Effect of food matrix interaction between dietary fibre and polyphenols on their metabolism by colonic bacteria

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A Thesis submitted for the degree of Doctor of Philosophy to

The School of Medicine, University of Glasgow (December 2013)

From research conducted in the

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AUTHOR'S DECLARATION

I declare that the work contained within this thesis is original and is the work of the author Bahareh Mansoorian. I have been solely responsible for the organisation and day to day running of this study as well as laboratory measurements and data processing, unless otherwise referenced.

Bahareh Mansoorian

ABSTRACT

Background: The consumption of plant based foods has demonstrated an inverse association with disease prevalence. Among the components of plant based foods, polyphenol and fibre are two of the main contenders for many health benefits.

The majority of polyphenols and fibre pass through the small intestine unabsorbed, reaching the colon where they are subjected to the action of the colonic microbiota; resulting in the production of the potentially bioactive metabolites: phenolic acids and SCFA. These metabolites are potentially responsible for many of the health benefits exerted by polyphenols rich foods and fibre.

Given the recent advances in understanding the role of colonic microbiota in metabolic and immune responses, factors, which may positively or negatively modify the composition of the colonic bacteria have also received much attention. Foods rich in dietary fibre and polyphenols have the potential to modify colonic bacteria through prebiotic and antibiotic action. The potential bacterial inhibition by polyphenolics and the stimulation of bacterial growth by fibre and polyphenols means potential for both sets of compounds to influence metabolite production from each other.

Polyphenols and fibre are most often present in the same foods and may be found together in plant cell walls. Thus they most often enter the colon together. We aimed to explore how the presence of these two components in the diet may impact on the metabolite production from the other by the colonic microbiota.

Methods: The food matrix interaction of fibres and polyphenols was assessed using the fibres: raftiline, pectin and ispaghula, having different physio-chemical properties (rate of fermentation and viscosity) and the polyphenols: rutin and catechin, epicatechin and other polyphenols present in cocoa in vitro models of phenolic acid and short chain fatty acid (SCFA) production. The impact of ispaghula on urinary phenolic acids after cocoa ingestion was then investigated in an acute human study.

In *Chapter-3* the impact of the fermentable fibres on phenolic acid production from isolated parent compound: rutin in-vitro using 24 hour batch cultures using human faecal samples from volunteers (n=10) after being on a 3-day low polyphenol diet was investigated. Using the same model the impact of rutin, quercetin and their metabolites on SCFA production from raftiline, ispaghula and pectin was then investigate. The SCFA were measured by GC-FID and phenolic acids by GCMS. pH and gas were also measured.

Using the same methodology the matrix interaction between raftiline, ispaghula and pectin separately on phenolic acid production from their parent compounds within their food matrix was investigated using cocoa as a rich source of polyphenols, as well as the impact of cocoa polyphenols and their metabolites on SCFA production from the fermentable fibres (*Chapter-4*)

In *Chapter-5*, 24-hour urinary polyphenolic acids were measured in 5 batches (0, 0-2, 2-5, 5-8, 8-24 hour) in 12 human volunteers after ingestion of 1g paracetamol with 20g cocoa (*extra brute Cocoa-Cacao Barry, Barry Callebaut, Hardricourt, France*) with water, 15g of ispaghula with water or the combination of the two. Urine samples were also used for total phenol and antioxidant capacity measurement. Plasma was collected over six hours (every half hour for 4 hours and at 6^{th} hour) and used for the measurement of total phenols as well as paracetamol concentrations for the estimation of gastric emptying rate. Breath hydrogen was used for estimation of small bowel transit time and visual analogue scales (VAS) were used for the estimation of subjective appetite response to meals.

Results: The faecal fermentation of rutin resulted in the production of the following phenolic acids: PAA, 4-HBA, 3-HPAA, 4-HPAA, 3,4-DHPAA, 3-HPPA and 4-HPPA. All of these phenolic acids were significantly reduced by at least one of the three fibres, with the exception of 3-HPPA and 4-HPPA. The extent of inhibition of total sum of phenolic acids from raftiline and pectin was similar ($p < 0.01$) and ispaghula demonstrated the least inhibitory effect ($p=0.03$). Rutin and quercetin had no impact on the SCFA production from the fermentable fibres.

The phenolic acids identified from cocoa faecal incubations consisted of: of PAA, 3-HPAA, 4-HPAA, 3,4-DHPAA, 3-HPPA, 4-HPPA, 3,4-DHPPA, 4-HBA, 3,4-DHBA, hippuric acid and vanillic acid. Unlike the rutin study where majority of phenolic acids were significantly reduced, in this study only four of eleven phenolic acids were affected (PAA, 3-HPAA, 4-HPAA, 4-HBA: also inhibited in the rutin study).The extent of phenolic acid reduction was the highest for pectin ($p < 0.01$), followed by raftiline ($p < 0.01$) and ispaghula ($p=0.03$). These phenolic acids or their parent compounds had no impact on SCFA production from the fermentable fibres.

The consumption of cocoa resulted in the urinary excretion of the following phenolic acids: 3-HPAA, 4-HPAA, 3,4-DHPAA, Hippuric, 4-HPA, 4-HBA, 3,4-DHBA, Vanillic, 4-HVA, Mandelic and 4-HMA. All of which, with the exception of vanillic acid and 3,4-DHPAA, were reduced by ispaghula (*Table-I*). Ispaghula accelerated gastric emptying rate but had no impact on small bowel transit time.

The analysis of total phenol (TP assay) concentration (plasma and urine) and antioxidant capacity (urine) did not demonstrate any difference between cocoa and ispaghula, which were both high.

However when they were ingested together there was a signification reduction in both total phenol and antioxidant capacity ($p < 0.01$).

Given that urinary and plasma concentration of total phenols was no different for ispaghula and cocoa we analysed the free phenolic and bound phenolics in both ispaghula and cocoa, showing that cocoa has significantly higher free phenolics than ispaghula, whereas bound phenolics were higher in ispaghula. The sum of bound and free total phenols was higher in cocoa than ispaghula (approximately 10 fold). Urinary, faecal SCFA were not measured as they are not validated to represent in-vivo production.

Conclusion: there is a strong inhibition of phenolic acid production from polyphenol by the fermentable fibres and their metabolites. This inhibition is stronger in-vivo than in-vitro for ispaghula, which may reflect the longer interaction time in the colon and potential small bowel interaction. The production of SCFA from fermentable fibres was not inhibited by the polyphenols or their metabolites. These interactions need to be considered when assessing the bioavailability of phenolic acid production and their potential health benefits.

ACKNOWLEDGEMENTS

To my parents, for supporting me throughout, without whom this PhD would not have been possible, “Thank you” will never suffice for the future you have offered me and for believing in me.

To my brother, who has been my go-to person regardless of distance and time difference, thank you for being there during my many panics about results, time, life.

To my supervisors Prof. Christine Edwards and Dr. Ada Garcia for offering me this PhD and their time, support, encouragement and patience throughout. I have learned a lot, thank you for the opportunity.

During this PhD, I have become indebted to many people who have kindly offered me their time, support and knowledge

To Dr. Emilie Combet for her help, support and answers to my endless questions.

To Dr. Dalia Malkova for her help and advice on the bioavailability study, her kind words and concern.

To Jodi Hampson, for being a friend and my go to person in the department, and for having so patiently dealt with my forever need of supporting documents.

To Dr. Douglas Morrison for his help and support regarding data analysis

To Dr. Bill Mullen for his time, knowledge and use of his lab

To Dr. Gina Borges and Dr. Angelique Stalmach for their time and support

To Dr. David Young for statistical advice.

To my friend Antonis Vlassopoulos, for being there at all times. I couldn't have asked for a better friend to share this journey with.

To Jezz Jones for being a friend, his encouragement and always offering his advice.

To Ms. Areej Alkhaldy for our work together.

To everyone in the Human Nutrition Department and the Child Health Department who have never hesitated to offer their help.

To all my friends, for their help, encouragement and patience. Thank you for accepting me at my worst and being there.

Dedicated to my parents

Despite everything I have learned and achieved through my academic education

I would only feel accomplished if I could achieve even a fraction of the strength and selflessness you represent.

PUBLICATIONS

(Mansoorian et al., 2012):

Impact of cocoa on short chain fatty acid production from ispaghula by colonic bacteria in vitro

B. Mansoorian, A. Garcia, E. Combet and C. A. Edwards

ABBREVIATIONS

- 3,4-DHBA 3,4-Dihydroxybenzoic acid
- 3,4-DHPAA 3,4-Dihydroxyphenyl acetic acid
- 3,4-DHPAA 3,4-Dihydroxyphenylpropionic acid
- 3-HPAA 3-Hydroxyphenylacetic acid
- 3-HPPA 3-Hydroxyphenylpropionic acid
- 4-HBA 4-Hydroxybenzoic acid
- 4-HHA 4-Hydroxyhippuric acid
- 4-HPAA 4-Hydroxyphenylacetic acid
- 4-HPPA 4-Hydroxyphenylpropionic acid
- AUC Area under the curve
- CVD Cardiovascular disease
- F Female
- FS Faecal slurry
- GalpA D-galactopyranosyluronic acid
- HDL High-denisty lipoprotein
- HMA Hydroxymandelic acid
- HVA Homo-vanillic acid
- ISP Ispaghula
- LDL Low-density lipoprotein
- M Male
- MCTT Mouth to caecum transit time
- PAA Phenylacetic acid
- PEC Pectin
- PRED Predicted
- Q Quercetin
- R Rutin
- RAF Raftiline
- SCFA Short Chain Fatty Acid
- TAG Triacylglyceride
- Yrs Years

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CHAPTER 1

Literature review

1.1 Plant based foods and health

Diet is one of the most important modifiable life style factors influencing health and prevention of chronic diseases (Eyre et al., 2004). One element of the diet with the highest consensus for possible health benefits are plant based foods. The prospective cohort study of women from the Nurse's Health study (n=84,521) and men from the Health professional study (n=42, 148) demonstrated an inverse association between the intake of fruits and vegetables with the risk of coronary heart disease as determined by nonfatal myocardial infarction or fatal coronary disease (Joshipura et al., 2001). This was later supported in a review of studies recommending only increase in fruit and vegetable intake with clinical CVD events (mortality, myocardial infarction, coronary bypass grafting etc.) as the end point, as a method of cardiovascular disease (CVD) prevention (Hartley et al., 2013).

Fruit and vegetables contain many possible bioactive constituents, including vitamins, minerals, sterols, but the studies in this thesis relate to dietary fibres and polyphenols, which are two of the main contenders for many health benefits and are present together in fruits and vegetables. There has been considerable interest in the potential health effects such as reduced risk of CVD and cancer, of plant based polyphenolics such as epi/catechins and quercetin, with stronger evidence for catechin/ epicatechin in regard to CVD risk, as they have been associated with a reduction in the inflammatory markers of CVD (Khan et al., 2014) and quercetin and rutin in regard to reduced risk of colon cancer through anti-proliferative properties and increasing apoptosis (Delgado et al., 2014, Chen et al., 2013). These effects may be related to their potential antioxidant and anti–inflammatory effects (the latter having more emphasis in recent years) and most studies have considered only the effects of the parent compounds, which may never reach the target tissues (evidence summarised in *Table 3-1* and *Table 4-1*).

The majority of polyphenols pass through the human small intestine unabsorbed, reaching the colon where they are subjected to metabolism by the colonic microbiota. This results in their conversion to phenolic acids which are absorbed but for which there is little evidence on bioactivity. Dietary fibre often consumed in the same foods as polyphenols will impact on the gut microbiota and many polyphenolics have also been reported to have both pre and antibiotic properties (Parkar et al., 2008, Tzounis et al., 2008a, Lee et al., 2006) and thus it is important to consider the possible colonic interactions of these two significant components in plant based foods.

In addition, the proposed health benefits of polyphenolic compounds and fibre such as prevention of cancer (Gonzalez-Vallinas et al., 2013, Aune et al., 2011), cardiovascular disease (Fuentes and Palomo,

2013, Threapleton et al., 2013b), diabetes (Munir et al., 2013, Hall and Flinkman, 2012) and obesity (Trigueros et al., 2013) has led to potential health claims by the food industry for food products containing one or both of these compounds. Many of these food products such as breakfast cereals and nutrition bars, claiming health benefits contain both of these compounds.

Moreover, there has been considerable level of interest in the role of the gut bacteria in health after the characterisation of the human gut microbiome (Qin et al., 2010) and its association with a range of chronic diseases and conditions such as CVD (Howitt and Garrett, 2012), obesity (Zhao, 2013), IBD (Mazmanian et al., 2008), allergy (Hanski et al., 2012) and auto immune disease (Tlaskalová-Hogenová et al., 2011) and even autism (Theoharides and Doyle, 2008). Thus it is very important to consider how dietary factors, which influence the microbiota interact and affect bioactive molecule production.

1.2 Carbohydrates

Carbohydrates are a major component of plant based foods and they can be divided into digestible carbohydrates such as starch and sucrose or non-digestible carbohydrates which mostly make up the plant cell walls (pectin, cellulose, etc.), or have specific roles such as ispaghula which is present in the seed husk of *Plantago ovata* and due to its great water holding capacity expands greatly when it rains allowing the seed to germinate. Other non-digestible carbohydrates may act as storage molecules such as inulin, which consists of polysaccharides (greater than 8 sugar units) and oligosaccharides (greater than 3 and less than 8 sugar units) such as raftilose in the roots of chicory and other plants. Non-digestible carbohydrates often co-exist alongside plant polyphenolics in these locations.

1.2.1 Digestible carbohydrates

The human digestive enzymes can break only certain carbohydrate bonds such as the α (1-4) and α (1-6) glycosidic bonds. Carbohydrates containing other bonds such as β (1-4) are not digested by the human digestive enzymes. Some carbohydrates made of α- linkage bonds are either directly absorbed by the epithelial cells such as monomers or easily broken down into their constituent monomers by the human digestive enzymes, such as sucrose and starch. The ingested galactose and fructose are mostly converted to glucose by the intestinal epithelial cells and liver, resulting in glucose being the main circulating monomer in the body (Whitney et al., 1998).

However, not all starch is digestible. The main components of starch are amylose (14-27%) and amylopectin (73-86%). Amylopectin is made of over 10,000 α (1-4) and α (1-6) linked glucose moieties in a branched manner. Amylose is smaller in size and is made of α (1-4) links in a helical manner, making it less accessible to α-amylase action as compared to the open and branched amylopectin (Cummings et al., 1996). Hence, based on digestibility, starch can be classified into 3 groups (Goñi et al., 1996): rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS).

RDS can be found in freshly cooked foods such as potato and bread and is rapidly digested in the human small intestine. SDS is present in partially milled seeds and in denser textured foods such as pasta, these type of starch are completely digested in the human small intestine, however at a much slower rate than RDS. Unlike the other two types of starch, RS is not digested in the human small intestine and is instead subjected to colonic microbiota action in the colon (discussed in more detail in section *1.2.2.1 Resistant starch*).

1.2.2 Non-digestible carbohydrates

These carbohydrates resist digestion in the human small intestine and reach the colon where they are fermented by the gut microbiota to different extents. It is estimated that 10 - 60g of non-digestible carbohydrates enter the colon daily and are used as a substrate for colonic bacteria (Cummings and Macfarlane, 1991).

Some simple carbohydrates can act as non-digestible carbohydrates conditionally. This is mostly dependent on genetic background of individuals and disorders of the gastrointestinal tract. An example of this is the simple sugar lactose. In a case of lactose intolerance caused either by insufficient lactase production or lack of genetic expression of lactase, lactose is subjected to fermentation by colonic microbiota (Wiggins, 1984). Fructose may be poorly absorbed as it is not associated with active transport and the facilitated carrier can be overloaded.

Oligosaccharides such as raftilose, raffinose, fructo and galacto-oligosaccharides are also non-digestible. They are present in foods such as artichoke, asparagus, chicory roots, onion, garlic, leeks and banana (Campbell et al., 1997). This group consists of mainly fructosyl-fructose glycosidic bonds of either β-2,1 linked inulins or β-2,6 linked levans. Due to their low sweetness (0.3- 0.6 that of sucrose) and due to their higher molecular weight, they are used as bulking agents in the food industry (Voragen, 1998). These non-digestible oligosaccharides received much attention due to their ability to alter the microbial population of the colon promoting the growth of lactic acid bacteria such as

lactobacilli and particularly bifidobacteria (Tokunaga et al., 1993, Gibson et al., 2004). When they are fermented they tend to produce more of the short chain fatty acid (SCFA) butyrate (Roberfroid and Delzenne, 1998, Roberfroid, 2005) which has many possible health benefits detailed in section 1.2.6.1.3 (page 25). They were termed prebiotics defined as "a food ingredient that is composed of oligosaccharides that are not digestible by the host and that has a beneficial effect on host health through selective stimulation of the growth and/or activity of specific members of the gut microbiota" (Gibson et al., 2004). It has been proposed that any type of dietary food that can promote the growth of probiotics and consequently promote health and homeostasis in the gut can be considered a prebiotic (Vieira et al., 2013).

Non-digestible polysaccharides are mostly comprised of the two classes, resistant starch, contributing towards 8-40g of non-digestible carbohydrates reaching the colon and non-starch polysaccharides contributing 8-18 grams of non-digestible carbohydrates reaching the colon daily (Cummings and Macfarlane, 1991).

1.2.2.1 Resistant starch

Starch is the main form of energy storage for most plants and also one of the main contributors to the amount of carbohydrates reaching the colon. The partially crystalline form of starch gives rise to the discrete x-ray diffraction patterns A, B and C that can be used for the classification of starch into groups having similar properties (Gallant et al., 1992).

Group-A starch is mostly found in cereals, group-B starch is mostly found in potatoes, bananas and other tubers and Groups-C starch is found in legumes. Out of these three groups, B and C are more resistant to pancreatic and salivary α-amylase making them more resistant to digestion in the human small intestine, thus available as substrates for colonic microbiota. This is due to the three dimensional structure of the starch which makes it harder for amylase to penetrate as well as differences in the surface area to volume ration of the starch granules.

The method of starch preparation also impacts its digestibility. Gelatinisation of starches occur during the process of cooking, making them more accessible to α-amylase, those with type B and C structures tend to re-crystallise upon cooling by forming hydrogen bonds between the polymers (retrogradation) most often in the form of the more resistant B-group eg. potato starch.

Hence, resistant starch is defined as "the sum of starch and starch degradation products that pass into the large intestine"(Asp and Björck, 1992). Resistant starch can hence be further classified into:

(**RS-1):** encased starch, referring to starch granules that are physically entrapped in the food matrix. (**RS-2):** resistant starches, referring to type-B starch that resists gelatinisation during cooking and (**RS-3):** retrograded starches, referring to re-crystallised starch after cooling (Englyst et al., 1992). **(RS-4):** chemically modified resistant starch, not present in nature.

1.2.2.2 Non-starch polysaccharide / Dietary fibre

The definition of dietary fibre, especially in relation to its classification is highly debated, usually based on analytical methods rather than physiological effects and has been under review for decades (DeVries et al., 1999, Howlett et al., 2010), with various definitions being proposed over the years. The lack of an accurate and universal definition, as well as classification of dietary fibre is problematic in the context of food health claims by the food industry and epidemiological interpretation of health benefits from fibre.

The original definition of fibre known as "crude fibre" was based on its resistance to hydrolysis by acid and alkaline. The term dietary fibre was first used by Hipsley (1953) in reference to the components of cell walls that resisted digestion in the human small intestine. A supporting definition was later proposed in 1974 by (Trowell, 1974) as: "plant cell wall polysaccharides and lignin". This was later developed as the non-starch polysaccharides in the UK (Trowell et al., 1978). This definition excluded resistant / modified starch and non-digestible oligosaccharides as dietary fibre. Later, the definition of dietary fibre in the UK was stated by the Department of Health (1991) as: "*dietary fibre should be defined as non-starch polysaccharides (NSP) where this refers to non-alpha-glucans as measured by the technique of Englyst and Cummings or other comparable techniques."* This was then followed by the proposed definition by the American association of Cereal Chemist in 2000:

"Dietary fibre is the edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine. Dietary fibre includes polysaccharides, oligosaccharides, lignin, and associated plants substances. Dietary fibres promote beneficial physiological effects including laxation, and/or blood cholesterol attenuation, and/or blood glucose attenuation."

This definition remains the most widely used and emphasises the physiological health benefits of dietary fibre. However these health benefits are dependent on the type of dietary fibre. There have since been various proposed sub classifications of fibre to differentiate plant cell wall associated fibre from those added as food ingredients. The Institute of Medicine (IOM) of the National Academies (2001) proposed the following classification: (i)-endogenous to a food (dietary fibre) and (ii)-fibre

extracted and/or synthesized (functional fibre). The sum of these two make up the total dietary fibre content of food. A similar classification was proposed by the Codex Alimentarius Commission (2009) as: (i)- naturally occurring in the food as consumed, (ii)- obtained from food raw material by physical, enzymatic or chemical means and (iii)- synthetic carbohydrate polymers. In this regard category (ii) and (iii) must display a physiological property inducing health benefits. The main difference between the two classifications is that in the latter classification, synthesised fibre is also considered as dietary fibre as opposed to functional fibre.

Classification of dietary fibres with different properties is also subject to debate, the most useful clinically and most relevant for the purpose of this PhD is based on their solubility and their fermentability. Based on this, the two main subclasses are: insoluble and soluble fibre. These subclasses are expected to exert different health benefits due to their different physical and chemical properties. Some of the factors that play a role in this classification are spatial arrangement and intermolecular bonds of these complex carbohydrates. Some carbohydrates such as pectin are composed of charged groups which act as repellents to similar charged groups, resulting in expansion and ultimately destabilisation of the molecule, favouring solubility.

1.2.2.3 Health benefits of dietary fibre

The interest in the health benefits of dietary fibre began after observations in Africa, by Burkitt, Trowell and Walker, linking the lack of fibre intake to prominent disorders in western and developed countries such as: constipation, diverticulitis, colon cancer, risk of obesity, type 2 diabetes and CVD (Champ et al., 2003). This was attributed to the high consumption of refined and meat based diets.

Dietary fibre has been associated with modification of key risk factors in the development of CHD such as hypertension, hyperlipidemia and insulin resistance (Threapleton et al., 2013a). Additionally viscous fibre has demonstrated a reduction in the rate of cholesterol absorption from the small intestine resulting in the regulation of blood cholesterol levels (James et al., 2003).

Fibre has also been associated with decreased risk of cancer based on systematic reviews from nine independent academic centres, assessed in the second WCRF expert report (Wiseman, 2008). The EPIC cohort study by Bingham et al. (2002) analysed the association between colorectal cancer and dietary fibre consumption in individuals aged 25-70 years (n=519, 978). They found an inverse association between the consumption of dietary fibre in food with large bowel cancer adjusted relative risk (0·75 [95% CI 0·59–0·95] for the highest versus lowest quintile of intake). They also found that the protective effect was the greatest for the left side of the colon where most dietary fibre is fermented

and least for the rectum. This could be attributed to fewer fermentation products reaching the right side of the colon and rectum. In this regard, the type of fibre and its rate of fermentation play an important role.

Fibre can induce its health benefits through various mechanisms related to its viscosity (regulation of plasma cholesterol and glucose), fermentation (actions of SCFA and low pH) modification of gut transit time and stool weight (reducing exposure to carcinogens). These will be discussed later.

1.2.3 Insoluble fibre

Insoluble fibres, such as cellulose, pass through the human small intestine intact and it is believed that they undergo very little fermentation compared with soluble fibres. However this may vary depending on method of investigation (*in-vitro* vs. *in-vivo*) and level of crystallisation (Edwards and Rowland, 1992).

They act mostly by speeding colonic transit and increasing faecal bulk (Delargy et al., 1997, Dikeman and Fahey, 2006) and exhibit most of their health benefits through a 'mop and sponge' effect (Shomik Sengupta MB et al., 2001). The 'sponge' binding of bile acids and carcinogens, as well as increasing stool bulk and the 'mop' sweeping material along and out of the gut. Insoluble fibres include bran and cellulose and are predominant in cereal grains and vegetables (Englyst et al., 1989, Dikeman and Fahey, 2006).

1.2.4 Soluble fibre

Soluble fibres can be further divided into viscous and non-viscous. Soluble fibres exert their health benefits through water holding capacity, viscosity and fermentability. These properties can vary depending on the type of fibre.

Through their viscosity they have shown to reduce cholesterol levels by decreasing the re-absorption of bile salts from the small intestine, thus increasing excretion of bile acids through faeces. Consequently 7α-hydroxylase is activated to rapidly catabolise cholesterol and replenish the bile acid pool (Chawla and Patil, 2010). A review by Brown et al. (1999) has shown that 2-10 g/ day consumption of soluble fibre can lead to 0.57 mmol/l reduction in LDL-C per gram of viscous fibre. Fat absorption may also be reduced (Ganji and Kies, 1994). Another role of viscous soluble fibre is reducing glycaemic response by altering gastric emptying/ mouth-caecum transit time (MCTT), slowing down the transport and mixing of digestive enzymes, resulting in a slow or reduced absorption of glucose and other macronutrients consequently resulting in a reduction of insulin levels (Bourdon et al., 1999). As insulin

has shown to play a role in the activation of 3-Hydroxy 3-methylglutaryl- coenzyme A reductase (HMG-Co-AR) which in turn is involved in cholesterol synthesis, the lower insulin levels could also contribute to lower blood cholesterol levels (Lakshmanan et al., 1973).

Through their fermentation, soluble fibres (and some insoluble fibres) produce short chain fatty acids, mainly acetic, propionic and butyric acids, which have many putative health effects. For example, it has been proposed that propionic acid is involved in gluconeogensis (Nishina and Freedland, 1990, Rémésy et al., 1992) and aids in the reduction of LDL-C by inhibiting hepatic cholesterol metabolism through mechanism such as reduction of HMG-Co-AR activity and inhibition of Acetyl- coenzyme A reductase (Acetyl-Co-AR), which is responsible for catalysing the synthesis of acetyl-CoA from acetate (Levrat et al., 1994, Wright et al., 1990b).

The fermentation of different carbohydrates produces different amounts of the individual SCFA (Table 1-1), which in turn may determine the health benefits they induce.

Table 1-1 SCFA production from various carbohydrate sources

Author (Year)	**Method**	**Carbohydrate choice**	**acetate**	**propionate**	**butyrate**
(Edwards et al., 1992)	In-vivo (rats)	▪ 10%Bran	% 60.3	% 15.5	% 24
		▪ 5%Ispaghula	% 64.4	% 22.2	% 13.2
(Bindelle et al., 2007)	In-vitro fermentation	▪ Potato	% 48	% 22	% 30
		▪ Sugar-beet pulp	% 65.9	% 30.9	% 0.29
		▪ Wheat bran	% 60	% 30.9	% 0.39
		▪ Starch	% 50	% 45	% 4.9
		▪ Raftiline	% 46.9	% 46.9	% 4.9
		▪ Cellulose	% 44	% 54.9	% 0.9
		▪ Xylan	% 62.9	% 31.0	% 4.9
		▪ Citrus pectin	% 80.9	% 17.9	% 0.9
(Le Gall et al., 2009)	In-vivo (pigs)	Breads with:			
		▪ Wheat flour	% 70.1	% 23	% 4.6
		▪ Whole wheat grain	% 65.5	% 24.5	% 8.4
		▪ wheataleurone flour	% 61.8	% 25	% 11.6
		▪ Rye aleurone flour	% 66	% 24.2	% 9.0
(Timm et al., 2010)	In-vitro fermentation	▪ Wheat dextrin	% 46.4	% 27.2	% 27.1
		▪ Raftiline	% 42	% 42.8	% 16.0
		▪ Ispaghula	% 39.9	% 48.4	% 11.4
(Chen et al., 2010)	In-vivo (mice)	▪ No fibre	% 65.2	% 12.4	% 16.5
		▪ Cellulose	% 75.1	% 8.4	% 15.0
		▪ Pectin	% 74.4	% 9.5	% 15.2
		▪ Konjac Glucomannan	% 75.5	% 9.8	% 17.6
		▪ raftiline	% 59.5	% 13.6	% 25.5
(Paturi et al., 2012)	In-vivo (rats)	▪ Cellulose	% 58.0	% 12.9	% 9.6
		▪ Potato fibre	% 59.2	% 9.5	% 12.2
		▪ Potato- RS	% 56	% 12.8	% 8.8
(Van den Abbeele et al., 2013)	In-vitro fermentation	▪ Raftiline	% 65.9	% 6.05	% 27.9
		▪ Arabinoxylan (long chain)	% 41.6	% 49.8	% 8.4

In the studies in this thesis several soluble fibres were chosen for their different fermentation characteristics, physical chemical properties and potential health benefits. These are explored below.

1.2.4.1 Ispaghula

Ispaghula is a highly branched acidic arabinoxylan with the backbone containing β (1-4) and β (1-3) bonds and D-galactose, D-rhamnose, D-galacturonic acid, 4-*O*-methyl-D-glucuronic acid and 2-*O*- (2-D-galactopyranosyluronic acid)-L-rhamnose (Chan and Wypyszyk, 1988, Edwards et al., 1992).

Ispaghula, also known as isapgul and psyllium, is derived from seeds of the *Plantago ovata* plant native to Asia and the Mediterranean (Bernstein et al., 2013). The name is derived from the sanskrit words *asp* and *ghol* meaning horse flower, after the shape of the seeds. India produces 85% of ispaghula with the United States being the biggest importer of the product. Ispaghula has been used as a bowel regulator and its primary use is as a bulk laxative for the treatment of constipation (Chawla and Patil, 2010). This may be due to its high water holding capacity and speeding the flow of contents in the colon (Edwards et al., 1992, Edwards and Eastwood, 1992). One gram of ispaghula husk can increase faecal weight by 5.9g, compared with 4.9-5.4g for wheat bran and 3.4-4.5g for oat bran (Chen et al., 1998, Cummings, 2001, Chawla and Patil, 2010).

In four different meta-analyses (Brown et al., 1999, Anderson et al., 2000, Olson et al., 1997, Jenkins et al., 2000b) an average consumption of ispaghula husk (9g/day) resulted in 6-7% decrease in LDL-C concentrations. Additionally all studies demonstrated a mean 0.7 mmol/l reduction in LDL-C per gram of ispaghula husk regardless of using different studies in the meta-analysis with different methods of ispaghula consumption (Jenkins et al., 2000b). In the study by Everson et al. (1992), volunteers with moderate hypercholesterolemia (n=20) were fed 15g of ispaghula/day or placebo (cellulose) for 40 days. This study reported that bile acid synthesis was increased in volunteers showing an LDL reduction of 10% or more. This study also investigated the MCTT by measuring breath hydrogen for five hours after ispaghula / cellulose consumption. No definite peak of breath hydrogen was shown for one volunteer in the ispaghula arm and four volunteers in the placebo arm of the study. While ispaghula did not change MCTT, cellulose consumption reduced the MCTT ($p< 0.01$).

The Food and Drug Administration (FDA) has allowed health claims of foods containing oat, oat products and ispaghula husk in regard to cholesterol reduction. The FDA has since recommended the addition of 3g/day of b-glucan or 7g/day of soluble fibre from ispaghula husk along with low cholesterol diets (Gunness and Gidley, 2010, Bernstein et al., 2013). EFSA also allow claims for cholesterol reduction for ispaghula (EFSA- ID2508-2007). This effect on cholesterol could be due to the effects of viscosity in the small intestine or fermentation or transit times in the colon (*Chapter 5*).

Ispaghula is slowly and incompletely fermented (Wolever et al., 1994) producing higher proportions of popionic acid (*Table 1-1*). Unlike rapidly fermented fibres, which are fermented mostly in the proximal

colon, the benefits of slowly fermented fibres such as ispaghula can be extended to the distal colon. It is more likely for all of the colon epithelium to be exposed to fermentation products when a slowly fermented fibre is used (Timm et al., 2010).

Ispaghula feeding studies (Marteau et al., 1994, Marlett and Fischer, 2003) have recovered a gel in the excreted stool sample of volunteers (n=14) consuming ispaghula husk (8.8g in the form of 15g Metamucil for 7 days) and suggested that this was the fraction responsible for the laxative and cholesterol reducing impact of ispaghula. Ispaghula was divided into 3 fractions: alkali insoluble material (fraction A), gel forming fraction (fraction B) and a viscous but not gel forming fraction (fraction C). The gel forming fraction constituted over 50% of ispaghula composition.

The fermentability of each fraction was tested using an *in-vitro* fermentation model. Fraction-A was < 5% over 48 h, fraction-B 25% over 72h and fraction-C was the highest fermented > 80% of its sugars disappearing by 24 h.

Viscous fibres that have a higher molar ratio of acetate production through microbiota fermentation are not desirable for reduction of LDL-C as they could either have no impact or potentially even increase LDL-C concentrations, whereas viscous fibres that produce higher ratios of propionate to acetate are more desirable in this aspect (*Table 1-1*). Ispaghula husk is one of these viscous fibres (Jenkins et al., 2000a). The high viscosity of ispaghula, which is maintained throughout the colon plays a key role in its health impacts. A study (Davidson et al., 1998a) investigating the impact of low viscosity soluble fibres: pectin and gum arabic at doses of 5, 9 and 15 g/day on LDL-C concentrations, did not show any impact on cholesterol levels whereas another study using lower doses of 3.4, 6.8 and 10.23 g/day of ispaghula husk over 24 days demonstrated a 5.3% reduction in LDL-C concentration (Davidson et al., 1998b).

Some studies have suggested that the method of ispaghula administration might have a great impact on its ability to impart health benefits. Wolever et al. (1994) demonstrated that the consumption of 7.3 g of ispaghula in the form of an enriched breakfast cereal reduced serum total cholesterol, LDL and HDL ($p < 0.02$) but not when ispaghula was consumed separately from the breakfast cereal, 1-1.5 hours before meals. This was attributed to the reduced absorption of carbohydrates when ispaghula was mixed with the meal.

Another influencing factor in the health impact of ispaghula could be gender and hormonal status of the individual. A study by (Vega-López et al., 2001) demonstrated that while 15g/ day consumption of ispaghula for 4 weeks reduced ($p<0.01$) LDL-C for men (n=24), pre-menopausal women (n=23) and postmenopausal women (n=21) by 7-9%; TAG was reduced only for the men by 17% ($p < 0.01$) while

it increased in post-menopausal women by 16% ($p<0.01$) and had no impact on pre-menopausal women. It is interesting to see an impact of ispaghula on TAG only for men, as no other study stated in this section has demonstrated an impact of ispaghula on plasma TAG concentrations. However it is important to note that unlike total cholesterol, LDL and HDL, plasma TAG concentrations are greatly influenced by the diet, which is not mentioned in their discussion. The gender/hormonal status in relation to TAG reduction in this study could be explained by the variation in polyunsaturated fatty acid consumption between the different groups. The post-menopausal women had the highest consumption of polyunsaturated fatty acid. The observations made may be due to the sample size rather than the role of hormonal status and gender.

1.2.4.2 Pectin

Pectin is largely found in plant primary cell walls. Henri Braconnot discovered the gel forming complex polysaccharide in 1825 and named it pectic acid (Leclere et al., 2013b). Pectin is made of a group of complex polysaccharides that contain D-Galactosyluronic acid residues, bond by α (1-4) bonds (Leclere et al., 2013b, Ridley et al., 2001). Three main polysaccharides have been identified within the pectic group of galacturonic acids. Homogalacturonan, accounts for 65% of the pectin molecule which is a chain of D-galactopyranosyluronic acid (Gal*p*A) linked by α (1-4) bonds forming a linear chain. The carboxyl groups of the chain can be methyl-esterified or in some cases acetylated (Ishii, 1995, Leclere et al., 2013a). Rhamnogalacturonan-I contributes 20–35% of the molecule and is made of galacturonic acid and rhamnosyl disaccharides repetition bond by [→4)-α-D-Gal*p*A-(1→2)-α-L-Rhap-(1→] with the possible *O*-acetylation of the Gal*p*A back bone on C2 and/or C3 (O'NEILL et al., 1990, Ridley et al., 2001, Mohnen, 2008). Substituted galacturonans are the third constituent of pectin, made of various groups of polysaccharides with a linear chain of D-Gal*p*A bound by α-1-4 bonds. An example of this is Rhamnogalacturonan-II made of a Homogalacturonan chain and different from Rhamnogalacturonan-I structurally. RG-II has been identified in all higher plant cell walls to date (O'NEILL et al., 1990, Mohnen, 2008).

Citrus fruits can contain 0.5 – 3.5% pectin, largely present in the peel. Hence, pectin is mostly extracted from the peels of citrus fruits and apple (Lattimer and Haub, 2010). Within plants pectin plays an important role in the growth and development of plants, morphology and defence. In the food industry this viscous fibre is used as a stabilising and gelling agent (Mohnen, 2008). Apart from its functional properties in the food industry, pectin has also been associated with reducing the risk of

colon cancer and cardiovascular disease (Leclere et al., 2013a). Rats fed pectin from citrus and apple origin and exposed to azoxymethane had lower carcinogen levels and fewer tumours. Rats exposed to apple pectin additionally demonstrated decreased activity of β-glucuronidase produced from colonic bacteria and known to be associated with the development of colon cancer (Ohkami et al., 1995). Hence, it is possible that the impact of pectin administered is dependent on source of the pectin. However other studies demonstrated an increase in β-glucuronidase activity or no change in activity in the presence of different kinds of pectin (Bauer et al., 1981). Borisenkov et al. (2011), demonstrated that cabbage pectins increased the activity of this enzyme while sweet pepper pectins had no impact, again demonstrating a variation in impact depending on source of pectin. While both sources of pectin increased the adhesion of β-glucuronidase, increasing its excretion, cabbage pectins had a greater impact. In another study, 5% low methoxylated or high methoxylated pectin had no impact on the activity of β-glucuronidase (Bauer et al., 1981). The use of various sources of pectin makes it difficult to associate function and structural properties of pectin, as pectin extraction methods, size of generated fragments and degree of esterefication and nature of monomers play an important role in the health benefits pectin can induce (Leclere et al., 2013b).

However, pectins, which are viscous, in general have the functional ability to thicken solutions and this may include intestinal contents. Many of the health benefits exhibited by pectin such as prevention of diabetes and cardiovascular disease are attributed to this property (Lattimer and Haub, 2010). Pectin maintains its viscosity throughout the small intestine and hence it can enhance bile acid excretion and prevent its re-absorption (Leclere et al., 2013b). Additionally colonic fermentation of pectin results in high concentrations of SCFA production especially higher molar ratios of acetate and butyrate than some other fibres (*Table 1-1*). These properties of pectin and its complex structure have demonstrated many health benefits such as reduced risk of CVD and colon cancer (Leclere et al., 2013b).

Considering acetate is particularly increased by pectin fermentation, it is speculated that the presence of this SCFA in the peripheral circulation could be the mechanism behind the impact of pectin on fibrin (Lattimer and Haub, 2010). However other studies such as Miettinen and Tarpila (1977) investigating the impact of 40–50 g/day pectin for two weeks administered to 9 normolipidemic and hyperlipidemic patients, have attributed the impact of pectin on CVD prevention to reduced blood cholesterol through increased faecal excretion of bile acids. This study did not demonstrate any change in TAG concentrations but a significant decrease in total cholesterol levels ($p < 0.05$) especially in hyperlipidemic patients. These results are similar to that seen for ispaghula trials. It is possible that pectin reduces the risk of CVD in more than one way. The mechanism behind CVD prevention by

pectin could be through increasing fibrin permeability and decreasing its tensile strength which was demonstrated in hyperlipidaemic men in a study by Veldman et al. (1999).

In regard to pectin's potential role in prevention of colorectal cancer, there is increasing research in various forms of modified pectin, such as pH and/or heat modified pectin and its use as a colon cancer preventive compound (Maxwell et al., 2012, Leclere et al., 2013b). These reviews have demonstrated a promising dose-dependent role of modified citrus pectin in the prevention of cancer. A postulated mechanism for this cancer preventive property of modified citrus pectin was proposed to be the impairment of the interaction between cells as it is a rich source of galactosides and thus may compete with endogenous ligands of galactoside binding proteins, such as galectin-3 (Platt and Raz, 1992, Inohara and Raz, 1994) Further exploration of possible mechanism of action for modified citrus pectin in regard to cancer prevention is beyond the scope of this PhD.

