hesperidin metabolites over 5 h after orange juice intake in females after 4 weeks exercise programme.

Data obtained in this study are different from Medina's study shows fivefold urinary excretion of flavanone metabolites in athletes after consuming 200 ml of aronia – citrus juice, compared with sedentary volunteers who drunk 400 ml of the juice (Medina et al., 2012). The reason might be the one week diet (including plant based food) designed for athletes, but not

for non-trained volunteers. Gut bacteria influenced by diet (Vendrame et al., 2011, Roberfroid et al., 1995), might change bioavailability and metabolism of flavanone. In addition, exercising modifies physiological function, such as gastrointestinal hormones (O'connor et al., 1995), blood flow (Duncker and Bache, 2008, Flamm et al., 1990, Khazaeinia et al., 2000), in turn probably affected urinary excretion of flavanone metabolites. In addition, food sources affected metabolism of flavanone in the GI tract (chapter 3). Bioavailability of flavanones could be different, even though both of groups daily consumed similar amounts of flavanone.

What is more, flavanone sulfates are resistant to hydrolysis by glucuronidase. As a result, hydrolysis of urine might cause inaccurate evaluation of flavanone metabolites in Medina's study. However, it provided an insight that physical exercise tended to affect bioavailability of flavanone, perhaps due to physiological changes of the intestine and gut function (Lippi et al., 2008, Medina et al., 2012).

In our study, 4 weeks intensive exercise reduced 0-5 h urinary excretion of hesperetin metabolites (21.6 ± 2.8 μmol vs. 14.0 ± 2.0 μm, $p < 0.05$), and 24 h urinary excretion of hesperetin -O- glucuronide (10.4 ± 2.9 μmol vs. 6.2±1.7 μm, $p < 0.02$), and 3-hydroxyphenylacetic acid (11.8 ± 3.4 μmol vs. 6.0 ± 2.3 μmol, $p < 0.05$) in previously sedentary females after orange juice intake. Physico-chemical characteristics of flavonoid and their metabolites, hydroxyl groups of the A and B ring, their methylation and unsaturated bond, affinity to human serum albumin, their distribution to target tissues, and remaining in faeces influence their bioavailability in the host (Xiao and Kai, 2012, Bolli et al., 2010, Czank et al., 2013, Faria et al., 2013, Wang et al., 2013, Challa et al., 2013, Perez-Vizcaino et al., 2012, Gonzales et al., 2015). Whole bowel transit time was shortened in humans by chronic exercise (Koffler et al., 1992, Oettle, 1991).Thus, it is supposed that physical activity might influence distribution and elimination of flavanone metabolites in subjects, or decrease time of cleavage by gut bacteria in the colon.

On the other hand, the degradation of flavanones depends on cleavage activity of gut bacteria. Some species are responsible for demethylation, dihydroxylation, decarboxylation, C-ring fission of flavonoids (Aura, 2008, Selma et al., 2009). Ruminococcus, Eggertheilla, Bifidobacterium, Clostridium, Bacterioides, Eubacterium, Streptococcus and Lactobacillus

bacteria have been evidenced their capacities to degrade flavonoids (Clavel et al., 2005, Keppler and Humpf, 2005, Schneider et al., 1999, Blaut and Clavel, 2007, Kim et al., 1998).

The composition and amount of gut microbiota are individually variable due to diet, life-style, disease, and antibiotic use (Nicholson et al., 2012). In an animal study, the number of Lactobacillus, Bifidobacterium and B. coccoides–E. rectale group increased in rats subjected to a fixed 6 days exercise, in comparison with rats restricted to exercise (Queipo-Ortuño et al., 2013). In addition, 6 weeks of exercise increased Bifidobacterium spp. in normal mice, but not diabetic mice (Lambert et al., 2015). As mentioned before, higher diversity of gut microbiota has been observed in rugby players compared with sedentary subjects (Clarke et al., 2014). In this study, the 24 h urinary excretion of hesperetin-O-glucuronide and 3-hydroxyphenylacetic acid reduced in females after the 4 week exercise, which also indicated that gut bacteria was probably affected by the intensive exercise. In addition, Clark and colleagues revealed a higher diversity of gut bacteria in athletes than in sedentary controls when their diet was considered, Lactobacillaceae, Bacteroides and lactobacillus were similar between athlete and control (BMI < 25) (Clarke et al., 2014), which were confirmed their ability to hydrolyse flavonoids (Hawksworth et al., 1971, Kim et al., 1998). Thus, the production of metabolites over 24 h might be attributed to gut bacteria related to metabolism of flavanone that should be investigated in future.

In conclusion, 4 weeks physical exercise modified the dynamics of flavanone metabolism in the sedentary females to some extent but had no impact on the 24 h bioavailability of flavanone and phenolic metabolites. The 4 weeks moderate intensity physical exercise prolonged MCTT so that delay metabolism of flavanone by gut bacteria to reduce 0-5 h urinary excretion of hesperidin and 0-10 h urinary excretion of naringenin after the consumption of orange juice with bits. In this study, ~ 67 % of flavanone intake was not recovered in urine 24 h after consumption of orange juice ‘with bits’. It is worth investigating their pharmacokinetics in plasma and faecal samples to further reveal the effects of physical exercise on metabolism of flavanones. The composition of the gut microbiota should be further investigated to assess effects of physical exercise relevant to metabolism of flavanone. In addition, animal studies would be a good way to explore effects of physical exercise on tissue distribution of metabolites. Considering the complex profile of phenolic metabolites, it would be interesting to explore if modification of metabolites can be the mechanism which associates physical activity with benefits against chronic diseases and cancers.

CHAPTER 7 *Discussion*

Flavonoid metabolites as markers in plasma and/or urine are used to investigate the bioavailability of flavonoids and relate their intake to human health (Serra et al., 2012, Roura et al., 2007a, Perez-Jimenez et al., 2010, Day et al., 2001, Gao et al., 2006a, Grün et al., 2008, Keppler and Humpf, 2005, Mullen et al., 2008a, Jaganath et al., 2009). Flavonoids can be absorbed as the parent compound, conjugated metabolites or phenolic acids, which are produced in the large intestine. After consideration of the relevant literature, it was clear that food sources, food matrix, age, ethnicity and lifestyle (alcohol consumption, smoking, physical activity) (Figure 7-1) may have positive or negative effects on metabolism of dietary polyphenols and phenolic acids in the host.