Pectin has also shown to have some prebiotic properties. Olano-Martin et al. (2002) selectively stimulated the growth of certain strains of Bifidobacteria and Lactobacillus and reduced potentially harmful bacteria in an *in-vitro* batch culture study with controlled pH (6.8). It was also shown that the partial hydrolysis of this soluble fibre improved its prebiotic potential. This study may explain the outcomes of a study by Rabbani et al. (2001) showing that 4 g/kg pectin consumption in 5–12 month old children (n=19) with intestinal infection reduced both acute intestinal infection and diarrhoea. This was speculated to be due to a decrease in pathogenic bacteria such as Salmonella, Enterobacter and Shigella. However studies have demonstrated that the Bifidogenic impact of pectin is dependent on its degree of methylation and that the partial hydrolysis of this compound, converting it to pectic-oligosaccharides can enhance its Bifidogenic properties (Olano-Martin et al., 2002). However it is important to note that this may also impact other health benefits induced by pectin. Considering that pectin is not the first choice as a prebiotic, the hydrolysis of its structure, impacting its other health benefits induced through viscosity and molecular weight (Diaz et al., 2007), may be undesirable.

1.2.4.3 Inulin (Raftiline)

Raftiline, Orafti's inulin product was used in this thesis. Inulin, is a polymer of fructose monomers, containing up to 60 fructose units (Clark et al., 2012) and is naturally found in foods such as chicory, leek, onions, garlic, bananas, artichokes and wheat. The average consumption of inulin through the diet in the West is estimated around 2-12 g/day (Roberfroid, 1993). This estimation does not take into consideration the supplemented form of raftiline. This soluble fibre is commercially available as a

white powder supplement extracted from chicory roots (Sokiic et al., 2009). Inulin is more rapidly fermented than ispaghula and pectin which may result in more gas production causing discomfort. However 20g/ day of inulin was consumed without adverse gastro intestinal effects (Flamm et al., 2001). The consumption of inulin increased stool weight and frequency and lowered pH thus relieving constipation (Sokiic et al., 2009).

In the food industry, raftiline is used as a functional food and as a prebiotic. Due to its ability to enhance the taste and mouth feel of food products, it is used to replace fat and sugars. (Mortensen et al., 2002, Lattimer and Haub, 2010, Tungland and Meyer, 2002).

Due to the β-bonds constituting raftiline and only traces of glucose present, less than 10% of this fibre is digested in the small intestine, the rest is subjected to colonic bacterial fermentation (Lattimer and Haub, 2010) which favours higher molar ratio production of butyrate than fermentation of ispaghula and pectin. Stewart et al. (2008) in an *in-vitro* batch model for 24 hours found that the highest chain raftiline had the least production of butyrate, indicating that the degree of polymerization is an important factor in the production of SCFA from raftiline fermentation.

As seen for ispaghula and pectin, raftiline has also demonstrated the potential to prevent CVD through reduced blood cholesterol levels. Mortensen et al. (2002) investigated the impact of 0 or 10% raftiline supplementation to an isocaloric balanced fat diet of 40 $LDLR^{-/-}$ male mice for 16 weeks. 10% raftiline supplementation lowered body weight and total cholesterol, LDL, IDL and VLD, with no impact on HDL or TAG. However no impact was seen on plasma cholesterol in 8 healthy individuals after 3 weeks of raftiline (10g) and a high carbohydrate/low fat diet (Letexier et al. (2003). However there was a significantly lower concentration of plasma TAG. The lack of effect in cholesterol levels may be because healthy individuals were used. It has been shown in many studies that reducing levels from normal is not easily achievable and it is better to use hyperlipidaemic individuals. The change in TAG concentrations caused by raftiline supplementation can be explained by elevated TAG concentration caused by the high carbohydrate diet consumption (Jeppesen et al., 1997).

There are not sufficient studies investigating the impact of raftiline on blood cholesterol levels. A review by Williams and Jackson (2002) contained 9 human studies (normo- or hyperlipidaemic individuals) investigating the impact of raftiline on plasma cholesterol. Four out of nine studies demonstrated a mild reduction in total and LDL levels with raftiline supplementation, three studies demonstrated a reduction in TAG and the remaining three did not demonstrate any impact of raftiline on plasma cholesterol. Unlike ispaghula and pectin, raftiline is not viscous. Raftiline is a prebiotic selectively stimulating growth of bifidobacteria (Clark et al., 2012). Hence, it can be speculated that its

cholesterol lowering ability is through its fermentation products and its prebiotic nature. Animal studies have suggested that the mechanism of action for TAG reducing effect of raftiline is through inhibition of hepatic fatty acid synthesis, this pathway is of importance only when a high carbohydrate diet is consumed, else it is relatively inactive (Parks, 2002).

The high production of butyrate through raftiline fermentation has attracted much research in regards to colorectal cancer prevention potential of raftiline. The mechanisms of butyrate action in regard to colorectal cancer prevention are discussed in *1.2.6.1.3 Butyrate.*

1.2.5 Microbiota

The human colon has a diverse colony of metabolically active microbiota, with an approximate 1000 different species of bacteria ranging from anaerobic to aerobic and beneficial to pathogenic. The population of the microbiota in the colon can reach up to 10^{11}–10^{12} cfu/g of colonic content (Slavin, 2013) accounting for 35-40% of the volume of colonic contents (Tappenden and Deutsch, 2007).

In recent years the colonic bacteria have shown major influences on the body's metabolic and immune function and this has led to exciting scientific research to the extent that there has been an accelerating number of publications (10,592 publications since 1956) with 261 published between January 2014 to March 2014, ranging from the role of microbiota in health, obesity (Turnbaugh et al., 2006); Crohn's disease (Hofer, 2014), cancer (Schwabe and Jobin, 2013, Greenhill, 2014) and CVD (Howitt and Garrett, 2012, Wang et al., 2011) to its impact on brain function and human behaviour (Cryan and Dinan, 2012).

The gastrointestinal tract is colonised by bacteria at birth and its composition is highly dependent on birth method, environment, breast / formula feeding, genetics, diet, medication, disease (Tappenden and Deutsch, 2007, Scott et al., 2013) and gastrointestinal factors (*Figure 1-1*); leading to great inter-individual variation in microbial profile (Turnbaugh et al., 2007) . The individual specific microbiota profile is often compared to a finger print.

The role of diet in microbiota composition begins early in life. Breast fed infants have shown to have a composition dominated by Bifidobacteria (Roger and McCartney, 2010, Fallani et al., 2010) compared to formula fed infants having a more diverse microbiota composition (Edwards and Parrett, 2002, Rinne et al., 2005). After initiation of weaning healthy adult like composition is formed (Yatsunenko

et al., 2012). Due to these factors high inter-individual variability can be seen in bacterial composition resulting in different molar ratios of SCFA production (Edwards et al., 1994).

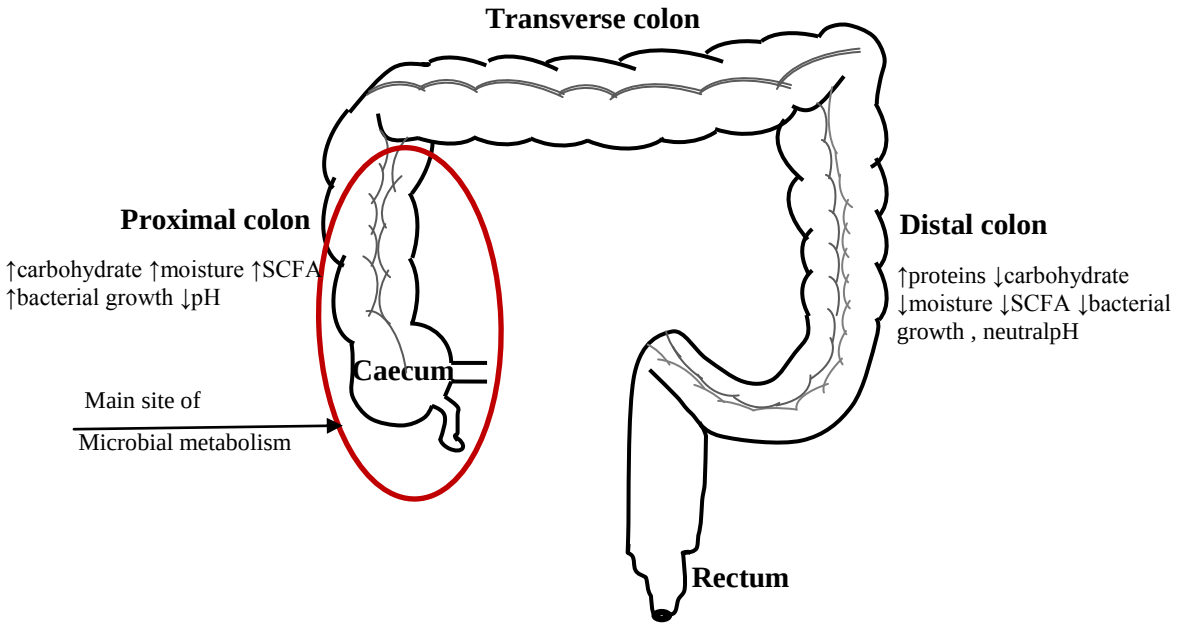


Figure 1-1 Regional differences in the human large bowel

Despite this great inter-individual diversity in the microbiota profile, four phyla have been identified to contribute most (98%) to the composition of the colony; Firmicutes (64%), followed by Bacteroidetes (23%), Proteobacteria (8%) and Actnobacteria (3%). The remaining 2% of the bacterial divisions are highly diverse and include Clostridium, Prevotella, Eubacterium, Ruminococcus, Fusobacterium, Peptococcus, Bifidobacterium, Escherichia and Lactobacillus (Matsuki et al., 2004, Zhu et al., 2010, Etxeberria et al., 2013).

The microbiota can exhibit both beneficial and pathogenic effects; depending on their metabolic activities and metabolites produced as a result. Some of the health benefits associated with the microbiota and the products of their activity include inhibition of pathogenic growth, prevention of colonic cancer, reducing risk of CVD, vitamin synthesis and immune-stimulation. Their harmful effects are due to the production of carcinogens, intestinal infection and diarrhoea (Saulnier et al., 2009). There has been much debate on the consequences, such as obesity and diabetes resulting from an imbalance between the two prominent bacterial phyla present in the gut; Bacteroidetes and Firmicutes (Burcelin et al., 2009, Cani and Delzenne, 2009). This has stimulated much interest in

dietary interventions to modify gut microbiota composition, increasing the ratio of beneficial to pathogenic bacteria.

In this regard, prebiotics have received much attention due to their low digestibility and bioavailability in the human upper gut, resulting in their availability for fermentation by selective commensal bacteria, leading to an improvement in bacterial composition and gut health (Gibson and Fuller, 2000). However, these prebiotics are mostly studied for their ability to promote beneficial bacteria and not their inhibitory impact on pathogenic bacteria. A study of flavanols in cocoa (Tzounis et al., 2011) demonstrated an increase in the growth of beneficial bacteria such as lactobacilli and Bifidobacteria both *in-vivo* and *in-vitro* ($p < 0.01$), as well as a reduction in the growth of pathogenic bacteria such as *C. histolyticum*. This opens grounds for research combining the well-known prebiotics such as fructo-oligosaccharides and some soluble fibres with flavanols to obtain greater bifidogenic effects.

Increased fibre consumption results in higher fermentation activity in the proximal colon, resulting in SCFA production and thus a drop in the pH to between 6.5 and 5.5 depending on type of fibre. This change in pH can greatly influence bacterial composition (Etxeberria et al., 2013) as demonstrated in a study by Duncan et al. (2009) where 33 bacterial species were incubated as a representation of colonic bacteria in an anaerobic continuous flow fermentor at pH 5.5, 6.2 and 6.7. They demonstrated a suppression of Bacteroides species at pH 5.5 with a promotion of gram positive bacteria favouring butyrate production.

The fate of both soluble fibres and polyphenols is greatly dependent on these gut microbiota, and the composition of the colony can determine the extent of impact fibre and polyphenols can have on human health.

The gut microbiota metabolism of polyphenols is essential for their absorption as well as production of more biologically active metabolites in the body. The majority of polyphenols reach the colon intact where they are hydrolysed to phenolic acids. However the metabolites of polyphenol are highly dependent on diet and vary amongst individuals (Hanhineva et al., 2010).

The microbiota can modify the phenolic skeletons through chemical reactions, making it possible for lower weight metabolites to be absorbed. This is made possible by the microbial enzymes that have the ability to cleave rings, hydrolyse glycosides, sulphates, glucoronides, lactones, amides and esters. These enzymes are also key to the reduction, dehydroxylation, demethylation and decarboxylation reactions (Aura, 2008).

Similarly soluble fibres escape digestion in the small intestine, reaching the colon where they can be partially or completely fermented.

Given the important role the gut microbiota play in the metabolism of carbohydrates and polyphenols, it is important to maintain the correct balance of host-microbiota and beneficial-pathogenic bacteria for human health.

1.2.6 Fermentation

The carbohydrates that have resisted digestion in the small intestine are the main source of carbon and energy for the colonic microbiota and are subjected to anaerobic degradation by these colonic organisms through which SCFA and gases are produced. This process is known as fermentation. The process of fermentation and production of SCFA allows for the salvage of energy from non-digestible substrates which would be wasted through excretion otherwise (Cummings and Macfarlane, 1991). (Tabernero et al., 2011, Macfarlane et al., 1992). The ileal content enters the caecum and proximal colon, staying there for 6-12 hours where the bacterial polysaccharidases and glycosidases are used to degrade the complex polysaccharides to oligomers and sugars, which are then used for fermentation by the anaerobic bacteria. Carbohydrate fermentation decreases as the material moves to the transverse colon and distal colon where they are stored until excretion (Macfarlane and Macfarlane, 2003, Cummings and Macfarlane, 1991). While the colonic content of nitrogen is approximately 6% of dry matter throughout the colon, the carbohydrate content is reduced from 20% at the proximal colon to 11% in the distal colon due to fermentation. Lower substrate availability in the distal colon results in lower SCFA production in this region (Cummings and Macfarlane, 1991). However, the type of carbohydrate is a determining factor as some carbohydrates are very fermentable, some less so or not fermentable at all, passing through the colon intact. Thus poorly fermented fibres may promote higher SCFA in more distal colon.

1.2.6.1 SCFA:

The major end products of polysaccharide, oligosaccharide, amino acids, proteins, peptides and glycoprotein metabolism by the gut microbiota are SCFA. Even though these metabolites can be produced through different substrate breakdown, polysaccharides are the biggest contributors to total SCFA production (Cummings and Macfarlane, 1991). The daily production of SCFA in humans amounts to 300mmol from which only 10 mmol/ day is excreted (Hoverstad, 1986).

The principal SCFA produced from all substrate fermentations, accounting for 85-95% of total SCFA produced are acetate, propionate and butyrate along with lesser produced formate, valerate, caproate, enanthate, caprylate, isobutyrate, isovalerate and isocaproate. Some of these branched SCFA such as isobutyrate and isovalerate can also be produced from the catabolism of branched chain amino acids such as valine, leucine and isoleucine (Macfarlane and Macfarlane, 2003, Flint et al., 2012).

As mentioned previously the production of these SCFA is highest in the proximal colon reducing towards the distal colon (*Figure 1-1*). However it was found that the molar ratio of these SCFA remains the same throughout the length of the colon (Cummings et al., 1987). This ratio depends on the type of carbohydrate/carbohydrates being fermented as different carbohydrates produce different molar rations of SCFA making the molar ratios, carbohydrate specific (*Table 1-1*).

After absorption, the colon the SCFA is removed at different rates by different tissues. Cummings et al. (1987) observed in sudden death victims that in the colon the molar ratio of acetate: propionate : butyrate was 57: 22 : 21. Most of the butyrate produced was absorbed through the colonic epithelium resulting in only 8% of butyrate in the portal blood. The concentration of propionate fell from 22% to 12% after transport to the liver, indicating the uptake of propionate by the liver. Acetate remained to be the only SCFA present in measurable quantities in the peripheral blood. Hence it is evident that the site of action and health benefit exhibit of each SCFA varies.

It is said that SCFA act mostly as an energy source; however they are also signalling molecules. The endogenous receptors of SCFA have been identified as G-protein coupled receptor free fatty acid receptor-2 (FFAR2, GPR43) as well as FFAR3 (GPR41). The receptor affinity for acetate is FFAR2, propionate FFAR2 and FFAR3 and butyrate FFAR3 (Le Poul et al., 2003). It was suggested that all three SCFA play a role in energy homeostasis by signalling through these receptors which can result in the modulation of gut transit time, adiposity and synthesis of glucagon like peptide-1 in the enteroendocrine cells, thus protecting against diet induced adiposity (Tolhurst et al., 2012, Samuel et al., 2008). This was not supported in a study by Lin et al. (2012) investigating the impact of these SCFA in FFAR3 deficient mice. It was shown that acetate and butyrate prevent weight gain independent from food intake, whereas propionate exhibited its impact by lowering food intake (Gao et al., 2009, Arora et al., 2011).

Knowing the molar ratio of the SCFA produced from a specific fibre, the site and health benefit of each SCFA, it may be possible to design diets for specific health effects. The role of SCFA in energy regulation may be of greater importance in regions where meeting daily energy requirements is a struggle.

1.2.6.1.1 Acetate

Acetate is the major SCFA produced through fermentation, mainly through the oxidative decarboxylation of pyruvate (Cummings, 1981) contributing ≈ 60% of total SCFA production and the only SCFA reaching the peripheral blood in any great amount (Scheppach et al., 1991, Cummings et al., 1987). Within the colonic environment, acetate stimulates cell turnover and heals damaged mucosal tissue (Sakata, 1987). Once it has entered the circulatory system it can be utilised by the hepatic tissues, indicated by significantly higher concentrations in portal blood than peripheral blood (Cummings et al., 1987). Mostly acetate is taken up by the peripheral tissues as a source of energy (Cummings and Macfarlane, 1991, Pomare et al., 1985) making it the primary method of energy salvation from non-digestible carbohydrates. Acetate is also the primary substrate used during cholesterol synthesis (Hijova and Chmelarova, 2007). Acetate produced from the colonic fermentation is not a major source of energy, but perhaps its role is of greater importance in regions of poverty where carbohydrates are the main source of energy.

Apart from the exogenous production of acetate by the colonic microflora, it is also produced endogenously. The actions of alcohol dehydrogenase (ADH) and cytochrome P450 2E1 (CYP2E1) metabolise ethanol into acetaldehyde which is then oxidised to acetate by acetaldehyde dehydrogenase (Shimazu et al., 2010). Acetate levels can rise during ethanol metabolism about 20 fold as well as during fatty acid oxidation as seen during long periods of starvation (Lundquist et al., 1962, Seufert et al., 1974, Pomare et al., 1985). It was shown by Pomare et al. (1985) that individuals (n=14) in a fasting state had significantly higher arterial concentrations of acetate (125.6 ± 13.5 umol/l) than in venous blood (61.1 ± 6.9 umol/l). Other endogenous sources of acetate are through its release in the nucleus and cytoplasm after the removal of acetyl groups by class I and class II histone deacetylases as well as after the release of acetylcholine in cholinergic nerve terminals by acetylcholine esterase (Tomaszewicz et al., 2003, Schweigert et al., 2004). The endogenous source of acetate is the more prominent source when compared to exogenous acetate production (Pomare et al., 1985).

The sum of exogenous and endogenous acetate can be converted to Acetyl-Co-A and used in various metabolic pathways. This process is facilitated by the two Acetyl-Co-A synthase enzymes. The AceCS1 enzyme is present in high concentrations in the kidney and liver where it facilitates the conversion of acetate, ATP and CoA to Acetyl-Co-A, which in turn is utilised in fatty acid and lipid synthesis. The sterol regulatory element-binding proteins regulate the expression of AceCS1genes and are insulin sensitive. Under fasting and starvation modes insulin levels decrease, resulting in lower

regulatory element-binding proteins and thus lower fatty acid synthesis (Luong et al., 2000, Fujino et al., 2001, Ikeda et al., 2001).

AceCS2 is present in high concentrations in the kidneys and cardiac muscles. The mitochondrial synthesis of Acetyl-Co-A in fed conditions is facilitated by Pyruvate dehydrogenase and generated from pyruvate. However under fasting conditions the Acetyl-Co-A produced from the liver cannot be completely oxidised and some of this Acetyl-Co-A is hydrolysed by acetyl-CoA hydrolases to yield acetate, which will enter peripheral circulation. This free acetate combined with ATP can be used by AceCS1 to generate acetyl-CoA (Luong et al., 2000, Yamashita et al., 2001).

In a study by Wolever et al. (1991) 180 mmol of rectally infused acetate increased blood acetate, cholesterol and glucagon levels and reduced free fatty acids as compared to the saline control. This fall in free fatty acids was considered to aid in the reduction of blood glucose, as free fatty acids and glucose compete for uptake by insulin sensitive tissues (Ferrannini et al., 1983, Wolever et al., 1989).

1.2.6.1.2 Propionate

Propionate is often the second most produced short chain fatty acid after acetate. The production of propionate can either be through the dicarboxylic acid pathway by the fixation of CO_2 forming succinate followed by decarboxylation or through the acrylate pathway produced from lactate and acrylate or even through propandiol pathway by processing deoxy sugars such as fructose (Cummings, 1981, Reichardt et al., 2014).

Propionate may contribute towards gluconeogenesis in humans (Sakata, 1989, Anderson and Bridges, 1984). As hepatic glucose production is a contributing factor to insulin resistance, there has been debate on the beneficial impact of propionate on health. It is important to note that the above two studies considering propionate as a gluconeogenic substrate were conducted previous to the identification of the intestine as a gluconeogenic organ (De Vadder et al., 2014, Mithieux et al., 2004a).

It is now hypothesised that propionate is a substrate of intestinal gluconeogenesis as it is produced in the gut lumen and its metabolites enter the gluconeogenic pathway through Krebs cycle (Mithieux et al., 2004b, Croset et al., 2001). The release of glucose through intestinal gluconeogenesis is detected by a glucose sensor of the portal vein, which in turn sends a signal to the brain through the peripheral nervous system, promoting beneficial effects on glucose metabolism and food intake (De Vadder et al., 2014, Delaere et al., 2012).

Propionate has also shown an impact on reducing food intake, however the mechanism behind this is not very clear and the available information is highly inconsistent. As explained previously, a hypothesised mechanism was through the FFAR-3. However substantial evidence on this mechanism is lacking. Human studies have demonstrated that the intake of sodium propionate in bread reduced the food intake in 6 participating individuals. Additionally insulin sensitivity was improved with lower glucose response after the consumption of the bread containing sodium propionate compared to control bread. In the same study *in-vitro* digestion of bread by saliva containing propionate demonstrated reduced digestibility (Todesco et al., 1991). This enzyme inhibiting property of propionate could be the contributing factor to lower glucose response and improved insulin sensitivity. These results were replicated in another study using various kinds of bread: whole meal bread, sourdough bread, whole meal bread with lactic acid, Calcium lactate or sodium propionate (62 or 185 g). Breads containing higher dose of sodium propionate resulted in higher satiety, improved insulin sensitivity and lower glucose levels. However in this study it was suggested that propionate reduces glucose response independent of starch digestibility as the amylolytic activity was only significantly reduced with Calcium lactate (Liljeberg et al., 1995). The same study was replicated with the addition of paracetamol as a measure of gastric emptying. It was found that breads containing sodium propionate slowed gastric emptying rate and hence this was proposed as the mechanism of action for propionate satiety properties. The same results were seen in rats fed propionate or lactate but not sodium propionate (Liljeberg and Björk, 1996). The addition of ultrasonography to a similar study model confirmed the above results, with sodium propionate induced delay of gastric emptying rate and reduced glucose response (Darwiche et al., 2001).

Similarly the mechanism of cholesterol lowering effect of propionate observed in animal and human studies has not been clearly understood. One of the suggested mechanisms is the change in propionate to acetate ratio. This was observed when rectally infused acetate (180 mmol) increased plasma cholesterol and decreased free fatty acids, whereas the same volume of propionate had no impact on cholesterol levels, but increased glucose, glucagon and delayed the fall in free fatty acids. When the two were combined no change was observed in cholesterol levels indicating the inhibitory impact of propionate on cholesterol synthesis from acetate (Wolever et al., 1991). However it is important to note that this study used equimolar ratios of acetate: propionate. Although the concentration of acetate is similar to physiological concentrations (57% of total 300mmol) this concentration is much higher than physiological concentration of propionate (≈12.5 mmol). The metabolism of propionate by the liver opposes that of acetate as propionate can reduce the oxaloacetate availability required for citrate

formation by using it for gluconeogenesis, thus inhibiting the oxidation of acetate in the hepatic tissues. This in turn influences carbohydrate and lipid metabolism (Wolever et al., 1991).

Hence the ratio of propionate to acetate may be critical in the physiological role of the SCFA in the body. Thus the selection of fibre type based on its molar ratio production of short chain fatty acids is of great importance. Slowly fermented viscous fibres producing higher relative propionate such as ispaghula may be preferred over rapidly fermented, high acetate producing fibres such as pectin for the purpose of reducing cholesterol synthesis.

There are many studies demonstrating the cholesterol lowering effect of propionate, however the mechanism proposed by the studies are numerous, such as: down regulation of the genes related to intestinal cholesterol biosynthesis pathway (Alvaro et al., 2008b), inhibition of 3-hydroxy 3-methylglutaryl CoA reductase a cholesterol synthesizing enzyme (Wright et al., 1990a), redistribution of cholesterol from plasma to liver (Illman et al., 1988), enhancement of bile acids synthesis and secretion and cholesterol 7α-hydroxylase activity *via* elevation of the mitochondrial succinyl-CoA (Imaizumi et al., 1992). However it is important to note that most of these studies are carried out in animal models. This might not necessarily be translatable to mechanism of action of propionate in humans. Studies have shown that rat hepatocytes can be affected by low concentration of propionate (0.1 mmol/L) while to see the same degree of inhibitions in humans a much higher concentration of 10-20 mmol/l propionate was required (Lin et al., 1995). Propionate in the colon may also stimulate cell turnover in the gut (Sakata, 1987).

1.2.6.1.3 Butyrate

Butyrate is usually the least abundant SCFA ranging from 5-20% of total SCFA production (Aschenbach et al., 2011). Butyrate is produced by the reduction of acetoacetate (Cummings, 1981) and it is the preferred source of energy for the colonocytes (Bergman, 1990, Arora et al., 2011). This was identified by Roediger (1980) in an *in-vitro* study demonstrating clear intra-colonic regional differences for butyrate uptake, the highest being in the distal lumen. This could be due to the lower pH in this part of the gut which promotes butyrate-producing bacteria to compete against gram-negative carbohydrate-utilizing bacteria, such as *Bacteroides sp* (Louis and Flint, 2009).

The higher concentration of butyrate in the colon than the portal veins suggested that most butyrate is utilised by the colonocytes after which it is metabolised by the hepatic tissues (Cummings et al., 1987). 70 – 90 % of Butyrate is metabolised by the colonocytes, it enters the mitochondria to undergo β-oxidation to Acetyl-coA, which enters the TCA cycle and results in ATP (Moco et al., 2012). Butyrate

may play an important role in increasing energy expenditure (Donohoe et al., 2011, Gao et al., 2009). This was displayed when a 5 week treatment of butyrate resulted in 10.2% of original body weight loss in obese mice, along with 30% reduction in fasting glucose and 50% reduction in insulin resistance (Gao et al., 2009). This is in contrast to the proposed association of butyrate production and obesity which has been attributed to the high energy salvage by the butyrate producing Firmicutes and Bacteroidetes (Turnbaugh et al., 2006), however other studies comparing the colonic composition of lean and obese individuals have found no association between body weight and butyrate producing bacteria (Duncan et al., 2008).

Apart from being the main source of energy for the colonocytes, butyrate plays an important role in colonic health as it can increase cell proliferation in normal intestinal cells (Kripke et al., 1989, Sakata and Engelhardt, 1983) and reduce proliferation in cancer cells (Canani et al., 2011, Clarke et al., 2012, GIBSON et al., 1992) as well as inducing apoptosis (Scharlau et al., 2009b, Hague et al., 1995). The butyrate paradox termed after the contradictory patterns of butyrate action are observed as butyrate stimulates the physiological pattern of proliferation in the colon basal crypt, simultaneously reducing the earliest detectable neoplastic lesions by reducing the number and the size of aberrant crypt focus in the colon (Alrawi et al., 2006, Comalada et al., 2006). Butyrate has shown to promote the induction of differentiation of colonic epithelial cells, improvement of tight junction barrier function, suppression of epithelial proliferation and tumorogenesis (James et al., 2003, Zoran et al., 1997, Basson et al., 2000). Sodium butyrate can act as an antiproliferative on many cell types, demonstrating prevention of colon cancer and adenoma development (Bornet et al., 2002). Butyrate can also modify gene expression at a molecular level via the phosphorylation and acylation of histone proteins (Archer and Hodint, 1999).

The passage of toxic and pro-inflammatory molecules into the sub-mucosa and systemic circulation is prevented by the barrier forming epithelial cells. The tight junction between these epithelial cells forms a barrier which can be disturbed by signalling from inflammatory cytokines that cause a change in tight junction proteins (Turner, 2006, Plöger et al., 2012). In this regard butyrate has demonstrated a modulatory impact on the expression of these tight junction proteins and the up-regulation of the dominant colonic mucin-2 in *in-vitro* models, thus improving the intestinal barrier (Plöger et al., 2012).

An imbalance of histone acetylation can lead to the deregulation in transcriptional genes involved in the cell cycle progression, differentiation and apoptosis; potentially contributing to the development of cancer. In this regard butyrate inhibits histone deacetylase causing hyperacetylation of histones (Scharlau et al., 2009a, Davie, 2003).

Butyrate induces its anti-inflammatory properties mostly by inhibiting nuclear factor κB (NF-κB) activation in inflammatory cells of the colon (Inan et al., 2000). NF-κB is often dysregulated in individuals with colon cancer, inflammatory bowel diseases, Crohn's disease or ulcerative colitis. NF-κB plays a key role in the regulation of cellular genes involved in early immune inflammatory responses, such as IL-6, IL-8 and TNF-α (Shao et al., 2000, Lind et al., 2001).

Additionally butyrate may be involved in colonic motility as demonstrated by Soret et al. (2010). In this study the proportion of choline acetyltransferase but not nitric oxide synthase, immunoreactive myenteric neurons was increased by butyrate *in-vitro* and *in-vivo*. An increase in the cholinergic-mediated colonic circular muscle contractile response *in-vitro* was also seen.

Most of the butyrate related health benefits are induced within the colonic environment. However some studies have suggested that butyrate may induce health benefits outside the colon. This includes increase in energy expenditure as previously mentioned as well as down regulation of nine genes involved in intestinal cholesterol biosynthesis which is seen in hypercholesterolemia, when the intestine becomes the major site of cholesterol biosynthesis, due to this process being inhibited in the other organs by fasting (Alvaro et al., 2008a).

1.2.6.2 pH

With the proximal colon being the site of extensive carbohydrate fermentation, a large amount of SCFA is produced causing a low pH environment. The absorption of these SCFA accompanied by the production of bicarbonate keeps the caecum pH at approximately 5.6 but it can go as low as 4.5 (Cummings et al., 1987). The pH increases gradually towards the distal colon (approximately 6.6) with less SCFA being present (Cummings et al., 1987). This lower luminal pH can inhibit the growth of pathogenic pH sensitive bacteria while promoting the growth of beneficial bacteria such as bifidobacteria and lactobacilli (James et al., 2003, Kiely et al., 2001). This was demonstrated in a continuous anaerobic culture where a number of bacteroides species survived a range of pH from 5-7, whereas lactobacilli and bifidobacteria surviving at pH 5 disappeared with increasing pH from 5 to 6 and then pH 7 (Edwards et al., 1985). In a study by Duncan et al. (2009) 19 out of 23 gram-positive anaerobes demonstrated growth by reducing pH from 6.7 to 5.5. These butyrate producing bacteria comprised 50% of population at pH 5.5 whereas they were not detectable at pH 6.7. The 8 bacteroides representative species although present at pH 5.5, were 27% of population as compared to 86% at pH 6.7. This indicates that the bacteriodes species can survive a range of pH but favour higher colonic pH.

Higher faecal pH has been identified and reviewed as a risk factor of colon cancer by many studies (Thornton, 1981, Walker et al., 1986, Kato et al., 2013, Van Dokkum et al., 1983). One of the proposed mechanisms behind this is less solubility of bile acids at lower pH resulting in less degradation of bile acid into co-carcinogens through the action of 7α-dehydroxylase (Thornton, 1981). Another proposed mechanism is that lower pH allows ammonia from urea degradation to remain in its ionised form (NH_4^+) which is less absorbed from the colon as compared to NH_3 (Newmark and Lupton, 1990).

Changes in colonic pH can also lead to a change in metabolic pathways of microbiota independent of numeric values of species. Lower pH may result in the promotion of propionate and butyrate production instead of acetate (Louis and Flint, 2009, Edwards et al., 1985). The lower pH may also enhance the absorption of minerals such as calcium and magnesium up to 6 fold as seen in a study by Younes et al. (1996).

Hence SCFA may exhibit some of their health benefits indirectly by lowering the pH in the colonic environment. Slowly fermentable fibres may be preferable as they can regulate the colonic pH over a longer period of time and this beneficial impact may be extended to the distal colon and not limited to the proximal colon. This is of great importance as many of the colonic diseases are associated with the distal colon.

1.2.6.3 Gases

Along with other products of fermentation, the gases hydrogen, carbon dioxide and methane are produced. Due to the activity of hydrogen utilising species (methanogenic, acetogenic and sulphate reducing species) hydrogen is normally kept at low levels in the colon. Non-methanogenic individuals lack the ability to produce methane and poses large numbers of sulphate reducing bacteria which use sulphate during oxidative reactions as an electron acceptor. Examples of these are desulfovibrio, desulfobacte and desulfomonas (Moco et al., 2012).

The composition of gas produced from microbiota is greatly varied and dependent on the diet. However individuals on the same diet have demonstrated great inter-individual variation in composition of gas produced, indication that gas production is influenced by diet and composition and metabolic activity of colonic microbiota (Manichanh et al., 2013). The absence of polysaccharides from the diet results in low production of hydrogen and methane (less than 50 ml/ day). When fermentable material is available to the microbiota the production of hydrogen and methane increases, the produced gas can be eliminated either as flatus or from the lungs. Hence breath hydrogen can be a

good measure of fermentation as 65% of gas produced at rates of 200 ml/day is excreted through breath (Cummings and Macfarlane, 1991).

Hydrogen is produced as a means of reducing power disposal generated during fermentation and can be produced from the oxidation of pyruvate, formate or reduced pyridine nucleotide. However pyruvate is thermodynamically more favourable to form hydrogen and can produce hydrogen through being cleaved by pyruvate-formate lyase in Enterobacteria or via ferredoxin by Saccharolytic Clostridia (Cummings and Macfarlane, 1991).

Highly fermentable fibres consequently result in higher gas production which leads to abdominal discomfort and pain in individuals (Manichanh et al., 2013). This may lead to non-compliance to intervention in long-term studies. Hence to incorporate soluble fibres in diet and life style of individuals slower fermented fibres such as ispaghula are preferred as rapidly fermented fibres can result in a high volume of gas produced rapidly.

1.3 Polyphenols

Polyphenols are one of the most common groups of bioactive compounds derived from plants. They greatly contribute to the colour and taste of fruits and vegetables (Duda-Chodak, 2012).

Chemically they are characterised by the hydroxylated phenyl moieties and are naturally occurring as their glycosylated forms (Moco et al., 2012). Dietary intake of polyphenols has been estimated to range on average between 0.15 and 1 g/day and represents approximately two-thirds of the total daily phenolic intake, with approximately one third as phenolic acids (Lee et al., 2006).

Polyphenols are only partially absorbed in the small intestine. The glycosidic form of these compounds undergo de-conjugation by hydrolases, β-glucosidases, β-glucoronidases and α-rhamnosidases (Selma et al., 2009). Upon reaching the colon, the gut microbiota are able to metabolise polyphenols by the following means: fermentation of the flavonoid backbone, deglucoronidation of flavonoids as well as *O*- and *C*-deglycosilation, ester and amide hydrolysis. The aromatic moieties of aglycones can undergo dehydroxylation, demethoxylation and demethylation, as well as ring rupture, hydrogenation, α and β-oxidation of the substituted aliphatic groups (Williamson and Clifford, 2010).

The backbone rupture of the flavonoids produce hydroxylated forms of phenyl acetate or phenyl propionate derived from the B-ring and phloroglucinol from the A-ring. The simple phenolic acids produced as a result of this backbone rupture of flavonoids can be metabolised to non-aromatic compounds, including SCFA, lactate, gases (CO_2 and H_2), ethanol and oxaloacetate (Moco et al., 2012). These phenolic acids undergo phase I and phase II metabolism in the liver, where they are converted to less toxic compounds through glycine-conjugation, sulphation, glucoronidation and methylation.

The microbial catabolism of polyphenols has been associated with the production of benzoic acid which is glycine-conjugated in the liver and converted to hippuric acid, which can be easily detected in urine (Lord and Bralley, 2008, Bazzocco et al., 2008) but naturally this hepatic metabolite will not be detected in *in-vitro* fermentation models (Bazzocco et al., 2008).

1.3.1 Health implications of polyphenols

Most of the health claims for polyphenols in humans are obtained from epidemiological studies investigating the impact of fruit and vegetable consumption (Riboli and Norat, 2003). This is through the hypothesis that fruits and vegetables are enriched with flavonoids which have a beneficiary impact on health, independent from other known micronutrients (Arts and Hollman, 2005). These epidemiological studies have investigated the relationship between food consumption with risk of disease. However due to the lack of an accurate food composition data only few foods were assessed for flavonoid content and their association with risk of disease. In these studies it cannot be said with certainty which compound in the food was responsible for the observed association, as increased intake of fruits and vegetables results in an increased consumption of fibre and plant sterols as well. Furthermore, causation cannot be claimed by epidemiological studies, only an association. Additionally increased intake does not necessarily increase bioavailability considering some factors such as food matrix interaction may reduce the bioavailability, thus further complicating the implications for cause-effect. Even though there is a poor understanding of their mechanism of action, in the field of nutrition, foods rich in polyphenols are considered to be healthy *in-vitro*.

While epidemiological studies have linked the consumption of plant based foods with reduced risk of diseases such as CVD, based on a reduction in blood pressure and improved microvascular function (Woodside et al., 2013, Dauchet et al., 2009b), further studies have attempted at identifying various components of plant based foods as a source of these health benefits. There are conflicting opinions on

the impact of polyphenols as a component of plant based foods on health, with some studies demonstrating a protective effect of these compounds against chronic diseases.

The Hertog et al. (1993b) study was one of the first studies to demonstrate a reduced incidence of CVD linked mortality to flavonoid intake. While a review of epidemiological and intervention studies by Tangney and Rasmussen (2013) has attributed the vasodilatory effect of polyphenols to its prevention of CHD risk. Other studies such as the seven countries study (Hertog et al., 1995) have shown that foods such as tea, red wine, onions and apples are the biggest contributors to this observed health benefit of polyphenols.

While a recent meta-analysis (184 intervention trials) has demonstrated a vasodilatory and blood pressure lowering impact of the six flavonoid subgroups combined (Kay et al., 2012); other studies have focused on specific polyphenols and their health benefits. Studies investigating individual polyphenols may be more valid than a meta-analysis on the 6 subgroups combined; given that different polyphenols (discussed below) have demonstrated different outcomes and impact on health. Some of such studies looking at individual polyphenols are the combination of The Nurses' Health study I, II and the Health Professional study follow up; indicating a reduction of 8% for hypertension through high anthocyanin consumption (Cassidy et al., 2011). Similarly the high consumption of flavan-3-ols was linked to a reduction in atherosclerosis in middle aged men (Dauchet et al., 2009a) and the consumption of proanthocyanidins to reduced risk of coronary artery disease in postmenopausal women (Mink et al., 2007).

Some of the mechanisms associated with this health benefit of polyphenols has been suggested to be prevention of platelet adhesion, secretion and aggregation through anti-inflammatory properties (El Haouari and Rosado, 2011), reduction of oxidative stress and cholesterol ester accumulation in macrophages through up-regulation of antioxidant enzymes and reduction of oxidative stress (Mulvihill and Huff, 2010).

Similar to the evidence for CVD prevention, the evidence on polyphenol consumption linked to reduced risk of cancer is modest. Epidemiological studies have demonstrated chemo-preventive properties for polyphenols (knekt et al., 2002). These chemo-preventive properties are dependent on type of polyphenol and food source. In a review by Thomasset et al. (2007), it was shown that while the evidence on the chemo-preventive properties of tea polyphenols qualify them for advancement in phase III clinical trials, the same may not be applied for the evidence on curcumin or soya isoflavones, and further investigation in pre-malignancies studies is required. In addition to type of polyphenol and food source, the impact of polyphenols may vary dependent on the site of cancer occurrence. As seen

in the seven countries study, while a strong correlation was found between the flavonoid intake and stomach cancer, there was no evidence for the same link to lung cancer. Contrary to this study, The Iowa women's study (Cutler et al., 2008) demonstrated that women with higher flavanone and proanthocyanidin intake had a lower incidence of lung cancer (32% and 25% respectively). There has also been an increased interest in the role of polyohenols in the prevention of colorectal cancer. A Meta-analysis of seven case-controlled studies has demonstrated an inverse association between flavonol and procyanidin intake and the development of colorectal cancer (White et al., 2014).

As previously mentioned the evidence on the health benefits of polyphenols is contradicting. They have shown both pro-oxidant and antioxidant effects in-vitro (Halliwell, 2007, Halliwell, 2008, Skibola and Smith, 2000). The pro-oxidant properties of polyphenols may be due to their oxidation in cell culture media of in-vitro studies or their presence in hot beverages resulting in the production of hydrogen peroxide as demonstrated for green tea (Forester and Lambert, 2011). However it has been suggested that this pro-oxidant property of polyphenols may contribute to their chemo-preventive properties as hydrogen peroxide may induce cytotoxicity and induce apoptosis in malignant cells (Nakazato et al., 2005). Low levels of pro-oxidant activity may even raise xenobiotic-metabolizing and antioxidant defence enzymes (Li et al., 2010). The anti / pro- oxidant properties of polyphenols may be dependent on the concentration used. This was demonstrated by Hong et al. (2002) by incubating 10 uM [3H] epigallocatechin 3-gallate (ECGC) with human colon adenocarcinoma cells. The further addition of 50 uM of ECGC to the plates resulted in the production of hydrogen peroxide and further uptake of the compound by the cells indicating a passive diffusion process.

Additionally these health implications induced by polyphenols are highly dependent on the polyphenols reaching the site of action. Studies investigating accumulation and distribution of polyphenols using radiolabelled compounds have been discussed in 1.3.3Tissue uptake of polyphenolic compounds. Studies have demonstrated that polyphenols such as quercetin-3-O-glucuronide accumulate in macrophage derived foam cells, where they may be de-conjugated to the aglycones quercetin (Kawai et al., 2008a). In the same study quercetin-3-O-glucuronide supressed the formation of foam cells by supressing the expression of mRNA class-A scavenger receptor and CD36. Similar evidence has been shown for (-)-epicatechin-3-O-gallate (Kawai et al., 2008b).

However, as demonstrated in a study by Mullen et al. (2002) Majority of the polyphenols are metabolised in the intestine with only 7.2% of ingested polyphenols found in blood and tissues such as 0.0017% ingested radioactivity found in the brain. Thus it is important to investigate the health impacts of the polyphenol metabolites from their colonic metabolism by the microbiota. However, there is very

limited evidence on the bioactivity of phenolic acids (discussed in 1.3.5.3Bioactivity of phenolic acids:). They are considered anti-oxidant agents due to their ability to stabilise unpaired electrons which is dependent on the number and position of the hydroxyl groups (Goufo and Trindade, 2014).

1.3.2 Bioavailability, absorption and metabolism

1.3.2.1 Bioavailability

The bioavailability of polyphenols can be greatly modified by factors such as food matrix interaction, gastric emptying, MCTT and method of administration (Erlund, 2004).

As the majority of polyphenols escape digestion and absorption in the small intestine, they are catabolised by the colonic microflora to phenolic acids, through which they may exhibit some of their health benefits, however information on the health benefits of phenolic acids is very limited. Polyphenol catabolism and carbohydrate fermentation both take place in the colon. Hence, food matrix interaction not only in the upper digestive system, but the interaction of food metabolites and catabolites in the colon can be a key factor in the bioavailability of phenolic acids from polyphenol breakdown.

The term bioavailability was first coined by the pharmacology industry and defined by the FDA as the "rate and extent to which a drug reaches its site of action". However the accurate quantification of a compound in its site of action is not possible and hence the definition was modified to allow for the fraction of an oral dose of a substance or its metabolites that reach the systemic circulation (Stahl et al., 2002).

Based on this definition bioavailability can be measured by the peak blood concentration of the substance (C_{max}), the time taken to reach the peak concentration (T_{max}) and the area under the curve (AUC).

However the majority of polyphenols are not directly absorbed but reach the colon where they are broken down into smaller fragments known as phenolic acids. Thus bioavailability of polyphenols in the blood is not an accurate representation as it is more a representation of intestinal absorption, hydrolysis, uptake by tissue and release from body stores (Yeum and Russell, 2002). Additionally a large dose of polyphenol consumption, above that of daily intake is required to detect an increase over plasma baseline levels due to their low signal to noise ratio (Déprez and Scalbert, 1999, Yeum and Russell, 2002, Heaney, 2001). Another method of assessing bioavailability of polyphenols is the balance method, using the difference between known ingested concentration of compound and the

concentration of the compound excreted (Heaney, 2001). Although this method might not be accurate for the bioavailability of polyphenols it is a useful estimation of phenolic acid production from a known concentration of polyphenols. Similarly the urine increment method can be used for estimation of the compound bioavailability, assuming that the urinary excretion of the compound is proportionate to their plasma concentrations (Heaney, 2001).

The most accurate method for assessing bioavailability of phenolic compounds is the use of radioactive isotopes. Using this method the distribution of labelled compounds as well as possible metabolites of compounds can be traced through their absorption. However the positioning and the number of the radiolabelled atoms in the compound is crucial in determining the strength of the signal as well as identification of potential metabolites and catabolites of the compound (Heaney 2001).

1.3.2.2 Absorption

For polyphenols to exhibit any health impact it is essential for them to be absorbed, either as the parent compound or as their metabolites. There are several factors that influence the absorption of polyphenols such as their molecular size and inter molecular linkage, solubility and lipophilicity (Spencer et al., 2001a).

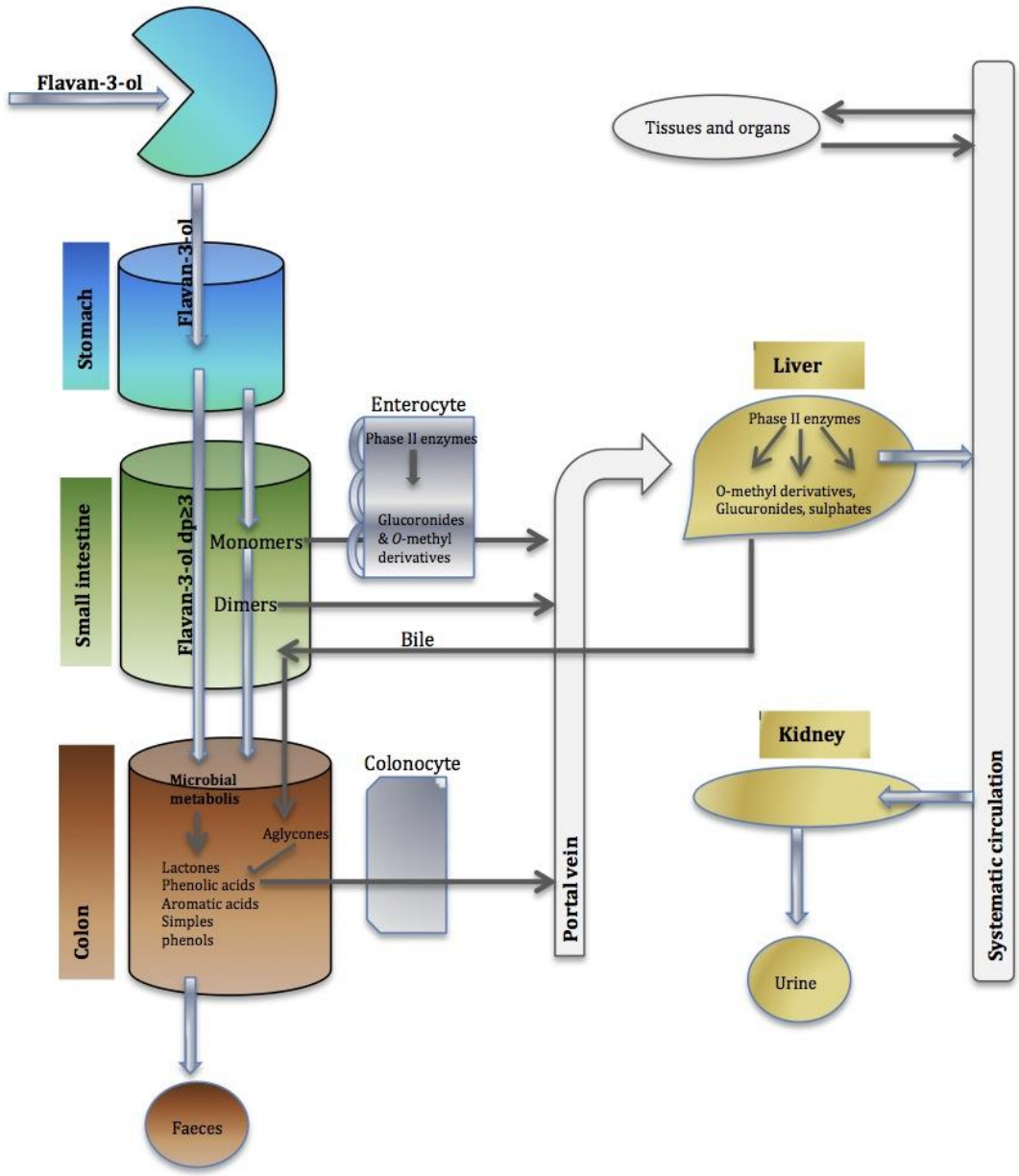
The majority of polyphenols present in the diet are in the form of polymers, glycosides and esters. These compounds cannot be readily absorbed and require hydrolysis by the intestinal enzymes or by the glycosidases of colonic microbiota (Manach et al., 2004). Polyphenols present as aglycones and few glucosides may be absorbed from the small intestine, whereas glycosides composed of more complex moieties such as rhamnose may only be absorbed after hydrolysis in the colon. This was demonstrated in an acute bioavailability study (Graefe et al., 2001) where no difference was found between the peak plasma concentrations of quercetin after ingestion in the form of onion or quercetin-4-glucoside (C_{max} = 2.31 ± 1.46 vs. 2.12 ± 1.63 ug/ml and T_{max} 0.68 ± 0.22 vs. 0.70 ± 0.31 h after ingestion). Whereas T_{max} was delayed to 6.98 ± 2.94 hours after ingestion of rutin, suggesting its absorption from the colon rather than the small intestine and the C_{max} was reduced to 1.0 – 2.0 uM. The reduction in C_{max} could be explained by the production of phenolic acids from released quercetin in the colon as well as less surface area for absorption as compared to the small intestine.

The rate of absorption of 18 polyphenols was reviewed by Manach et al. (2005) using C_{max} and AUC as an indication. There was a large variation in plasma C_{max} after consumption of 50mg aglycone equivalents from 0.4-4 uM. gallic acid and isoflavones were found to be the most readily absorbed,

followed by catechins, flavanones and quercetin glucosides. The least absorbed polyphenols were found to be proanthocyanidins, tea galloylated catechins and anthocyanins.

The oral absorption of flavanols has displayed large variability, ranging from 1-50% of ingested flavanols (Baba et al., 2000, Rein et al., 2000, Hollman et al., 2001). Some of this variation has been attributed to the complex *in-vivo* system such as gastric pH, the type and activity of microbiota. However, in a study (Schramm et al., 2003) investigating the impact of the antibiotic Famotidine on oral absorption of cocoa flavanols, changes in gastric pH failed to demonstrate any impact on the oral absorption (C_{max} or AUC) of flavanols. The absorption and metabolism of polyphenols has been illustrated in *Figure 1-2*, with the use of Flavan-3-ols as an example.

Figure 1-2 absorption and metabolism of polyphenols



Adapted using information from Monagas et al. (2010) and Moco et al. (2012) and with the use of flavan-3-ols as an example.

1.3.2.2.1 Mechanism of absorption

Human ileostomy studies, use of human Caco-2 cells cultures and the use of rat intestinal perfusion (Walle, 2004) are some of the possible methods available for the understanding of polyphenol absorption:

Most polyphenols present in the diet are hydrophilic, thus their penetration of the gut through passive diffusion in unlikely. The active transport mechanism proposed is the Na+ dependent saturable transport. According to this method glucosides can be transported into the enterocytes by SGLT1. However intact flavanol glucosides such as quercetin glucosides were not present in plasma samples after ingestion (Day et al., 2001, Mullen et al., 2004). This was further supported in a study by (Walgren et al., 2000b), when the absorbed quercetin glucosides by SGLT1 were efluxed by the proposed MRP2 transporter. Since then studies using rat intestinal perfusion and ileostomy patients have suggested that quercetin glucosides are hydrolysed allowing for the absorption of quercetin from the intestine (Walle et al., 2000, Sesink et al., 2003).

1.3.2.2.2 Factors affecting absorption:

- Glycosylation

  Glycosylation of a compound can affect both site and extent of absorption. As shown by Graefe et al. (2001) no differences were found between quercetin and quercetin-4-glucoside C_{max} or T_{max}. However when consumed in the form of quercetin-3-rutinoside the C_{max} was significantly reduced and T_{max} was delayed. These results were replicated in more recent studies by Jaganath et al. (2006b) and Mullen et al. (2006). However it is important to note that this lower absorption does not necessarily translate into lower bioavailability. As the glycosidic compounds enter the colon, where they are hydrolysed by the gut microbiota and made available in the form of phenolic acids.

- Structure

  The structure of the compound can also be a key factor influencing its absorption as reviewed by Manach et al. (2005), 50 mg of aglycone equivalents have resulted in a range of 0-4 uM in plasma. This was also demonstrated for compounds containing a glycoside moiety where absorption takes place from the colon. (Jaganath et al., 2006a, Mullen et al., 2008a) The C_{max}/dose values for hesperetin-7-O-rutinoside appeared to be significantly higher (16.5 Nm/Umol) as compared to that of quercetin-3-*O*-rutinoside (0.09 Nm/Umol).

- **Food matrix**

  Polyphenols are not consumed in isolation from other food components. Polyphenols present in the diet are present in fruits and vegetables along with macronutrients, fibre, plant sterols, minerals etc. It is hence important to assess the impact of food matrix interactions on the bioavailability of polyphenol compounds. The food matrix interaction of foods commonly consumed alongside have been previously investigated and summarised in the relevant section (*1.4 Food matrix interaction, page:74*). These combinations may include cocoa and milk, strawberry and cream, orange juice and yoghurt or carbohydrates and polyphenols. We aimed to further investigate the food matrix interaction between two functional food groups; fibre and polyphenols that are present together in plant foods.

1.3.2.3 Metabolism

Following absorption of the phenolic compounds they are metabolised and conjugated. This can take place in the gastrointestinal lumen, intestinal cells or in the liver. The metabolism of these compounds can play a key role in the impact they exhibit in the body and their ability to enter cells (Kroon et al., 2004).

The phase I and phase II metabolising enzymes involved in the metabolism of polyphenolic compounds are the enzymes responsible for catalysing detoxification reactions as part of the primary defence mechanism against xenobiotic and toxic compounds (Sanchez et al., 2001).

Once absorbed the polyphenolic compounds can be metabolised by the phase II metabolising enzymes (Manach et al., 2004): the uridine-5-diphosphate glucuronosyl transferase (UGTs), cytosolic phenol-sulfotransferase (SULTs) and catechol-*O*-methyltransferase (COMTs). The result of phase II metabolism is glucuronide and sulphate conjugates with or without methylation across the catechol functional group with the possibility of multiple conjugates. Phase II enzymes are distributed among tissues such as intestines, liver and kidney. The intestinal conjugated metabolites can be taken up by the liver and partially secreted in bile. The detection of these metabolites in the bile suggests that they undergo eneterohepatic recycling after which they are secreted back into the intestinal lumen (Crespy et al., 1999, Crespy et al., 2003).

1.3.2.4 Colonic metabolism

Polyphenol compounds that have escaped absorption in the small intestine (80-90%), or have been returned to the colon through enterohepatic recycling will be subjected to gut microbiota metabolism

(Spencer et al., 2000). The range of catabolites produced, known as phenolic acids is specific to type of polyphenol compound and their physio-chemical properties. Many studies have tried to identify phenolic acids produced by the action of the colonic microbiota, either by *ex-vivo* faecal fermentations or analysis of urinary excreted phenolic acids after the consumption of polyphenol rich foods (Olthof et al., 2003, Aura et al., 2002, Rechner et al., 2004, Labib et al., 2004, Bravo et al., 1994).

The mechanism of action for the catalytic and hydrolytic microbiota is suggested to be the splitting of the polyphenol nucleus through ring fission, as well as hydrolysis, dehydroxylation, demethylation, decarboxylation and deconjugation (Scheline, 1999).

The concentration of phenolic acids in the colon is much higher (~15- 410 uM) than that of flavonoids (~7uM), suggesting that these phenolic acids may have direct protective effects in the colon through scavenging of nitrogen and oxygen species before entering circulation (Halliwell, 2007, Halliwell et al., 2005).

As most of the polyphenol compounds are catabolised to phenolic acids, and as it has been shown that the plasma concentration of these phenolic acids is higher than conjugated metabolites (Gonthier et al., 2003, Rechner et al., 2002), it is important to further investigate the factors that can potentially modify the production of these phenolic acids in the colon.

1.3.3 Tissue uptake of polyphenolic compounds

Polyphenols and their metabolites must reach their target tissue in order to exhibit any beneficial effect; hence their actual bioavailability should be assessed when they reach their site of action. Despite the importance of their site of accumulation and action, the information in this regard is limited. One way to assess this information is by using radio labelled compounds.

Most of such studies are feeding studies in animal models, such as the ingestion of [2-^{14}C]-quercetin-4-*O*-glucoside in rats which resulted in high accumulation of the metabolite in the liver and kidneys, indicating that these organs are the main site of metabolism and excretion (Graf et al., 2005). In another study, rats were fed a supplemented diet of blueberry extract containing anthocyanins for 10 weeks, this resulted in the detection of trace levels of anthocyanins in the brain (Andres-Lacueva et al., 2005). A study by Abd El Mohsen et al. (2002) demonstrated that the consumption of epicatehin (100 mg/kg body weight) in male wistar rats resulted in the appearance of the two metabolites epicatechin glucoronide and 3-*O*-methyl epicatechin glucuronide in the brain tissue. The presence of epicatechin in the brain tissue was suggested to protect against neuronal cell death induced by oxidative stress. The same authors detected 0.2 nmol/g unmetabolised anthocyanidin in the brain tissue of rats 18 hours after

feeding with 184 umol/kg of pelargonidin (El Mohsen et al., 2006). Similarly the supplementation of rats with 50 mg/kg and 200 mg/kg quercetin resulted in the accumulation of trace amounts of quercetin in the brain (Ishisaka et al., 2011). Such radioactivity in the brain was also detected after the consumption of ^{14}C labelled grape polyphenols (Janle et al., 2010).

In contrast, Suganuma et al. (1998) detected radioactivity in organs such as the brain, liver, lungs, kidney, uterus, ovary and testes after the administration of [^{3}H](-)-epigallocatechin gallate into the stomach of male and female mice. Similar results were found for the accumulation of anthocyanins in plasma, liver, kidney, testes and the lungs of rats after a bilberry extract diet for two weeks (Sakakibara et al., 2009).

However, a study using ^{14}C labelled quercetin-4-glucosides detected only 6.4 % of the ingested dose outside the gastrointestinal tract 60 minutes after administration (Mullen et al., 2002). These results were supported by a later study from the same authors (Mullen et al., 2008c) when rats were supplemented with 4 mg/kg body weight [2-^{14}C] quercetin-4-glucosides. Majority of the radioactivity was found in the caecum and colon, after which it was degraded to phenolic acids and excreted through urine over 72 hours without any tissue accumulation. Similarly the supplementation of rats by gavage with 2.8ml of raspberry juice did not result in any anthocyanin accumulation in the brain (Borges et al., 2007).

Similarly, These tissue accumulation results were not replicated in a human study (n=6) where flavan-3-ol metabolites were only detected in plasma and not in the cerebrospinal fluid after the ingestion of green tea (Zini et al., 2006). However there is some evidence for the accumulation of flavan-3-ols in prostate biopsy tissue after the consumption of 1.42 L of green / black tea (Henning et al., 2004). Similarly, Daidzein, genistein and their metabolites were found in prostate tissue after isoflavone ingestion, and in breast tissue after 300 umol of daidzein ingestion (Crozier et al., 2009).

1.3.4 Elimination of polyphenolic compounds

Phenolic acids can be excreted through three different pathways, bile, urine and faeces. The highly conjugated and large metabolites are mostly excreted through bile whereas smaller conjugates are most likely to be excreted through urine (Manach et al., 2004).

1.3.5 Phenolic acids

Phenolic acids even though present in the diet (see below) are not very abundant. Most of the phenolic acids available for tissue uptake are derived from the metabolic action of the gut microbiota on larger polyphenolic compounds such as the flavanoids. Phenolic acids are present as two main classes:

1.3.5.1 Hydroxy benzoic acid derivatives

This group of phenolic acids are mostly present as conjugates but may be found in the free form after the processing of fruits. The main hydroxy benzoic acid derivative is gallic acid, found in tea. The dimer of gallic acids known as ellagic acid is an important component of the berry variety fruits such as strawberries, raspberries and blackberries (Tomás-Barberán and Clifford, 2000). The esterification of these hydroxybenzoic acid derivatives forms hydrolysable tannins; gallotannins (present in mangoes) and ellagitannins (present in strawberries, raspberries and pomegranates) which can be hydrolysed in the presence of acids or bases (Tomás-Barberán and Clifford, 2000, Manach et al., 2004).

1.3.5.2 Hydroxycinnamic acid derivative

This group of phenolic acids are only found in the free form if they have undergone processing, fermentation, sterilisation or freezing. They are mostly present as the esters of quinic acids, shikimic acid and tartaric acid. The most common hydroxycinnamic acids are: caffeic acid (3,4-dihydroxycinnamic acid), *p*-coumaric (4-hydroxy) and ferulic acid (3-methoxy, 4-hydroxy) (Clifford, 2000). Esters formed between certain *trans*-cinnamic acids and quinic acid (1L-1 (OH),3,4/5-tetrahydroxycyclohexane carboxylic acid) are known as chlorogenic acids. The *trans* isomers of chlorogenic acid may be converted to *cis*-isomers during processing. 5-*O*-caffeoylquinic acid is the most common of the chlorogenic acid family (Clifford, 2000).

1.3.5.3 Bioactivity of phenolic acids:

Phenolic acids are mostly produced as the result of the colonic degradation of polyphenols by the microbiota and contribute greatly to the bioavailability of polyphenols, as it is more likely that these lower weight metabolites would get absorbed than their more complex parent compounds. However there is a large gap in the literature on the catabolic pathway of polyphenols resulting in phenolic acid production, the fate of the phenolic acids once absorbed and their bioactivity.

There has been some investigation on the antioxidant activity of these phenolic acids, which may suggest their potential impact on health. Franck et al. (2013) investigated the antioxidant potential of several phenolic acids *in-vitro* including benzoic acid, 3,4-DHBA, 2,6-DHBA, gallic acid, etc. The antioxidant potential was determined by their potential to scavenge reactive oxygen species (ROS) and the radiacal cation ABTS [2,2-azinobis (3-ethylbenzothiazoline-6-sulphonic acid)], which were produced from stimulated neutrophils (equine). The phenolic acids demonstrated a strong antioxidant potential which was found to be inversely correlated with their redox potential. Phenolic acids which were more easily oxidised such as gallic acid (redox potential 0.29 v) and 3,4-DHBA (redox potential 0.46 v) had a higher antioxidant potential than benzoic acid, which is not easily oxidised (redox potential 1.1 v). It was also found that the efficiency of the phenolic acid to scavenge ATBS/ROS was enhanced when three hydroxyl groups were present on the aromatic ring. This property of the phenolic acids was shown to be dose dependent. Li et al. (2011) also investigated the antioxidant capacity of protocatechuic acid, 3,4-DHPAA, hydro caffeic acid, caffeic acid, gallic acid, 3,4,5-trihydroxyphenylacetic acid and 3-(3,4,5-trihydroxy-phenyl) propanoic acid determined by their hydroxyl and superoxide scavenging potential and metal chelation. 3,4,5-trihydroxyphenylacetic acid was shown to have the highest antioxidant potential.

The above studies were conducted *in-vitro* using pure phenolic compounds. A similar study (Henning et al., 2013) was conducted by treating colon cancer cells *in-vitro* with phenolic acids (at concentrations of 10^{-4}, $10^{-5,}$ 10^{-6} M) that had significantly increased after the consumption of green tea and black tea. The consumption of green tea resulted in increased urinary excretion of 4-HPAA, 3-HPAA and polyhydroxy-ɣ-valerolactone. Whereas the consumption of black tea resulted in increased urinary excretion of 3-*O*-methylgallic acid. They also investigated the increase in serum phenolic acid, which was 3,4-DHPAA and 4-HPAA after green tea and 3,4-DHPAA after black tea consumption. The anti-proliferative effect of 3,4-DHPAA and 3-*O*-methylgallic acid was found to be additive.

Similar studies undertaking the determination and comparison of antioxidant capacity of various phenolic acids have been summarised in Table 1-2. It is however difficult to draw conclusion from the results of such studies due to use of different phenolic acids with differing structure, various analytical methods and end-point determinants.

As mentioned above, it has been proposed that the structural modification of the phenolic acids may lead to higher antioxidant properties. Phenolic acids with more hydroxyl groups (eg. Trihydroxy) have been shown to have higher antioxidant properties than those with fewer hydroxyl groups (eg. Dihydroxy or monohydroxy) (Wu et al., 2007, Dziedzic and Hudson, 1984, Siquet et al., 2006, Zhu et

al., 2006, Castelluccio et al., 1995). However this may not always be valid as seen in lipid peroxidation assays where lipophilicity plays an important role. In these assays, phenolic acids with fewer number of hydroxyl group, having higher lipophilicity may be more potent antioxidant agents (Siquet et al., 2006, Wu et al., 2007). Additionally the presence of ortho-dihydroxy groups or 4-hydroxy 3-methoxy may contribute to higher antioxidant properties (Cheng et al., 2007).

However, lipophilicity may not necessarily predict the bioactivity of the phenolic acids. Castelluccio et al. (1996) investigated the distribution of ferulic acid in plasma using ^{14}C-radiolabelled ferulic acid and compared the antioxidant potential of cinnamic acid and ferulic acid to the more hydrophilic ascorbic acid against lipid oxidation. Ferulic acid was primarily bound to the albumin fraction of the plasma. The remaining radiolabelled ferulic acid was partitioned between the LDL fraction and the aqueous fraction of the plasma. Interestingly ferulic acid was not found associated with the lipid fraction of LDL, thus suggesting that it exhibits most of its antioxidant potential through the aqueous layer. Despite this observations, ferulic acid exhibited higher antioxidant potential than the more hydrophilic ascorbic acid, with cinammic acid displaying the least potential.

It has also been shown that the ester derivatives of phenolic acids may be stronger antioxidant agents than the parent compound (Chigorimbo-Murefu et al., 2009, MeRkl et al., 2010, Garrido et al., 2012), however some other studies have shown that esterification may not have any impact (Menezes et al., 2011) or may even reduce the antioxidant properties (Gaspar et al., 2010). Interestingly the two studies demonstrating a higher impact of the ester derivatives have investigated lipid peroxidation (Stamatis et al., 2001) and antioxidant properties of sunflower oil (MeRkl et al., 2010) which may be linked to higher lipophilicity of these derivatives (Gaspar et al., 2010, Kikuzaki et al., 2002).

Similar to their parent compounds (*1.3.1* Health implications of polyphenols), phenolic acids may also act as pro-oxidant agents (Maurya and Devasagayam, 2010). This may depend on various factors such as pH (Medina et al., 2012), chelating capacity (Maurya and Devasagayam, 2010), presence of redox active metals (Sakihama et al., 2002, Zheng et al., 2008) and the concentration of the compounds; as lower concentrations of phenolic acids act as antioxidant agents; the higher concentration of the same compounds have shown to be pro-oxidants (Yen et al., 2002, Maurya and Devasagayam, 2010).

In addition to these antioxidant properties, it has been shown that phenolic acids such as 3,4-DHPAA and 4-HPAA are more effective in anti-platelet aggregation activity than their parent compounds rutin and quercetin (Kim et al., 1998). Additionally these phenolic acids may prove to have stronger chemo-preventive properties as has been shown by Kampa et al. (2004) for 3,4-DHPAA and 3,4- DHBA through anti-proliferative and apoptotic potential as seen on T47D human breast cancer cells. A study

by Scazzocchio et al. (2011) has also shown that phenolic acids may have an insulin like effect. In this study, both cyaniding-3-*O*-β-glucoside and protocatechuic acid increased adipocyte glucose uptake, GLUT4 membrane translocation, adiponectin and GLUT4 expressions and nuclear PPARγ activity.

Even though these findings appear promising regarding the role of phenolic acids in health, the results of *in-vitro* studies, especially in regard to the actions of phenolics must take into consideration the amount of polyphenol consumption required to produce sufficient phenolic acids in circulation to exert any health impact.

Table 1-2 Antioxidant potential of phenolic acids

Author year	Study method	Measurements	Findings (phenolic acid production)	Comments
Franck et al 2013	In-vitro benzoic acid, 3,4-DHBA, 2,6-DHBA, gallic acid, salicylic acid, caffeic acid, syringic acid, ferulic acid, propyl gallate	Antioxidant potential determined by ROS and the radiacal cation ABTS scavenging capacity produced from produced from stimulated equine neutrophils.	Antioxidant potential of phenolic acids related to their redox potential Number of hydroxyl groups and their position were related to their efficacy as antioxidant agents Scavenging activity towards radical cation ABTS: Gallic acid > propyl gallate> 3,4 –DHBA ~ ferulic acid, syringic acid > caffeic acid ~ 2,6-DHBA < salicylic acid > benzoic acid Scavenging activity towards ROS: propyl gallate> Gallic acid > caffeic acid > 3,4 –DHBA > syringic acid > Ferulic acid > 2,6-DHBA > salicylic acid > benzoic acid	
(Li et al., 2011)	In-Vitro Protocatechuic acid Positive controls: 3,4-dihydroxyphenylacetic acid, hydrocaffeic acid, caffeic acid, gallic acid, 3,4,5-trihydroxyphenylacetic acid, and 3-(3,4,5-trihydroxy-phenyl) propanoic acid	1,1-diphenyl-2-picryl-hydrazyl (DPPH•), ABTS+, superoxide anion radicals (O2−•) and hydroxyl radical (•OH) scavenging activity, ferric ions (Fe3+) and cupric ions (Cu2+) reducing power, ferrous ions (Fe2+), and cupric ions (Cu2+) chelating activity, compared with the positive controls Trolox or BHT	3, 4, 5-trihydroxy-phenylacetic acid was the most potent radical scavenger generated by AAPH in liposomes 3, 4-dihydroxyphenylacetic acid was most effective in the lipid peroxidation assay Protocatechuic acid showed dose-dependently antioxidant ability	
(Medina et	In-vitro	Antioxidant potential (FRAP) and	Reducing capacity: Caffeic acid > ferulic acid > galocatechin	

al., 2007)	Phenolic acids: Caffeic acid, chlorogenic acid, o-coumaric acid, and ferulic acid Polyphenols: catechin, gallocatechin, catechin gallate, and gallocatechin gallate	chelating capacity (Kolayli et al. method)	gallate > catechin gallate > gallocatechin > catechin > chlorogenic acid > o-coumaric acid Chelating capacity: galocatechin gallate > catechin gallate > Caffeic acid > chlorogenic acid > gallocatechin > catechin > ferulic acid > o-coumaric acid Chelating capacity is dependent on number of hydroxillic groups in ortho position	
(Cheng et al., 2007)	In-vitro caffeic acid, chlorogenic acid, sinapic acid, ferulic acid and p-coumaric acid	Human LDL peroxidation model induced by 2, 2′-azobis(2-amidinopropane hydrochloride (AAPH) or cupric ion (Cu2) using TBARS assay	Antioxidant capacity against AAPH induced LDL peroxidation: caffeic acid ~ chlorogenic acid > sinapic acid > ferulic acid > p-coumaric acid Antioxidant capacity against Cu2 induced LDL peroxidation: caffeic acid ~ chlorogenic acid > sinapic acid ~ ferulic acid ~ p-coumaric acid	ortho-dihydroxyl and 4-hydroxy-3-methoxyl groups increased antioxidant properties
(Yeh and Yen, 2003)	In-vitro Gentisic acid, chlorogenic acid, syringic acid, protocatechuic acid, p-hydroxybenzoic acid, vanillic acid, ferulic acid, gallic acid, caffeic acid, sinapic acid, o-coumaric acid, p-coumaric acid, m-coumaric acid (6.7 μM)	Mesurement of phenolsulfotransferase-phenols (PST-P) and phenolsulfotransferase-monoamines (PST-M) activity by Folds and Meek method. Antioxidant potential by ORAC, TEAC method.	↓ PST-P and PST-M activity (21-30%) by chlorogenic acid, syringic acid, protocatechuic acid, vanillic acid, sinapic acid, and caffeic acid significantly ($p < 0.05$) ↑ PST-P activity by p-hydroxybenzoic acid, gallic acid, gentisic acid, o-coumaric acid, p-coumaric acid, and m-coumaric acid ↑ PST-M activity by gentisic acid, gallic acid, p-hydroxybenzoic acid, and ferulic acid. ↑ antioxidant capacity by all phenolic acids, higher for gallic acid, p-hydroxybenzoic acid, gentisic acid, and coumaric acid impact of phenolic acids on PST-P and PST-M activity related	

			to antioxidant activity for ORAC (r = 0.71, p < 0.01; and r = 0.66, p < 0.01)	
(Chen and Ho, 1997)	In-vitro caffeic acid (CA), caffeic acid phenethyl ester (CAPE), ferulic acid (FA), ferulic acid phenethyl ester (FAPE), rosmarinic acid (RA), and chlorogenic acid (CHA)	lipid oxidation inhibition activity using Metrohm 679 Rancimat instrument, Scavenging effect on DPPH radicals, Antioxidant activity in oil-water imulsion	↑ induction time of lipid oxidation in lard CA > CAPE ~ RA > CHA > FA ~ FAPE ↑ induction time of lipid oxidation in corn oil RA > CA ~ CAPE ~ CHA ↑ The 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) scavenging activity: RA ≫ CAPE > CA > CHA > FA > FAPE ↓ oil-in-water emulsion oxidation was CA > CAPE > RA > FA > CHA > FAPE	
(Castellucci o et al., 1996)	In-vitro Cinnamic acid, ferulic acid, ascorbic acid	LDL oxidation induced by hydroperoxide dpendent oxidative stress Distribution of ferulic acid in plasma tested with 14C-labelled ferulic acid	Antioxidant capacity: ferulic acid > ascorbic acid> cinnamic acid Majority of radiolabelled ferulic acid found bound to albumin rich fraction of plasma, other fraction found partitioned between LDL fraction and aqueous fraction.	Ferulic acid does not bind to lipid fraction of LDL
(Laranjinha et al., 1994)	In-vitro Chlorogenic acid, caffeic acids, ellagic acid and protocatechuic Control : trolox	Human LDL oxidation induced by hydrophilic azo initiator. Oxygen consumption and the fluorescence quenching of cis-parinaric acid measured	Antioxidant capacity : Chlorogenic acid > caffeic acid > trolox > ellagic acid.	Protocatechuic acid excluded due to non-ideal oxidation inhibition

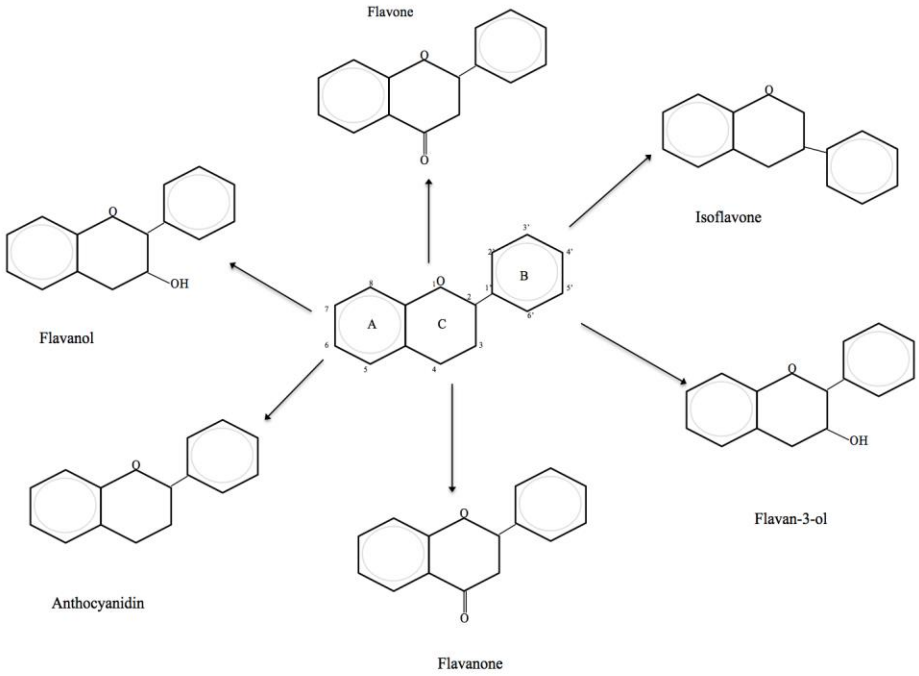
1.3.6 Flavanoids:

Flavonoids are one of the largest groups of natural products identified; widely distributed in plants and contribute greatly to their pigmentation. Over 5000 different naturally occurring flavonoids have already been identified and the list is still growing. The pigmentation of flavonoids is associated with their biological selectivity. Apart from energy capture these pigments may play a role in attracting suitable pollinators and selective activation of light sensitive genes. Flavonoids are only exogenously provided to man as it is only plants that are capable of their synthesis (Havsteen, 2002, Kirby and Styles, 1970). The initial interest in flavonoids began in 1930s with an observation that flavonoids present in citrus fruits displayed strong antioxidant capacity and were able to decrease capillary permeability (Bentsath et al., 1936). With the increase in CHD prevalence in the 1990s more research was dedicated to flavonoids after an inverse association was observed between flavanol / flavone consumption and mortality from incidence of myocardial infarction in 805 individuals. The major sources of flavonoid consumption were identified to be tea, onions and apples (Hertog et al., 1993a). It is important to note that the increased consumption in flavanoids could also be associated with increased fruit and vegetable intake, thus increased fibre consumption.