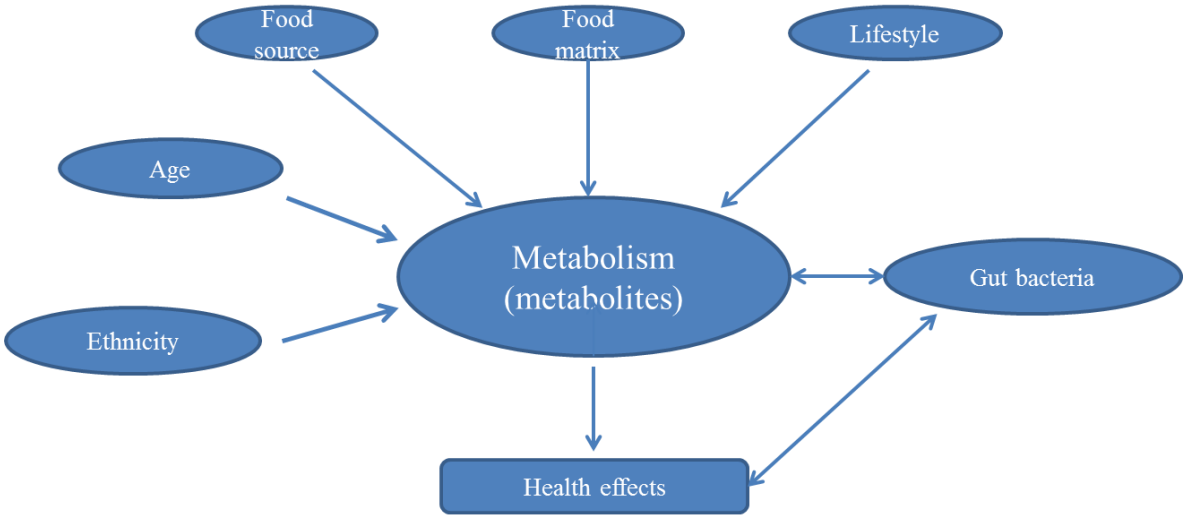


Figure 7-1 Factors affect metabolism of flavonoids in the host

7.1 Research questions

The research questions addressed in this thesis build on previously established evidence that bioavailability of dietary flavanones varied substantially ranging from 0.8 ~ 16 % in humans after consumption of orange juice (Pereira-Caro et al., 2014b, Brett et al., 2009, Bredsdorff et al., 2010, Erlund et al., 2001c, Silveira et al., 2014, Vallejo et al., 2010c, Tomas-Navarro et al., 2014, Manach et al., 2003), and how to enhance bioavailability of flavanone to enhance health in humans. On the other hand, inclusion of phenolic acids (ring fission products of flavanone) in the calculation to estimate bioavailability resulted in urinary excretion values ranging from 37 to 88 % after orange juice intake (Pereira-Caro et al., 2014a, Roowi et al., 2009a). In the past, studies reported individually different bioavailability of flavonoids in humans (Alkhaldy et al., 2012b, Halm et al., 2007, Alkhaldy et al., 2012a). Thus, in this

thesis, we focused on other factors. One factor highly likely to affect the colonic bacterial metabolism of flavanones and production of phenolic acids is the presence of dietary fibre. Very little has been published on the impact of dietary fibre in this context although there is evidence that inclusion of fermentable carbohydrate in *in vitro* fermentations can alter the phenolic acids produced but the exact effect depended on the type of carbohydrate included. In previous studies, glucose enhanced rate of rutin disappearance in *in vitro* fermentation (Jaganath et al., 2009), but it did not affect metabolism of hesperidin (Hou et al., 2015). However, dietary fibre (pectin, raftiline and ispaghula) reduced phenolic acid production from rutin (Mansoorian et al., 2015).

In addition, Mullen *et al* reported reduced urinary excretion of flavanone metabolites 0-5 h after orange juice consumption combined with intake full fat natural yoghurt (Mullen et al., 2008a). The yoghurt also reduced urinary excretion of five phenolic acids including 3-hydroxyphenylacetic acid, 3-hydroxyphenylhydracrylic acid, dihydroferulic acid, 3-methoxy-4-hydroxyphenylhydracrylic acid and 3-hydroxyhippuric acid from 62 ± 18 μmol to 6.7 ± 1.8 μmol (Roowi et al., 2009a). However, mechanisms responsible for yoghurt affects bioavailability of flavanone in humans remain unclear.

There is some evidence to suggest that the level of physical activity is also among host factors influencing bioavailability of flavanone metabolites. The study of Medina et al reported that urinary excretion of flavanone metabolites after consumption aronia juice intake was five times greater in triathletes than sedentary individuals (Medina et al. 2012). Indirect evidence suggests that increased bioavailability of polyphenols reported in trained athletes might be related to greater diversity in the gut bacteria of athletic individuals (Clark et al, 2014). However, it should be noted that the cross sectional design of the Medina's study did not allow control for rather large inter-individual variation in urinary excretion of flavanone metabolites and may provide misleading information. Thus, more and better designed studies are needed. Indeed, the expectation that enhanced participation in exercise should increase bioavailability might be misleading since several mechanisms operate and might interact with each other to influence of bioavailability under trained and sedentary conditions. For example, a study using radio-opaque markers to measure whole gut transit time reported that both cycling and running for one week significantly reduces total gut transit time (how long it takes for the food to move from mouth to the end of intestine) (Oettle, 1991) and thus

indirectly suggests that exercise training can be expected to reduce rather than increase flavanone metabolite bioavailability.

The aim of the research in this thesis therefore was to understand how bioavailability of flavanones is low and variable in humans, and increase awareness of these issues for designing future studies. This led to the following questions:

- Does the food matrix (in this case fermentable fibre and yoghurt) affect the digestion and degradation of flavanones in the gastrointestinal tract?
- How variable is the content of flavanones in different brands of orange juice and how is affected by the pulp content or fermentable carbohydrate?
- Does yoghurt affect bioavailability of flavanones and if so what is the mechanism-, eg probiotic bacteria, and are the effects in the small intestine or in the colon by changing bacterial degradation?
- Does participation in physical exercise affect the bioavailability and metabolism of orange juice flavanone in healthy sedentary humans?

7.1.1 Does the food matrix affect digestion and degradation of flavanones in the gastrointestinal tract?

The digestion and degradation of flavanone was investigated by using in vitro and in vivo models. The metabolites detected in urine in humans after ingesting orange juice enriched with pulp were, hesperetin-*O*-glucuronide (3 isomers), naringenin-*O*-glucuronide (2 isomers), naringenin-*O*-sulfate (2 isomers), hesperetin-*O*-diglucuronide, hesperetin-*O*-sulfate-*O*-glucuronide, hesperetin-*O*-sulfate-*O*-glucoside, hesperetin-*O*-sulfate and naringenin-*O*-diglucuronide. Urinary excretion of flavanones metabolites in this study was 9.4 % of flavanone intake. When this was compared with other published studies it was clear that there was a large variability in reported bioavailability ranging from 0.8 % to 16 % from different sources (Vallejo et al., 2010c, Mullen et al., 2008a, Manach et al., 2003, Bredsdorff et al., 2010, Perez-Jimenez et al., 2010, Ameer et al., 1996). In these studies there was very little detail of the source or composition of the orange juice used. Silveira et al compared freshly-squeezed and commercially processed orange juices and showed processed orange juice had lower bioavailability (4.1 % and 3.8 % of hesperidin intake, 3.8 % and 8.7 % of naringenin intake) (Silveira et al., 2014). However, the reasons for the large variability in bioavailability

in published studies have not been investigated. This was why in this thesis. I chose to study different factors that may affect the variability in gut metabolism of flavanones and phenolic acid production.