Flavonoids have a polycyclic structure with a C6-C3-C6 skeleton. The two phenyl rings (A and B) are connected by the 3 carbon bridge (ring C). It is possible for the heterocyclic ring-C to be replaced by a 5-carbon ring such as chalcones or to be present in the isomeric open form. Flavonoids are present in plants mostly as their glycosidic conjugated form. Conjugation with hydroxyl, methoxyl, methyl and/or glycosyl substitute groups may occur in plants with occasional conjugation with aromatic/ aliphatic acids, sulphate prenyl, isoprenyl and methylenedioxyl groups. Sugar or hydroxyl groups present in the rings increase the solubility of flavonoids whereas presence of methyl groups increases lipophilicity. There have been 4000 compounds so far classified as flavonoids (Iwashina, 2000).

Flavonoids can be classified into six main subcategories (*Figure 1-3*) Flavones, Isoflavones, Flavan-3-ols, Flavanone, Anthocyanins and Flavonols. This is based on cyclisation and degree of unsaturation / oxidation of the three carbon segments.

The flavonols and flavan-3-ol groups have been discussed in more detail below as the compounds of interest for this PhD belong to these sub-groups, which are found in abundance in the diet.



1.3.6.1 Flavones

This subcategory of flavonoids is not widely distributed, thus their intake through diet is not significant. They are mostly consumed through cereals, celery, parsley and sweet red pepper. They are very similar in structure to flavonols only lacking the hydroxylation at the third position of the C-ring. The main flavones are luteolin and apigenin (Crozier, 2003).

1.3.6.2 Flavanones

This subcategory of flavonoids is mostly present in citrus fruits contributing to the citrus flavour mostly in the form of hesperitin-7-*O*-rutinoside (hesperidin). They are also present in tomatoes and tomato products in the form of naringinin, or naringinin chalcone present in tomato peel. In tomato products such as ketchup naringinin chalcones are converted to naringin (Krause and Galensa, 1992).

Flavanone structure lacks the $\Delta^{2,3}$ double bond and instead has a chiral centre at the C-2 position. This structure is greatly reactive allowing hydroxylation, glycosylation and *O*-methylation (Iwashina, 2000, Crozier, 2003).

1.3.6.3 Anthocyanidins

Nineteen types of anthocyanidins have been identified with the most common being pelargonidin, cyanidin, malvidin, peonidin, delphinidin and petunidin (Iwashina, 2000).

These flavonoids are mostly present in higher plants in their glycosidic form known as anthocyanins. The glycosidic form of anthocyanidins are water soluble and contribute greatly to the pigmentation of fruits, flowers and leaves; hence playing a key role in the selection of pollinators (Clifford, 2000, Crozier, 2003). They also contribute to the violet / blue / red pigmentation in some commonly consumed fruits such as plums, berries and grapes; increasing as the fruit ripens (Peterson and Dwyer, 1998).

1.3.6.4 Isoflavones

Similar to flavones, this subcategory of flavonoids is not widely distributed and is mostly consumed in the diet from legume sources. The most common isoflavones identified are: genistein, daidzein and glycitein present mostly in soy beans, black beans and split peas (Dixon and Steele, 1999, Graham, 1991, Barnes, 2004). The estrogenic property of isoflavones created great interested in their role towards breast cancer prevention (Barnes, 2004).

The B-ring of isoflavones is attached to the C-ring by C-3 rather than C-2 and in this way it differs from the general structure of flavonoids. The possible polymerisation, methylation and hydroxylation of isoflavones give rise to various subcategories such as isoflavans, isoflavanols and isoflavanones.

1.3.6.5 Flavonols

Flavonols are ubiquitous in plants. The most common flavonol in the diet is quercetin (onions) followed by kaempferol (broccoli), isorhamnetin (pears /onions) and myricetin (maize/ berries).

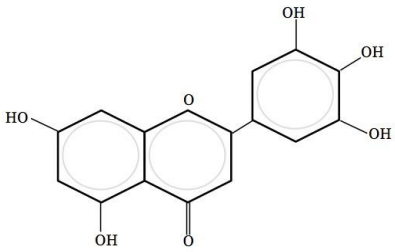


Figure 1-4 Quercetin

Quercetin can be found in various fruits and vegetable but the most important dietary source of quercetin is onions followed by capers (Hertog, 1992). The quercetin present in onions can be found in the form of quercetin-4-glucoside or quercetin-3,4-diglucoside. The most common glycoside of quercetin is quercetin-3-glucosyl-rhamnoside also known as rutin; mostly present in tomatoes, buckweed and asparagus. Quercetin can also be found in apples in the form of quercetin galactosides and in berries as quercetin arabinosides (Peterson and Dwyer, 1998).

1.3.6.5.1 Rutin and Quercetin

Quercetin, usually conjugated to glucose, is of interest due to its ubiquitous nature in the diet especially in onion, pepper, kale and tea. Quercetin can also be found in its glycosidic form rutin, in foods such as buckwheat, dark berries, green pepper, citrus fruits and apricots. Rutin is also known as quercetin-3-rutinoside. The disaccharide moiety of rutin is made of rhamnose (6-deoxy-Lmannose) and glucose (Jaganath et al., 2009). Rutin is pale yellow in colour, slightly yet more soluble in water than its aglycone, quercetin.

Normal plasma quercetin concentration is the lower nM range, and supplementation with quercetin pushes the concentration to the higher nM or lower uM range (Hollman et al., 1996). The half-life of quercetin and its metabolites in plasma are relatively high, ranging from 11-28 hours (Hollman et al., 1997a, Manach et al., 2005). Other studies have reported even longer half lives of quercetin metabolites ranging from 20-72 hours (Day et al., 2001). Hence continuous supplementation with quercetin may raise plasma concentrations of this compound substantially. The tissue distribution of quercetin and its metabolites were shown to be the highest in the lungs, liver and kidneys.

Quercetin has displayed strong antioxidant property due to its ability to scavenge oxygen free radicals, chelate metals and prevent oxidation of LDL *in-vitro* (Kandaswami and Middleton Jr, 1994, Bors et al., 1990, Navab et al., 1996). The oxidation of LDL is a risk factor in the development of atherosclerosis, hence quercetin may also play a role in the prevention of CVD (Navab et al., 1996, Steinberg et al., 1989).

However some of these properties of quercetin may be affected by the presence of the rutinose disaccharide in rutin. In a recent study it was demonstrated that this disaccharide moiety reduces the anti-inflammatory properties of quercetin *in-vitro*. Both quercetin and rutin demonstrated strong anti-inflammatory action, however this was found to be higher in quercetin than rutin (Cai et al., 2014).

Quercetin has also demonstrated anti-cancer properties *in-vivo* and *in-vitro* by induction of apoptosis and inhibition of growth of pancreatic cancer cells (Angst et al., 2013). In another animal model study a 20 week quercetin diet (0.02% wt/wt) delayed the incidence of skin tumour by 2 weeks in mice with over expression of IGF-1 in the skin, known to be a risk factor of tumour incidence and progression. Mice fed quercetin also had lower tumour multiplicity by 35% (Jung et al., 2013). However these studies lack information on required dose in human for impact, and possible toxicity of high doses of quercetin consumption as pharmaceutical supplements (Harwood et al., 2007).

These observations made *in-vitro* and in animal studies have been met with doubt in their translation to health benefits in humans due to the low solubility, absorption and thus bioavailability of these compounds.

1.3.6.5.1.1 Absorption and bioavailability of rutin and quercetin

The absorption of quercetin is greatly influenced by the sugar moiety attached. The sugar-flavanol bond is a β- glycoside bond, which is mostly resistant to hydrolysis by the pancreatic enzymes (Hollman et al., 1997b, Day et al., 2000). The deglycosylation step involves β-glucosidases that releases the aglycone for passive diffusion. The intestinal lactase phlorizin hydrolase (LPH) has demonstrated a specific activity in the deglycosilation of flavonoids and in particular quercetin, as shown in an in situ rat small intestine perfusion model. The carrier mediated transport of quercetin glycosides may involve the sodium dependent glucose transporter-1 (Arts et al., 2002) and / or the multi-drug resistance protein 2 (Walgren et al., 2000a, Walgren et al., 2000b). Hydrolysis by the β-glucosidases follows after the carrier mediated transport (Németh et al., 2003).

Once absorbed, quercetin is metabolised in the small intestine, liver and kidney through phase II metabolism or it is subjected to ring fission by the colon microbiota, resulting in the formation of phenolic acids (Hollman and Katan, 1998, Day et al., 2000). Rutin on the other hand, escapes absorption in the small intestine and is subjected to hydrolysis by the colonic microbiota.

In a study (Hollman et al., 1995) with ileostomy patients (n=9), quercetin glucosides provided in the form of fried onions had the highest absorption (52 ± 15% of ingested) compared with pure quercetin aglycones (24 ± 9 % of ingested) and pure quercetin-rutinosides (17±15% of ingested). While in this study glucosides seem to increase absorption of quercetin, the opposite is seen for glycosides. However it is important to note that in this study quercetin glucoside was provided in the form of fried onions compared to pure aglycone or glycoside form. More recent studies have demonstrated that fried and baked onions have 7-25% increase in quercetin, and boiled onions have a loss of 18% in quercetin concentration (Lombard et al., 2005). This may have influenced the results of this study.

The study was repeated again (Hollman et al., 1997b) to investigate the absorption of quercetin-glucosides from onion ingestion, quercetin-glycosides from apple or pure quercetin-rutinoside. They found similar bioavailability of quercetin from apples and rutin as 30% relative to onions. T_{max} was also delayed for plasma quercetin after ingestion of apples (2.5h) and rutin (9h) as compared to 0.7h after ingestion of onions.

The increased absorption of quercetin in the presence of the glucoside could be due either to the facilitation of glycoside absorption by deglycosylation (Day et al., 2000, Németh et al., 2003) or carrier mediated transport (Gee et al., 2000, Walgren et al., 2000a).

The presence of rutinose not only impacts extent of absorption but also site of absorption. While quercetin can be absorbed from the small intestine, the fate of rutin is determined by the colonic bacteria. This has been indicated by delayed T_{max} after consumption of rutin compared to quercetin, suggesting the colon as the site of absorption (Graefe et al., 2001, Jaganath et al., 2006b).

Hence, rutin escapes absorption in the small intestine and is subjected to colonic microbiota action, where it is hydrolysed to phenolic acids. Based on this observation, it may not be correct to state that the disaccharide moiety of rutin reduced the bioavailability of quercetin, but rather it made quercetin available in the form of phenolic acids.

1.3.6.5.1.2 Phenolic acid production from rutin and quercetin

The phenolic acid production from rutin and quercetin has been analysed in plasma, urine and faecal incubations. For the scope of this PhD we are interested in the phenolic acid production from rutin in faecal incubations. As rutin escapes absorption in the small intestine it enters the colon where it is hydrolysed to quercetin. The released quercetin is then catabolised to phenolic acids.

There are various factors that can influence the type and concentration of phenolic acids produced from rutin in the colon. Higher doses of rutin were shown to deglycolise less easily to quercetin as compared to lower doses of rutin (Rechner et al., 2004). Hence administration of high doses of rutin may not necessarily translate into higher production of phenolic acids from this compound.

The presence of glucose (as a model of fermentable carbohydrate) was also found to alter the metabolites produced as well as increase deglycosylation of rutin and hence phenolic acid production (Jaganath et al., 2009). In the absence of glucose 3,4-DHBA was converted into 4-HBA, whereas this pathway was directed towards the production of HPAA derivatives in the presence of glucose. Thus stimulating the growth/metabolism of the colonic bacteria may influence the metabolites seen in the colon.

Despite the differences in methodology and concentration of compounds used in the studies summarised in *Table 1-3,* they all identified 3,4-DHPAA as the major metabolite from quercetin breakdown. This phenolic acid was produced early in the fermentation and declined between 6-8 hours after incubation, concomitantly the production of 3-HPAA was observed. The phenolic acids produced were: 3,4-DHPAA, 3-DHPPA, 3,4-DHBA, 4-HBA, 3-HPPA, 3,4-Dihydroxy toluene, 3,4-Dihydroxy

Benzaldehyde, phloroglucinol and 3,4-dihydroxytoluene. These studies demonstrated a high donor dependent variation in metabolite production. The speculated catabolic pathway of rutin has been illustrated in *Figure 1-5.*

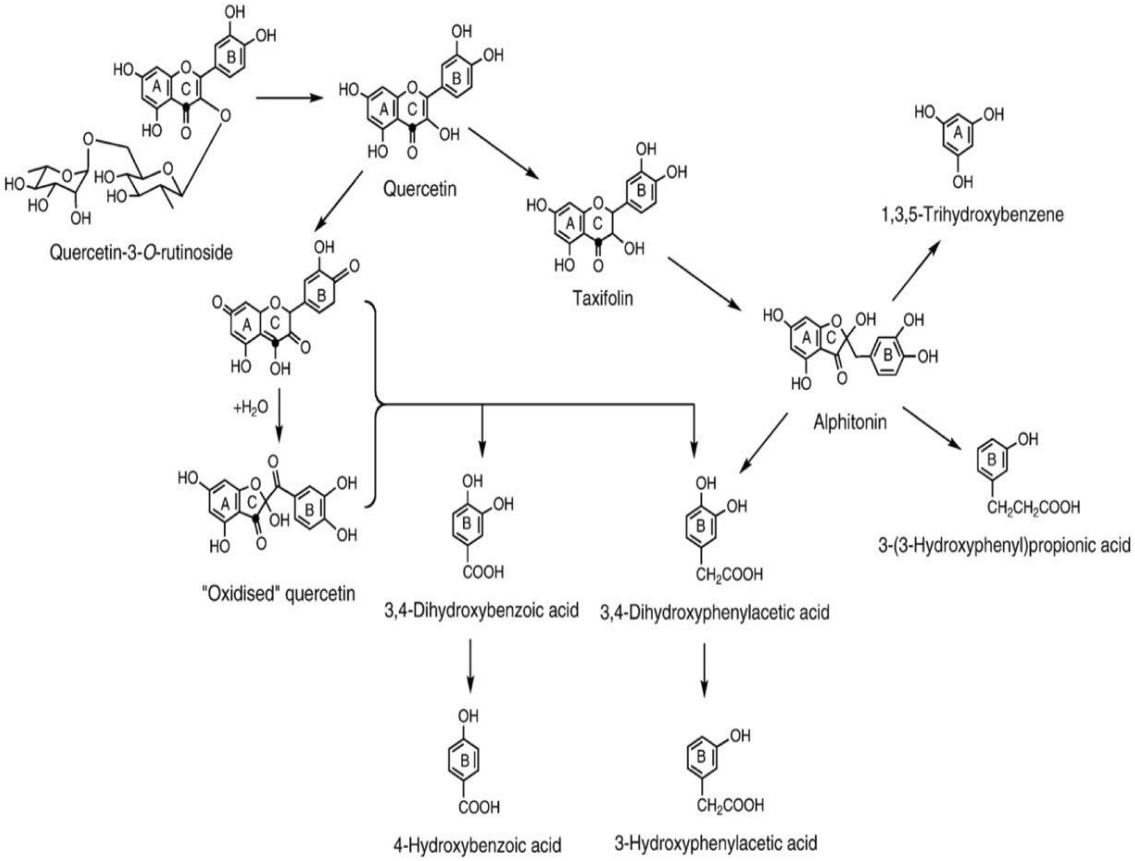


Figure 1-5 Speculated catabolic pathway of rutin by colonic microbiota - Adapted from Jaganath et al. (2010)

Table 1-3 phenolic acid production from rutin and quercetin

Author (year)	Study method	Measurements of phenolic acids	Results	Comments
(Justesen et al., 2000)	*in-vitro* fermentation N=3 (i)-12.5 mg/ml rutin (ii)-12.5 mg/ml quercetin	LCMS	Phenolic acids: 3,4-DHPAA > 3 or 4- HPAA, 3 or 4-HPPA, 3,4-Dihydroxy toluene, 3,4-Dihydroxy Benzaldehyde	Rutin and quercetin were added as 100mg to 8ml of fermentation buffer directly =12.5 mg/ml
(Aura et al., 2002)	*in-vitro* fermentation N=4 (i)-1 umol rutin (ii)-1 umol isoquercitrin or (iii) 0.20 umol mixture of quercetin glucoronides	HPLC-MS	Phenolic acids: 3,4-DHPAA > 3-HPAA, 3,4-DHPPA	3,4-DHPAA appeared in 2h 3-HPAA appeared at 8h
(Rechner et al., 2004)	*in-vitro* fermentation N=4 (i)-180 mg/l rutin (ii)-350 mg/l rutin	LCMS and GCMS	Phenolic acids: 3,4-DHPAA > 3-HPAA, 3-HPPA	Faster deglycosylation of rutin to quercetin at lower concentrations of rutin
(Labib et al., 2004)	*in-vitro* Pig caecum model N=3 (i)-250 mM quercetin	HPLC-DAD	Phenolic acids: phloroglucinol, 3,4-dihydroxyphenylacetic acid, and 3,4-dihydroxytoluene	3,4-DHPAA highest up to 6h.
(Jaganath et al., 2006a)	*in-vitro* fermentation N=3 (i)-28 umol rutin (ii)-55 umol quercetin or (iii) 18×10^6 dpm of [4-^{14}C]quercetin + / - 0.5g glucose	HPLC-PDA-MS^2	Phenolic acids: 3,4-DHPAA > 3-HPAA, 3,4-DHBA, 4-HBA, 3-HPPA -presence of glucose enhanced deglycosilation of rutin -presence of glucose increased phenolic acid production	3,4-DHPAA appeared in 2h 3-HPAA appeared at 24h

1.3.6.6 Flavan-3-ols

Flavan-3-ols are ubiquitous in the plant kingdom and can be found in high concentrations in cocoa, blackberries, sour cherries, apricots and grapes; contributing to the astringent taste of some foods such as tea and wine. They are present in nature either in monomeric forms such as (+)-catechin (*Figure 1-7)* and (-)-epicatechin (*Figure 1-6)* or in the oligomer and polymer formations such as proanthocyanidins / condensed tannin (Porter, 1988). Additionally esterification with gallic acid gives rise to compounds such as catechin gallates and gallocatechin through hydroxylation; present highly in green tea in the forms (-)-epigallocatechin gallate and (-)-epigallocatechin (Stewart et al., 2005).

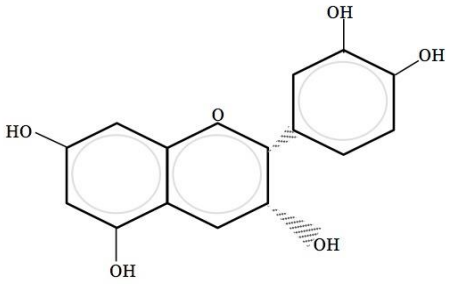


Figure 1-6 Epicatechin

It is estimated that the flavn-3-ol intake from the diet varies within a range of 100- 550 mg (Auger et al., 2004, Hammerstone et al., 2000). The reported estimation of daily intake varies and is most often based on average wine consumption. Auger et al. (2004) reported an average of 100.4 mg flavanol units based on moderate wine consumption (180 ml/ day). Whereas Hammerstone et al. (2000) reported an estimated range of 147.1- 164.7 mg per daily serving of food.

According to Guyton and Hall (2010) the amount of chyme reaching the caecum from the ileum averages 1500ml daily. Hence the concentration of flavan 3-ols present in the caecum would on average range between 60-360 ug/ml.

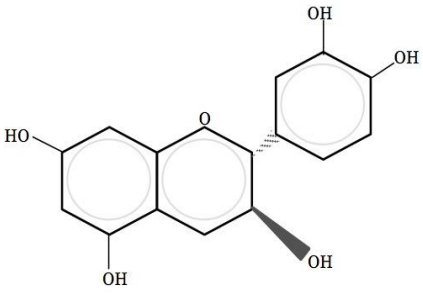


Figure 1-7 Epicatechin

Once in the colon, the first step in the catabolism of flavan-3-ols has been suggested as the production of hydroxyphenyl valerolactones and valeric acids. This takes place through the decarboxylation, dehydroxylation, lactonization and oxidation reactions following the opening of procyanidin C-ring (Selma et al., 2009). The production of these metabolites from monomeric flavan-3-ols is thought to be through the cleavage of the monomers by microbial esterases, the opening of the C-ring results in the production of diphenylpropan-2-ol which is then converted to 5-(3,4-DHP)- γ-valerolactone in the case of epi/catechins (Roowi et al., 2009, Groenewoud and Hundt, 1986). The breaking of the valerolactone ring eventually gives rise to 5-(3,4-DHP)-valeric acid and/or 4-H -5-(3,4-DHP)-valeric acid (Monagas et al., 2010). The β-oxidation of the side chains of these metabolites subsequently results in the formation of HPPAs and HBAs (Meselhy et al., 1997). Studies have shown that procyanidin dimers may also initially be depolymerised into monomeric units prior to further degradation (Stoupi et al., 2010b, Appeldoorn et al., 2009), however this is seen as less than 10% in the case of B2 procyanidins (Stoupi.,2010).

The catabolic pathway of 3,4-DHPAA has been greatly debated. It was initially proposed that this metabolite was mainly produced through the breakdown of procyanidins, with the cleavage of the upper unit of procyanidin dimers, with the lower unit giving rise to above described production of valerolactones. However it is now debated that 3,4-DHPAA may also be produced through the α-oxidation of 3,4- DHPPA produced through the catabolism of the monomeric compounds. The further preferential dehydroxylation of 3,4-DHPAA at C-3 and C-4 in the case of epi/catechins gives rise to 4-HPAA and 3-HPAA respectively (Gonthier et al., 2003, Stoupi et al., 2010a). It has been suggested that the production of 3-HPAA may also be formed through the α-oxidation of 3-HPPA and 3,4-DHBA may be produced through the rapid degradation of 3,4-DHPAA (Stoupi et al., 2010a, Fogliano et al., 2011).

The understanding of the catabolic pathway of these flavan-3-ol metabolites is limited due to the use of different analytical methods and as previously demonstrated, great variations in faecal microbiota composition. Depending on the catabolic capacity of these microbiota it is possible that different pathways exist, with some more predominant than others.

1.3.6.6.1 Cocoa

Cocoa tree originated in the South African tropical regions. There are a few subspecies of the cocoa tree. The main cultivated subspecies accounting for 90% of the worlds usage is Theobroma cacao var. Forastero, this subspecies was developed in the Amazon basin (Tomás-Barbéran et al., 2012).

The use of cocoa for treatment of ailments can be traced back to the Maya and Aztecs; where cocoa was claimed to relieve fever and diarrhoea, sooth stomach and intestinal ache and promote strength in the "weak and fainthearted". In later years the use of cocoa for its medicinal value was extended to prolonging longevity. The medicinal use of cocoa in Europe is traced back to 1500s and has been recorded ever since the 17th century (Dillinger et al., 2000).

Cocoa is now widely consumed in the form of confectionary/ chocolate products and beverages. The annual consumption of cocoa in the European Union ranged from 1.3 kg per head in Portugal to 8.8 kg per head in Germany (Wollgast and Anklam, 2000) and in 2010 the world wide consumption was estimated at over 3.5 million tons in 2010 (FAO, 2010). The high concentration of polyphenols in cocoa beans results in a bitter taste making them unpalatable in their natural state. Hence they are processed to produce more palatable products.

During this process the cacao pods are broken open and covered by large leaves, allowing the pulp to ferment for 6-8 days: during which invertase converts sucrose to fructose and glucose. The glucose released is taken up during the fermentation process. The ethanol yield is converted into acetic acid during this process. The proteins are converted to peptides and amino acids while phenolic compounds are converted to quinines, which through polymerisation yield highly insoluble compounds responsible for the brown pigmentation of cocoa. The seeds are then dried to reduce moisture from 55% to 7.5%.
During cocoa powder manufacturing the cocoa beans are roasted at 150 °C, followed by the mechanical separation of shell (hull) from the meat (nib). The nib containing 55% cocoa powder are ground while hot to produce the liquor, which is then pressed to produce the cocoa butter used in chocolate manufacturing. The cocoa powder is produced by pulverising the residual cake (Bixler and Morgan, 1999).

Cocoa products have displayed a variety of health benefits but they are mainly studied due to the antioxidant and anti-inflammatory properties of their polyphenols. Consumption of cocoa has been associated with increased levels of plasma antioxidants, thus preventing LDL oxidation (Kurosawa et al., 2005). Additionally cocoa flavanols have demonstrated the ability to increase nitric oxide synthesis, suppress platelet activation, positively modulate eicosanoid synthesis, inhibit the production of certain pro-inflammatory cytokines and stimulate the production of anti-inflammatory cytokines

(Zhu et al., 2002, Schramm et al., 2001, Karim et al., 2000, Andújar et al., 2012, Rein et al., 2000). Short term consumption (15 day) of dark chocolate has also increased insulin sensitivity and reduced blood pressure (Grassi et al., 2005). Cocoa procyanidins with a higher degree of polymerisation (pemtomers) selectively inhibited proliferation of breast cancer cells without affecting normal cell proliferation (Ramljak et al., 2005).

All of these potent biological properties of cocoa polyphenols contribute towards their health benefits such as protective mechanism in heart disease and anti-carcinogenic properties. However this is highly dependent on concentration of polyphenols required to exhibit these effects and the relative bioavailability of these polyphenols.

The impact of a cocoa drink on gut microbiota was investigated in a study by Tzounis et al. (2011). In this study 22 volunteers were supplemented with either a high cocoa flavonol drink (494mg total cocoa flavanols) or a low cocoa flavonol drink (29mg total cocoa flavanols). Cocoa was consumed mixed in 150ml of water for a period of 4 weeks, allowing 4 weeks of wash out between the two arms of the study. Faecal samples were collected before and after each intervention. This study demonstrated that while consumption of high cocoa flavanol drink increased certain bacterial species such as bifidobacteria ($p<0.01$) and lactobacilli ($p<0.01$), other bacterial species were decreased such as clostridia ($p<0.01$). This study also demonstrated a reduction in plasma TAG and C-reactive protein (CRP) ($p<0.05$) as well as a correlation between changes in the lactobacilli to a decrease in CRP levels ($p<0.05$, $R^2= 0.33$).

1.3.6.6.2 Polyphenols in cocoa

Cocoa is a great source of polyphenols and contains flavanols mostly in the form of monomers (+)-catechin, (-)-epicatechin and the polymers proanthocyanidins as well as anthocyanins, flavonol glycosides, phenylethylamine, N-oleoyl, N-linoleoylethanolamine, clovamide and theobromine (Rios et al., 2003). It is estimated that cocoa contributes towards 20% of total catechin consumption in the West (Arts et al., 1999). Cocoa has shown to have a higher concentration of polyphenols per dose of consumption than tea or red wine (Lee et al., 2003) and higher procyanidin content than that of cranberries and blue berries on a dry weight basis (Gu et al., 2002).

The bitter taste resulting from high concentrations of polyphenols in cocoa makes them unpalatable in their natural form. They are processed in order to make them palatable. The process of fermentation / dutching and chocolate manufacturing results in the reduction of polyphenol content of cocoa from 100% to approximately 10% (Andújar et al., 2012, Miller et al., 2008, Rusconi and Conti, 2010).

Cocoa polyphenols are comprised of the monomers (+)-catechin and (-)-epicatechin, the combination of the two form proanthocyanidins, which is present in dark cocoa in high concentrations. 100 g dark chocolate contains, on average, 42 mg (−) -epicatechin and 12 mg (+)-catechin (Tzounis et al., 2008a). Catechin and epicatechin have high solubility in water relative to most other flavonoids (Schramm et al., 2003). Procyanidins represent 50% of the approximate 1g of daily polyphenol consumption (Gonthier et al., 2003). The procyanidins are mainly present as B1 – B5, C1 and D procyanidins made up of dimers, trimers and oligomers of the flavan-3,4-diols linked through 4→ 6 and 4→ 8 bonds. Whereas, the anthocyanidins are present in the 2 forms of cyanidin-3-α-L-arabinoside and cyanidin-3-D-galactoside. Some traces of dimeric epicatechin glycosides such as 3-*O*-arabinoside and 3-*O*-galactoside conjugates of (+)-epicatechin-(2α→7, 4α→8)-(+)-epicatechin have also been reported. The proportion of polyphenol content in cocoa is displayed in the image (Figure 1-8) below (Andújar et al., 2012, Gu et al., 2006).

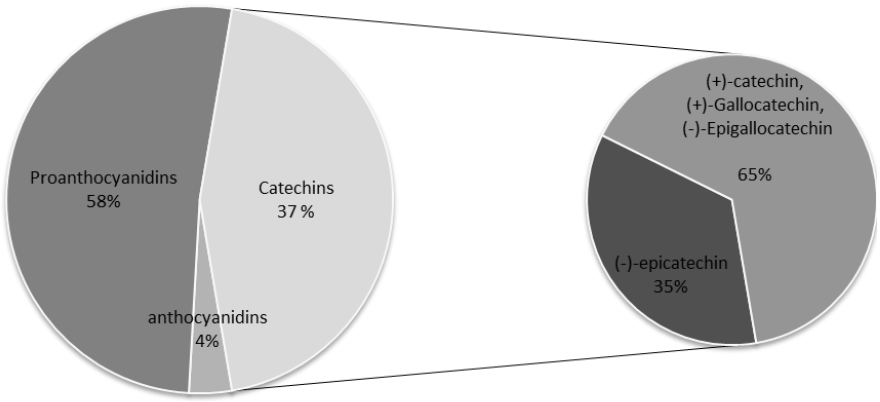


Figure 1-8 Polyphenol composition of cocoa

Recent studies have demonstrated that unlike previously thought, the (-)-catechin enantiomer is the most abundant form of catechin found in chocolate products (Donovan et al., 2006). While (-)-catechin is found to be more abundant in the cocoa products, (+)-catechin is most abundant in the natural state of the cocoa (Gotti et al., 2006). This could be a result of processing and the epimerisation at the 2 positions of (-)-epicatechin. However, regardless of its abundance, (+)-catechin remains to be the most bioavailable form of catechin enantiomers in cocoa products. Donovan et al. (2006) analysed the bioavailability of 10, 30 or 50 umol/l of (-)-catechin and (+)-catechin in male wistar rats (n=15). The plasma concentration of (-)-catechin was found to be 2-8 fold lower than that of (+)-catechin ($p<0.005$) for all concentrations. Similar results were seen for intestinal absorption ($p<0.01$). (-)-catechin

constitutes 89% of total catechins according to other studies (Neilson et al., 2009). Cocoa also contains phenolic acid derivatives in the form of N-caffeoyl-3-*O* -hydroxytyrosine also known as clovamide and N-*p*-coumaroyl-tyrosine (deoxyclovamide).

The total polyphenol content of cocoa beans is estimated to be 6-8% of dry weight (Zumbé, 1998). Due to the variation in cocoa products and analytical methods, there is a great variation in reported concentrations of polyphenols quantified in cocoa. An example of this is seen in *Table 1-4* as mg/g dry weight of de-fatted cocoa.

Table 1-4 cocoa polyphenol concentration

Author (year)	Measurement	**Dark chocolate**	**Milk chocolate**	**Cocoa powder**	**Method**
(Waterhouse et al., 1996)	Total Phenol	8.4	5.0	20.0	Folin-ciocalteau, **Gallic** acid eqv.
(Vinson et al., 1999)	Total Phenol	36.5±5	15±5.8	65±19	Folin-ciocalteau, **Catechin** eqv.
(Adamson et al.,1999)	Procyanidins	1.7±0.08	0.7±0.17	-	NP-HPLC
(Adamson et al., 1999)	Catechins+epicatechins	0.8±0.08	0.2±0.05	-	NP-HPLC
(Arts et al., 1999)	Catechins+epicatechins	0.5	0.16	-	RP-HPLC

1.3.6.6.3 Bioavailability of cocoa polyphenols

The health benefits derived from cocoa have been associated with its flavan-3-ol content especially circulating (-)-epicatechin in relation to prevention of CVD (Schroeter et al., 2006, Rasmussen et al., 2005). These health benefits are thus dependent on the bioavailability of these polyphenolic compounds from cocoa.

The fate of flavan-3-ols post consumption is very much the same as the fate of flavanols described above. Ingested (-)-epicatechins are absorbed from the small intestine and appear in circulation 30 minutes after ingestion mostly in the form of sulphate, glucuronide and methylated substances due to the action of sulphotransferases, uridine-5-diphosphate glucuronosyltransferases and catechin-*O*-methyltransferases as they are transported to the portal vein. These compounds may then undergo phase II metabolism after which they may be transported to the colon through bile. Most studies on bioavailability of the flavan-3-ols, catechin and epicatechin use green tea as a source of these compounds. Typically volunteers follow a low polyphenol diet for a period of 24-48 hours, baseline blood and urine samples are collected after an overnight fast, after which volunteers ingest dietary

source of the compound with repeated measures of blood and urine taken over the following 24-48 hours while volunteers remain on the low polyphenol diet. Bioavailability measurement of compounds can also take place using animal models (Baba et al., 2001). Baba et al demonstrated that (-)-epicatechin was more bioavailable in rats than (+)-catechin, which was shown to be more bioavailable than (-)-catechin (Donovan et al., 2006).

The events that occur pre-absorption are of importance in determining the compounds and concentration available for absorption (Spencer et al., 2001a). Saliva has displayed little to no impact on catechin and procyanidin modification (Tsuchiya et al., 1997). Epicatechin was found to be stable under stimulated gastric conditions as well (Auger et al., 2008). There is however on-going debate on the stability of cocoa procyanidins in the stomach and small intestine and whether or not they are cleaved to produce epicatechin before reaching the colon. The stability of these compounds is highly dependent on the surrounding pH. Procyanidins were shown to be unstable at a pH of 2.0 when they were incubated in acid and simulated gastric juice; 60-80% of the oligomers were decomposed to monomeric and dimeric units of epicatechin during the first 90 minutes and were almost completely decomposed by 3.5h (Spencer et al., 2000, Spencer et al., 2001b). It was also demonstrated that higher polymerised index of monomers were more readily cleaved. Similar results were seen in a study by zhou et al. (2002) demonstrating instability of dimers in simulated gastric juice. However they also demonstrated a higher stability for the monomers (+)-catechin and (-)-epicatechin in simulated gastric juice as well as an inverse relationship between pH levels and stability of monomeric and dimeric units. Monomeric compounds were found to be relatively stable at a pH of 5.0 with increasing instability as the pH was increased to 9.0 in the following order of relative stability (+)-catechin > (-)-epicatechin > Dimers.

However Rios et al. (2002) demonstrated that proanthocyanidins were stable in the stomach of healthy volunteers (n=6) fed 500 ml of a cocoa beverage containing 733 mg procyanidin polymers and 351 mg structurally related flavanol monomers using a nasal-gastric tube. The contents of the stomach were collected every 10 minutes for the quantification of flavanols and procyanidins. It is important to note that in such studies, the composition of cocoa may have a buffering effect on the gastric pH and affect observations.

The degree of polyphenol absorption from the small intestine can be studied in ileostomy patients. Such studies have found that 20% of ingested catechin, epicatechin and metabolites in the form of tea extract (polyphenol E) are found in the ileal fluid (Auger et al., 2008). In similar studies the ingestion of apple juice in 11 ileostomy patients demonstrated 90% recovery of ingested procyanidins in

ileostomy bags, even though they were present at a lower degree of polymerisation (Kahle et al., 2007). The absorption of flavanols has displayed large variability, ranging from 1-50% of ingested flavanols (Baba et al., 2000, Rein et al., 2000, Schramm et al., 2003, Hollman et al., 2001). Some of this variation has been attributed to the complex *in-vivo* system such as gastric pH, the type and activity of microbiota. However, in a study by Schramm et al (2003) investigating the impact of the Famotidine on oral absorption of cocoa flavanols, changes in gastric pH failed to demonstrate any impact on the absorption (C_{max} and AUC) of flavanols. Another factor influencing the variation in bioavailability and metabolites produced is the composition of the cocoa product and the degree of processing. As discussed previously, processing may result in higher concentrations of the less bioavailable (-)-catechin than (+)-catechin. In commercially available cocoa products, taste and palatability of the product are key factors and hence, these products may be subjected to higher degrees of processing such as dutching, subsequently making their phenolic content less available. An example of this is seen in the study by Mullen et al. (2009) where commercially available Green & Blacks cocoa was used, displaying higher concentrations of (-)-catechin compared to (+)-catechin. Some of the studies Investigating Bioavailability of cocoa polyphenols are summarised in *Table 1-4.*

Author	Study method	Measurements	Results	Comments
(Richelle et al., 1999)	Acute *in-vivo* (n=8) (i)-40g black chocolate (Nestle noir) having 282 umol (-)-epicatechin (ii)-80g black chocolate (Nestle noir)	HPLC (plasma epicatechin)	Plasma concentration of epicatechin ↑significantly with 40g of chocolate, C_{max}= 355 nmol/L and T_{max}= 2.0 h 80g of chocolate ↑ epicatechin significantly, C_{max}= 676 nmol/L and T_{max}= 2.6 h	Delay in T_{max} due to ad libitum consumption of bread Bitter taste of high dose cocoa may result in changes of gastric emptying.
(Baba et al., 2000)	Acute *in-vivo* (n=5) (i)-35g chocolate containing 760umol (-)-epicatechin and 240umol of catechin (ii)-35g cocoa containing 760umol (-)-epicatechin and 240umol of catechin	HPLC and LCMS (plasma & urineepicatechin)	C_{max} of (-)-epicatechin after 2 hours of chocolate consumption = 4.77 ± 0.94 umol/L C_{max} of (-)-epicatechin after 2 hours of cocoa consumption = 4.94 ± 0.94 umol/l	-
(Wang et al., 2000)	Acute *in-vivo* parallel (n=20) Chocolate = 5.3mg total procyanidin/g [1.3 mg/g= (-)-epicatechin] +40g bread (i)-0g chocolate (ii)-27g (iii)-53g (iv)-80g	HPLC (plasma epicatechin)	↑ (-)-epicatechin respectively : 0g chocolate= 19 ± 14, 27g chocolate=133 ± 27 53g chocolate=258 ± 29, 53g chocolate=355 ± 49	Was not determined if catechin content was (+)-catechin or (-)-catechin
(Holt et al., 2002)	Acute *in-vivo* (n=20) (i)- 0.375 g cocoa/kg body weight +300ml water 1g cocoa = 12.2 mg monomers, 9.7 mg dimmers and 28.2mg polymers	HPLC (plasma monomers and polymers)	Procyanidin dimer, (−)-epicatechin, and (+)-catechin detected in the plasma at 0.5 h (16 ± 5 nmol/L, 2.61 ± 0.46 μmol/L, and 0.13 ± 0.03 μmol/L, respectively). And by 2 h it reached (41 ± 4 nmol/L, 5.92 ± 0.60 μmol/L, and 0.16 ± 0.03 μmol/L, respectively).	
(Mullen et al., 2009a)	Acute *in-vivo* (n=9) Cocoa drink=10g cocoa powder + Hot water cocoa= 22.3 umol (-)-catechin mostly 23 umol (-)-epicatechin and 70mg procyanidins	HPLC-PDA-MS2 (plasma and urine metabolites)	24 hour urine excretion of (epi)catechin metabolites= 7.32 ± 0.82 umol = 16.3 ±1.8% of ingested 2 metabolites in plasma and 4 in urine Urine: (epi)catechin-O-sulphate (RT 15.5 min), (-)-epicatechin-O-glucuronide Urine and plasma : (epi)catechin-O-sulphate (RT 18.6 min), O-methyl-(epi)catechin-o-sulphate	Majority of catechins were the (-) isomer which are less bioavailable

Table 1-5 Bioavailability of cocoa polyphenols

1.3.6.6.3.1 Phenolic acid production from cocoa

As previously discussed, 20% of catechins and 90% of procyanidins reach the colon; where they are subjected to colonic microbiota degradation. Moreover, the very low amounts of different flavan-3-ols monomers, proanthocyanidin dimers and trimmers detected in human plasma as glucuronidated, sulphated and methylated conjugates as well as sulphate metabolites in rat urine after the consumption of red wine or cocoa is an indication that most of these compounds reach the colon intact where they are subjected to microbial degradation and converted to phenolic acids (Holt et al., 2002, Sano et al., 2003).

Considering that procyanidins make up the largest concentration of polyphenols in cocoa, it could be postulated that cocoa exhibits most of its health benefits through the colonic metabolites of its polyphenols. However, the bioactivity of phenolic acids and how they are metabolised by colonic bacteria has not been thoroughly studied. Studies on colonic production of phenolic acids include

Colonic production of phenolic acids has been studied in various forms:

- In-vivo measurement of metabolites excreted through urine and plasma after ingestion of foods containing polyphenols of interest: for this method volunteers typically follow a low polyphenol diet ranging from 24-72 hours, followed by an overnight fast, collection of baseline urine / plasma samples. They are then given the food source of interest followed by the collection of blood and urine samples. Urine samples are typically collected in batches over 24-48 hours post ingestion or as 24 hour urine samples. Blood is typically taken through canulation over 6 hours after food ingestion. Volunteers remain on a low polyphenol diet throughout the sample collection period. Although this is a good method of phenolic acid measurement, there is the possibility of non-compliance to low polyphenol diet, or consumption of different diet on different arms of the study (in the case of cross over models), resulting in phenolic acid production from food sources other than that of investigation.
- Animal study models: This model is typically used for the incorporation of native compound in the diet of animals such as rats/mice and pigs. This is later corrected for the amount of food consumed, however the distribution of compounds in the diet are not even and hence percentage of ingestion will not be precise. Secondly some animals might have more potential for hydrolysing and degrading native compounds than possible in the human caecum.

- In-vitro faecal fermentations (ex-vivo): In this model fresh faecal sample from human volunteers is used as a source of colonic microbiota in a simulation model of the colon. Faecal samples are either used for individual fermentations or pooled as a diverse source of microbiota. In this model native source of the phenolics or the food source as a whole matrix can be incubated with the faecal inocula. Samples are taken typically at baseline and every 2 hours for 6 hours and at 24 and 48 hours post incubation. Ensuring anaerobic conditions in this model is key. This model is ideal for understanding pathways of production and conversion of phenolic acids. However as absorption and eneterohepatic circulation is not modelled this limits the representativeness of the model.

A number of studies using the above models have been summarised in *Table 1-6.* In An *in-vitro* study by Roowi et al. (2010), colonic metabolites of (-)-epicatechin (50 umol) were found to be mostly transitory: 3-HPPA, 3,4-DHP-γ-valerolactone and 3,4-DHP-γ-valeric acid. Lower concentrations of 4-HPAA and 3-HPAA were also identified. However high concentrations of 3-HPAA were present in the faecal slurry alone with no added substrate incubations (control), suggesting that this phenolic acid is derived mostly from the background diet. Presence of transitory traces of (2,4,6-trihydroxy)-propan-2-ol in this study suggested its production as a first catabolite from (-)-epicatechin degradation, and its later conversion to 3,4-DHP-γ-valerolactone → 3,4-DHP-γ-valeric acid → 3-HPPA →4-HPAA. The method used for the identification and quantification of these phenolic acids is key. Some phase II metabolites such as the glucuronides and sulphates of 3,4-DHP-γ-valerolactone can go undetected by the GCMS (Llorach et al., 2009).

The findings of Roowi et al. (2009) was supported by a similar study incubating (-)-epicatechin and (+)-catechin at concentrations of 150 mg/L and 1000 mg/L. This study in addition demonstrated that the degradation of (+)-catechin required its conversion to (+)-epicatechin before the production of 3,4-DHPAA (Tzounis et al., 2008a). The rate of (+)-catechin degradation was found to be significantly higher than that of (-)-epicatechin at 4 and 8 hours for the lower concentration and at 8, 10 and 17h for the higher concentration. However both monomers produced the same final catabolites regardless of different steps in the pathway.

The majority of ingested procyanidins reach the colon intact. Very few studies have detected procyanidins in plasma samples. Holt et al. (2002) identified dimers B2 but not B5 present in the purified cocoa standard in plasma as early as 0.5 hours after cocoa ingestions in humans. A significant increase from baseline values for catechins and epicatechins was also observed within 0.5h after ingestion of cocoa, demonstrating much higher concentrations of catechin (2.61 ± 0.46 μmol/L

compared to 0.08 ± 0.46 μmo/L baseline) than epicatechin (0.13 ± 0.03μmo/L compared to 0.00 μmo/L baseline). Other studies have failed to demonstrate the presence of procyanidins in plasma. Although T_{max} appeared the same for monomers and dimer B2, the C_{max} for dimer B2 was 100 fold lower than that of the monomers. Unlike Holts's observation, procyanidin dimers, trimers, tetramers and pentamers were identified in the plasma of rat fed procyanidins (Shoji et al., 2006). The dose administered was of a much higher range (1g/kg body weight), considering total polyphenol consumption in humans is estimated as 1g. Additionally administration through gavage might have had an impact on the observed results. It was suggested by shoji et al. (2006) that the extraction method using 8 μmol/L of urea as compared to the more common extraction method of using methanol / acetonitrile prevented the irreversible binding of plasma proteins to procyanidins. While these animal and human studies suggest the absorption of procyanidins other studies have not replicated these results.

Unlike the controversy around the absorption of intact procyanidins, the fate of the procyanidins reaching the colon is clearer. Recent studies have demonstrated extensive degradation of procyanidins by colonic microbiota. Procyanidins are known to be metabolised into hydroxyl benzoic acids, phenyl acetic acids (PAA), mono/ di-phenyl acetic acids and mono/ di-phenyl propionic acids (PPA) (Rios et al., 2003).

Gonthier et al. (2003), fed rats a diet containing catechin. The abundant excreted urinary compounds comprised of catechin and its 3′-*O*-methylated derivative, when rats were fed a dimer B_3 or trimer C_2 diet, neither intact catechin nor its methylated forms were detected.

The oral dosing of rats with [^{14}C] procyanidin B2 resulted in 60% urinary excretion in 96 hours of radioactivity greatly varying from the intact compound consumed. This is further supported by *in-vitro* faecal fermentation of [^{14}C] procyanidin B2 in studies that have identified the following as the main phenolic acids produced from this compounds *in-vitro*: 3,4-DHPAA, 3,4-DHPVA, 3,4-DHP-γ-valerolactone, 3-HPAA, 4-HPAA, 3-HPPA, PVA, 3,4-DHPPA,

Monohydroxylated-phenylvalerolactone and 1-(3,4-dihydroxyphenyl)-3-(2,4,6-trihydroxyphenyl)-propan-2-ol, 1-(HP)-3-(2,4,6-THP)-propan-2-ol (Stoupi et al., 2010b, Appeldoorn et al., 2009).

While conjugated forms resulting from phase II metabolism are absent in faecal fermentation results, there is on-going debate on the urinary excretion of (-)-epicatechin metabolites in free or conjugated forms. While majority of studies have suggested the former, other studies such as (Llorach et al., 2009) have identified glucuronide and sulphate conjugates of valerolactone in urinary excretion.

It is clear from above studies that more research is needed on the *in-vivo* fate of cocoa polyphenols and their colonic catabolites. The speculated catabolic pathway of cocoa polyphenol catabolism by colonic microbiota has been illustrated in *Figure 1-9.* The majority of studies investigating phenolic acid production from cocoa have utilised the parent compounds (catechin/ epicatechin/ procyanidins) in the fermentation model. It is however more important to investigate the production of these phenolic acids from cocoa as a whole matrix as this can greatly impact the production of phenolic acids. Moreover, these compounds are not consumed as independent nutrients but as part of a food source. Such studies have demonstrated 3-HPAA, 3-HPPA and 3,4-DHBA as the main metabolites of cocoa *in-vitro* fermentation (Fogliano et al., 2011).

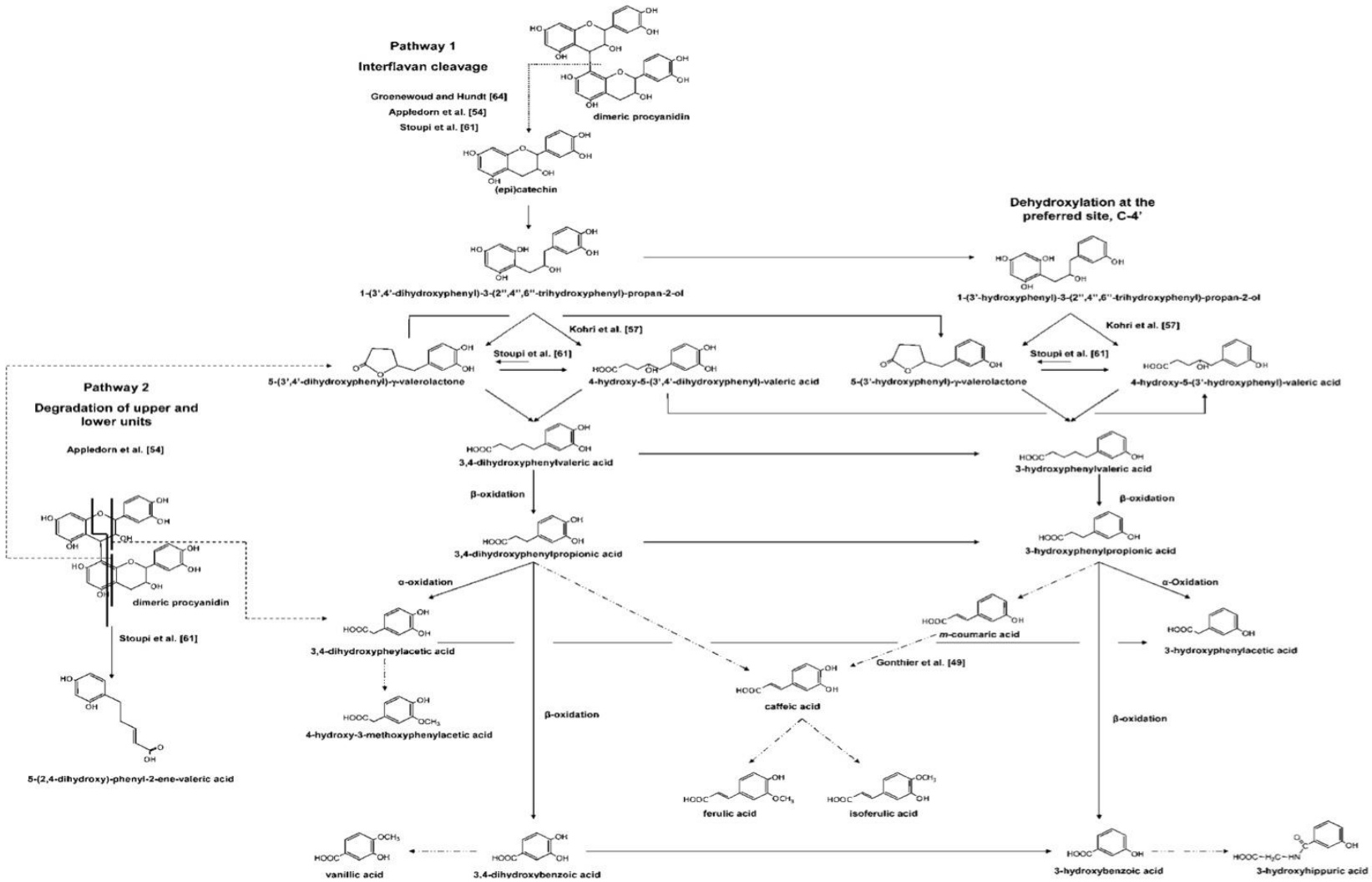


Figure 1-9 Speculated catabolic pathway of cocoa polyphenols by colonic microbiota - Adapted from Monagas et al. (2010)

Table 1-6 Phenolic acid production from cocoa polyphenols

Author year	Study method	Measurements	Findings (phenolic acid production)	comments
Holt et al (2002)	n=5 Age: 23-34 yrs, average b.d.w = 70.5 ± 4.6 Cocoa 0.374 g/kg b.d.w + 300ml water + white bread 1g cocoa = 12.2mg monomers, 9.7mg dimers and 28.2 mg polymers	LCMS	Procyanidins B2 and B5 identified in purified cocoa standards. Significant increase in Dimer B2 concentration at 0.5, 2 and 6h ($p<0.05$). B5 not detected in plasma samples. Significant increase from baseline in catechin (2.61 ± 0.46 μmol/L compared to 0.08 ± 0.46 μmo/L) and epicatechin (0.13 ± 0.03μmo/L, 0.00 μmo/L) concentrations within 0.5 hours of consumption $p<0.05$	Average consumption: 26.4 *g* cocoa, = 323 mg monomers and 256 mg dimers
(Rios et al., 2003)	n=11 2 day polyphenol-free diet 80g of chocolate (439 mg proanthocyanidins and 147 mg catechin). Urine collected for 24h, at 0, 3, 6, 9, 24 and 48h.	GCMS	3-HPAA, Ferulic, 3,4-DHPAA, 3-HPPA VanilliC. 3-HBA	
Mullen et al 2009	n=9 cross over Age: 20-43, BMI:24.7 1g paracetamol + 5g lactulose + 250ml of a cocoa drink with hot water or hot milk (250ml +10g green and blacks cocoa). Cocoa = 45 umol (-)epicatechin and (-)catechin	HPLC-MS	3,4-DHPPA, 3,4-DHPAA, 3-Methoxy-4-HPAA, 3,HPAA, PAA, protocatechuic, 4-HBA, 3-HBA, 4-HPA, Hippuric, Vanillic, Caffeic, Ferulic, p-coumaric, m-coumaric	
Sarda et al 2009	n=42 cross over Long term study Age: 69.7 ± 11.5 At risk of CVD 20g cocoa *2 day +250ml skimmed milk 4 weeks	LCMS-MS	Vanillic, 3,4-DHPAA, 3-HPAA, 5-(3,4-DDHP)-γ – valerolactone, 3,4-DHPPA, 3-HPPA, HVA, PAA, m-coumaric, p-coumaric, caffeic, ferulic, protocatechuic, Vanillic, 4-HBA, 3-HBA, 4-HHA, 3-HHA	
Llorach et al (2009)	Acute- in-vivo randomised control crossover n=10 Age: 18-50 yrs BMI: 21.6 2.1 Cocoa: 0.71 ± 0.09 mg/g (−)-epicatechin, 0.21 ± 0.01 mg/g (+)-catechin, 0.64 ± 0.06 mg/g of procyanidin B2. (i)-40g cocoa +250ml milk (ii)-40g cocoa +250ml water (iii)- 250ml milk	LCMS	5-(3,4-dihydroxyphenyl)- γ-Valerolactone sulphate, 5-(3,4-dihydroxyphenyl)- γ-Valerolactone glucuronide, *O*-Methylepicatechin, Epicatechin-O-Sulphate, 3-Methoxy-4-hydroxyphenylvalerolactone, 3-Methoxy-4-hydroxyphenylvalerolactone glucuronide, 4-hydroxy-5-(3,4-dihydroxyphenyl)-valeric acid, Vanillic acid	Milk did not affect cocoa polyphenol bioavailability
(Tzounis et al., 2011)	In-vivo n=22 Age: 30.2 ± 11yr, BMI 23.2 ± 2.5)	LCMS - with fluorescent	No flavanols present in the non-inoculated Faecal sample ↑ Plasma epicatechin after HFC from 24.7 ± 6.6 to 72.9 ± 12.7 mmol/L	Drinks matched for fibre

	Faecal samples collected before and after a High cocoa flavanol diet (494 mg cocoa flavanols/d) or Low cocoa flavanol diet (23 mg cocoa flavanols/d) 4 weeks + 4 weeks washout period	detector	($p<0.01$) but not for catechin 3- and 4- *O*- methyl- epicatechin Epicatechin and 3- and 4- *O*- methyl- epicatechin detectable in 24 urine sample for HFC.	
(Tzounis et al., 2011)	In-vivo n=22 Age: 30.2 ± 11yr, BMI: 23.2 ± 2.5) Faecal samples collected before and after a High cocoa flavanol diet (494 mg cocoa flavanols/d) or Low cocoa flavanol diet (23 mg cocoa flavanols/d) 4 weeks + 4 weeks washout peri	Reverse phase HPLC	↓ monomers, dimmers and oligomers after 6h of incubation ↑transient increase of trimers at 6h ↓time dependent, epicatechin, catechin and dimer B2 ↑ 5-(3,4-dihydroxyphenyly)-γ-valerolactone	Batch culture fermentation with 10g Faeces (with 10ml phosphate buffer.
Gonthier et al (2003)	Animal model-Male wistar rats (n=25) /Parallel /5 days Weight= 228 ± 3 g (i) - semipurified diet (20g/d) (ii)-semipurified diet (20g/d) + 1% (w/w) catechin (iii)-semipurified diet (20g/d) + 1% (w/w) procyanidin B_3 (iv)-semipurified diet (20g/d) + 1% (w/w) procyanidin C_2 (v) semipurified diet (20g/d) + 1% (w/w) polymers	HPLC-MS	metabolites from catechin diet only: catechin, 3-O-methyl catechin, 3,4-DHVA, ferulic acid Metabolites from catechin and dimer: 3,4-DHPPA, 3-HBA, 3-HHA Metabolites from catechin, dimer, trimer diet: 3-HVA, Metabolites from catechin, dimer, trimer, polymer diet: 3-HPPA, m-coumaric, p-coumaric, 3,4-DHPAA, 3-HPAA, protocatechuic, 4-HBA, vanillic, 4-HPPA, HA	
Stoupi et al 2010	Animal model, wistar rats (n=5) Weight =200-250g (i)-Intravenous (~150ul/animal) adjusted to 21 mg/kg b.d.w (ii)-oral (~500ul/animal) adjusted to 21 mg/kg b.d.w (iii)- to 10.5 mg/kg b.d.w Urine and faeces collected @ 0, 24, 48, 72, and 96 h after dosing Blood collected (0.1 ml) @ 0.5, 1, 2, 3, 4, 5, 6, 7, 9, 12, 15, 18, 20, 22, and 24 h		24h urine: 69% excreted for intravenous group, 58% for oral groups Total urinary excretion 76% for intravenous group, 62% and 63% for oral groups 24h faeces: 25% excreted for intravenous group, 39% and 40% for oral groups Total urinary excretion 28% for intravenous group, 41% for oral groups	Excretion of radioactivity higher in urine than faeces
(Déprez et al., 2000)	n=1 *In-vitro* fermentation (48h) with procyanidin 150 ul of 100 mmol/L expressed as catechin unit equivalents, i.e., 29 g/L) final concentration of 5 mmol/L	GCMS	4-HPAA, 3-HPAA, 3-HP-valeric, PPA, 4-HPPA and 3-HPPA	both labelled and non-labelled procyanidins
(Bazzocco et al., 2008)	Digestion followed by in-vitro fermentation Marie-mernard apples – short PA chains Avrolle apples- long PA chains	Phenolic acids SCFA production	Marie mernard apples more microbial metabolites than avrolle Phenolics produced = 3,4-DHPPA, 3-HPPA, 3-PPA, BA, 3,4-DHPAA, 3-HPAA.	Ciders had the lowest average degree of

			Primary metabolite =3,4-DHPPA Followed by : 3-HPPA, 3-PPA, BA, PAA ↑BA from pre-digested apples than non-digested ↑ PAA from non-digested than digested Cider with the shortest PA had the highest profile of PPA, PA BA for cider was lower than that of digested apple Cider had the most extent of conversion (44% for avrolle cider and 62% for marie.m cider) Maximal extent was reached much later (6-8h) with the marie.m apples than the extracts (2h) Delay in time needed for maximal extent of conversion when avrolle.p fermented alone (4h) than fermented with cell wall preparation (8h)	polymerisation. 3,4-DHPPA from ring-fission of catechins Rest from dehydroxylation of 3,4-DHPPA No phenolic acid produced from cell wall preparations.
Tzounis et al (2008)	In-vitro fermentation (pH controlled stirred batch-culture) n=3 (i)-[FS + (+)-catechin (150mg/L or 1000 mg/L)] ± 1% (w/v) sucrose or FOS (ii)-[FS + (-)-epicatechin (150mg/L or 1000 mg/L)] ± 1% (w/v) sucrose or FOS		<u>Phenolic acids from (-)-epicatechin:</u> 5-(3,4-DHP)—valerolactone, PPA 5-phenyl—valerolactone <u>Phenolic acids from (+)-catechin</u>: (+)-epicatechin 5-(3,4-DHP)—valerolactone 5-phenyl—valerolactone Rate of (+)-catechin metabolism much more than (-)-epicatechin at 4 and 8 hours for 150mg/L and at 8, 10 and 17h for 1000 mg/L concentration ($p<0.001$) (-)-epicatechin bacterial metabolism was higher in the presence of both sucrose and FOS at 8 and 10h ($p<0.01$). (+)-catechin bacterial metabolism was higher in the presence of both sucrose and FOS at 4 ($p<0.01$), 8 and 10h ($p<0.001$).	-volunteers were not put on low polyphenol diet
Appeldoorn et al (2009)	In-vitro fermentation (n=4 pooled) Mixture of dimmers (5umol) incubated as : (i)- FS + 1% (w/v) suspension of procyanidins (ii)-FS +5 % (w/v) suspension of procyanidins (iii)-FS	HPLC analysis of phenolics	Phenolic acids produced: Main phenolics: 3,4-DHPAA, 3,4-DHPVA, Other phenolics: 3-HPAA, 4-HPAA, 3-HPPA, PVA, Monohydroxylated phenylvalerolactone and 1-(3,4-dihydroxyphenyl)-3-(2,4,6-trihydroxyphenyl)propan-2-ol	Volunteers were not on a low polyphenol diet

Roowi et al 2009	In-vitro fermentation N=3 (2male, 1 female) (i)- FS + 1ml of epicatechin [50umol (-)-epicatechin] + 50g glucose (ii)-FS alone	GCMS analysis of phenolic acids in faecal slurries	Phenolic acids produced : 4-HPAA, 3-HPAA, 3-HPPA (54%) , 5-(3,4-dihydroxyphenyl)-γ-Valerolactone (38%) , 5-(3,4-dihydroxyphenyl)-valeric acid (32%).	4-HPAA very small amounts
Stoupi et al (2010)	In-vitro fermentation (N=1) (i)-FS + 5mM (-)-epicatechin (ii)-FS + 5mM Dimer B_2	LCMS analysis of phenolics	<u>Phenolic acid production from (-)-epicatechin and procyanidin B2@6h:</u> 1-(3,4-DHP)-3-(2,4,6-THP)- propan-2-ol, 1-(HP)-3-(2,4,6-THP)-propan-2-ol, 5-(3,4-DHP)-γ-valerolactone, 3-HPPA <u>Phenolic acid production from (-)-epicatechin only @12h:</u> 5-(3-HP)-γ-valerolactone, 5-(4-hydroxy)-(3′,4′-DH)PVA, 3-HPPA, 3-HPAA, 3,4-DHPPA <u>Phenolic acid production from procyanidin B2 only @24h:</u> 5-(3-HP)-γ-valerolactone, 5-(4-hydroxy)-(3′,4′-DH)PVA, 3-HPPA, 3-HPAA, 3,4-DHPPA <u>Phenolic acid production from (-)-epicatechin and procyanidin B2</u> @24h: 5-(3,4-DHP)VA, 5-(3′-HP) VA	Only 1 volunteer used
(Fogliano et al., 2011)	In-vitro fermentation (n=1) Continuous fermentation model after digestion Insoluble-fraction of cocoa 20g of cocoa in 120ml of water digested, followed by dialysis and freeze drying A concentration of 1% w/v used.	LC/MS/MS	3-HPAA 3-HPPA 3,4-DHBA -and an unknown compound with MRM fragmentation peak of *m/z* 289→245	
Dall'Asta et al (2012)	In-vitro fermentation (n=3) (i)- FS+ 9% chocolate extract (90% chocolate)	LCMS analysis of polyphenols and phenolic acids	Phenolic precursors: epicatechin, catechin, procyanidin dimmers-B, procyanidin trimers-B, procyanidin tetramers-B Phenolic acids: 5-(3,4-DHP)- γ-valerolactone, 3,4-DHPAA, protocatechuic, HBA, salicylic	Concentration not given

1.4 Food matrix

The food matrix is defined by the USDA as "The nutrient and non-nutrient components of foods and their molecular relationships, i.e. chemical bonds, to each other". Food matrix interaction therefore refers to when components of such matrices possess the physio-chemical properties to influence the metabolism of another component in the food matrix. This may lead to the enhancement or inhibition of the absorption and or metabolism of one or both of the components. Food matrix interactions may be the result of direct chemical interaction such as the mineral-oxalate binding which may reduce mineral absorption (eg. Spinach oxalate and calcium) or indirect, such as the influence of polyphenol or fibre on the colonic microbiota resulting in the potential modification of each other's metabolism by the bacteria.

As discussed in various sections above, the majority of polyphenolic compounds enter the colon either as the native compounds present in food or as the metabolites of small intestinal hydrolysis and/or conjugates of phase II metabolism. It is becoming increasingly evident that most of the absorption occurs via lower molecular weight catabolites produced through colonic microbiota action but the bioactivity of these in human tissues and their role in disease prevention is not certain, although there have been studies (summarised in Table 1-2) demonstrating antioxidant properties *in-vitro* (Rice-Evans et al., 1996), protection from *in-vitro* lipid peroxidation (Sroka and Cisowski, 2003) and chemo-protective effects in cancer cell lines (Henning et al., 2013). The same bacterial ecosystem is responsible for some of the health benefits induced by dietary soluble fibre, by fermentation and production of bioactive compounds: SCFA. Nutrients present in the diet can alter gut physiology and hence impact the metabolite production from the microbiota. Alteration of GI physiology can include effect on gastric emptying time, intestinal motility, acid, bile and enzyme secretion, blood and lymph flow as well as changes in the function of the endocrine and nervous system, depending on type of food and matrix in which it is ingested (Schramm et al., 2003).

Most studies have focused on the fermentation end products of non-digestible carbohydrates, proteins and their health benefits, studies on the active metabolites of polyphenol degradation by these microbiota are more scarce.

Studies investigating the metabolite production from these food components have demonstrated a high inter-individual variation in metabolites produced from colonic microbiota action. These variations can be attributed to genetics, age, body weight, gastrointestinal disease, ethnicity and diet. There has been extensive research on how diet can modify colonic microbiota by selectively stimulating growth of

specific bacterial strains as seen for prebiotics or by selectively inhibiting growth of certain bacterial strains as seen by some phenolic compounds. Changes in bacterial composition should subsequently affect metabolite production from these food components. Dietary compounds can also influence the metabolite production by altering small intestinal absorption. This is seen in the case of viscous fibres.

These two food components: polyphenols and fibre not only have the ability to alter the composition of colonic microbiota, but are both present in the same foods. Most studies have investigated the health benefits exhibited by each of these food components separately; however, their food matrix interaction is key to their ability to induce a beneficial health impact. Hence it is of great importance to determine if this food matrix interaction results in a synergistic combination of health benefits from fibre and polyphenols or if there is any inhibitory impact.

Polyphenols in plants can be found either in the free form (extractable) or bound (non-extractable) to the protein and /or fibre components of the plants. The free polyphenols, which are easily extracted are usually low molecular weight phenolics while the high molecular weight polyphenols that are non-extractable can be found both in the free and in the bound forms (Bravo et al., 1994, Saura-Calixto et al., 1991, Terrill et al., 1992). The importance of considering the amount of bound phenolics in food is not well known, thus most studies have not taken into consideration the presence of these polyphenols and their contribution to health *in-vivo*. In plant based foods these bound phenolics may be associated with dietary fibre and, similar to the fibre, they pass through the digestive system and reach the colon intact where they are subjected to microbial action (Perez-Jimenez et al., 2013). An example is longer chain proanthocyanidins that have shown a binding affinity towards plant cell walls which contain the dietary fibre pectin (Bazzocco et al., 2008, Le Bourvellec et al., 2007). The concentration of these bound polyphenolics can range from 26 mg/100g as measured in whole grain (Hatcher and Kruger, 1997) to 1240 mg/100g in acai fruit (Rufino et al., 2011). These would normally not be included in studies on health risks.

Thus dietary fibres and polyphenols are not only consumed together in a diet as a result of simultaneous ingestion of a mixture of foods but they can also be found in a bound form in plant based foods, ensuring they both reach the colon. Once in the colon the bound polyphenols can be released from their food matrix (Perez-Jimenez et al., 2013) and can impart prebiotic or antibiotic properties on the colonic microbiota, which would subsequently impact the metabolism of other components of the food matrix which rely on the colonic bacteria for their metabolism.

The chemical structure of carbohydrates makes a range of physiochemical properties possible, resulting in matrix interactions with other carbohydrates, fats, proteins and minerals. Carbohydrate rich

foods, especially major dietary fibre sources, may contain anti-nutrient components such as phytic acid and tannins. Phytic acid has been shown to reduce the absorption of trace element such as zinc and copper by forming insoluble complexes through divalent cations (Gibson, 1994). Turnlund et al. (1984) demonstrated that the absorption of zinc was reduced from 34% to 17.5% when 2.34 g of phytate was added. Furthermore when foods containing oxalate, such as cocoa or spinach are present with phytic acid and minerals, they form a fibre- nutrient-oxalate matrix, which is more difficult to breakdown than the fibre-nutrient or oxalate-nutrient bond alone. This was demonstrated in a 4 week study by Kelsay and Prather (1983) when volunteers were fed a high fibre -high oxalate diet (fruits/vegetable + spinach), low fibre-high oxalate diet (spinach) or high fibre-low oxalate diet (fruits/veg+ cauliflower). A negative apparent balance of Ca, Mg and zinc was reported when high fibre foods were added to high oxalate diets ($p<0.05$).

Food matrix interactions involving polyphenols have been reported typically involving protein-polyphenol binding and investigation of the impact of milk based products on bioavailability of polyphenols from food sources. Milk commonly consumed with cocoa in the form of cocoa beverage or milk chocolate on cocoa polyphenol bioavailability has shown controversial effects.

Interest in the impact of milk on cocoa polyphenol bioavailability was initiated when Serafini et al. (2003) demonstrated that the increased plasma antioxidant levels [from 100± 3.5% to 118.4 ± 3.5% ($p<0.01$)] brought about by the consumption of 100g of dark chocolate was no longer observed when the cocoa was consumed along with 200ml of milk or as milk chocolate. Furthermore, the consumption of milk and milk chocolate also reduced the absorption of (-)–epicatechin ($p<0.01$). The antioxidant analysis of defatted dark cocoa and milk cocoa demonstrated that twice the quantity of milk chocolate needs to be consumed to match dark chocolate antioxidant levels.

However, other studies such as Roura et al (2007) investigated the impact of milk on flavan-3-ol metabolites from 40g of cocoa (128umol flavan-3-ol monomers) consumed with milk or water in a crossover model (n=21) and found no significant effect on urinary excretion of metabolites. Similarly Keogh et al. (2007) investigated the impact of milk proteins on cocoa in the form of polyphenol extracts in a cross over study (n=24). They did not find a difference in average plasma polyphenols between no-milk cocoa beverage [2.7g polyphenols + 7g non-dairy creamer+7g sugar (33.4g powder) + 200ml water] and milk cocoa beverage [2.7g polyphenols+ 7g skimmed milk powder (35% pr, 54% lactose), 2.5g cocoa butter and 4.5g sugar +200ml water]. The cocoa in this study contained 293 mg/g total flavanols, 50.8g of which were catechin+epicatechin (1:7).

Following the controversy created by these studies, Mullen et al (2009) investigated the impact of 10g of cocoa [45 umol (-)-epicatechin + (-)-catechin] consumed with hot milk vs. hot water for 9 volunteers in a cross over model. Four metabolites were detected in urine peak-1: (epi)catechin-*O*-sulphate (Rt 15.5), peak-2: (-)-epicatechin-*O*-glucoronide (Rt 17.4), peak-3: this peak shared the same mass spectrum as peak-1 and thus it was identified as (epi)catechin-*O*-sulphate, however it eluted with a delay (Rt 18.6), peak-4: *O*-methyl-(epi)catechin-o-sulphate (Rt 22.1), and the two main metabolites identified in urine were also detected in plasma peak-3: (epi)catechin-*O*-sulphate (Rt 18.6) and peak-4: *O*-methyl-(epi)catechin-o-sulphate (Rt 22.1). When cocoa was consumed with water the combined urinary excretion of the metabolites for the 0–2-h (3938 ± 538 nmol vs. 2115 ± 323 nmol, $p<0.01$) and 2–5-h (2690 ± 470 nmol vs. 1914 ± 322 nmol, $P<0.05$) was significantly higher for the cocoa-water beverage compared to the cocoa-milk beverage. However the impact of milk on plasma pharmokinetics was more modest compared to its impact on urine pharmokinetics. Milk extended the $t_{1/2}$ of plasma (epi)catechin-*O*-sulphate from 1.5 ± 0.1 to 2.0 ± 0.2 h ($P<0.05$) and reduced AUC for plasma (epi)catechin-*O*-sulphate from 173 ± 27 to 135 ± 16 nmol· h/L.

Studies using high concentrations of cocoa polyphenols reported no inhibitory impact of milk on the bioavailability of these phenolics, whereas studies using concentrations of polyphenols present in commercially available cocoa demonstrated an inhibitory impact. Thus the impact of milk appears to be saturatable suggesting some sort of binding.

Impact of milk- based foods have been seen for other polyphenol rich foods. The impact of milk on the bioavailability of tea polyphenols which are similar to those found in cocoa has also been a topic of interest. Reddy et al. (2005) demonstrated that the consumption of black tea (7g tea leaves in 350ml boiling water+ sugar with or without milk) resulted in significantly higher plasma catechin levels, the addition of milk to the tea reduced the catechin AUC ($p<0.05$). However the antioxidant capacity of tea did not change when milk was added, interestingly a correlation was found between plasma catechin levels and antioxidant potential only when tea was consumed without milk ($p<0.01$).

Some studies have suggested that the brewing method of black tea may be of importance when considering the impact of milk on bioavailability of black tea. Kyle et al. (2007) tested the impact of infusion time of six different tea brands (3g tea leaves in 300ml boiling water with or without milk) with different infusion time: 3, 5, 7 and 10 minutes. The antioxidant capacity, total phenolics and catechin levels of plasma increased with infusion time and none of these results were affected by the addition of milk. Van der Burg-Koorevaar et al. (2011) also investigated the impact of brewing methods such as amount of milk and sugar on the catechin concentrations from 1 tea bag (Indian tea

and English tea in 170ml boiling water) in an in-vitro simulation of digestion. 5.6% of milk resulted in a 59% recovery of total catechins whereas higher amounts of milk (15-40%) resulted in the 40% recovery. When sugar was added to 25% milk with English tea the impact of milk was no longer seen. The authors suggested that sucrose had a stabilising impact on the milk protein and catechin interaction. However Keogh et al. (2007) did not demonstrate any impact of 2.45g of milk proteins on the bioavailability of catechins after the consumption of 2g of chocolate polyphenols, sugar and cocoa butter (in 200ml water).

Most of the above studies have investigated the impact of milk on black tea which has a 3-fold lower concentration of catechins than green tea (van het Hof et al., 1998). The impact of milk on green and black tea was investigated by Leenen et al. (2000). Green tea increased plasma antioxidant capacity significantly more than black tea, however milk had no impact on the antioxidant capacity of either. Plasma catechins were not measured in this study. It may be that the plasma catechins were reduced but there was no impact on antioxidant capacity as seen by Reddy et al. (2005).

Mullen et al. (2008) investigated the impact of cream on polyphenol bioavailability from strawberries. In this study cream delayed gastric emptying and small bowel transit time resulting in more than one hour delay in T_{max} for pelargonidin-3-O-glucuronide but did not affect total absorption of this compound and its urinary excretion. If anything there was a trend for increased absorption probably due to the greater time in the small intestine.

In a study on yoghurt and orange polyphenolics, there was no impact on plasma polyphenol levels or gastric emptying and mouth-caecum transit time (Mullen et al 2008) but the yoghurt appeared to delay the appearance of polyphenols in the first 5 hours after ingestion and reduced the amount of phenolic acids (sum of 5 phenolic acids identified as colonic metabolites) in urine tenfold (6.7 umol vs 62 umol) (Roowi et al 2009). The mechanism for this effect is unclear.

Table 1-7 food matrix interactions

Author year	Study method	Measurements	Findings	comments
(Serafini et al., 2003)	Cross over study (n=12)- Milk on cocoa Dark chocolate and milk chocolate – defatted twice age 32.2 ±1.0 yrs, weight of 65.8 ± 3.1 BMI 21.9 ± 0.4 100g dark chocolate with or without 200ml full fat milk, or 200g milk chocolate (40ml milk)	FRAP one hour after cocoa consumption	Cocoa FRAP: Dark chocolate 147±4.5 , Milk chocolate 78.3 ± 3.4 Plasma FRAP : Dark chocolate from 100 ± 3.5% to 118.4 ± 3.5% ($p < 0.01$). returning to baseline after 4h No sig increase with milk or milk chocolate (-)-Epicatechin measurement Absorption was much less if cocoa was consumed with milk or as milk chocolate ($p<0.001$)	
(Roura et al., 2007)	cross over – 1 week wash out (n=21) 40g cocoa [(–)-Ec 70.5 mg/100 g, procyanidin B 2 63.75 mg/100 g, catechin 21 mg/100 g, and 5 mg/100 g of flavonols, including isoquercitrin, quercetin, quercetin-3-glucoside, and quercetin-3-arabinoside] +250ml water / milk	LCMS-MS 2 hours after cocoa consumption	(–)-Ec-glucuronide was the only (–)-Ec metabolite detected, showing a mean (SD) plasma concentration of 330.44 nmol/l (156.1) and 273.7 nmol/l (138.42) for CC-W and CC-M, respectively ($p<0.01$)	
(Keogh et al., 2007)	N=24 Age 50 – 65 yrs. BMI 18 – 30 (i)-Milk- free beverage = 2.7g polyphenols + 7g non-dairy creamer +7g sugar (33.4g powder) + 200ml water (ii)-Milk beverage: 2.7g polyphenols+ 7g skimmed milk powder (35% pr, 54% lactose), 2.5g cocoa butter and 4.5g sugar +200ml water Cocoa : total flavanols=293 mg/g catechin+epicatechin = 50.8g (1:7~)	HPLC-MS	No impact of milk protein on average plasma polyphenol Milk protein significantly reduced T_{max} for epicatechin with differences at 1 and 2 h ($p<0.01$) and marginally sig at 4h and 5h ($p<0.05$) Low polyphenol meal provided 4h after dosing Catechin was sig lower at 4h with milk powder ($p<0.01$)	Skimmed milk used
(Mullen et., al 2009)	Cross over- Milk on cocoa (n=9) Age 20-43 yrs, Bmi 24.7 2.7 1g paracetamol 5g lactulose 250ml cocoa drink with hot water or hot milk (250ml +10g green and blacks cocoa). Cocoa (45 umol (-)epicatechin and (-)catechin	HPLC-MS	Gastric emptying and transit time not affected by milk 2 metabolites in plasma and 4 in urine Minor effects on plasma pharmokinetics of (epi)catechin-o-sulphate and no effects on o-methyl-(epi)catechin-o-sulphate However milk sig decreased the excretion of 4 urinary metabolites from 18.3% to 10.5% of ingested dose Urine: (epi)catechin-o-sulphate (-)-epicatechin-o-glucoronide (epi)catechin-o-sulphate	

			o-methyl-(epi)catechin-o-sulphate plasma : (epi)catechin-o-sulphate o-methyl-(epi)catechin-o-sulphate Plasma: significant effect on the C_{max} for (epi)catechin-*O*-sulphate. significantly extend the $t_{1/2}$ of (epi)catechin-*O*-sulphate from 1.5 ± 0.1 to 2.0 ± 0.2 h (P = 0.02). Milk also significantly reduced the AUC for (epi)catechin-*O*-sulphate from 173 ± 27 to 135 ± 16 nmol · h/L Urine: the combined urinary excretion of the metabolites for the 0–2-h and 2–5-h samples was significantly higher for the cocoa-water drink than for the cocoa-milk beverage. These values were 3938 ± 538 nmol compared with 2115 ± 323 nmol for the 0–2-h samples (P< 0.01) and 2690 ± 470 nmol compared with 1914 ± 322 nmol for the 2–5-h samples (P = 0.04)	
(Mullen et al.,2008)	Acute in-vivo (n=8). Age: 23-48yrs and BMI= 23.7 ± 1.2 5g lactulose+1g paracetamol + (i)-200g strawberries (222 µmol of pelargonidin-3-O-glucoside) or (ii)-200g strawberries + 100ml double cream	HPLC-PDA-MS2 analysis (urine and plasma)	strawberry alone: C_{max} of pelargonidin-3-O-glucoside = 274 ± 24 nmol/L and T_{max} = 1.1 ± 0.4 h when strawberry+cream C_{max}= 227 ± 35 nmol/L and T_{max} = 2.4 ± 0.5 h tmax significantly lower for the combination than strawberry alone (p<0.01) C_{max} only significantly lower between 0-2 hours and only for hydroxyl cinnamis acid	
(Roowi et., al 2009)	Acute in-vivo cross over (n=5) Age: 21 – 50 years, average BMI: 23.1 orange juice fortified with 131 lmol of hesperetin-7-O-rutinoside (i)- 250 ml orange juice (ii)250ml orange juice +150 ml full fat yoghurt (3.8%) (iii)- water Control group: (N=5) Age: 31 – 38 years of age, average BMI 30.9 (24.2 – 46.9)	GCMS (urine)	Phenolic acids produced: 3-HPAA, 3-hydroxyphenylhydracrylic acid, dihydroferulic acid, 3-methoxy-4-hydroxyphenylhydracrylic acid and 3-HHA Total pheolic acid production from orange juice= 62 ± 18 µmol orange juice + yoghurt= 9.3 ± 4.4 , water=6.7 ± 1.8 µmol Yoghurt reduced total phenolic acid produced from orange juice (p<0.05 No change in gastric emptying- mouth-caecum transit time	Volunteers consuming water not same as other 2 arms

1.4.1 Impact of carbohydrates on polyphenol bioavailability

The impact of carbohydrates on polyphenolic bioavailability depends not only on their physio-chemical properties but also on the location in the gut of the interaction with other compounds (upper/lower digestive system). Previous studies have investigated the impact of simple carbohydrates such as sugars on the bioavailability of polyphenols. However sugars are mostly absorbed in the small intestine. The food matrix interaction between soluble fibres and polyphenols is more likely as soluble fibres retain their structure in the upper gut and they enter the colon together where they are subjected to degradation by the colonic microbiota. The possible impacts of fibre on the digestive system have been adapted from Edwards et al. (2012) and elucidated below.