I used a simulated GI tract model which mimicked the stomach, small intestine and large intestine of humans. This model is often used in studies of digestion (Pérez-Vicente et al., 2002, McDougall et al., 2005, Sengul et al., 2014, Gil-Izquierdo et al., 2003, Gil-Izquierdo et al., 2001) and fermentation (Saura-Calixto et al., 2010, Serra et al., 2012) and was a good way to consider any factors which influenced the release of metabolites from foods and the colonic bacterial degradation of flavanones. In this thesis, in vitro model used gastrointestinal digestion and colonic fermentation with gut bacteria to reveal stability / release of flavanone in orange juice, degradation of flavanone and production of phenolic acids by gut bacteria, and effects of food matrix on these indexes.

In our study of the metabolism and degradation by gut bacteria of orange juice flavanones from different sources (Chapter 3), we had thought that orange juice with bits may be affected by the presence of dietary fibre in the bits. However, the juices with and without bits had no difference in their fibre content and indeed not much difference in the flavanone content. In addition we thought that there might be non-extractable polyphenols that are linked to cell wall constituents (polysaccharides and protein) or can bind with food matrix by hydrophobic characteristics (Arranz et al., 2010, Hasni et al., 2011, Yuksel et al., 2010) in the ‘ bits’ that were not detected by the usual analytical procedures (Perez-Jimenez et al., 2013). However, there were similar amounts of polyphenols in orange juice with bits after enzymatic hydrolysis with pectinase / cellulose or driselase compared with the extraction process by measuring total phenols. Hesperidin can be transformed to chalcones under GI physiological conditions and precipitated to interact with proteins, pectins, or other carbohydrates in orange juice (Gil-Izquierdo et al., 2001). In chapter 3, hesperidin was found to be more stable in smooth orange juice than orange juice ‘with bits’ in the upper GI tract, especially the gastric phase, while narirutin was similar in both juices. Thus, these ‘bits’ indeed affected passage of hesperidin to the colon for fermentation, but it was not due to dietary fibre which was not found in significant amounts in the juice. Higher concentrations of hesperidin and total flavanones (hesperidin, hesperetin, narirutin and naringenin) were found from orange juice with ‘bits’ after 2 h fermentation with gut bacteria than orange juice without bits. Significant higher phenolic acids were observed at 6 h of fermentation by gut bacteria from orange juice

with bits. In this study, the metabolism of flavanone was therefore increased, but not due to the fibre content in orange juice. This may be related to transformed chalcones degraded by gut bacteria in the models.

Fermentable carbohydrates (dietary fibre) however in the food matrix may affect the bacterial metabolism of flavanones, such as hesperidin that is insoluble and abundant in orange juice, as they provide an energy source for the gut bacteria and may change the physico chemical properties of the colonic contents, eg by reducing pH. For instance, raftiline reduced phenolic acid production from rutin (Mansoorian et al., 2015).Thus, we studied the impact of raftiline as a soluble fibre which selectively stimulates the growth of lactic acid bacteria and is not viscous, on colonic bacterial metabolism of hesperidin and compared its effects to glucose which is fermented by most bacteria. In this study, raftiline also reduced production of phenolic acids by gut bacteria. In addition, the levels of aglycone remained higher during the fermentation. The gut microflora plays an essential role in the metabolism and bioavailability of flavonoids. They were deconjugated to release the aglycone and then produce metabolites such as phenolic acids (Selma et al., 2009). In several studies, dietary fibre has been shown to impact on intestinal microbiota composition, such as stimulated growth of *Bifidobacterium* and *Lactobacillus, E. rectale, Roseburia intestinalis,* and *Anaerostipes caccae* (Bouhnik et al., 1997, Holscher et al., 2015, Steer et al., 2003, Turner, 2008, Yang et al., 2013, Duncan et al., 2002, Hamaker and Tuncil, 2014). *Bacteroides*, *Bifidobacteria,* and *Lactobacilli* have the highest activity of the β-glucosidases and sulfatases responsible for glycoside hydrolysis and subsequent aglycone cleavage (Steer et al., 2003, Turner, 2008). In this study, raftiline significantly decreased production of phenolic acids from 220.3 ± 46.8 μM to 126.6 ± 44.1 μM by the 24 h fermentation of hesperidin by gut bacteria ($p < 0.05$). However, glucose as energy source for most of bacteria fastedcleavage of hesperidin during fermentation, but did not affect the production of phenolic acids after 24 h of fermentation. Thus, dietary fibre should be considered as a factor to influence metabolism of flavanone in the colon.

7.1.2 Does yoghurt affect flavanone release from orange juices in the gastrointestinal tract?

Full fat natural yoghurt reduced urinary excretion of flavanone metabolites 0-5 hours after orange juice ingestion and 24 h urinary excretion of five phenolic acids in previous studies (Mullen et al., 2008a, Roowi et al., 2009b). In this thesis, the mechanism of yoghurt affecting

metabolism of flavanone in the GI tract was investigated by using GI digestion and fermentation models (Chapter 4). Our study was inconsistent with Roowi study that full fat natural yoghurt reduced urinary excretion of phenolic acids six fold in healthy subjects after consumption of orange juice. In our study, full fat natural yoghurt enhanced the recovery of hesperidin content in smooth orange juice and orange juice with bits during the upper GI digestion, but did not affect the concentration of hesperetin and naringenin derived from hesperidin and naringenin in orange juice with bits by gut bacteria, and phenolic acids production during the colonic fermentation.

Yoghurt also contains lactic acid bacteria, *Streptococcus thermophilus* and *Lactobacillus delbrueckii spp. Bulgaricus* (Ng et al., 2011), as probiotic yoghurt for health properties. These bacteria might also impact metabolism of flavanone in orange juice when consumed with this type of yoghurt. At the same time, In a previous study, passion fruit or mixed berry improved survival of *L. acidophilus* LAFTI L10 during storage (Kailasapathy et al., 2008). In addition, fruits in yoghurt provide extra polyphenols to orange juice. Thus, fruit yoghurt might have different effects on release / stability of flavanone and production of phenolic acids during the upper GI digestion. In our study, the yoghurt significantly increased four phenolic acids in OJ during the upper GI digestion, but not in OJ bits. It indicated that types of yoghurt has different effects of phenolic acids, even yoghurt provided *Bifidobacterium spp* and *Lactobacillus acidophilus* that might enhance production of phenolic acids derived from flavanones during the upper GI digestion. Moreover, full fat natural yoghurt increased phenolic acids in OJ bits. Variable yoghurt had different effects on phenolic acids during the digestion *in vitro*. Even though yoghurt starter culture bacteria could not survive in the acidic stomach condition, as shown by lack of growth on selective plates after the gastric phase *in vitro* model, but survival of probiotics and probiotics in the presence of polyphenols under the upper GI conditions has not been verified. Thus, survival of probiotics should be assessed so that potential effects of probiotics on metabolism of flavanone could be uncovered and reveal if these bacteria can play their role in the human colon and potential interaction between polyphenols and probiotics in the GI tract. Furthermore, the effect of yoghurt on absorption should be considered due to production of phenolic acids between *in vivo* and *in vitro* study.