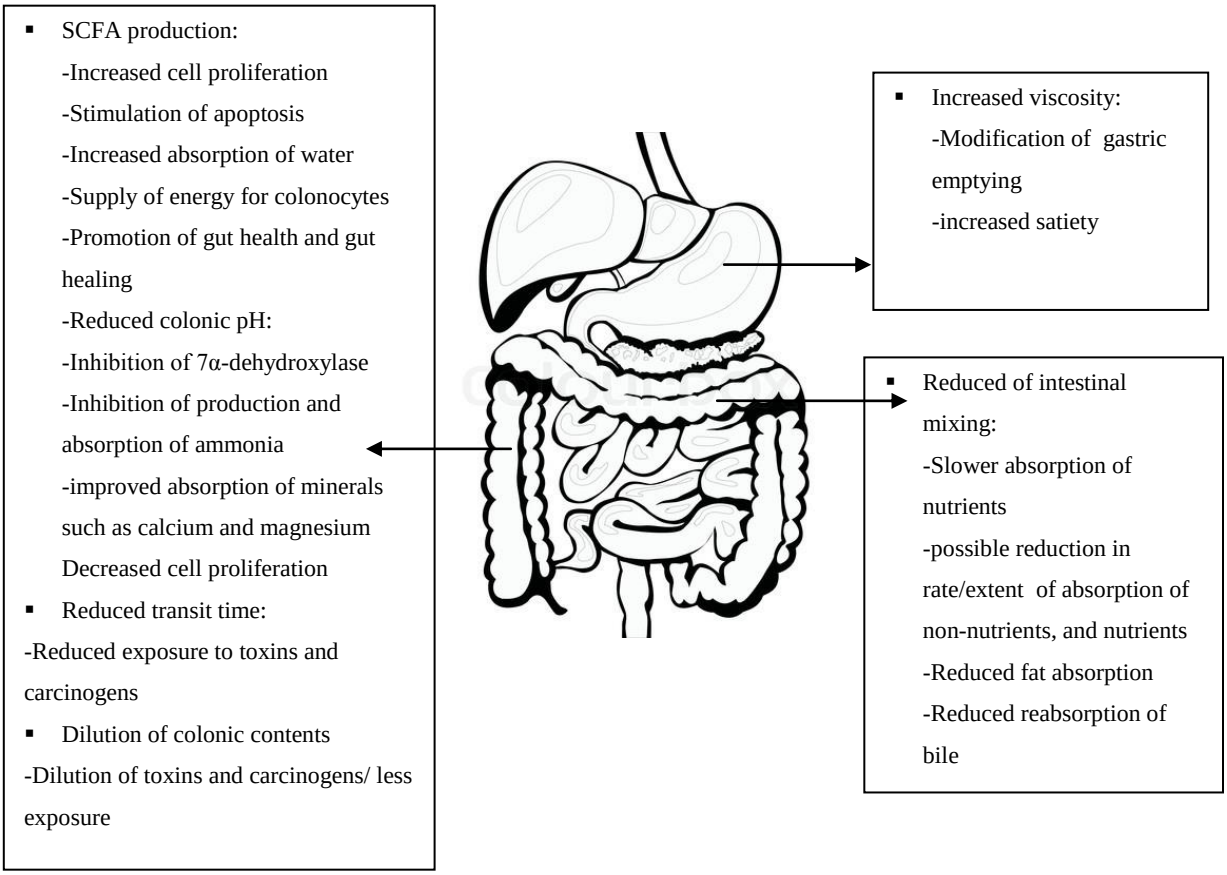


Figure 1-10 Potential impact of fibre on the digestive system- Adapted from Edwards et al., (2012)

Viscous soluble fibres such as ispaghula may impact polyphenol metabolism and phenolic acid production before and after these polyphenols reach the colon. The matrix formed by the viscous fibre may entrap the polyphenols in the upper gut making them less accessible for absorption. Hence greater

amounts of these polyphenols are transferred to the colon and excreted. Once in the colon, the impact will depend on the fermentability of the fibre. Those mostly or completely fermented, such as pectin, may interact by altering bacterial activity (Scott et al., 2013) and changing pH whereas less fermentable fibres such as ispaghula dilute colonic content and decrease transit time (Edwards et al., 2012). The latter may result in increased faecal excretion of phenolic and polyphenolic acids.

1.4.1.1 The evidence:

Few studies have investigated the impact of carbohydrates on bioavailability of cocoa polyphenols. Schramm et al (2003) investigated the effect of carbohydrates in the form of table sugar (0, 8.75, 17.5 kj/kg body weight) and bread on the bioavailability of catechin and epicatechin of a flavanol rich, sugar free cocoa (0.125 g/kg body) mixed with water (2ml water /kg body weight). Sugar increased C_{max} and AUC of flavanols ($p<0.05$), also resulting in a faster rate of flavanol elimination ($p<0.05$). When cocoa was consumed with bread, the absorption AUC of flavanols increased from 3.3% to 4.4% resulting in an increased flavanol C_{max} ($p<0.05$). The impact of whole milk and butter was also investigated, not displaying any interaction with flavanol bioavailability. T_{max} was not affected by any food group.

A similar study was conducted by Neilson et al (2009) investigating the impact of different matrices in the form of beverage and confectionary on epicatechin bioavailability in a 6h crossover model. These included dark chocolate (CDK), high sucrose (CHS), high milk protein (CMP) confections and sucrose milk protein (BSMP), non-nutritive sweetener milk protein (BNMP) cocoa beverages. All matrices contained 94 umol (-)-epicatechin and 32 umol catechin. There were no differences in AUC, C_{max} or T_{max} within the confectionary group or the beverage group. However, the beverage group had higher C_{max} compared to the confectionary group (45 ± 4.3 BSMP, 41.6± 2.1 BNMP, 31.6± 2.8 CDK, 24.7 ± 1.9 nM CMP) but not different from CHS (34 ± 3.3) as well as lower T_{max} (0.9, 1.1 hours for beverage and 2.3, 1.8 and 2.3 for confections respectively); suggesting that the physical form of the cocoa could have an impact on the bioavailability of epicatechin ($p<0.05$).

Renouf et al. (2010) demonstrated that the addition of non-dairy creamer and sugar or milk to coffee did not impact total absorption of metabolites as AUC did not differ between the 3 different arms of the study. However the addition of non-dairy creamer (containing vegetable fat but no lactose) did reduce the C_{max} of hydroxycinnamic acid ($p=0.039$) and increase the T_{max} of ferulic ($p=0.009$) and isoferulic acid ($p=0.014$). It is difficult to attribute this effect to the creamer or sugar content of the non-dairy creamer. The delay in T_{max} may be due to the vegetable fat content of the creamer.

There are several hypotheses explaining the increased polyphenolic absorption aided by simple carbohydrates such as sugar *in-vivo*. Some of these include controlled cellular flavanol absorption by SGLT1 transporters and lactase phlorizin hydrolase enzymes (Gee et al., 2000). This was further demonstrated when specific quercetin glycosides were absorbed at a higher rate than their aglycones or diglycosides (Gee et al., 2000).

In the colon, carbohydrates may impact on polyphenol metabolism by altering colonic bacterial activity. Jaganath et al. (2006) measured the colonic metabolism of 28 umol rutin or 55 umol quercetin or 18×10^6 dpm of [4-^{14}C]quercetin with or without 0.5g of glucose. In this study the addition of glucose to faecal incubation vessels increased the extent of rutin deglycosilation and subsequent phenolic acid production. Interestingly this study demonstrated that the presence of glucose can alter the decomposition pathway and metabolite production. Glucose influenced the catabolic pathway by directing the conversion of 3,4-DHBA towards HPAA derivative production instead of 4-HBA production seen in the absence of glucose. Glucose was used to mimic the presence of fibre, most often present in the gut along with the polyphenol. Glucose is a common constituent of cellulose, beta glucan and other dietary fibres. However, simple carbohydrates lack the physio-chemical properties of fibre such as viscosity and slower fermentation rates for less rapidly fermented soluble fibres such as ispaghula.

Tzounis et al (2008) conducted a continuous *in-vitro* fermentation model incubating 150 mg/L or 1000 mg/L of (+)-catechin and (-)-epicatechin in the presence or absence of sucrose and / or fructo-oligosaccharides. Both catechin and epicatechin had a higher rate and total production of colonic bacterial metabolites in the presence of both sucrose and fructo-oligosaccharides. This was seen at 9 and 10 hours after incubation for (-)-epicatechin ($p<0.01$) and at 4 ($p<0.01$), 8 and 10 hours ($p<0.01$) for catechin.

Unfortunately there is not much evidence on the impact of soluble fibre on the bioavailability of polyphenols and phenolic acid production from these compounds. In a study by Bazzocco et al. (2008) two different apple varieties were used: Marie-mernard apples contain short chain proanthocyanidins and avrolle apples containing long chain procyanidins. The freeze-dried apples were digested and incubated in a faecal fermentation model, additionally the respective cider and proanthocyanidin extracts of the relevant apples were incubated separately. The proanthocyanidn extract of avrolle apples was also incubated along with 100mg of cell wall preparation containing pectin. The prominent phenolic acids produced from these apple proanthocyanidins were found to be 3,4-DHPPA followed by HPPA, 3-PPA, BA, 3,4-DHPAA and 3-HPAA. There was no phenolic acid production from cell wall

preparations alone. When cell wall preparations were added to proanthocyanidin extract incubation the maximal extent of conversion was delayed from 4 hours to 8hours post incubation. However, there was no difference in the total concentration of phenolic acid production. Additionally the maximum extent of conversion for marie-mernard apples was much later (6hours) as compared to their proanthocyanidin extracts (2hours). This study also demonstrated that the maximum conversion and proanthocyanidins occurred in cider samples having the shortest proanthocyanidin chains.

While simple sugars and prebiotics can enhance the deglycosilation of parent compounds reaching the colon and increase phenolic acid production, there is not enough evidence on the impact of more complex carbohydrates such as soluble fibres on phenolic acid production from polyphenols.

Table 1-8 Impact of carbohydrates on polyphenol bioavailability

Author year	Study method	Measurements	Findings	comments
(Schramm et al., 2003)	cross over study- in-vivo (n=6) Study-1 : effect of table sugar sugar (0, 8.75, 17.5 kj/kg body weight) + 0.125 sugar free flavanol rich cocoa (in 2ml water /kg body weight) Study-2: effect of nutrient rich foods Better, bread, steak and water (8.75, 17.5 and 8 ml/kg body weight) + 0.125 sugar free flavanol rich cocoa (in 2ml water /kg body weight) Study-3: effect of miscellaneous foods Water, bread, grapefruit juice, whole milk (8.75, 17.5 and 8 ml/kg body weight) + 0.125 sugar free flavanol rich cocoa (in 2ml water /kg body weight) Study-4: effect of Famotidine (a) Water and cocoa (b) Water, cocoa and 20mg famotidine (1h before water and cocoa)	HPLC of non-(plasma) Plasma antioxidant capacity	Study-1: Sugar ↑ plasma Cmax (8.75 kj/kg) and ↑ plasma AUC (8.75 and 17.5 kj/kg) As well as faster rate of flavanol elimination (E1/2: 8.75 and 17.5 KJ/KG) P <0.05 Study-2 CHO rich foods increased flavanol absorption % of flavanol absorption Cocoa alone 3.3% +bread 4.4% ↑ AUC and ↑Cmax p<0.05 +steak 3.1% ↑ E1/2 p<0.05 +butter 3.7% Study-3 Cocoa alone +bread ↑ AUC and ↑Cmax ↑ E1/2 p<0.05 +milk - +grape juice ↑ AUC ↑ E1/2 p<0.05 Study-4 No effects on flavanols No correlation between ability of foods to buffer pH and flavanols Correlation between CHO content of foods and flavanol absorption r=0.83 p<0.02	No changes in T_{max} However their data points were limited which could have lost sensitivity to T_{max} Displayed very large STDEV
(Neilson et al., 2009)	Study-1 In-vivo N=6 (3male, 3 female) cross over 7 day wash out Mean age: 23.8 ± 1.7 Mean BMI: 25.5 ± 0.7 40g Confections: -dark chocolate – high sucrose – high milk protein 250 ml Beverage: -sucrose milk protein – non-nutritive sweetener milk protein (solid ingredients dissolved in	HPLC (plasma)	Higher bioavailability from cocoa beverages than chocolates High sucrose slightly increased epicat AUC but not significantly and milk proteins slightly reduced epicat AUC but not significantly NO difference in bioavailability within groups but the state in which the cocoa was consumed resulted in a difference in epicat bioavailability (p<0.05) T_{max} lower for beverage than confections but not significant	Limited time points Milk protein limits bioavailability while sucrose increases based on cmax this was

	water and consumed) Cocoa = 36mg cat+epicat Study-2 In-vitro digestion		No difference in T_{max} between groups Cmax sig higher for beverages $p<0.05$ than confections (45 ± 4.3 BSMP , 41.6± 2.1 BNMP 31.6± 2.8 CDK 24.7 ± 1.9 nM CMP but not different from CHS (34 ± 3.3) Sucrose slightly increased Cmax CHS higher Cmax than CMP	seen in the confections but not beverages
Renouf et al (2010)	Acute in-vivo cross over (n=9) Age 21-50 yrs (i)-4g soluble instant coffee+400ml water (ii)- 4g soluble instant coffee+360ml water+40ml whole milk (iii)- 30.5 g premixed instant coffee with sugar and non-dairy creamer =4 g of instant coffee 4g coffee =335 mg (900 μmol) of total chlorogenic acid ingested	LCMS (plasma)	No difference in AUC for coffee ± milk or NDC/sugar No impact of milk on C_{max} and T_{max} Non-dairy creamer reduced C_{max} of Hydroxycinnamic acid ($p<0.01$) and isoferulic acid ($p= 0.02$). Non-dairy creamer reduced Increase Tmax for ferulic ($p<0.01$) and isoferulic ($P=0.01$).	
Jaganath (2006)	*In-vitro* fermentation N=3 (i) 28 umol rutin (ii) 55 umol quercetin or (iii) 18×10^6 dpm of [4-^{14}C]quercetin + / - 0.5g glucose	HPLC-PDA-MS^2 analysis of phenolic acids	Phenolic acids: 3,4-DHPAA > 3-HPAA, 3,4-DHBA, 4-HBA, 3-HPPA -presence of glucose enhanced deglycosilation of rutin -presence of glucose increased phenolic acid production	3,4-DHPAA appeared @ 2h 3-HPAA @ 24h
(Bazzocco et al., 2008)	Digestion followed by in-vitro fermentation Experiment-1 (n=4) (i)-100mg Marie-mernard apples – short PA chains (ii)-25mg of Proanthocyanidin extract from Marie-mernard apples (iii)-100mg cider (iv)- 100mg of Cell wall preparation containing pectin Experiment-2 (i)-100mg Avrolle apples- long PA chains 25mg or 100mg (ii)-25mg proanthocyanidin extract	Phenolic acids SCFA production	Marie mernard apples more microbial metabolites than avrolle Phenolics produced = 3,4-DHPPA > 3-HPPA, 3-PPA, BA, 3,4-DHPAA, 3-HPAA. Primary metabolite =3,4-DHPPA ↑BA from pre digested apples than non-digested ↑ phenyl acetic acid from non-digested than digested Cider with the shortest PA had the highest profile of PPr, PA BA for cider was lower than that of digested apple Cider had the most extent of conversion (44% for avrolle cider and 62% for marie.m cider)	Differences between benzoic acid and phenyl acetic acid between digested and non-digested apples.

	(iii)-25m 25mg proanthocyanidin extract + 100mg cell wall preparation (iv)-100mg cider (v)-100mg of Cell wall preparation containing pectin	Maximal extent was reached much later (6-8h) with the marie.m apples than the extracts (2h) when PA from avrolle.p was fermented with cell wall preparation the maximal extent was much later (8h) compared to when they were fermented alone (4h) No phenolic acid produced from cell wall preparations.	
Tzounis et al (2008)	In-vitro fermentation (pH controlled stirred batch-culture) (N=3) (i)-[FS + (+)-catechin (150mg/L or 1000 mg/L)] ± 1% (w/v) sucrose or FOS (ii)-[FS + (-)-epicatechin (150mg/L or 1000 mg/L)]± 1% (w/v) sucrose or FOS	(-)-epicatechin bacterial metabolism was much higher in the presence of both sucrose and FOS at 8 and 10h ($p<0.01$). (+)-catechin bacterial metabolism was much higher in the presence of both sucrose and FOS at 4 ($p<0.01$), 8 and 10h ($p<0.01$).	volunteers were not put on low polyphenol diet
Neilson et al., 2009)	Study-2 In-vitro followed by digestion Matrices were diluted to provide equivalent of one serving as the in-vivo study Confections were melted at 50 C for 15 mins to mimic the melting and chewing process.	Influence of product matrix on flavan-3-ol bioavailability was modest Sig differences seen between relative rate of absorption (T_{max}) and C_{max} of epicatechin seen between beverages and confections No differences in EC recovery from beverages or confections but C had sig higher recovery from beverages than from confections.	Recovery for EC similar in all forms but different for C Hence the physical form might not have an impact on EC but might affect C

1.4.2 Impact of polyphenols on fibre

The composition and activity of the microbiota is the main influencing factor in the health benefits induced by fibre and polyphenols reaching the colon; as the microbiota's capacity to ferment and metabolise compounds determines the final metabolite products, their bioavailability and their impact on human health.

In addition to all other health benefits induced by polyphenols they have also demonstrated antibacterial activity. This property of polyphenols may indirectly impact their own bioavailability as well as the bioavailability of active metabolites from compounds such as soluble fibre. However most evidence of this property of polyphenols has been obtained from studies conducted on specific pathogenic bacteria *in-vitro* rather than complex ecosystems such as that in the colon a clear understanding of their ability to impact the complex gut microbiota and the mechanism behind this antimicrobial property is needed (Duda-Chodak, 2012).

1.4.2.1 Mechanism of action

There have been many hypotheses on the mechanisms of the antimicrobial actions of polyphenols. The lack of consistency in the proposed mechanisms is due to the large family of polyphenols and the structural minor structural differences among them, resulting in different mechanisms of action. Most studies have investigated the impact of 1-2 compounds of a certain polyphenol sub-group.

A general hypothesis that could be applicable to most polyphenolic compounds is that polyphenols bind to bacterial cell membranes, thus disrupting membrane function and inhibiting cell growth (Sirk et al., 2009). It has also been suggested that the antimicrobial property may be attributed to the ability of polyphenols to form strong bonds with heavy metals. As bacteria contain metalloenzymes it is possible that this interaction of polyphenols with heavy metals causes inhibition of enzymes, and impairment of ion channels. The deficiency in available iron in the gut may affect certain sensitive bacterial species. The mechanisms of action proposed from 1987-2004 have been reviewed by Cushnie and Lamb (2005), summarising three possible mechanism of action:

- Damage of cytoplasmic membrane caused by perforation and/ or reduction in the fluidity of the membrane (Ikigai et al., 1993, Tsuchiya and Iinuma, 2000).
- Nucleic acid synthesis inhibition caused by the inhibition of topoisomerase (Mori et al., 1987, Bernard et al., 1997, Plaper et al., 2003).

- Energy metabolism inhibition caused by the inhibition of NADH-cytochrome c reductase (Haraguchi et al., 1998).

Since 2005 more studies have been presented supporting the mechanisms summarised by Cushnie and Lamb. (2005). These include damage of cytoplasmic membrane by the flavan-3-ols, flavonol and flavonal, inhibition of nucleic acids by flavan-3-ols and isoflavones as well as inhibition of energy metabolism by the flavan-3-ol and flavanol group. In addition inhibition of cell wall synthesis and the inhibition of cell membrane synthesis have also been proposed (Cushnie and Lamb, 2011). Some compound specific mechanisms of action have also been proposed such as enzyme inhibition and reactive oxygen generation for epigallocatechin gallate and the inhibition of DNA gyrase by quercetin have been proposed (Cushnie and Lamb, 2005).

The evidence provided could suggest that polyophenolic compounds either have several methods of action or that the method of action is dependent on the structure of the compound.

1.4.2.2 Impact of compound structure

Duda Chodak. (2012) demonstrated that the aglycone quercetin had a strong antimicrobial impact, while the aglycone catechin did not demonstrate much antimicrobial ability. This was attributed to the double bond between C2-C3 and presence of a carbonyl group in quercetin. However, the flavanones hesperetin and naringenin having a saturated C-ring also exhibited antimicrobial activity, these flavanones also contain a carbonyl group. Hence, it can be postulated that the carbonyl group is responsible for the antimicrobial activity of these compounds. Interestingly it has been shown that the presence of a carboxyl group on C4 of the A-ring of chalcones increases their aqueous solubility by 60 folds without affecting their antimicrobial activity, additionally the hydroxylation at position 2 of their A-ring is a determining factor in their antimicrobial properties, however these have been attributed to their increased structural stability (Nielsen et al., 2004, Ávila et al., 2008). Batovska et al. (2009) demonstrated that even though the presence of a hydroxyl group on the C-4 of the B-ring could be important, it is not sufficient for the antimicrobial action and the presence of this hydroxyl group on the A ring is the determining factor. Other studies have demonstrated that in the case of flavones the presence of *O*-acyl or *O*-alkylamino chain on the C-7, and hydroxylation at C-5 of the A-ring enhance the antimicrobial activity (Li et al., 2009, Suresh Babu et al., 2006). The same was seen for the presence of O-acyl or O-alkylamino chain on the C-3 of the C-ring for flavan-3-ols and flavonols (Park and Cho, 2010, Ávila et al., 2008, Otsuka et al., 2008). It was also found that the antimicrobial activity of flavones can be improved by replacing the oxygen atom at C-4 with nitrogen or sulphur (Ullah

Mughal et al., 2006). These structure-related enhancements in antimicrobial activity have lead many researchers to modify the structure of polyphenols in order to use them as strong antimicrobial agents. However the discussion of these modifications and their implications are beyond the scope of this PhD. The use of natural compounds such as polyphenols as antibiotics is becoming increasingly important in the light of antibiotic resistance of many bacterial species.

1.4.2.3 The evidence:

The antimicrobial properties discussed above cannot be generalised to all polyphenol categories. Duda Chodak et al. (2005) investigated the impact of various polyphenols on *in-vitro* cultures of individual bacteria representing the gut microbiota (*Table 1-9*). While the aglycones inhibited bacterial growth, this was not true for their glycosidic forms. Rutin had no effect but quercetin inhibited growth of all bacterial species tested at concentrations as low as 20 ug/ml. The authors stated then that the glycosidated polyphenol would have no effect but of course in the colon, rutin is first degraded to quercetin which would have antibacterial effects even though its presence may be short lived. It is unlikely however that many polyphenol aglycones are present in the gut as they are ingested mainly in conjugated forms. While the glycosides demonstrated an inhibitory impact on certain strains of bacteria, this was seen at MIC $\geq$ 250 ug/ml. Only compounds demonstrating MIC $\leq$ 100 are considered noteworthy and those demonstrating MIC $\leq$ 10 are of great interest (Rios and Recio, 2005).

In a study (Tzounis et al., 2008a) the impact of catechin and epicatechin at concentrations of 150 mg/L and 1000 mg/L were investigated in an *in-vitro* fermentation model with or without the addition of 1% (w/v) sucrose / FOS. Even though there were no differences in the total bacterial count it was found that (-)-epicatechin increased the number of *Eubacterium rectale-C* at 150 mg/L only, while (+)-catechin increased the growth of *Lactobacillus* spp. and *Bifidobacterium* spp. at 150 mg/L and *coccoides-Eubacterium rectale* at both150 and 1000 mg/L. (+)-catechin was also found to inhibit the growth of *C. histolyticum* group at 1000 mg/L. Although authors have stated that these polyphenolics may have a prebiotic impact, the increase in bacterial species was seen only at lower concentrations of 150 mg/l. and a possible explanation for this could be that the inhibition of other bacterial species allowed the these species to grow more, as the total number of bacteria remained constant. Additionally higher concentrations demonstrated an inhibitory and not prebiotic impact on the bacterial growth.

The same authors (2011) carried out an *in-vivo* study comparing the impact of a high polyphenol cocoa supplementation (494 mg cocoa flavanols/day) to a low polyphenol cocoa supplementation (23 mg

cocoa flavanols/day) for four weeks, collecting faecal samples before and after interventions and measuring bacterial growth. Supporting their previous study, they found no change in total bacterial count while the high polyphenol cocoa diet resulted in the increase of selective bacterial species: lactobacillus and Enteroccocus ($p<0.01$) for both diets, Bifidobacteria ($p<0.01$) only for the high flavanol cocoa diet and a reduction in *C. histolyticum* counts ($p<0.001$). Furthermore this study found a reduction in TAG and CRP levels after cocoa supplementation. Interestingly the reduction in CRP values was inversely related to the Lactobacillus spp. count ($p<0.05$, $R^2= -0.33$). This study suggests that the long-term consumption of cocoa can have a prebiotic impact on the gut microflora. However it is important to note that cocoa products contain relatively high fibre content and it is difficult to separate the polyphenol or fibre content of the cocoa as being responsible for these observations. These results were supported in a study by Parkar et al. (2008) investigating the impact of various polyphenolics at a concentration of 62.5-1000 ug/m on 4 different bacterial strains: Escherichia coli, *Staphylococcus aureus* , *Salmonella typhimurium*, *Lactobacillus rhamnosus*. This study did not demonstrate any antibacterial impact of the glycosides rutin or phloridzin, while quercetin and naringenin had the lowest MIC for all bacterial strains. Catechin and epicatechin demonstrated the same MIC for all bacterial strains except for *L.rhamnosus*, with catechin having an MIC of $\leq$ 250 ug/ml and epicatechin requiring a higher concentration of 500 ug/ml. The MIC of the polyphenols was compared to that of Gentamicin. *L. rhamnosus* demonstrated less sensitivity to the polyphenol and Gentamicin, requiring higher MIC. Thus it is possible that this probiotic may be less affected by polyphenols in the gut. The lowest antimicrobial impact was seen for isoflavones, flavonols and glycosides while phenolic acids had an intermediate impact. Unlike the previous study, this study did not find any structural association with antimicrobial activity, with the exception of glycosilation, which is not of relevance in *in-vivo* studies as discussed before. The lack of structure–growth inhibition association was also demonstrated in a study by Lee et al. (2006) investigating the impact of tea polyphenols and their colonic phenolic acid derivatives (epicatechin, catechin, 3-*O*-methyl gallic acid, gallic acid and caffeic acid, 3-PPA, 4-HPAA, 4-HPPA) on 28 colonic microbiota species. The results of this study supported previous studies demonstrating a general inhibition of pathogenic bacteria (*Clostridium perfrigens*, *Clostridium difficile* and *Bacteroides. spp*) especially by the phenolic acid derivatives and a small to no impact on the commensal and probiotic bacteria (as *Clostridium spp*, *Bifidobacterium spp* and *Lactobacillus spp*).

Most of the above studies have investigated the impact of polyphenolic compounds *in-vitro* and very little is known on their impact after long-term consumption. Interestingly a study by Smith and Mackie (2004) demonstrated that long term (3.5 weeks) consumption of 0.7% and 2% proanthocyanidins in

rats significantly increased the number of tannin resistant bacteria from 0.3% ±5.5% to 25.3%± 8.3% and from 0.3% ±5.5% to 47.2%± 5.1% respectively ($p<0.05$), while gram-positive bacteria decreased in the presence of the proanthocyanidins. The post-proanthocyanidin diet analysis of faecal bacteria demonstrated a return of tannin resistant bacteria to pre-treatment numbers.

Even though polyphenols demonstrated an anti-bacterial impact, they are mostly consumed as food components and not as independent compounds. In a study investigating the impact of (10% w/w) cocoa on faecal microbiota of wistar rats, a reduction in Bacteroides, Clostridium and Staphylococcus ($p<0.05$), without the total bacterial count being affected, was observed. In addition the cocoa diet resulted in a lower body weight ($p<0.01$), which was correlated to the reduction of Clostridium species. There was no difference in food consumption between the two groups (Massot-Cladera et al., 2012).

None of the above studies have investigated the impact of this antimicrobial activity on metabolite production from the colonic bacteria. Kemperman et al. (2013) investigated the impact of 1000 mg/day of tea extract polyphenols on the growth of gut microbiota and their metabolite production for 2 weeks in a Simulator of Human Intestinal Microbial Ecosystem (SHIME). In addition to the antimicrobial activity seen by tea extract, a reduction in butyrate concentration was demonstrated, which was attributed to the inhibition of butyrate producing bacteria. This was accompanied by an initial increase in acetate concentration. Towards the end of the study a drastic reduction in total SCFA and acetate concentration was demonstrated. This reduction in SCFA production corresponded to reduction in bacterial numbers observed. Even though this in-vitro model is ideal for studying the longer-term impact of polyphenols on gut bacteria *in-vitro*, there is still a lack of other factors such as host-bacteria interrelationship and presence of other influencing food matrix interactions.

Etxeberria et al. (2013) extensively reviewed the previous evidence (*in-vitro* and *in-vivo)* on the impact of various food sources and food extracts, as well as pure compounds on bacterial populations which is not the core of this PhD. They elucidated the variation in antibacterial properties of different polyphenolic compounds as well as the variation in sensitivity to polyphenolic compounds by the bacterial populations. Polyphenolic compounds demonstrated an antimicrobial impact especially towards pathogenic bacteria both *in-vitro* and *in-vivo*, with less impact on the commensal bacteria. However, the authors concluded that the implications of these changes as well as the interaction of these polyphenolic compounds with other compounds *in-vivo* or *in-vitro* have not been sufficiently investigated and understood.

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Table 1-9 Antibacterial activity of polyphenolics

Author year	Study method	Measurements	Findings	comments
(Arima et al., 2002)	*In-vitro* Incubated *Bacillus cereus* and *Salmonella enteritidis* with or without Quercetin, Quercitrin, Rutin, Kaempherol, Morin, Thymidine and Uridine	Growth of bacteria measured by paper disk method	Quercetin and morin inhibited growth at 100 ug/disk and Rutin no inhibitory impact even at 400 ug/disk or at concentration 1000 ug/ml MIC of quercetin on *S. enteritidis* and *B.cereus* alone = 250 ug/ml and 350 mg/ml MIC of quercetin+ Rutin on S. *enteritidis* and *B.cereus* alone = 100 ug/ml and 200 mg/ml MIC of morin or kaempherol on *S. enteritidis* and *B.cereus* alone = (150 and 400 ug/ml) and (300 and 800) mg/ml MIC of morin or kaempherol + Rutin on *S. enteritidis* and *B.cereus* alone = (50 and 150 ug/ml) and (100 and 400) mg/ml	
(Smith and Mackie, 2004)	*In-vivo* long term Adult wistar rats (n=18) Proanthocyanidins extracted from *A.angustissima* added to the diet at concentrations of 0.7% And 2% for 3.5 weeks Faecal samples collected weekly.	DNA fingerprinting of bacterial populations	Increase in both total bacterial count and tannin-resistant bacterial count 0.7% proanthocyanidin diet: increase in tannin resistant bacteria from 0.3% ±5.5% to 25.3%± 8.3% $p<0.05$) 2% proanthocyanidin diet: increase in tannin resistant bacteria from 0.3% ±5.5% to 47.2%± 5.1% ($p<0.05$)	
(Vaquero et al., 2007)	*In-vitro* Bacteria obtained from human source*: Escherichia coli, Proteus mirabilis, Serratia marcescens, Flavobacterium sp.* and *Klebsiella pneumonia* Incubated along with 5, 10, 25, 50, 100, 200, 500 and 1000 ug/ml of Gallic, Vanillic, Protocatechuic, caffeic acids, Rutin, Quercetin, Catechin.	Agar diffusion test	-Rutin: weak impact on *S.marcescens* with MIC > 10 ug/ml and moderate impact on E.coli with MIC > 25 -Quercetin: strong impact on *S.marcescens, P.mirabilis, E.coli and K.pneaumoniae* at MIC> 2, 10 and 25 respectively -Catechin: moderate impact on *S.marcescens and E.coli* with MIC > 100	

	(+)-control: Chloramphenicol and (-)-control:ethanol		and 50 ug/ml respectively. Strong impact on *P.mirabilis* MIC > 500 The phenolic acids only had a weak to moderate impact on *E.coli* (MIC> 25-50) and a moderate to strong impact on *K.pneumoniae* (Gallic and Vanillic acid only) with MIC> 10 ug/ml	
(Lee et al., 2006)	*In-vitro* incubation 28 strains of pathogenic and prebiotic strains (5% inoculum) incubated in a broth with or without 1% polyphenol extracts of tea: epicatechin, catechin, 3-O-methyl gallic acid, gallic acid and caffeic acid, 3-PPA, 4-HPAA, 4-HPPA	Monitoring growth of bacteria using spectrophotometry	The growth of pathogenic bacteria such as *Clostridium perfrigens*, *Clostridium difficile* and *Bacteroides spp* was inhibited especially by the phenolic acid derivatives. Commensal anaerobes and probiotics such as *Clostridium spp*, *Bifidobacterium spp* and *Lactobacillius* not affected much. Caffeic acid had the strongest inhibition especially for *E. coli, Salmonella, Pseudomonas, Clostridium and Bacteroides*.	
(Tzounis et al., 2008a)	In-vitro fermentation (pH controlled stirred batch-culture) N=3 (i)-[FS + (+)-catechin (150mg/L or 1000 mg/L)] ± 1% (w/v) sucrose or FOS (ii)-[FS + (-)-epicatechin (150mg/L or 1000 mg/L)]± 1% (w/v) sucrose or FOS	FISH	Impact of (-)-epicatechin ↑ *Eubacterium rectale-C* at 150 mg/L only Impact of (+)-catechin: ↓*C. histolyticum* group (1000 mg/L) ↑*C. coccoides-Eubacterium rectale* (150 and 1000 mg/L) ↑ *Lactobacillus spp.* And *Bifidobacterium spp* (150 mg/L) No difference in total number of bacteria.	-volunteers were not put on low polyphenol diet
(Parkar et al., 2008)	In-vitro incubation of bacteria with polyphenols Representative of gut microbiota: *Escherichia coli*, *Staphylococcus aureus* , *Salmonella typhimurium* , *Lactobacillus rhamnosus* . bacterial suspension (50 µl, 1×10^5 cfu/ml) Polyphenols : caffeic acid, chlorogenic acid, *o*-coumaric acid, *p*-coumaric acid, catechin , epicatechin, phloridzin, rutin, quercetin, naringenin, daidzein and genistein at a concentration ranging from 62.5-1000 ug/ml	Bacterial viability tested using broth micro-dilution method	All polyohenols except rutin had an inhibitory impact on bacteria at different MIC. Phloridzin only inhibited Staphylococcus aureus at MIC 125 ug/ml Quercetin and naringenin had the lowest MIC varying depending on bacterial strain. Required MIC (ug/ml): -*Escherichia coli*, catechin=1000, epicatechin=1000, rutin>1000, quercetin=125, Gentamicin=8 -Staphylococcus aureus catechin=125, epicatechin=125, rutin>1000,	

			quercetin=62.5, Gentamicin=1 -Salmonella typhimurium catechin=1000, epicatechin=1000, rutin>1000, quercetin=125, Gentamicin=8 -Lactobacillus rhamnosus catechin ≤ 250, epicatechin=500, rutin>1000, quercetin=250, Gentamicin=32	
(Vaquero et al., 2007)	In-vitro incubation *Escherichia coli* ATCC 35218 tested against 50 or 100 ug/ml of Gallic, Protocatechuic, Vanillic, Caffeic acids, Rutin, Quercetin and Catechin in 99.8% ethanol (ethanol added to all media toobtain 5% (v/v) Control: media + 5% ethanol	Bacterial growth measured in a microplate reader	-Quercetin allowed the least growth at 50 and 100 ug/ml (0.44±0.05 and 0.19±0.02) having the most growth reduction (1.91 and 2.16 respectively) -Rutin allowed for growth at 50 and 100 ug/ml (1.12±0.07 and 0.7 ±0.05) having the growth reduction 1.23 and 1.65 respectively -catechin had the most growth of flavonoids at 50 and 100 ug/ml (1.4±0.09 and 1.31 ±1.04) having the growth reduction 0.95 and 1.04 cfu/ml respectively) - Gallic acid: growth at 50 and 100 ug/ml : 1.77±0.08 and 1.4 ±0.09 having the growth reduction 0.58 and 0.95 cfu/ml respectively - protocatechuic acid: growth at 50 and 100 ug/ml : 2.05±0.09 and 1.8 ±0.08 having the growth reduction 0.3 and 0.55 (cfu/ml) respectively - vanillic acid: growth at 50 and 100 ug/ml : 2.2±0.07 and 2.08 ±0.05 having the growth reduction 0.15 and 0.27 cfu/ml respectively - caffeic acid: growth at 50 and 100 ug/ml : 0.85±0.05 and 0.2 ±0.03 having the growth reduction 1.5 and 2.15 cfu/ml respectively When rutin and quercetin were mixed their impact was synergistic, when rutin and catechin were mixed their impact was additive	
(Tzounis et al., 2011)	N=22 healthy humans (12 men, 10 women, 30.2 ± 11yr, BMI 23.2 ± 2.5) Faecal samples collected before and after (i)-High cocoa flavanol diet (494 mg cocoa flavanols/d) or (ii)-Low cocoa flavanol diet (23 mg cocoa flavanols/d) for 4 weeks + 4 weeks washout period	FISH	↑ lactobacillus and Enteroccocus ($p<0.01$) ↑ Bifidobacteia post intervention ($p<0.01$) only for HFC ↓ C.Histolyticum counts ($p<0.01$) ↓ TAG and CRP ($p<0.05$) ↓ lactobacilli ≈ ↓CRP ($p<0.05$, $R^2= -0.33$) No change in total bacterial count, bacteroides or Ecoli	drinks matched for fibre

	Batch culture fermentation was conducted with 10g Faeces (with 10ml phosphate buffer) Cocoa contained <1% overall fibre (10mg/ml)			
(Duda-Chodak, 2012)	Bacterial culture of microbiota representatives were used in a bacterial suspension of 1.5×10^8 units/ml: *Bacteroides galacturonics, Lactobacillus sp, Enterococcus caccae, Bifidibacterium catenulatum, Ruminococcus gauvereauii, Escherichia coli* were used in a bacterial suspension of 1.5×10^8 units/ml Impact of 20, 200, 250 ug/ml of naringenin, naringin, hesperetin, hesperidin, rutin, catechin and 4,20, 50 ug/ml ug/ml quercetin on 1ml of bacterial suspension after 24 hours of incubation at 37 C	Measurement of turbidity	Quercetin MIC ≥ 50 for all species except for *Ruminococcus gauvereauii at 20 ug/ml* Rutin, no inhibitory impact except for *Enterococcus caccae and Ruminococcus gauvereauii* at MIC > 250 ug/ml Catechin: inhibitory impact only for Bifidibacterium catenulatum at MIC > 250 ug/ml Naringenin inhibition of all species at MIC ≥ 250 ug/ml Nringin: no inhibitory impact Hesperidin: no inhibitory impact Hesperetin minimum inhibitory impact at ≥ 250 ug/ml except for Lactobacillus sp.	Aglycones had an impact but glycosides didn't
(Massot-Cladera et al., 2012)	Female wistar rats (n=) Cocoa enriched diets [10% (w/w)] containing 10.62 mg/g polyphenols for 6 weeks Faecal samples collected before and after 6 weeks.	FISH - FCM	Total bacterial count not affected but there was a reduction in Bacteroides, Clostridium and Staphylococcus($p<0.05$) Body weight significantly lower ($p<0.01$) for cocoa diet (167.36 ± 0.40 g) compared to control group 193.84 ± 0.25 g	
(Kemperman et al., 2013)	In-vitro SHIME (2 weeks) Impact of black-tea extract (1000 mg/day)	SHIME and qPCR / pyrosequencing.	Reduction in Bifidobacteria, Blautia coccoides, Anaeroglobus and Victivallis Increase in: *Klebsiella sp. Enterococci Akkermansia* SCFA: Reduction of butyrate concentration, Initial increase in acetate concentration. Towards the end of the study a sharp decrease in total SCFA and acetate was demonstrated	

After careful consideration of the relevant literature, it was clear that the metabolism of fibre and polyphenolics by colonic bacteria will involve some element of interaction. It was therefore important to ask whether the interaction of these compounds and their metabolites result in synergistic, additive or modification of their metabolism and how this may impact on their potential health benefit. There is great need, given the increased awareness of the role of gut bacteria in health to have a better understanding of colonic bacterial release of bioactive molecules under real life conditions, and to the best of our knowledge there have been no previous studies investigating the impact of food matrix interaction between fermentable fibres and polyphenols.

In this PhD we aimed to investigate the impact of the antimicrobial properties of polyphenols on the production of bioactive molecules essential for human health such as SCFA from fibre fermentation, as well as the impact of prebiotic food components such as soluble fibre on phenolic acid produced from polyphenol degradation by these colonic microbiota. Given that majority of polyphenols and fibre reach the colon intact, it is of great interest to evaluate their matrix interaction in the presence of the colonic microbiota and the final metabolites produced as the result of this microbial metabolism. Given the fact that very low concentrations of polyphenols are readily absorbed and that they are heavily metabolised in the colon, it can be said that their impact on health is largely dependant on these metabolites and thus any factor inhibiting the production of phenolic acids, prevents the health impacts induced by polyphenols by reducing their bioavailability. In a similar manner, most of the health impact exhibited by soluble fibres is dependent on the SCFAs produced through microbial fermentation, and thus any factor affecting the microbial population may in turn influence the composition and amount of SCFAs produced. Understanding the food matrix interactions between fibres and polyphenol, particularly those related to the production and absorption of their colonic metabolites will help draw a clearer picture of the biological and health implications induced by each of these food components.

CHAPTER 2

General Methods

This chapter details the methods and materials common to many of the experiments making up this PhD, or variations of a protocol used in more than one study/experiment. As fermentation products are the primary outcomes in most of the studies, the methods relevant to production and analysis of these outcomes are detailed in this chapter as well as the ex-vivo fermentation model and preparation of samples for ex-vivo experiments.

The methods described in this chapter are relevant to:

- Chapter-3: Matrix interaction between the polyphenols rutin and quercetin and the soluble fibres raftiline, ispaghula and pectin, on their metabolism by colonic bacteria- *ex-vivo*
- Chapter-4: Matrix interaction between cocoa polyphenols and the soluble fibres raftiline, ispaghula and pectin, on their metabolism by colonic bacteria- *ex-vivo*
- Chapter-5: Impact of ispaghula on the phenolic acid production from cocoa *in-vivo*

2.1 Faecal sample collection

The colonic microbiota responsible for the fermentation of fibre and polyphenols are a complex community with approximately 1000 different species of bacteria (Slavin, 2013) many of which (>60%) are not able to be isolated and cultured. The process of fermentation in the gut is difficult to assess directly due to inaccessibility of the proximal colon where most bacteria activity occurs (Edwards and Rowland, 1992). Thus indirect measurements, in faeces, urine, plasma and breath have to suffice but these are limited in their usefulness.

In-vivo models of colonic fermentation are expensive and often non-ethical. Human studies where colonic fermentation is measured directly include: intubation, which is invasive, expensive and requires medical supervision. This model provides accurate measurements of the metabolites but may not be suitable for assessing transit time (Florent et al., 1985). Animal models may allow assessing the impact of a restricted diet on the bacteria and metabolite production; however they are expensive, invasive and may not necessarily be translatable to human physiology (Martin et al., 1998).

Indirect measures include the measurement of acetate in the peripheral blood and breath hydrogen. Peripheral acetate measurement, although convenient, may not only be reflective of bacterial action but also endogenous production of acetate (Rumessen et al., 1992). Breath hydrogen measurement provides an estimated rate of fermentation but no qualitative information on metabolite production (Rumessen, 1992). This method is often used for the estimation of mouth-caecum transit time (MCTT).

Hence, faecal samples are often used as a representation of the colonic bacteria population. However this representation of the complex microbial colony may not necessarily be accurate as they are found in larger numbers towards the end of the ileum and caecum which may be due to the optimum acidic environment for their growth; as the pH changes (more neautral) towards the distal colon, so might the composition of the bacteria. Little is known about the composition of caecal bacteria due to their inaccessibility and often it is relied upon the faecal sample to provide a representation of this colony for the mimicking of their metabolic action in the colon. Marteau et al. (2001a) have shown that the composition of caecal microbiota contains 25% anaerobes vs. 1% in faeces. Despite this limitation the faecal bacteria are still widely used in in-vitro fermentation models to understand their metabolic action and products of fermentation as direct metabolite measurement from the faeces does not take into consideration the absorption of the metabolites in the proximal colon.

Even though there have been doubts regarding the accuracy of this representation (Marteau et al., 2001b), the use of faecal sample in the *ex-vivo* fermentation model for the measurement of metabolites was found to be the most feasible, ethical non-invasive method as well as being the most simple and effective way of obtaining these bacteria (Edwards and Rowland, 1992). We did not use faecal measurements for any in vivo study as these provide less useful information as most fermentation and other products have been absorbed before the faeces was passed.

In this thesis, fresh faecal samples were obtained from healthy non-smoker male and female volunteers who had not experienced any gastrointestinal infection or received antibiotics and laxatives for at least 3 months prior to start of the study. The volunteers were asked to follow a low polyphenol diet for 3 days prior to study (Appendix-1). This was meant to reduce the concentrations of polyphenols and their metabolites at baseline and thus improve the comparison of outcomes.

Volunteers were asked to pass the entire bowel movement into single use plastic tubs with the help of paper bedpans placed on the toilet seat. The sample was placed in a gas proof bag immediately after passage, along with a kit to induce anaerobic conditions *(AnaeroGen-Anaerocult A, Merck,* Germany) and an Anaerob indicator *(*Anaerotest- Merck, Germany*)* to check the anaerobic conditions. These were then placed in insulated bags with ice packs. Samples were either collected from the residence or work of the volunteer or transported to the reception of Yorkhill hospital by taxi service. In all cases, samples were transported to the lab immediately and processed within 2 hours of passage.

This method is relevant to chapters 3 and 4.

2.2 *Ex-vivo* Modelling of anaerobic colonic fermentation

Due to difficulties mentioned in section 2.1 regarding the study of colonic fermentation in-*vivo*, an in vitro or it could be classed *ex-vivo* model of the gut was adopted to examine the fermentation of fibre and polyphenols by the microbiota. There are three main models of ex-vivo anaerobic fermentation that use faeces as a source of the gut microbiota population, these are: batch (static), semi-continuous and continuous culture.

Semi-continuous and continuous models are better representatives of the colonic environment. They model the influx of digestive substrates from the upper gastrointestinal tract, pH and dilution of media as well as addition of nutrients to the culture periodically; thus keeping the microbiota in a steady state. Hence it is a better representation of in-vivo conditions where the bacteria are mostly kept at a steady state. However, these methods are not feasible for the number of substrates investigated in this thesis as they are more time consuming and expensive than the batch culture models. Additionally this model does not allow the study of multiple samples at the same time, which was the objective of this PhD.

A batch culture model therefore, was used in the studies conducted for this PhD. This feasible and easily reproducible model (Edwards and Parrett, 1999) allowed for the measurement of physiochemical changes, extent of fermentation and consequently SCFA and phenolic acids produced. Like most models, the batch model has limitations such as accumulation of fermentation products resulting in more drastic pH reduction which in turn can inhibit the microbiota population (Edwards and Rowland, 1992). Despite being suitable for the survival of anaerobic microbiota (Edwards et al., 1996).

To replicate the model described by Edwards et al (1996), the fermentation medium was prepared by mixing 2.25g of tryptone in 450 ml of distilled water and 112.5 ul of micromineral solution (consisting of 13.2 g of $CaCl_2 \cdot 2H_2O$, 10.0 g of $MnCl_2 \cdot 4H_2O$, 1.0 g $CoCl_2 \cdot 6H_2O$, $FeCl_3 \cdot 6H_2O$, and distilled water up to 100 ml). This solution was agitated to dissolve the chemicals followed by the addition of 225 ml of buffer solution (2 g of $NH_3 \cdot CO_3$, 17.5 g of $Na_2 \cdot 2CO_3$, and 500 ml of distilled water), 225 ml of macromineral solution (2.85 g of $Na_2HPO_4 \cdot H_2O$, 3.1 g of $KH_2PO_4 \cdot H_2O$, 0.3 g of $MgSO_4 \cdot 7H_2O$, and 500 ml of distilled water), and 1 ml of 0.1% (w/v) resazurin solution (a redox indicator). This medium was adjusted to pH 7 using 6M HCl, after which it was sterilised by being boiled for 5 minutes. This also allowed the removal of oxygen from the medium. Reducing solution prepared on the day of fermentation (312.5 mg of cysteine hydrochloride, 2 ml of 1 M NaOH, 312.5 mg of sodium sulfide, and 47.5 ml distilled water) was added at 0.5 ml per 10 ml of medium after the solution was cooled to 37°C under oxygen-free nitrogen (OFN) until anaerobic conditions were achieved as indicated by a

colour change from pale indigo to colourless resulting from the reduction of resazurin (Jaganath et al., 2009)

A 32% faecal slurry was prepared for each subject in phosphate buffer (pH=7), homogenised in a household blender for 2 minutes and strained through a nylon stocking. 5ml of the slurry was added to 44 ml of the pre-reduced fermentation medium in 100-ml autoclaved fermentation bottles. Each batch culture consisted of 50 ml of the above slurry mixture to which 1g of fibre and/or polyphenol was added (respective to the study). The small scale (50ml) and 32% slurry were used to provide higher amount of bacteria at the start of each culture and a better chance of survival (Jaganath et al., 2009).

The fermentation bottles were then purged with OFN for 2 minutes/bottle and sealed air-tight using gas tight aluminium crimp tops with septum. A purging tube was designed, making it possible to purge multiple bottles with OFN simultaneously. They were then placed in a shaking water bath at 37°C and 60 strokes/min. Incubate took place over 24 hours. Aliquots of the fermented faecal samples (6 ml) were collected after measuring gas production and pH at 0, 2, 4, 6 and 24 hours and stored immediately at −80°C.

This method is relevant to chapters 3 and 4.

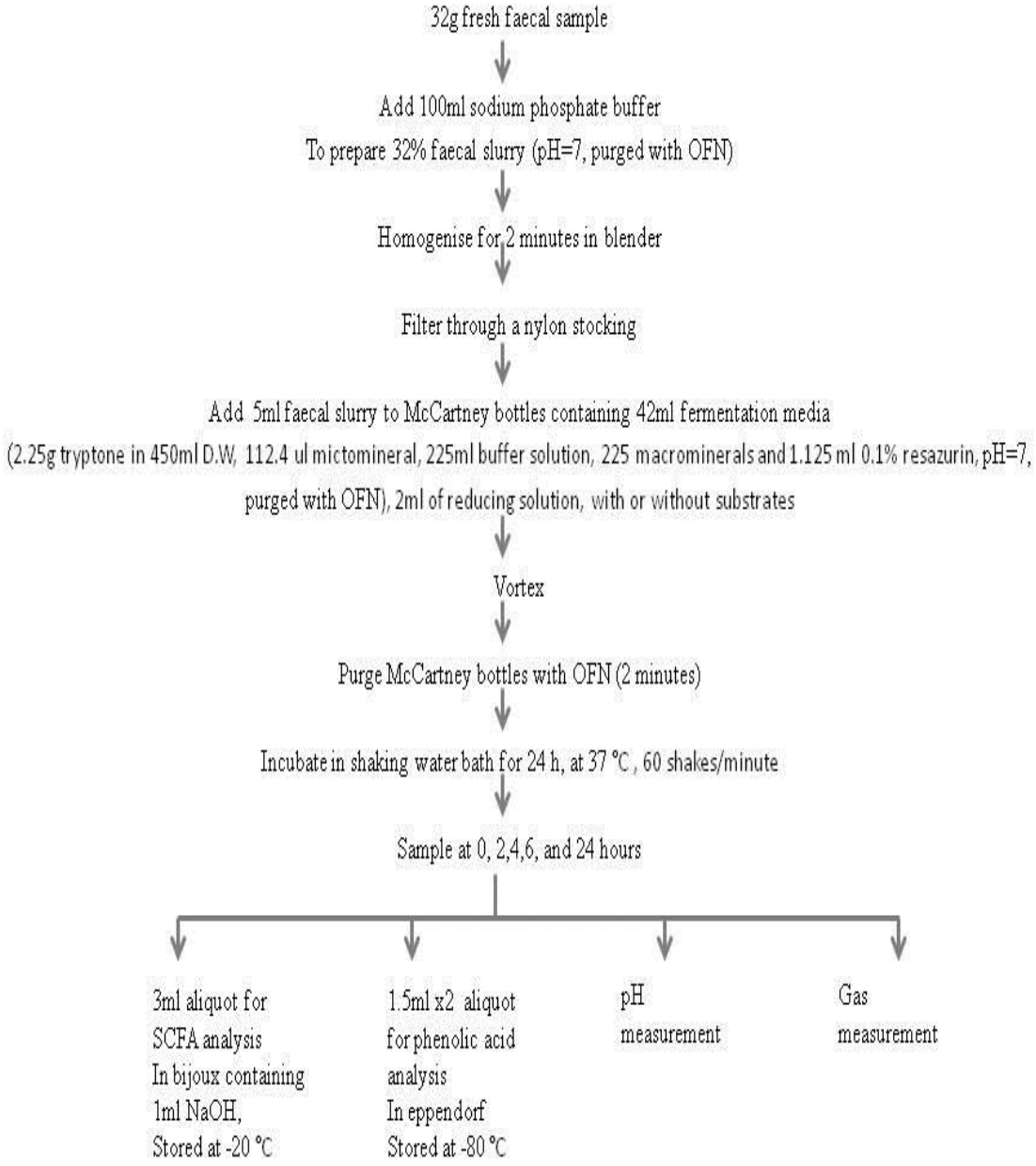


Figure 2-1 Ex-vivo fermentation method

2.2.1 Gas production

One of the inevitable by-products of colonic fermentation is the production of gases such as carbon dioxide (CO_2), hydrogen (H_2) and Methane (CH_4). Hence, production of gas can be an indication of rate and extent of fermentation. These gases are produced in different volumes depending on the CHO subjected to fermentation and the bacteria present in the faeces. Rapidly fermented gases can result in an initial high volume of gas, whereas slowly fermented fibres demonstrate a more steady production of gas over time (Cummings et al., 2001). Some individuals are more likely to produce hydrogen, CH_4 or hydrogen sulfide (H_2S).

The gas produced through fermentation may be of importance when translating the fermentation of fibres in vitro to predicting in vivo gas. Rapidly fermented fibres might cause discomfort to the consumer due to high volumes of gas produced in a short period of time.

Gas was measured using 3 way valves ensuring the fermentation media was not exposed to oxygen. A needle was inserted into the 3-way valve and into the septa sealing the bottle tight. A 50 ml graduated syringe was then inserted into one opening of the valve while the other openings were tightly closed.

After securing the syringe the valve was opened, allowing the gas produced in the bottle to escape into the syringe, pushing the plunger of the syringe out as a result of accumulated pressure (Kaur et al., 2011).

The amount of gas in the syringe was noted, the valve was tightly shut, the syringe was removed and the gas was emptied into a flask containing water. If the gas produced was over 50 ml, once the syringe was full the valve was shut, the syringe was emptied and the procedure was repeated to measure the remaining gas in the bottle.

Figure 2-2 measurement of gas produced in fermentation bottles

2.2.2 Measurement of pH

The accumulation of SCFA and phenolic acids produced through colonic fermentation can result in changes in pH levels. In the batch culture model, it is possible for the pH to be reduced to levels that can inhibit microbial activity (Clausen et al., 1991, Rumney and Rowland, 1992). In a batch culture model pH may also be used as an indication of rate and extent of fermentation.

In our studies aliquots for SCFA measurement were placed in pre-weighed bijoux bottles containing 1ml NaOH. As sodium hydroxide changes the pH of the faecal slurry these samples were not used for measuring pH. The quantity of sample suitable for pH measurement was relatively small thus pH was measured with a micro combination pH electrode (Shelfscientific- Lazer Research Laboratories, Inc - USA) suitable for measuring pH in small volumes.

A comparison was also made for pH indicator paper ranging from a scale of 1-14 (Fisherbrand 33041*)* vs. the micro combination pH electrode. This resulted in similar values for both measurement methods.

2.3 SCFA analysis

Measurement of the production of SCFA in the ex-vivo studies was conducted according to the method by Laurentin & Edwards (2004), which is well established in the Department of Human Nutrition at the University of Glasgow.

Aliquots for SCFA measurement were placed in pre-weighed bijoux bottles containing 1ml NaOH (ratio 2:1) immediately after sampling and storage at -20°C. This was done due to the volatile nature of SCFA (Martin et al., 2010). The strong alkaline environment reduced their volatility as well as inhibiting further metabolic activity by the microbiota.

SCFA were estimated by gas liquid chromatography using a TRACE 2000 gas chromatogram equipped with a flame ionisation detector (250 °C) and an oven programmed at an initial temperature of 80 °C going up to 210 °C; using a Zebron ZB-Wax capillary column (15 m x 0.53 mm id x 1 μm film thickness). Nitrogen (flow rate: 30ml/min) was used as the carrier gas.

0.1 ml of internal standard (2-ethylbutyrate 73.8 mmol/L) and 0.1 ml orthophosphoric acid were added to 0.8 ml of faecal slurry aliquots (homogenised by vortex for 15 seconds before sampling).

Extraction was done three consecutive times with 3ml of diethyl ether and vortexed for 1 minute each time. The diethyl ether layer was recovered and pooled into a clean tube. 1ul of the pooled extract was injected onto the column using an autosampler. Extractions were done in duplicate for each sample.

The chromatograph and peak integrals were analysed by chrom-card 32-bit version 2.2 *(Thermofinnigan, Milan, Italy)* using an averaged (n=5) response factor for each external standard *(Table 2-2)* for calibration. All standards were of analytical grade and purchased from Sigma-Aldrich Ltd *(Dorset, UK),* except for acetic acid (glacial), which was purchased from Fisher Scientific *(Loughborough, UK).* Calibrators were run in 6 different concentrations (10ul, 25 ul, 50 ul, 100 ul, 200 ul, 300 ul) at the start and end of each run with concentration 100 being repeated every 24 samples as a measure of quality control. This Method is relevant to chapter 3 and 4.

Table 2-1 Preparation of external standard for SCFA analysis

SCFA	Acid name	Conc. (mM)	g / 200ml of NaOH
C2	Acetic	183.2	2.2014
C3	Propionic	133.2	1.9735
iC4	Iso-butyric	104.2	1.8362
C4	Butyric	86.3	1.7628
iC5	Iso-pantanoic	74	1.7192
C5	Pantanoic	64.7	1.6847
iC6	Iso-hexanoic	57.5	1.6584
C6	Hexanoic	102	1.7974
C7	Heptanoic	86	1.7566
C8	Octanoic	50	1.1616
Internal std	2-ethylbutyrate	73.8	1.71452

Figure 2-3 SCFA external standard run using standard 300 (mg/ml)

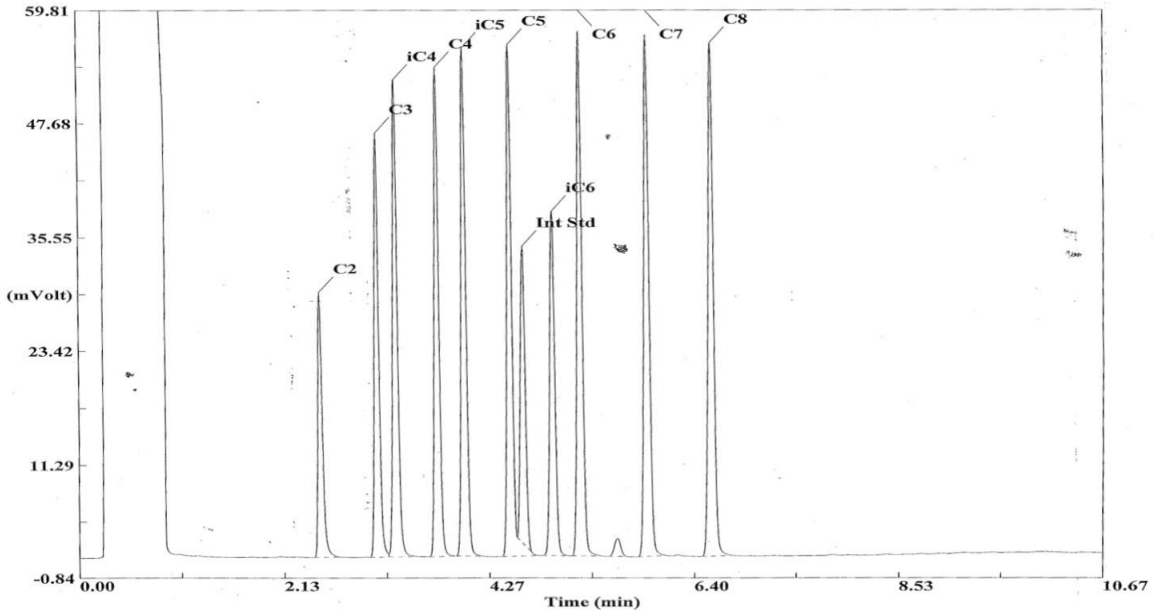


Table 2-2 Reproducibility and repeatability of SCFA measurement

Acid name	Response factor* reproducibility	CV** Reproducibility	Response factor* repeatability	CV** repeatability
Acetic	0.16 ± 0.01	4.86	0.15 ± 0.0001	0.077
Propionic	0.36 ± 0.02	4.94	0.35 ± 0.0004	0.13
Iso-butyric	0.55 ± 0.03	5.05	0.54 ± 0.0003	0.07
Butyric	0.54 ± 0.03	4.97	0.53 ± 0.0004	0.07
Iso-pantanoic	0.72 ± 0.03	4.81	0.71 ± 0.0005	0.06
Pantanoic	0.71 ± 0.03	4.88	0.71 ± 0.0003	0.04
Iso-hexanic	0.88 ± 0.05	5.11	0.88 ± 0.001	0.11
Hexanoic	0.90 ± 0.04	4.82	0.89 ± 0.0005	0.06
Heptanoic	1.06 ± 0.05	5.17	1.06 ± 0.0008	0.07
Octanoic	1.22 ± 0.06	5.31	1.23 ± 0.003	0.28

Values are mean ± standard deviation (n=15 for reproducibility, n=5 repeatability)
*The response factor (Rf) for each SCFA was calculated asRf=(AUCES/AUCIS)/(ConcES/ConcIS)
Where AUC= area under the curve, Conc=concentration, ES=external standard, IS=Internal standard.
** Coefficient of variance = (standard deviation/mean)*10

2.4 Phenolic acid analysis

Phenolic acids are not completely stable and are easily prone to oxidation. Hence, aliquots for phenolic acid analysis from both ex-vivo and in-vivo studies were stored at -80°C in 2ml eppendorf tubes to inhibit further metabolic activity of the microbiota and oxidation of phenolic acids.

The protocol used for the analysis of phenolic acids in this thesis was adapted from Combet et al (2011). 1ml of faecal slurry, 30ul of internal standard (2,4,5-Trimethoxycinnamic acid) was added and samples were acidified by adding 60ul of 1M HCL. They were vortexed and placed at 4°C for 10 minutes. They were partitioned twice against 1.5ml ethyl acetate. Each time, after addition of 1.5ml ethyl acetate, samples were centrifuged for 10 minutes at 1426 × g at 4°C. Upper organic phases were combined and transferred into an amber glass and dried at 37°C under a gentle flow of nitrogen. 200ul of dichloromethane was added to rinse the wall of the vials and dried at 37°C under a gentle flow of nitrogen. 50ul N,O-Bistrifluoroacetamide (BSTFA) was added as a derivatisation reagent, vials were flushed with a gentle flow of nitrogen, sealed and placed on a hot block at 80 °C for 4 hours, during which they were vortexed every 30 minutes in order to achieve complete silanisation.

Hexene (450 ul) was added to the derivatised samples. Samples were analysed by a Trace GC interfaced to a DSQ mass spectrometer equipped with a split/splitless injector and an AI3000 autosampler (Thermo Fisher, Hemel Hampsted, UK). Samples (1ul) were injected in a split mode with a 25:1 ratio. A 220 °C inlet temperature was maintained while the oven was programmed with an initial temp of 45°C going up to 300°C (ramp: 45- 160- 200- 250- 300 °C). The helium carrier gas flow rate was at 1.2 ml/min. hexane was used as prewash solution and acetonitrile as post wash solution. Extraction was done in duplicate for each sample.

Phenolic acids present in the external standard were also analysed individually to determine retention time and identifying ions (*Table 2-3*) This library was used to identify phenolic acids present in the urine and faecal slurry samples using the software Xcalibur for windows XP. Retention time, target ion and reference standard comparison were used for identification.

Table 2-3 Phenolic acid standards

Phenolic acid name	Retention time	Target ion	Other qualifying ions	Molecular weight
Benzoic	6.91	179	105, 135	122
Phenyl acetic	7.45	164	73, 91, 164	136
Mandelic	9.97	179	71, 147, 253	152
3-Hydroxy benzoic	12.09	267	223,73.193,282	138
3-Hydroxy phenyl acetic	13.22	164	73, 147, 75	152
4-Hydroxy benzoic	13.82	267	73, 267, 193	138
4-Hydroxyphenyl acetic	14.15	179	73, 164, 281, 252	152
3-Hydroxy phenyl propionic	17.17	205	192, 177, 73, 310	166
4-Hydroxy phenyl propionic	18.44	179	192, 73, 75, 177	166
Vanillic	18.55	297	73, 267, 223, 126	166
Homo vanillic	18.79	209	73, 179, 326, 267	168
4-Hydroxy mandelic	19.08	267	73, 268, 147	182
3,4-Dihydroxy benzoic	20.87	193	73, 370, 194	168
3,4-Dihydroxy phenyl acetic	21.1	179	73, 179, 267. 384	154
Hippuric	21.88	206	105, 73, 207	168
m-coumaric	23.38	203	73, 293, 308, 249	164
4-Hydroxy 3-methoxy phenyl propionic	24.16	209	73, 192, 340, 179	196
p-coumaric	26.4	219	73, 293, 308, 249	164
3,4-Dihydroxy phenyl propionic	26.51	179	73, 267, 398	182
Gallic	27021	281	73, 458. 179	170
Ferulic	33.79	338	73, 249, 323, 308	194
Caffeic	35.16	219	73, 396, 191	180
4-Hydroxy hipuric	37	193	73, 294	195

Figure 2-42 phenolic acid external standards

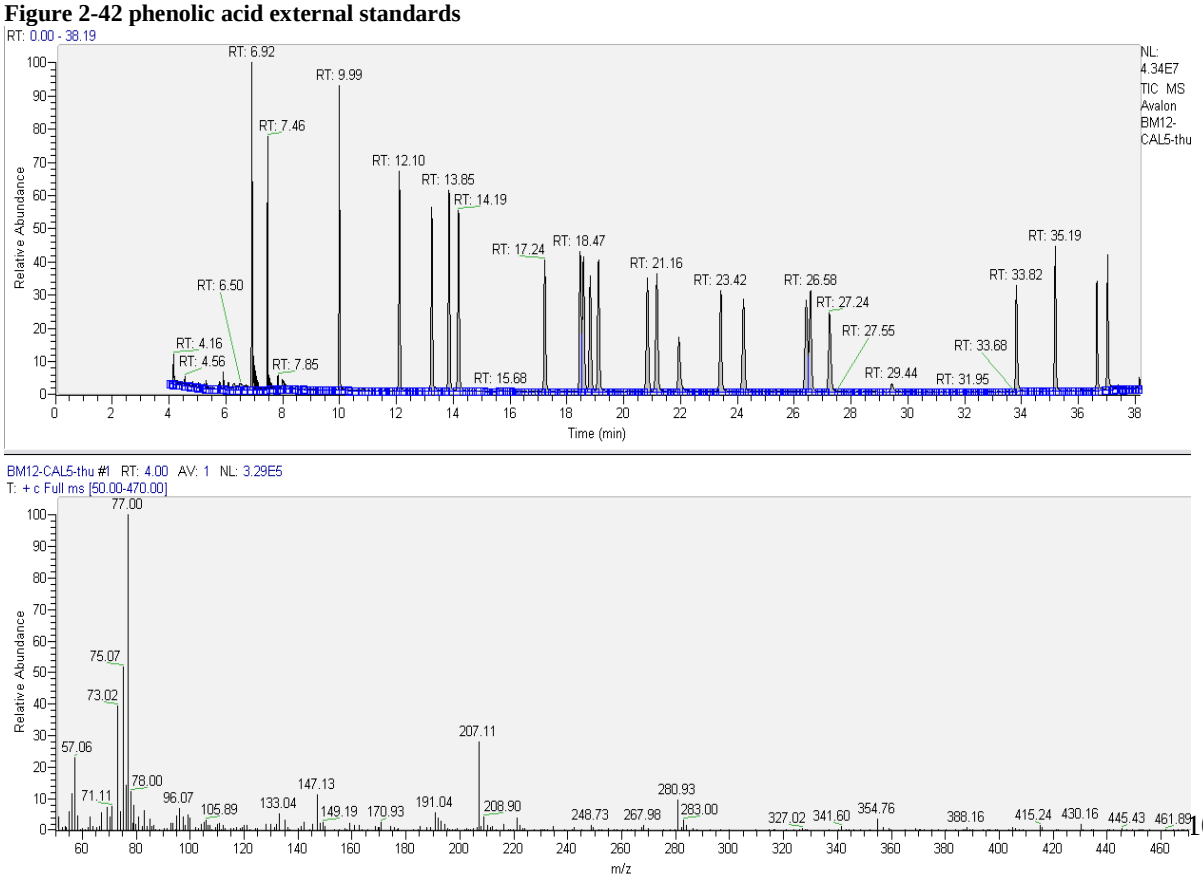
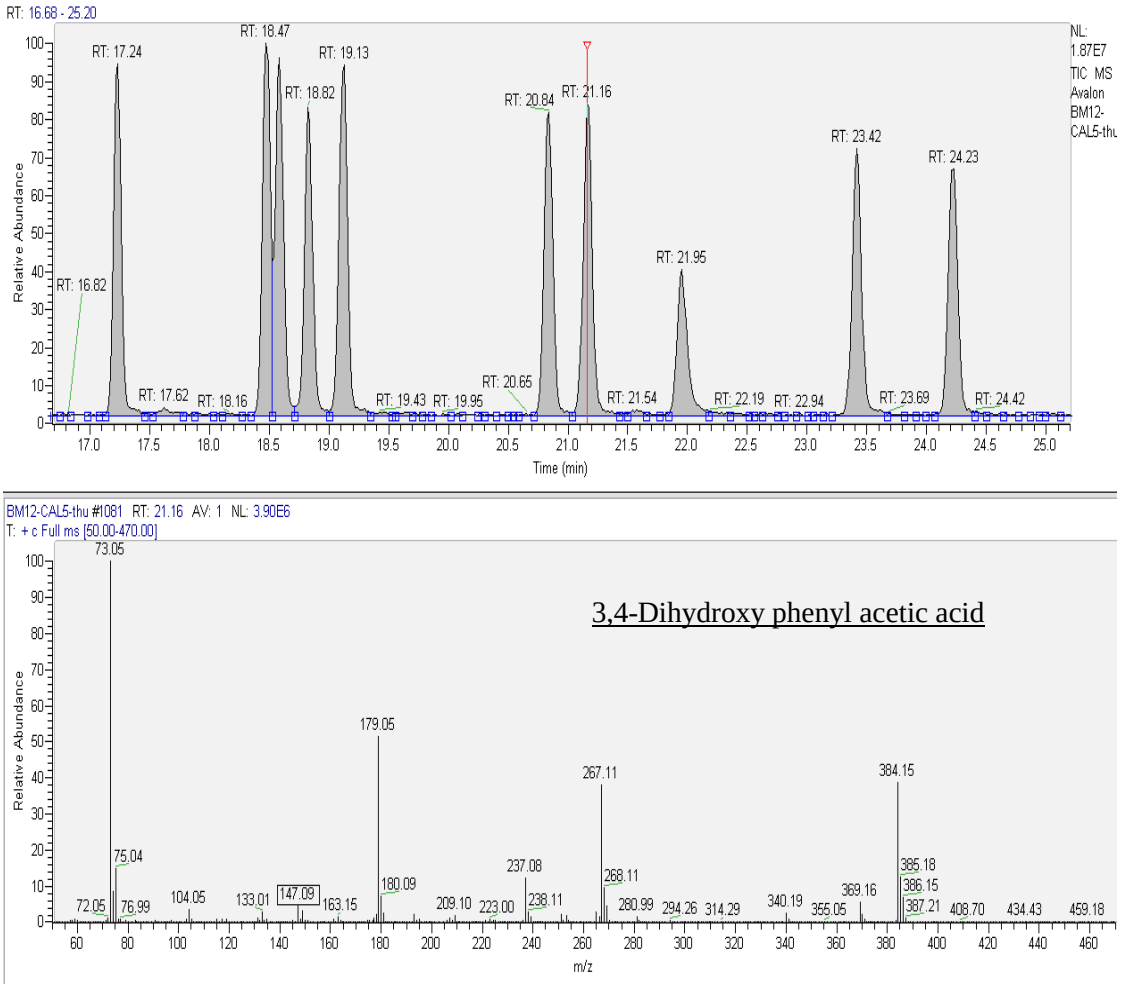


Figure 2-5 2 5 phenolic acid peak identification



For quantification the internal standard was referred back to an external standard, which was analysed in concentration of 4, 8, 15, 27, 32 ul/ml. Area ratio was calculated as **area of standard or sample/ area of internal standard**. A calibration curve was constructed for each phenolic acid using the external/internal standard ratios. The computer calculated gradient of the calibration curve was then used to quantify the concentration of phenolic acids.

This method is relevant to chapter 3, 4 and 5. The other methods used in chapter 5 are included within the chapter.

CHAPTER 3

Matrix interaction between the polyphenols rutin and quercetin and the soluble fibres raftiline, ispaghula and pectin, on their metabolism by colonic bacteria- *ex-vivo*

Flavonoid containing foods also contain dietary fibre. As both types of compounds can affect the metabolism and growth of colonic bacteria it is important to consider how they affect each other's metabolism in the colon. The study described in this chapter investigated the impact of the flavonoids rutin and quercetin on the SCFA production from the fermentation of the soluble fibres ispaghula, pectin and raftiline, as well as the impact of the individual fibres on the colonic bacterial metabolism of rutin. The use of isolated components from their food matrix such as rutin and quercetin allows better understanding of mechanisms.

3.1 Introduction

Fibres and polyphenols are two major contenders of health benefits induced by fruits and vegetables. The majority of both components escape absorption in the small intestine and reach the colon intact where they are heavily metabolised by bacteria in the colon but so far their metabolism by these colonic bacteria has been studied separately. However these are most likely consumed together as part of the daily diet. Both components also have the potential to alter the growth and population of the gut microbiota by which they are both catabolised. The fibres may increase the growth of the bacterial population while there is evidence for both antibacterial effect and a prebiotic effect for several polyphenol compounds. Thus it is important to understand the interaction of these compounds on bacterial activity and the release of bioactive molecules.

Rutin and quercetin are amongst the most abundant flavonoids found in fruits and vegetables. Studies have shown that when quercetin is ingested in its glycosidic form (rutin), 83-86% is recovered in the ileal fluid of ileostomy patients, as intact rutin (Hollman et al., 1995, Jaganath et al., 2006a). These compounds would be made available to the colonic microbiota of healthy individuals; here these compounds will be initially hydrolysed to yield quercetin, which is further degraded to produce lower molecular weight phenolic acids. Rutin is the least absorbed in the human small intestine compared to quercetin-glucosides or the aglycone quercetin. In fact, unlike the glycosidic moiety, the glucoside moiety has been found to increase the absorption of quercetin with only 24-44% of ingested quercetin-glucosides reaching the colon which is less than that observed for quercetin aglycones (~77%) (Hollman et al., 1995, Walle et al., 2000). It was hypothesised that the absorption of quercetin is facilitated by its glucose moiety which is drawn into the enterocyte by the glucose carrier; the lack of the glucose moiety on the aglycone quercetin may be a factor contributing to lower absorption (Hollman et al., 1995).