7.1.3 Does physical exercise affect bioavailability and metabolism of orange juice flavanone in healthy sedentary humans?

In a cross sectional study, it was found that 24 h urinary excretion of naringenin, eriodictyol, and hesperetin metabolites were 92 %, 88 %, and 53 % higher in very well trained athletes than in sedentary individuals after ingestion of aronia-citrus juice (a mixture of citrus juice (95%) with 5% of *A. melanocarpa* juice containing 86.1 mg of flavanone per 400 ml) (Medina et al., 2012). Clark et al reported that there were 48 taxa significantly greater abundant in athletes than in high BMI (> 28) controls excluding *Bacteroidetes,* and 40 taxa excluding *Lactobacillaceae, Bacteroides* and *Lactobacillus* than low BMI (< 25) controls by measuring 16S rRNA of fresh stool samples (Clarke et al., 2014). Thus, the hypothesis in this thesis was that intensive physical activity affects flavanone bioavailability in sedentary humans. However, several previous studies focused on cross sectional populations with different lifestyles (active and sedentary), or animal models. Up to now, no study investigated effects of increased physical activity on bioavailability of flavanones, and phenolic metabolites in sedentary people. In our study, physical exercise involved a 4 week intervention in sedentary females, which would be better to provide information for general population and discover mechanism how physical activity affects bioavailability.

It was found that participation in 4 week moderate intensity exercise programme reduced urinary excretion of one isomer of hesperetin-*O*-glucuronide and 3-hydroxyphenylacetic acids, even though there was no effects on total flavanone metabolites and phenolic acids over 24 h after orange juice intake in females. Even though the results were contrary with Medina's study it should be noted that the current study allowed to control for large inter-individual variation in urinary excretion of flavanone metabolites and had longitudinal design allowing to compare bioavailability before and after exercise training. Even though higher diversity of gut bacteria was found in athletes, Lactobacillaceae, Bacteroides and Lactobacillus were similar with low BMI (< 25) sedentary controls (Clarke et al., 2014). In our study, finding of negative effect of 4 weeks of exercise programme on 24 h urinary excretions after orange juice intake in previously sedentary females may indicated that gut bacteria responsible for metabolism of flavanone was affected by 4 weeks moderate intensity physical exercise.

The 4 week exercise programme significantly reduced 0-5 urinary excretion of hesperetin metabolites after consumption of orange juice, and prolonged the mouth to caecum transit time, In addition to studies that the shortened whole bowel transit time (time from mouth to the end of intestine) by chronic exercise (Koffler et al., 1992, Oettle, 1991), which implies that gut physiological function potentially affect metabolism and absorption of flavanone.

In general, this study provides an insight that rate of flavanone metabolism tends to change directly by physical activity via intestinal physiological function. In relation to gut bacteria, extent of flavanone degradation was not observed. However, the pharmacokinetics of flavanone metabolites and phenolic metabolites and composition of gut bacteria have not been investigated in this thesis due to time constraints. Thus, we should be very cautious to conclude a limited effect of physical activity on the bioavailability of orange juice flavanone against Medina's and Clark's studies between well trained and sedentary populations.

7.2 Relevance and applications of findings:

This thesis provides information about some of the factors affecting metabolism and bioavailability of orange juice flavanone which may be linked to benefits of flavonoids and flavonoids enrich food in humans. These data may inform future research in epidemiological studies, experimental studies, and nutritional industries.

7.2.1 Implication for epidemiological and clinical researches

Many studies have demonstrated an association of polyphenols consumption with reduced risk of several chronic diseases and cancers. Diet, nutritional status, and lifestyle constitute comprehensive factors to act incidence of cancer and other chronic diseases (Edmands et al., 2015). In this thesis, food sources, food matrix (yoghurts and dietary fibre), and physical activities affected metabolism of flavanones, which might influence protective property of dietary flavonoids. Health properties of dietary polyphenols in studies might be misunderstood without considering food source, interaction with food matrix, and physical exercise. In addition, the details of sources of orange juice, or other polyphenol enrich food and food matrix should be carefully considered. Thus, these factors might enhance accuracy of future studies.

On the other hand, some clinical studies used pure polyphenol and randomly chosen polyphenol enriched diets without consideration of the food sources and aspects of the food matrix which may affect the final amounts of bioactive metabolites that will reach the tissues. This may impact on the possibility for correctly assessing the role of flavanones in reducing disease risk and cause error. Based on this thesis, these factors should be noted in future studies. In addition, metabolites are more abundant than their mother compounds. The amounts and types of metabolites released in the gut and reaching the tissues should also be considered for their bioactivity and such consideration may strengthen the association between flavanone intake and reduced incidence of diseases and cancers.

7.2.2 Implication for experimental and animal studies

Epidemiological and human clinical studies are important to understand the benefits of both polyphenol and polyphenol-rich foods. However, due to their expensive cost and time consuming nature, cell-culture techniques and animal models have been widely conducted to investigate mechanisms of polyphenols lowering risk of chronic disease. However, these studies usually use pure polyphenols rather than their metabolites to evaluate biological properties. These pure compounds or mixtures will not reflect real physiological bioactivities through consumption of flavonoids or flavonoids contained diet. Metabolites more complex than single or few metabolites used in those *in vitro* studies, which might display synergistic and / or antagonistic effects of metabolites in the host.

On the other hand, in animal studies, high doses of polyphenols are often fed with other foods, such as dietary fibre, to observe biological effects. Their interaction in metabolism might cause error in bioactivity of flavonoids in humans. In addition, both of humans and animals have some differences in metabolic processes, especially conjugation process (Manach et al., 2004). Composition of polyphenol intake regarding to metabolites level in blood and / or urine might be more useful and valuable to evaluate health effects by using animal models. Thus, this thesis might be able to provide some information for these studies to improve study design to obtain valuable achievements.

7.2.3 Implication for nutrition and food industry

Polyphenols have been increasingly introduced in foods by the nutritional and food industries. In this thesis, it has been found that the bioavailability of polyphenols is influenced by food matrix, food matrix and lifestyles. In this thesis, the effects of food sources, food matrix and physical activities have been demonstrated in order to provide some information for nutrition and food industries to enhance polyphenols properties when they design new supplements or food products for consumers.

In general, this thesis has important data to improve other relevant flavonoid research, and other bioactive food components. These efforts hopefully provide scientific foundation for researches on potential health effects of flavonoids, and put academia, industry and the public together to improve human health.

7.3 Strengths and limitations

Strengths of this thesis was that the *in vitro* GI metabolism model was conducted to investigate mechanisms of factors (food sources and food matrix) affecting bioavailability of orange juice flavanone, including various stability /release of flavanone from different orange juice sources during the upper GI digestion, effects of yoghurts and carbohydrates on colonic metabolism. The physical activity intervention study explored effects of physical activity on the bioavailability of flavanones in a sedentary population through modification of physiological functions and/or gut bacteria.

Limitations and challenges in this PhD study included the stimulated GI models, recruitment of volunteers and other confounding factors, time limitations as followed. Firstly, it was very difficult to stimulate real physiological environment of GI tract by using *in vitro* model to reveal the bioavailability of flavanone in the gastrointestinal tract involving absorption. The limitation was that all types of yoghurt were not compared their effects on colonic metabolism by gut bacteria due to time limitation. In addition, intestinal absorption of flavanone *in vitro* was not involved by *in vitro* models in this thesis, which could reveal more information about effects of food sources and food matrix. Study recruitment took longer than expected as it was difficult to find females who were healthy Caucasian with sedentary lifestyle (less than 1h of physical activity), normotensive, non-smokers, and not taking any

medications, and were willing to change lifestyle for 4 weeks. In addition, many potential participants refused to participate in this study because of restricted intake of fruit, vegetables, dietary fibre, coffee, and tea for 3 days before and after the intervention. Some participants were not willing to change their lifestyle, because they did not want to spend 4 weeks on moderate intensity exercise. In addition, it was difficult for participants to stay in the metabolic room for 8 h to wait for sample collection during trial days.

Finally, survival of probiotics in probiotic yoghurt was not measured, even though it had a similar effect with full fat natural yoghurt on stability / release of flavanone during the upper GI digestion, where yoghurt starter culture bacteria could not be resistant to the gastric digestion. In addition, measurements of flavanone metabolites in control group and flavanone metabolites and phenolic metabolites in plasma and faeces were limited due to deadline of thesis submission, which potentially illustrate overall flavonoid metabolism in the host to reveal more details for academic, industrial and individual recommendation. In general, based on results in this thesis, the effects of food sources, food matrix and lifestyle should be considered as preliminary data to build up a bridge between polyphenols and their health effects in humans in future works.

7.4 Future work

Although this thesis investigated effects of food sources and food matrix on metabolism of orange juice flavanones, absorption was not assessed in this thesis. Absorption of flavonoids is linked to bioactivity of dietary flavonoids in the host (Gonzales et al., 2015). Thus, it is necessary to investigate if these factors influence absorption of flavonoids and phenolic acids. In this thesis, food sources, food matrix and physical activity affected metabolism of orange juice flavanone (flavanone and phenolic acids). In addition, bioactive concentration of metabolites in tissues should be established. If it can be built up, the effects of food sources, food matrix and physical activity on health will be confirmed so that these factors must be considered and provided in future epidemiological, experimental and clinical studies.

Although physical exercise was shown to affect urinary excretion of flavanone metabolites and phenolic acids, pharmacokinetic and faecal data have not investigated yet. These results will provide more details about the effects of physical exercise related on dietary flavonoids. The composition of gut bacteria should be estimated in order to explore if physical exercise

can modify gut bacteria relevant to metabolism of dietary flavanones. Thus, the following further research might help to provide more information:

- *In vitro* studies:
    1. To stimulate absorption of hesperetin, naringenin and phenolic acids through the intestinal epithelium cells by using cell lines, such as Caco 2, to investigate if absorption is synchronous with dynamic metabolism of orange juice flavanone so that influence concentration of metabolites in the host
    2. To investigate bioactivity of flavanone and phenolic acids in a range of physiological concentrations by using cellular techniques.
    3. To test survival of probiotics in probiotic yoghurt and polyphenol containing yogurt during the gastric digestion
- Intervention studies:
    1. Metabolites in plasma and faeces could be measured to investigate pharmacokinetics of metabolites and metabolite profiles, and distribution of metabolites in the host
    2. Biomarkers (oxidative stress, glucose, insulin, CRP) could be measured to investigate effects of orange juice flavanone in humans induced by physical exercise
    3. Gut bacteria could be identified and quantified to reveal if physical exercise can link to metabolism and bioavailability of flavanone through gut bacteria

7.5 Conclusion

Food sources and food matrix (yoghurt and carbohydrates) affected stability / release of flavanone during the upper GI digestion, and cleavage of flavanone and production of phenolic acids in the colon. Physical exercise affected MCTT so there was a shorter time period of flavanone metabolism after consumption of orange juice. Phenolic acids accounted for the majority of metabolites. Based on these results, metabolism of orange juice flavanone could be modified to some extent, so that more details about tested food (orange juice, other flavonoid-rich food), food matrix (yoghurts and fibre) and exercise levels of participants, as well as some unknown factors (diet habits, physical conditions) should be provided to enhance outcomes in current and future flavonoid relevant studies. On the other hand, in the past, there were a number of studies revealing bioactive properties of conjugated flavonoid aglycones (Trzeciakiewicz et al., 2009, Suri et al., 2010, Dueñas et al., 2011, Yamazaki et al., 2014) , and phenolic acids (Kampa et al., 2004, Amin et al., 2015, Henning et al., 2013, Kim et al., 1998). However, these compounds are conjugated as sulphated, glucuronided and

methylated metabolites present in circulatory system (Pimpao et al., 2015, Heleno et al., 2015), which have been very little studied (Gesso et al., 2015). Thus, there is a need to consider factors above to assess bioactivity and beneficial effects of dietary flavonoids in *in vivo* studies, and take attention to physiological concentrations induced by factors in *in vitro* studies.

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Appendices

Appendix-1

Labelled ingredients of orange juice in bottles

		Energy (KJ/Kcal)	Fat(g)	saturates(g)	Carbohydrates(g)	sugars (g)	Fibre (g)	Protein(g)	Salt(g)	Vitamin C
Tropicana	**OJ**	201/48	0	0	10	10	0.8	0.8	0	33
	OJ bits	204/48	0	0	10	10	0.7	0.8	0	32
Tesco	**OJ**	186/44	<0.1	<0.1	10.1	7.8	<0.1	0.8	<0.01	30
	OJ bits	186	<0.1	<0.1	10.1	8.1	<0.1	0.8	<0.01	30
Innocent	**OJ**	180/42	0.1	0	9.4	8.1	0.6	0.7	0.01	21
	OJ bits	185/44	0.1	0	9.7	8.2	0.6	0.7	0.01	25
Morrison's	**OJ**	163/38	0.1	0	8.7	8.1	0.2	0.6	0	24
	OJ bits	164/39	0.1	0	8.8	8.3	0.2	0.5	0	24
Waitrose	**OJ**	181/43	0.1	--	9.5	9.3	0.5	0.7	0	46
	OJ bits	159/37	0.2	--	8.4	6.7	0.3	0.5	0.04	46
Sainsbury's	**OJ**	226/53	<0.5	<0.1	11.4	9.2	<0.5	0.7	0.03	40
	OJ bits	226/53	<0.5	<0.1	11.4	9.2	<0.5	0.7	0.03	40

Appendix-2

University of Glasgow

PARTICIPANT INFORMATION SHEET

Title of study

In vitro catabolism of Orange juice, hesperidin and narirutin by human faecal bacteria

Invitation to take part

Thank you for reading this.

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

What is the purpose of the study?

In previous study, the bioavailability of flavanones in orange juice, especially hesperidin and narirutin, was investigated in humans. Therefore this study will help to monitor the degradation products of dietary flavonoids and food matrix effects and to delineate the possible catabolic pathways involved in the degradation of flavonoids.

Why have I been chosen?

You are:

i) a healthy individual, aged 18-60, not obese, non-smoker, in good general health, not taking any supplement or medication

ii) you do not suffer from any allergy or condition affecting bowel health

Do I have to take part?

If you do decide to take part you will be given this information sheet to keep and will be asked to sign a consent form. If you decide to take part you are still free to withdraw at any

time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen to me if I take part?

You will meet a member of the Research Team from the University of Glasgow who will explain the study at a convenient time. All transport costs as well as costs associated to the study will be reimbursed, but we cannot offer any fees.

What do I have to do?

You will be invited to follow low polyphenol diet for two days (48 h). Diet sheets will be provided, and you will also be given a telephone number, to be able to call a member of the Research Team with any queries. You will be asked to tell us if you think you may have slipped up with any of the dietary advice.

Example of a typical intervention

Day 1	8 am		Start of the low phenolic diet (see examples)
Day 2	8 am	(24 hr)	Low phenolic diet
Day 3	8 am	(48h)	Return in the morning with faeces sample

The diet involves foods with *low polyphenol* content, such as meats, fish, pasta, bread (but avoiding tea, coffee, colourful fruits and vegetables). During this period, you will be asked to keep a dietary intake record.

You will be asked to record any possible slip-ups (e.g. eating high polyphenol foods by mistake during the low-polyphenol diet). It is very easy to make mistakes, but vital that we know in order to get reliable results.

At the end of the diet (after 48 h of low polyphenol diet) a faecal sample will be collected. The faecal sample should be collected in a special pot provided together with anaerobic pouch to keep the sample in anaerobic condition and send on the morning of day 3.

What are the possible disadvantages and risks of taking part?

There are no risks or disadvantages associated with this study other than time loss and inconvenience of following dietary restrictions.

What are the possible benefits of taking part?

There are no direct benefits to the volunteers associated with taking part. This study will provide us with a better understanding of the association between polyphenol-rich food consumption and colonic health. All participants who completed the study will be compensated.

Will my taking part in this study be kept confidential?

All information which is collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the hospital/university will have your name and address removed so that you cannot be recognised from it.

What will happen to the results of the research study?

Results will be presented at meetings of learned societies and published in scientific journals. Results will also be included in student project reports, when applicable. We will arrange a meeting to discuss the results with participant volunteers if they would like that.

Who is organising and funding the research?

This project is being organised by the Plant Product and Human Nutrition Group, at the University of Glasgow.

Who has reviewed the study?

This project has been reviewed by the University of Glasgow, Faculty of Medicine, Ethics Committee.

Contact for further information

If you require further information please contact

Thank you for reading this information sheet

Study Number:

Appendix -3

DIETARY GUIDELINES FOR LOW POLYPHENOL DIET | 2014

Baseline diet:

Avoid ***all fruits, vegetables, onions, coffee, tea, chocolate, fruit juices, soft drink (i.e. irn bru, coca cola), alcohol, vanilla and similar flavourings***, ***wholemeal products, spices (i.e. curry), tomato sauce, and beans (i.e. lentils, green beans, peas)***. Avoid all dietary supplements (***vitamins, mineral, and herbal products*****)** and fibre rich products **(*oats, wheat, bran, fibre cereal, wholemeal bread or biscuits*)**. Vegetable oil avoids ***olive oil***, Honey.

Examples of suitable breakfast include:

Eggs

Cheeses

Sausage

Bacon

Fish

White Toast

White Bread

Croissant (NO chocolate, no almonds)

Waffles, pancakes with butter and sugar (NO jam, no chocolate, no fruits)

Butter

Milk

Rice based cereals (NOT Coco pops)

Examples of suitable lunch & dinners include:

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 (NO ketchup)

Chicken fried rice with eggs (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 chips (No tomato sauce)

Examples of suitable snacks include:

Biscuits (NOT wholemeal, chocolate, or fruit containing)

Shortbreads

Custard rice puddings

Crisps (ready salted)

Cheese and crackers

Plain donut without chocolate or fruit fillings

Salted rice crackers

Rice cakes

Examples of suitable drinks include: Water and milk

Appendix -4

DIETARY INTAKE RECORD

During the day which you have to follow the diet described previously, we are going to ask you to record what you have been eaten at every meals.

By asking you to record your daily diet (quantities do not have to be written down), this may be easier for you to choose and plan what you can eat, and this may also help you remembering that you are on a `special diet'. Please write down and food/drink consumed

DAY -- Date:_____

Breakfast:

.....

.....

.....

.....

.....

Lunch:

.....

.....

.....

.....

Dinner:

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.....

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All-day snacks:

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.....

.....

Apendix-5

University of Glasgow | College of Medical, Veterinary & Life Sciences

PARTICIPANT INFORMATION SHEET

Impact of Exercise Training on Bioavailability of Flavanones from Orange Juice

You are being invited to take part in a research study. Before you decide whether or not to take part, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. If you feel that something is not entirely clear or if you would like more information, do not hesitate to contact us. Take time to decide whether or not you wish to take part. Thank you for reading this.

What is the purpose of the study?

Flavonoids are compounds that may have a beneficial effect on health, such as reducing the risk of developing cardiovascular disease and, in some cases, cancer. Orange juice has high flavonoid content. However, the absorption of flavanones into the body is limited. This study will investigate whether participation in physically active improves the absorption of flavanones into the body.

Why have you been chosen?

You have been chosen because you are 18-45 years old male or female and have been sedentary for at least 6 weeks. You also meet the following criteria: non-smoker, not taking any drug therapy, no gastrointestinal disease, non-vegetarian, non-pregnant.

Do I have to take part?

It is up to you to decide whether or not to take part. If you decide to take part, you will be given this information sheet to keep and you will be asked to sign a consent form. If you decide to take part, you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen once I decide to take part?

Screening Procedures

In order for us to determine whether you fall into the group of people we wish to study, you will be asked to attend a screening visit in which you will complete a Health Screening and Physical Readiness Questionnaires. We will also measure your height and weight and calculate body mass index.

Experimental Tests

You will be randomized to one of two groups. We will ask you to undertake 2 experimental tests: one before the intervention and the other after 4 weeks training intervention. On the day of each experimental test you will be asked to come to the metabolic investigation room between 8 and 9 am. We will place a tiny plastic tube called a ‘cannula’ into a vein in your forearm, from which we will take blood samples. This is no more painful than a simple blood test. Following this, you will be asked to consume 1000 mg of paracetamol (standard dose) with 100 ml of water. Then you will be asked to consume 500 ml of freshly squeezed orange juice. Further blood samples will be obtained at 1, 2, 3, 4, 5, 6, 7, 8 hours after the juice intake. A total of 90 ml of blood will be taken over the course of the day. Expiratory breath hydrogen levels will be monitored every 30 minutes for 8 hours after the orange juice intake. At 4 hours after juice consumption you will be provided with a white roll with butter and some cheese and at the end of the experimental test with a polyphenol-free dinner. You will be allowed to leave

after dinner and instructed to avoid polyphenol containing foods during the rest of the day and record all food and drink consumed. We will ask you to come back to the metabolic investigation room in the fasted state next morning for the last blood sampling. Then you will be provided with breakfast. Prior to the experimental test you will be asked to collect 24 hour urine in two fractions (day fraction and overnight fraction). You will be also asked to collect urine for 24 h after the juice ingestion, in different fractions: 0–2, 2–5, 5-8, 8-10, 10–20, 20-24 h.

Interventions

Exercise training will involve endurance type exercise (cycling , running or combination of both) for 30 minutes in week 1, 40 minutes in week 2, 50 minutes in week 3 and 60 minutes in week 4. The work load of cycling and running speed will correspond to the work load achieved at 70-80% of the predicted maximal heart rate. All training sessions will take place at Exercise and Energy Balance Laboratory (New Lister Building, the Royal Infirmary and be supervised by a researcher. The time and day of each training session will be agreed between the investigator and the participant and will be based around the participant's availability. Participants of the Control group will be asked to carry on with their habitual life style for 4 weeks. Following this they will be given access to exercise laboratory for physical training. Other than specific tasks described above, for 2 days prior each experimental test you will be asked to follow a polyphenol-free diet. A list with polyphenol-free food is provided bellow. We will ask you to record your food intake throughout these 2 days before the first experimental test and replicate this food intake prior the second one. List of the foods that should not be eaten and foods that you may eat during the polyphenol free diet will be provided. Prior to the experimental tests you will be asked to collect 24 hour urine. We will also ask you to collect a stool sample before and during one of the very last days of the intervention.

Will I be rewarded for taking part in this study?

After the completion of the study, the participants of the exercise group will be offered a financial remuneration of either £80 (participants of exercise group) or £40 (participants of the control group).

What are possible disadvantages and risks of taking part?

Blood sampling via cannula may cause minor bruising (a small accumulation of blood under the skin). Some people may feel faint when they give blood. Training intervention is not expected to cause any health related issues.

What if something goes wrong?

The chance of something going wrong is extremely small. In the unlikely event that you are harmed due to someone's negligence, then you may have grounds for legal action. If you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of the study, the University of Glasgow complaints mechanism may be available to you.

Will my taking part in this study be kept confidential?

Any information which is collected about you during the course of the research will be kept strictly confidential. You will be identified by an ID number. Furthermore, findings from the study that are published will contain only the results. We will not pass on confidential personal information to others.

Who has reviewed the study?

This study has been reviewed and approved by the College of Medical, Veterinary and Life Sciences Ethics Committee for Non Clinical Research Involving Human Subjects.

Contact for Further Information

Any questions about the procedures used in this study are encouraged. If you have any doubts or questions, please ask for further explanations by contacting

Min Hou:
E-mail: min-hou710@hotamil.com; m.hou.1@research.gla.ac.uk; Contact phone number: 07565398155
Dr Dalia Malkova:
E-mail: Dalia.Malkova@glasgow.ac.uk; Contact phone number: 0141 2010648
You will be given a copy of this information sheet and a signed consent form to keep for your records.

Apendix-6

University of Glasgow

College of Medical,
Veterinary & Life Sciences

Are you a healthy, non-smoking male or female looking for ways to enhance physical activity?

If you are a non-smoker, aged between 21 and 45, we may be able to help you!

We are investigating how enhanced physical activity helps absorption of beneficial antioxidants in orange juice

Participants of this study will benefit from:

. Free, supervised exercise training sessions for 4 weeks
. Information regarding your metabolic health status
. Assessment of body weight and composition
. Be a part of exiting new research project
. £60 incentive

If you , or someone you know may be interested in participating, please contact **Min Hou or Craig Wratten**

orangejuicestudy@gmail.com

07565398155

Appendix-7

FOOD INVENTORY INSTRUCTIONS

It is important that you weigh and record everything that you eat and drink for the **two** days prior to each oral fat tolerance test (OFTT). Please do not take any alcohol on these days. Your last food and drink should be taken 12 hours before your OFTT appointment.

Please (i) start a separate page for each day.

(ii) start a separate line for each item.

Column 1

Record meal and time and place of eating.

Column 2

Describe each item as accurately as possible, stating where relevant:

(i) type and brand

(ii) whether food is fresh, dried, canned, frozen, salted, smoked, etc.

(iii) whether food is cooked, if so give method of cooking e.g. fried, baked, etc.

Column 3

Record the weight of each item after cooking:

(i) place scales on a level surface

(ii) place plate or container on top of scales

(iii) press 'ON/Reset' button to turn on scales

(iv) once zero appears, add first item of food

(v) record weight displayed

(vi) press reset button before weighing next item

Wherever possible, record weights in grams. If this is not possible, record weights in household measures (e.g. sugar or jam in teaspoons, stating whether level, rounded, or heaped).

Column 4

Record the weight of any leftovers, such as food remaining on plate, weight of container in which food has been weighed, apple cores, etc.

Columns 5 and 6

Please leave blank.

If food consists of several items, please list each on a separate line i.e. instead of writing 'one cheese sandwich', record separately the weights of bread margarine, cheese, etc.

Please remember to record all drinks, as well as food, giving weights where possible, or volumes if these are known. Record separately the weights of added milk and sugar.
An example is shown overleaf.

Food Inventory

Name_____________________________________ **Date** ______________________

1. Time/Place	**2. Description of**	**3. Weight of food/drink (g)**	**4. Weight container/ leftovers**	**Leav Blan**	

Appendix-8

DIETARY GUIDELINES FOR LOW POLYPHENOL DIET | 2014

Baseline diet:

Avoid ***all fruits, vegetables, onions, coffee, tea, chocolate, fruit juices, soft drink (i.e. irn bru, coca cola), alcohol, vanilla and similar flavourings***, ***wholemeal products, spices (i.e. curry), tomato sauce, and beans (i.e. lentils, green beans, peas)***. Avoid all dietary supplements (***vitamins, mineral, and herbal products*****)** and fibre rich products **(*oats, wheat, bran, fibre cereal, wholemeal bread or biscuits*)**. Vegetable oil avoids ***olive oil***. Honey.

Examples of suitable breakfast include:

Eggs
Cheeses
Sausage
Bacon
Fish
White Toast
White Bread
Croissant (NO chocolate, no almonds)
Waffles, pancakes with butter and sugar (NO jam, no chocolate, no fruits)
Butter Milk
Rice based cereals (NOT Coco pops)

Examples of suitable lunch & dinners include:

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 (NO ketchup)
Chicken fried rice with eggs (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 chips (No tomato sauce)

Examples of suitable snacks include:

Biscuits (NOT wholemeal, chocolate, or fruit containing)
Shortbreads
Custard rice puddings
Crisps (ready salted)
Cheese and crackers
Plain donut without chocolate or fruit fillings
Salted rice crackers
Rice cakes

Examples of suitable drinks include: Water and milk

Appendix- 9

RATING OF PERCEIVED EXERTION-RPE

6	
7	**Very, very light**
8	
9	**Very light**
10	
11	**Fairly light**
12	
13	**Somewhat hard**
14	
15	**Hard**
16	
17	**Very hard**
18	
19	**Very, very hard**
20	

Appendix-10

HEALTH SCREEN FOR STUDY VOLUNTEERS

Name: …….

It is important that volunteers participating in research studies are currently in good health and have had no significant medical problems in the past. This is to ensure (i) their own continuing well-being and (ii) to avoid the possibility of individual health issues confounding study outcomes.

Please complete this brief questionnaire to confirm fitness to participate:

1. At present, **do you have any health problem for which you are:**

(a) on medication, prescribed or otherwise	**yes [ ]**		**no [ ]**	
(b) attending your general practitioner		**yes [ ]**		**no [ ]**
(c) on a hospital waiting list	**yes [ ]**		**no [ ]**	

2. **In the past two years,** have you had any illness which required you to:

(a) consult your GP		yes [ ]		no [ ]
(b) attend a hospital outpatient department		yes [ ]		no [ ]
(c) be admitted to hospital		yes [ ]		no [ ]

3. **Have you ever** had any of the following:

(a) Convulsions/epilepsy	yes [ ]		no [ ]	
(b) Asthma	yes [ ]		no [ ]	
(c) Eczema	yes [ ]		no [ ]	
(d) Diabetes	yes [ ]		no [ ]	
(e) A blood disorder	yes [ ]		no [ ]	
(f) Head injury	yes [ ]		no [ ]	
(g) Digestive problems	yes [ ]		no [ ]	
(h) Hearing problems	yes [ ]		no [ ]	
(i) Problems with bones or joints	yes [ ]		no [ ]	
(j) Disturbance of balance/co-ordination	yes [ ]		no [ ]	
(k) Numbness in hands or feet	yes [ ]		no [ ]	
(l) Disturbance of vision	yes [ ]		no [ ]	
(m) Thyroid problems	yes [ ]		no [ ]	
(n) Kidney or liver problems	yes [ ]		no [ ]	
(o) Chest pain or heart problems	yes [ ]		no [ ]	

(p) Any other health problems yes [ ] no [ ]

4. **For female volunteers only**

(a) Are you pregnant or think that you might be pregnant yes [ ] no [ ]

(b) Do you take the contraceptive pill or other hormone-based contraceptives yes [ ] no [

(c) Are you postmenopausal yes [ ] no [ ]

(d) Are you receiving Hormone Replacement Therapy (HRT) yes [ ] no [ ]

5. **Have any of your immediate family** ever had any of the following: (if yes please give details including age of first diagnosis)

(a) Any heart problems yes [ ] no [ ]

(b) Diabetes yes [ ] no [ ]

(c) Stroke yes [ ] no [ ]

(d) Any other family illnesses yes [ ] no [ ]

6. Do you currently smoke yes [ ] no [ ]

Have you ever smoked yes [ ] no [ ]

If so, for how long did you smoke and when did you stop? ……

5. How many units of alcohol do you typically drink in a week? …….

If YES to any question, please describe briefly if you wish (e.g. to confirm whether problem was short-lived, insignificant or well controlled.) (Use a separate sheet if necessary)

……

……

……

……

……

……

……

……

…….

Name and address of GP

……

……

Blood pressure measured at screening……..mm Hg

Appendix-11

Physical activity questionnaire

Physical Activity Readiness Questionnaire - PAR-Q (revised 2002)

PAR-Q & YOU

(A Questionnaire for People Aged 15 to 69)

Regular physical activity is fun and healthy, and increasingly more people are starting to become more active every day. Being more active is very safe for most people. However, some people should check with their doctor before they start becoming much more physically active.

If you are planning to become much more physically active than you are now, start by answering the seven questions in the box below. If you are between the ages of 15 and 69, the PAR-Q will tell you if you should check with your doctor before you start. If you are over 69 years of age, and you are not used to being very active, check with your doctor.

Common sense is your best guide when you answer these questions. Please read the questions carefully and answer each one honestly: check YES or NO.

YES	NO	
☐	☐	1. **Has your doctor ever said that you have a heart condition and that you should only do physical activity recommended by a doctor?**
☐	☐	2. **Do you feel pain in your chest when you do physical activity?**
☐	☐	3. **In the past month, have you had chest pain when you were not doing physical activity?**
☐	☐	4. **Do you lose your balance because of dizziness or do you ever lose consciousness?**
☐	☐	5. **Do you have a bone or joint problem (for example, back, knee or hip) that could be made worse by a change in your physical activity?**
☐	☐	6. **Is your doctor currently prescribing drugs (for example, water pills) for your blood pressure or heart condition?**
☐	☐	7. **Do you know of any other reason why you should not do physical activity?**

If you answered

YES to one or more questions

Talk with your doctor by phone or in person BEFORE you start becoming much more physically active or BEFORE you have a fitness appraisal. Tell your doctor about the PAR-Q and which questions you answered YES.

- You may be able to do any activity you want — as long as you start slowly and build up gradually. Or, you may need to restrict your activities to those which are safe for you. Talk with your doctor about the kinds of activities you wish to participate in and follow his/her advice.
- Find out which community programs are safe and helpful for you.

NO to all questions

If you answered NO honestly to all PAR-Q questions, you can be reasonably sure that you can:

- start becoming much more physically active – begin slowly and build up gradually. This is the safest and easiest way to go.
- take part in a fitness appraisal – this is an excellent way to determine your basic fitness so that you can plan the best way for you to live actively. It is also highly recommended that you have your blood pressure evaluated. If your reading is over 144/94, talk with your doctor before you start becoming much more physically active.

DELAY BECOMING MUCH MORE ACTIVE:

- if you are not feeling well because of a temporary illness such as a cold or a fever – wait until you feel better; or
- if you are or may be pregnant – talk to your doctor before you start becoming more active.

PLEASE NOTE: If your health changes so that you then answer YES to any of the above questions, tell your fitness or health professional. Ask whether you should change your physical activity plan.

Informed Use of the PAR-Q: The Canadian Society for Exercise Physiology, Health Canada, and their agents assume no liability for persons who undertake physical activity, and if in doubt after completing this questionnaire, consult your doctor prior to physical activity.

No changes permitted. You are encouraged to photocopy the PAR-Q but only if you use the entire form.

NOTE: If the PAR-Q is being given to a person before he or she participates in a physical activity program or a fitness appraisal, this section may be used for legal or administrative purposes.

"I have read, understood and completed this questionnaire. Any questions I had were answered to my full satisfaction."

NAME _____

SIGNATURE _____ DATE _____

SIGNATURE OF PARENT or GUARDIAN (for participants under the age of majority) _____ WITNESS _____

Note: This physical activity clearance is valid for a maximum of 12 months from the date it is completed and becomes invalid if your condition changes so that you would answer YES to any of the seven questions.

CSEP SEPE © Canadian Society for Exercise Physiology

Supported by: Health Canada Santé Canada

continued on other side...