Table 3-1 Type and amount of quercetin available to colonic microbiota in humans (Ileostomy studies)

Author	Feed	Main component in feed	Concentration of component in feed	Recovery of component in ileal fluid	% of ingested dose
Hollman et al (1995)	-Fried onion (=89mg aglycone)	Quercetin glucoside	89mg	44mg	49%
	-quercetin-rutinoside (=100mg aglycone)	Rutin	100mg	83mg	83%
	-quercetin aglycone 100mg	quercetin	100mg	66mg	77%
Walle et al (2000)	-Fried onion (76-150 g)	Quercetin-monoglucosides	4.7-29.7mg	29±19mg Quercetin-aglycones	~24%
		Quercetin diglucosides	5.9-21.7mg		
Kahle et al (2005)	-Apple juice (cloudy)	Quercetin glycoside:	(10.8 ug/ml)		~3%
		-glucosides	1.8 ug/ml	-	
		-rhamnosides	2.7 ug/ml	0.27 ug/ml	
		-galactosides	1.5 ug/ml	-	
		-xyloside	3.9 ug/ml	-	
		-arabinoside	0.9 ug/ml	0.05 ug/ml	
Jaganath et al (2006)	-Tomato juice supplemented with quercetin-rutinoside	Quercetin-rutinoside	176umol (~107.45 ug/ml)	151±5umol (~92.18 ug/ml)	86%

This is an indication that bioavailability of rutin and quercetin is greatly dependent on the action of the gut microbiota and its colonic metabolites, thus any factor with the potential to modify the gut microbiota can indirectly impact the bioavailability of rutin as well as its colonic metabolites, thus affecting its impact on health (Jaganath et al., 2009, Rechner et al., 2004).

As the colon was found to be the main site of rutin degradation and absorption, the importance of its colonic metabolites were emphasised. The metabolites resulting from the colonic degradation of rutin and quercetin have been studied in urine, plasma and faecal incubations and summarised in chapter-1. However these studies do not take into consideration the presence of other food matrices with microbiota modifying potential *in-vivo*, such as carbohydrates, especially fibre. Evidence on the food matrix interaction between carbohydrates and rutin/quercetin is modest. A study that merits attention is the one conducted by Jaganath et al. (2009); investigating the bioavailability of rutin and quercetin with or without 0.5g of the simple carbohydrate glucose in an *in-vitro* fermentation model. The addition of glucose to incubation vessels increased the extent of rutin deglycosilation, subsequent phenolic acid production and the decomposition pathway. Simple carbohydrates such as glucose may

act as a direct source of energy for the colonic microbiota, promoting their growth and hence their ability to hydrolyse and degrade substrates, as is seen with vessels containing glucose having more de-glycosylation of rutin. In addition, simple carbohydrates lack the physio-chemical properties of fibre such as viscosity. Moreover simple carbohydrates such as glucose are mostly absorbed in the small intestine, whereas fibre is most often present in the colon along with the polyphenols, where it is fermented and can induce changes in the microbiota composition. This modification may be induced by SCFA through the reduction of colonic pH (*chapter-1*). Additionally SCFA may have a direct impact on specific bacterial species, as seen that the growth of Bifidobacteria was enhanced in the presence of propionate (Arora et al., 2011).

Despite the great potential for food matrix interaction between soluble fibres and the abundant polyphenolics such as rutin and quercetin and their metabolites, studies in this area are few. Most such studies are based on the impact of dietary fibre on antioxidant bioavailability and have conflicting results. An example of this is an increased absorption of quercetin metabolites in plasma after chronic ingestion of pectin as demonstrated in animal studies (Nishijima et al., 2009, Tamura et al., 2007) compared to reduced β-carotene bioavailability in the presence of pectin, guar gum or cellulose (Hoffmann et al., 1999). Studies on antioxidant interaction with dietary fibre suggest that apart from increasing luminal viscosity, dietary fibre may reduce the release of antioxidants by physically trapping them within the fibre matrix (Palafox-Carlos et al., 2011). The chemical interaction between dietary fibre and polyphenols has been suggested as the formation of ordered junctions stabilised by arrays of non-covalent bonds between hydroxide groups from phenolic compounds and the polar groups from polysaccharide molecules (Eastwood and Morris, 1992). Unabsorbed low molecular weight phenolic and polyphenol compounds that are associated with dietary fibre constitute a major part of dietary polyphenols and are not absorbed in the human small intestine (Manach et al., 2005).

The health benefits from rutin, depends not only on its concentration in the diet but also on its bioavailability and bioaccessibility from the matrix after ingestion. Considering that rutin is not absorbed in the small intestine, its bioavailability is directly related to its bioaccessibility. Hence it is of importance when discussing the health benefits of rutin, to understand the impact of dietary fibre, most often present in the matrix on the bioaccessibility of rutin. This was demonstrated in a study by Hollman et al. (1995), investigating the recovery of quercetin and its conjugates when consumed in different forms. Quercetin ingested in the form of onions had higher recovery rates (1.39 ± 0.49 % proportion of intake) as compared to quercetin ingested as apple, which contains the soluble fibre pectin (0.44 ± 0.22 % proportion of intake). The least amount of recovery was from the consumption of

rutinoside (0.35 ± 0.41% proportion of intake), which was only 25% of that seen for quercetiningested from onions and 79% of quercetin from apple.

For better understanding of the impact of soluble fibres on the bioavailability of polyphenols and consequently their metabolites, three different soluble fibres; raftiline, ispaghula and pectin were chosen as they vary in viscosity and rates of fermentation. Rutin was used as a pure source of polyphenols to avoid any other matrix impact; allowing us to attribute any differences in rutin metabolite concentrations to the impact of soluble fibres. As suggested by Palafox-Carlos et al (2011) some of the inhibitory effect of fibre on polyphenol bioavailability can be attributed to their viscosity. This study design allowed us to investigate the impact of soluble fibres with low and high viscosity and fermentation rates on rutin degradation by colonic bacteria. This allowed for the better understanding of the role of viscosity or fermentability on the polyphenol metabolite bioavailability.

As soluble fibres exhibit many of their health benefits through the production of short chain fatty acids in the colon, it is also of importance to understand the impact that polyphenols, can have on their production through the modification of the colonic microbiota.

Many polyphenols have been shown to exhibit an inhibitory effect on the gut microbiota. However, there are very few studies investigating their impact on non-pathogenic bacteria in a complex ecosystem such as that in the human colon. Due to the large family of polyphenols and various methods used, there is a lack of consistency in the understanding of the inhibitory mechanism of action of polyphenols on gut microbiota (*Chapter 1*).

Quercetin has demonstrated stronger antibacterial properties more than other polyphenolic compounds tested in the same studies (Arima et al., 2002, Parkar et al., 2008), however its glycosidic form rutin did not exhibit any antibacterial properties. The same was seen in the case of aglycone naringenin but not its glycoside naringin (Parka et al., 2008) These studies were carried out *in-vitro,* which may not translate well *in-vivo* conditions, as the glycosidic forms of these polyphenols will be hydrolysed by the bacteria, releasing the aglycones. It is possible that the glycosidic forms may be of more importance in this regard as some aglycones may be absorbed in the small intestine but majority of the glycosidic bacteria reach the colon intact where they will be deconjugated by the bacteria.

For better understanding of the antibacterial properties of these polyphenolic compounds more studies are required, especially studies using *in-vivo* or *in-vitro* fermentation models, allowing the use of more than a few bacterial species and better investigation of glycosidic compounds. Even though rutin has not shown an antibacterial impact *in-vitro* it must not be ignored as an antibacterial agent as its role *in-vivo* may be of great importance given that larger numbers of rutin than quercetin reach the colon,

where it is initially converted to quercetin by the colonic bacteria (Jaganath et al., 2009) before its further degradation to lower molecular weight phenolic acids. Moreover phenolic acids have demonstrated to have stronger antibacterial properties than their parent compounds *in*-vitro (Lee et al., 2006).

The above mentioned studies investigating the impact of rutin and quercetin on bacteria were carried out *in-vitro* on pure cultures, not taking into consideration factors such as pH and other food components in the food matrix that may act as prebiotics, thus ameliorating the antibacterial properties of these phenolic compounds. Moreover, these studies demonstrated a shift in bacterial species but no change in total bacterial counts. A shift in bacterial species may result in a difference in their fermentation metabolites, which has not been investigated so far. This was previously shown in a study using tea extracts as an antibacterial agent, demonstrating a reduction in butyrate production accompanied by an initial increase in acetate production, which was attributed to the reduction in butyrogenic bacteria. By the end of two weeks, a drastic fall in total SCFA and acetate concentration was observed (Kemperman et al., 2013). However it is important to note that 1000 mg of polyphenol extracts were fed into the Simulator of Human Intestinal Microbial Ecosystem (SHIME) model three times daily, which is relatively high considering total polyphenol intake has been estimated as 1g/day. Secondly this study model does not take into consideration the ingestion of other food components, which may inhibit the antibacterial effect seen from the high dose of polyphenols.

In this chapter we investigated the impact of rutin and quercetin as well as their colonic metabolites on SCFA production from soluble fibres which may act as enhancers of bacterial growth. Quercetin has demonstrated antimicrobial activity (Vaquero et al., 2007, Rodríguez Vaquero et al., 2010, Duda-Chodak, 2012, Parkar et al., 2008), which in turn can affect the production of these SCFA and thus their impact on health.

3.1.1 Hypothesis

To test the hypothesis that soluble fibres change phenolic acid production from rutin in the colon by fermentation related mechanisms, and that the potential antibacterial properties of quercetin, rutin and their colonic metabolites inhibit the production of SCFA from the microbial fermentation of soluble fibres.

3.2 Materials and methods

3.2.1 Study design

The study used is an *ex-vivo* model of the gut using inoculated faeces from human volunteers (n=10) on 10 separate occasions. Volunteers followed a three day low polyphenol diet; avoiding fruits, vegetables and beverages high in polyphenols such as tea, coffee and cocoa milk. On the 4th day faeces was collected from volunteers according to the method described in *2.1 Faecal sample collection.*

To investigate the impact of fermentable fibres on phenolic acid production from rutin, three different fibres with varying fermentability rates were chosen (raftiline, pectin and ispaghula), resulting in 11 different combinations (detailed in 4.2.3).

3.2.2 Participants

For this *ex-vivo* batch fermentation, fresh faecal sample was collected from healthy volunteers (n=10) with a mean (± SD) BMI of 22.5 ± 3.0 (range, 18.4 to 27.4) and a mean (± SD) of 23 ± 3.8 years (range, 19 to 33). Volunteers were non-smokers who had not received antibiotics, constipation remedies or probiotics for at least 3 months prior to start of the study.

Table 3-2 volunteer characteristics

	Gender	Age (years)	Height (cm)	Weight (kg)	BMI (Kg/m^2)	Waist circumference (cm)	Blood pressure (mmHg)
1	M	22	175.5	84	19.7	96	125/60
2	F	19	161	47.9	27.4	77	109/71
3	F	21	158	63.9	18.4	73	129/67
4	F	21	156	54	25.6	80	119/71
5	F	26	174	57.2	22.2	78	107/59
6	F	22	160	50.5	18.9	73.5	112/80
7	F	25	161	54.3	20.9	83.5	112/80
8	M	23	183	77.8	23.2	91	143/83
9	M	33	175	76	24.8	93	165/63
10	M	23	176	72.2	23.3	93	114/72
Mean ± STDEV		23 ± 3.8	152.5 ± 53.7	63.8 ± 12.8	22. ± 2.9	83.8 ± 8.7	123.5 / 70.6 ± 18.2 / 8.5

Values are means (± SD), M: male, F: female, BMI: body mass index

3.2.3 Ex-vivo fermentation

The ex-vivo fermentation was carried out with faeces from each of ten volunteers on separate occasions, according to the method described in *2.2 Ex-vivo Modelling of anaerobic colonic fermentation.* The incubation bottles contained faecal slurry alone with or without one of 11 possible combinations of fibre and/or polyphenols. Incubation was allowed for 24 hours and samples for phenolic acid analysis were collected at 0, 2, 4, 6 and 24 hours; and stored at -80 °C immediately.

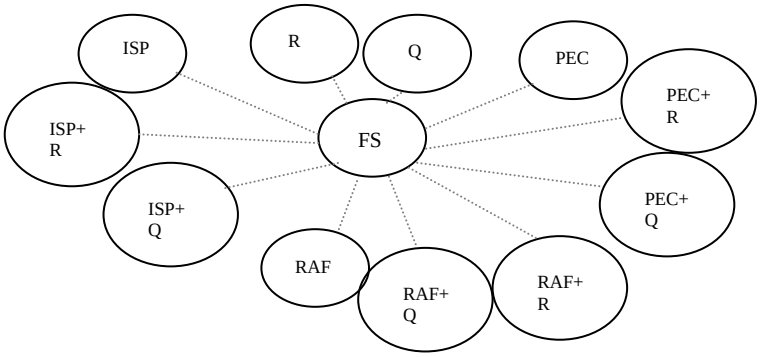


Figure 3-1 Substrate combinations for faecal fermentation.
FS: Faecal slurry, ISP: Ispaghula, PEC: Pectin, RAF: Raftiline, R: Rutin, Q: Quercetin

3.2.4 Materials

Rutin and quercetin were purchased from sigma-Aldrich *(Poole, UK),* final concentration: 28 umol/L in distilled water and incubated along with the soluble fibres, Ispaghula husk (*Whole ispaghula husk – myprotein.com)* pectin (*pectin apple- 250 grade, BDH laboratory supplies)* and raftiline (*Siber Hegner Ltd).* The concentration of rutin used in the study was based on the recovery of 28 umol/L rutin in ileal fluid of patients consuming tomato juice supplemented with 176 umol/L of rutin (Jaganath et al., 2006b). The amount of fibre used is based on 6g of fibre as moderate fibre consumption (Khossousi et al., 2008), present in 300ml of colonic content, resulting in 1g of fibre present in 50ml incubation bottles.

3.2.5 Phenolic acid analysis

Phenolic acid analysis was carried out for samples taken from the fermentation vessel at 0, 2, 6 and 24 hour time points using 23 phenolic acids (*Table 2-3*) as standard references, according to the method described in *2.4Phenolic acid analysis.*

Data for all identified phenolic acids were quantified and analysed separately and a sum of all identified phenolic acids was considered as the total sum of phenolic acid production.

3.2.6 SCFA analysis

SCFA were analysed in samples from the fermentation vessels at 0, 2, 4, 6 and 24 hours according to the method described in *2.3 SCFA analysis*. Analysis of the results focused mainly on acetic, propionic and butyric acid and the total sum production as the sum of acetate, propionate, butyrate, iso-butyric, iso-pentanoic, pentanoic, iso-hexanoic, hexanoic, heptanoic, octanoic.

3.2.7 pH measurement

pH was measured for all samples at 0, 2, 4, 6 and 24 hours according to the method described in 2.2.2 Measurement of pH.

3.2.8 Gas volume

Gas was measured for all samples at 0, 2, 4, 6 and 24 hours post incubation according to the method described in *2.2.1 Gas production*

3.2.9 Statistical analysis

To determine the impact of the soluble fibres raftiline, ispaghula and pectin on phenolic acid production from rutin, and the impact of rutin and quercetin on SCFA production from the fibres, a general linear model Anova was performed using the software Minitab-15. This statistical model allowed for the analysis of paired data with repeated measures. In this analysis phenolic acid concentration was used as the response, with volunteer, time, substrate and time*substrate included in the model. Volunteer was included in the random factors.

Further analysis was conducted for the data at 24 hours separately, using a paired t-test model or 1-way Wilcoxon test after assessing for normality. This analysis was conducted as the general linear model considers change over time and ignores differences at specific time points. Soluble fibres differ in their rate of fermentation, resulting in some slowly fermented fibres such as ispaghula to demonstrate an impact only at 24 hours. Hence, it was important to investigate the 24 hour time point separately.

3.3 Results

The soluble fibres used in this study are plant extracts. Hence, some phenolic acid production from these soluble fibre sources was expected. The main outcome investigated in this chapter was the difference in phenolic acid production from rutin incubated with soluble fibre (represented as fibre+R, where R represents rutin) to rutin incubated alone.

The phenolic acids identified in the rutin incubations are listed in *Table 3-3*. Phenolic acid production from soluble fibres is discussed in the relevant sections.

Table 3-3 Phenolic acids identified from Rutin incubation with human faecal bacteria

Phenolic acid	Abbreviation	Conc. at 24 hours (ug/ml)	Number of volunteers producing
4-Hydroxy benzoic acid	4-HBA	0.04 ± 0.03	2
Phenyl acetic acid	PAA	5.60 ± 3.35	10
3-Hydroxy phenyl acetic acid	3-HPAA	1.26 ± 1.60	9
4-hydroxy phenyl acetic acid	4-HPAA	0.79 ± 0.65	10
3,4- Dihydroxy phenyl acetic acid	3,4-DHPAA	3.11 ± 4.2	10
3-Hydroxy phenyl propionic acid	3-HPPA	0.43 ± 0.28	10
4-Hydroxy phenyl propionic acids	4-HPPA	0.39 ± 0.6	10
Total sum of phenolic acids	TP	11.98 ± 6.0	10
Concentration of rutin added	-	17.08	10

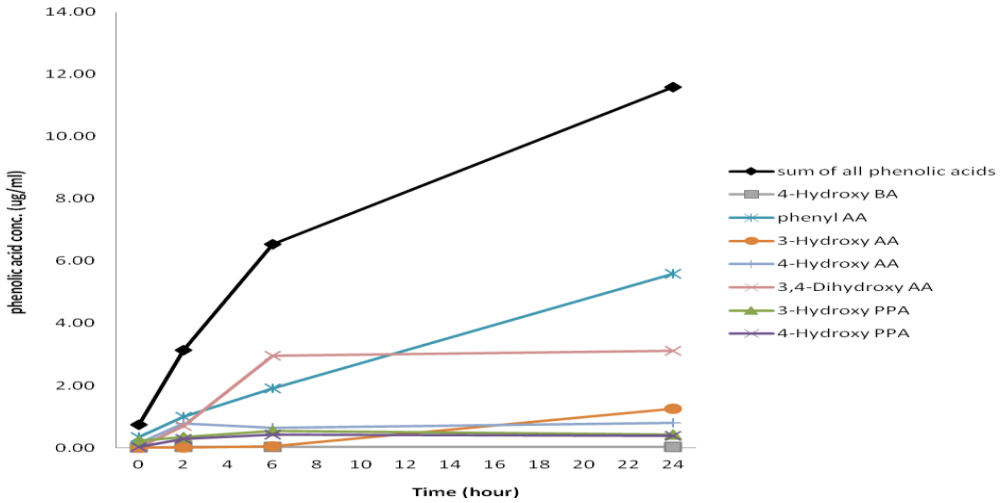


Figure 3-2 Phenolic acids identified in rutin only incubations with human faecal bacteria in comparison to total sum of phenolic acids produced.

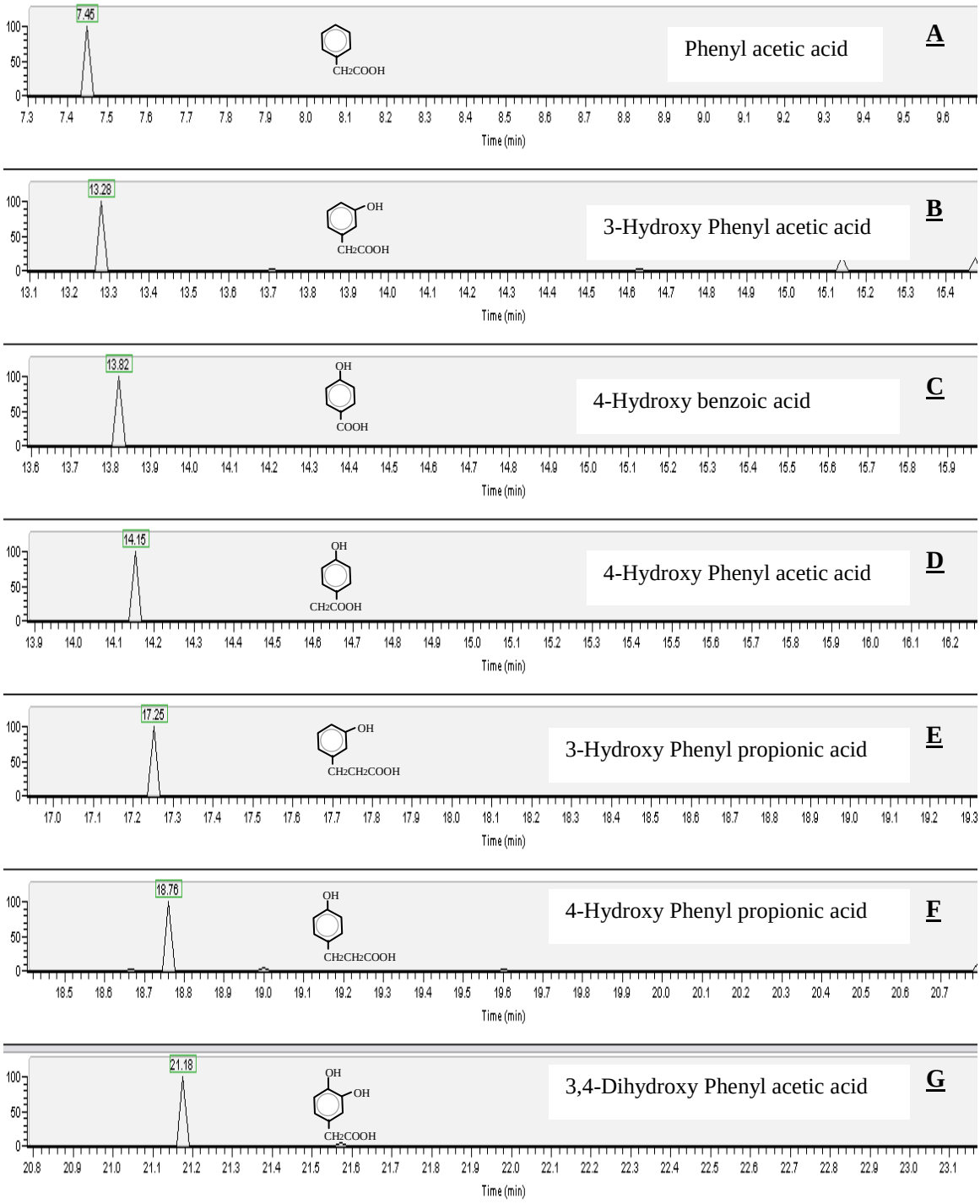


Figure 3-3 Phenolic acids identified in rutin incubations with human faecal bacteria

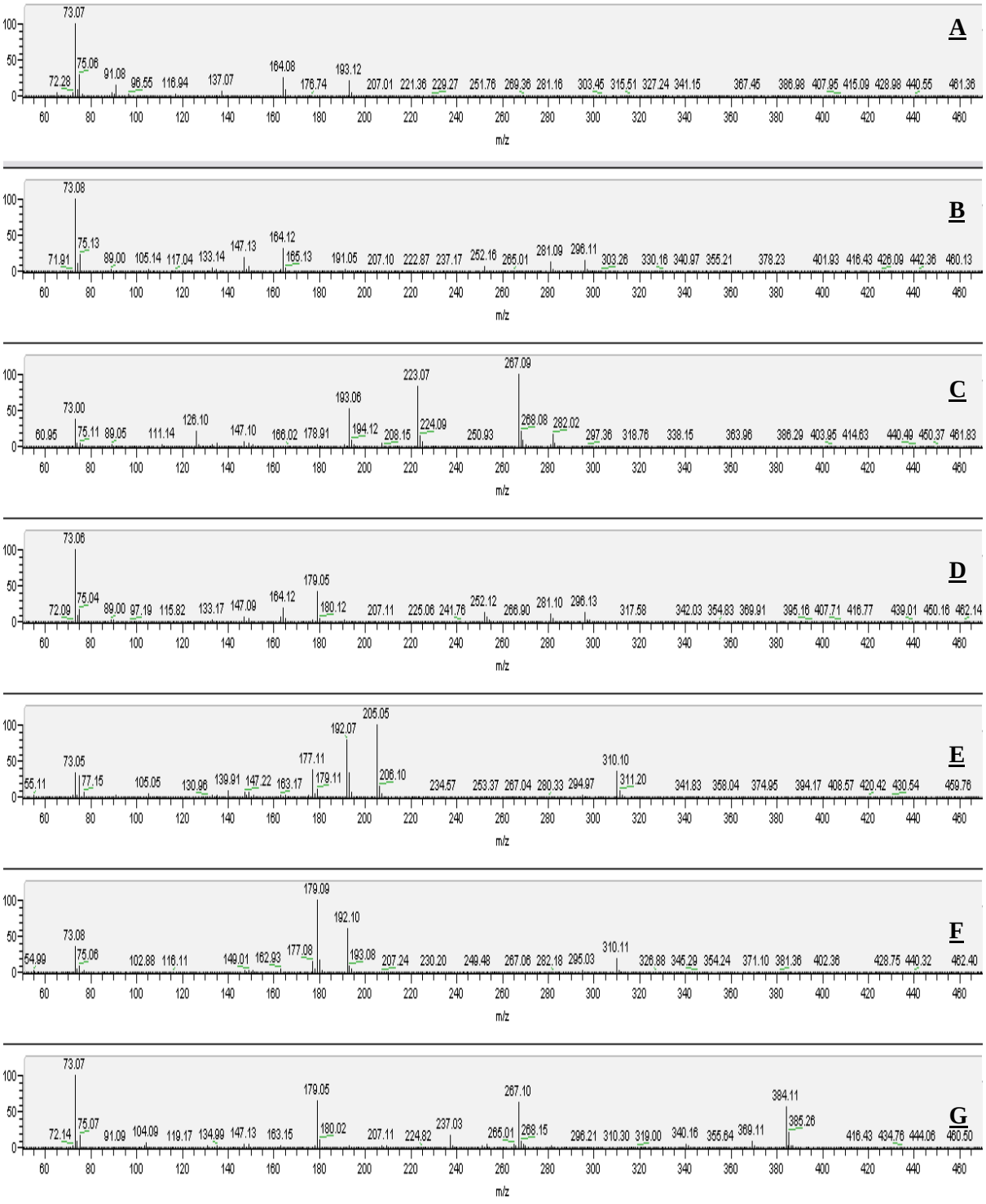


Figure 3-4 Identified phenolic acids in rutin incubations

3.3.1 Impact of raftiline on phenolic acid production from rutin

Raftiline fermentation resulted in the production of 3-HPAA in 1 volunteer and 4-HPAA in 8 out of 10 volunteers. Raftiline had an inhibitory effect on the production of both of these phenolic acids from rutin fermentation ($p < 0.01$ (98.4%), *Figure 3-5* and $p < 0.01$ (76%), *Figure 3-6* respectively).

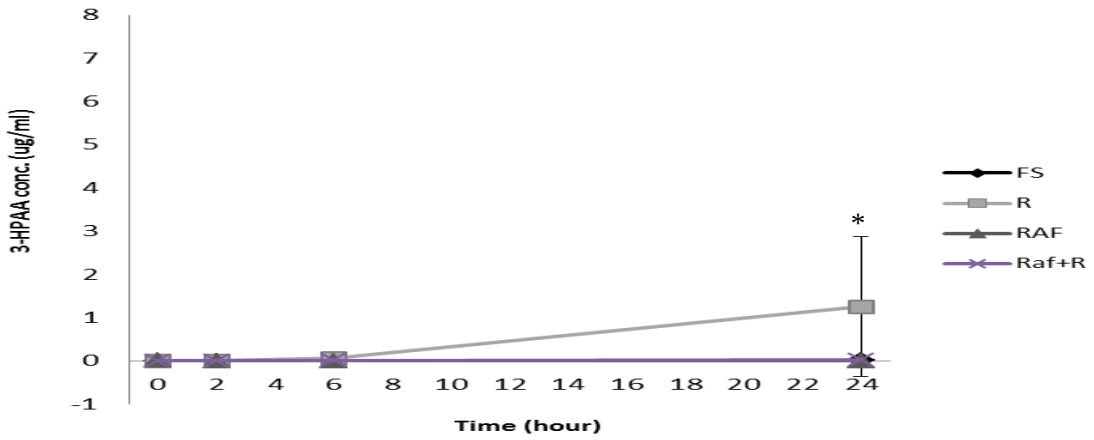


Figure 3-5 Impact of raftiline on 3-HPAA production from rutin incubation with human faecal bacteria
Values are displayed as mean (± SD) of 24 hour profile in faecal incubations having 28 umol/L rutin with 1g of RAF (n=10), FS: Faecal slurry, R: Rutin, RAF: Raftiline, Raf+R: Raftiline+Rutin *P < 0.01

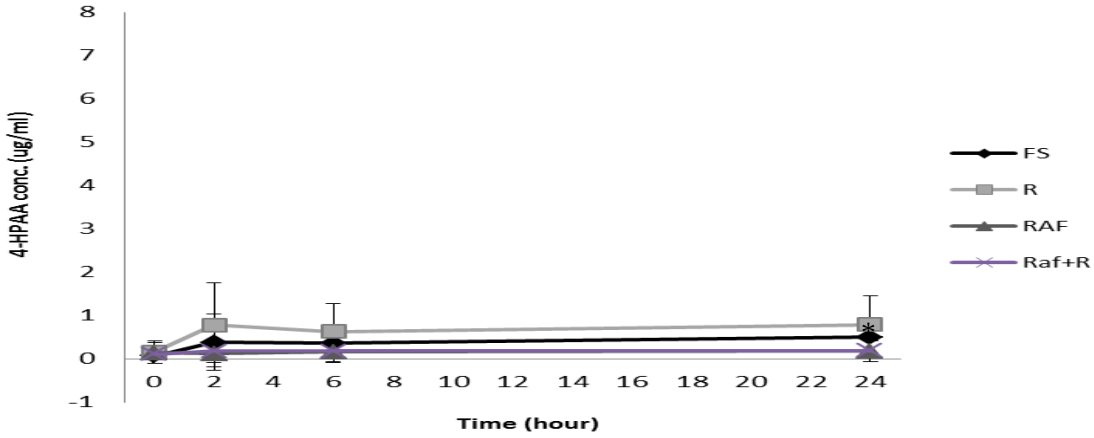


Figure 3-6 **Impact of raftiline on 4-HPAA production from rutin incubation with human faecal bacteria**
Values are displayed as mean **(± SD)** of 24 hour profile in 50ml faecal incubations having 28 umol/L rutin with 1g of RAF (n=10), FS: Faecal slurry, R: Rutin, RAF: Raftiline, Raf+R: Raftiline+Rutin, *P < 0.01

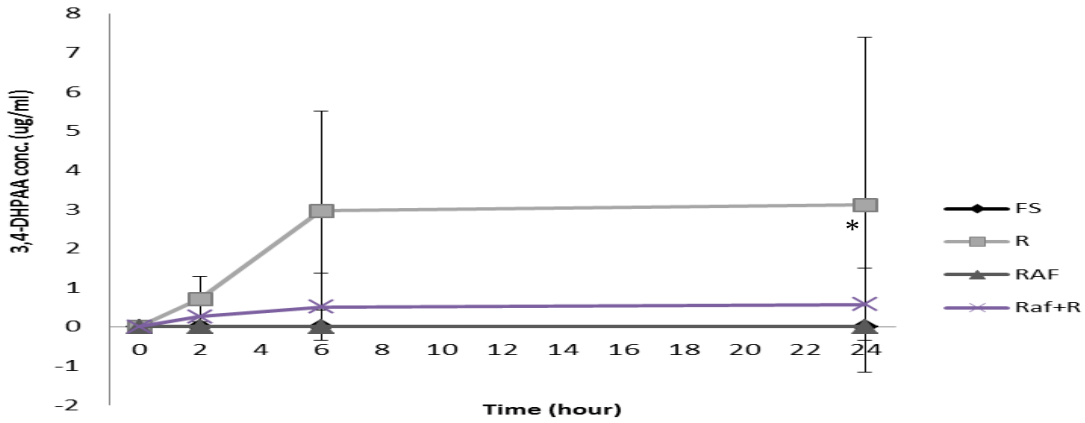


Figure 3-7 Impact of raftiline on 3,4-DHPAA production from rutin incubation with human faecal bacteria
Values are displayed as mean (± SD) of 24 hour profile in 50ml faecal incubations having 28 umol/L rutin with 1g of RAF (n=10), FS: Faecal slurry, R: Rutin, RAF: Raftiline, Raf+R: Raftiline+Rutin, *P < 0.01

A similar inhibitory impact (p < 0.01 (81.6%), *Figure 3-7)* was seen on the production of 3,4-DHPAA, which was not produced in any volunteers when raftiline was incubated separately.

The inhibitory effect of raftiline on 4-HPAA and 3,4-DHPAA was seen as early as 2 hours post fermentation. Unlike the other 2 phenolic acids, 3-HPAA was produced only after 6 hours of fermentation, suggesting a later release from the intermediate 3,4-DHPAA.

The total phenolic acid production from rutin was inhibited by 85.5% in the presence of raftiline (p <0.01, *Figure 3-8*), seen as early as 2 hours post fermentation. Raftiline also showed an inhibitory effect on the production of phenolic acids from polyphenolics present in the background diet of the volunteers, resulting in higher values for phenolic acids in the control than that of raftiline and rutin combined, or raftiline alone. The three-day dietary records of volunteers demonstrated compliance to the low polyphenol diet, hence the possible explanation for the phenolic acid production from faecal slurry alone incubation can be either due to inaccurate record of diets or the presence of insoluble phenolics bound to cereal cell-walls.

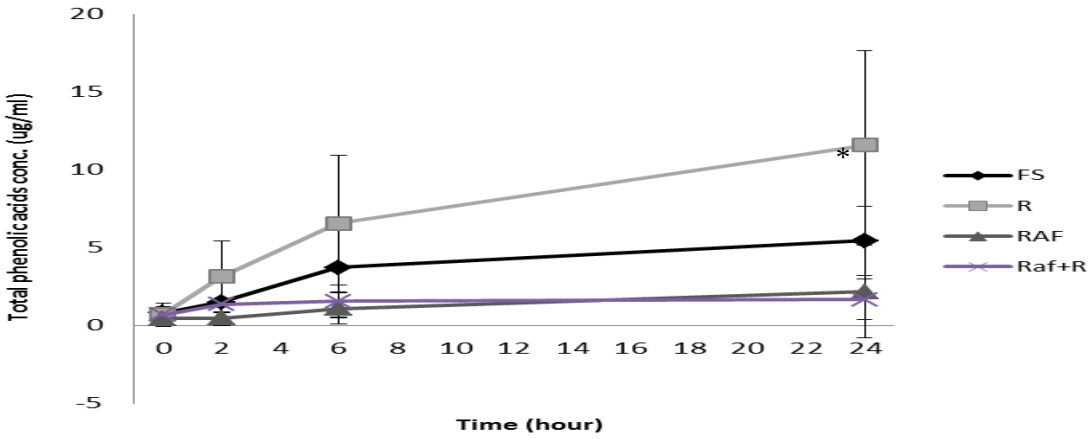


Figure 3-8 Impact of raftiline on total phenolic acid production from rutin incubation with human faecal bacteria
Values are displayed as mean (± SD) of 24 hour profile in 50ml faecal incubations having 28 umol/L rutin with 1g of RAF (n=10), FS: Faecal slurry, R: Rutin, RAF: Raftiline, Raf+R: Raftiline+Rutin, *p < 0.01

Raftiline inhibited the production of PAA, 3-HPAA, 4-H PAA, 3,4-DHPAA and total phenolic acids. This inhibitory effect was not seen for 3-HPPA and 4-HPPA. These two phenolic acids increased rapidly up to 6 hours post fermentation and plateaued thereafter. The analysis at 6 hours for both phenolic acids did not result in a statistical difference between raftiline and rutin to rutin alone. 4-HBA was produced in only 2 volunteers from rutin fermentation and was not affected by the presence of raftiline.

Table 3-4 inhibitory effect of raftiline on phenolic acid production from rutin incubation with human faecal bacteria

Substrate	Raf+R Conc. at 24h (ug/ml)	R Conc. at 24h (ug/ml)	P value
P AA	0.49 ± 1.0	5.60 ± 3.35	P < 0.01(92.5%)
3-HPPA	0.35 ± 0.15	0.43 ± 0.28	P > 0.05
4-H PPA	0.15 ± 0.12	0.42 ± 0.59	P > 0.05
4-HBA	0.05 ± 0.0	0.04 ± 0.02	P > 0.05

Results are shown as mean values (± SD) at 24 hours in 50ml faecal incubations having 28 umol/L rutin with 1g of raf (n=10), R: Rutin, RAF: Raftiline

3.3.2 Impact of ispaghula on phenolic acid production from rutin

Ispaghula was slowly and incompletely fermented; hence it exhibited any impact through fermentation at 24 hours post fermentation. The results were compared across time and at 24 hours separately.

When ispaghula was fermented alone, it resulted in the production of 3-HPAA in 1 volunteer, 4-HPAA in 9 volunteers and 3,4-DHPAA in none of the volunteers. This is similar to results seen from raftiline only fermentation.

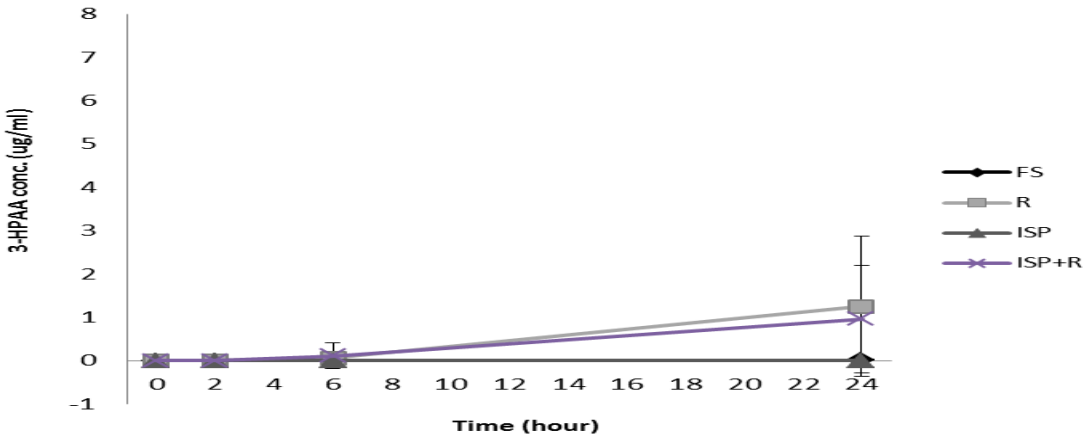


Figure 3-9 Impact of ispaghula on 3-HPAA production from rutin incubation with human faecal bacteria
Values are displayed as mean (± SD) of 24 hour profile in 50ml faecal incubations having 28 umol/L rutin with 1g of ISP (n=10), FS: Faecal slurry, R: Rutin, ISP: Ispaghula, ISP+R: Ispaghula+Rutin.

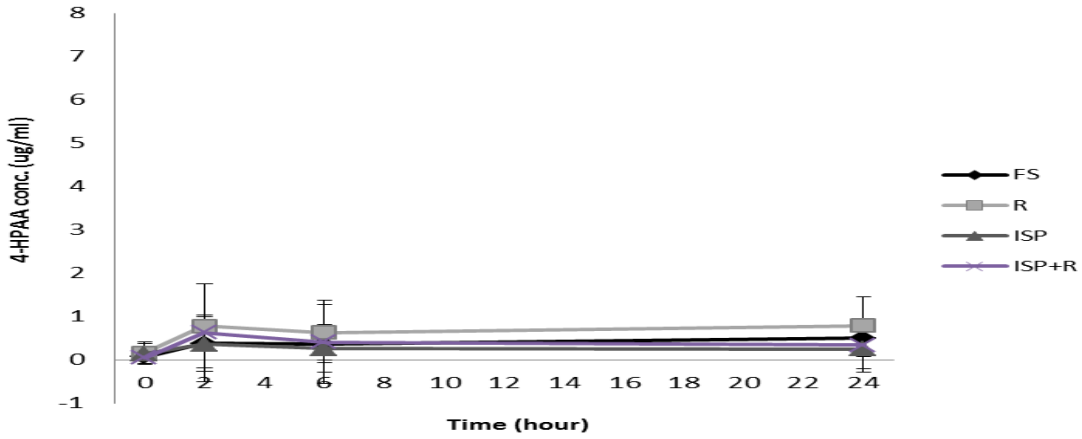


Figure 3-10 Impact of ispaghula on 4-HPAA production from rutin incubation with human faecal bacteria
Values are displayed as mean (± SD) of 24 hour profile in 50ml faecal incubations having 28 umol/L rutin with 1g of ISP (n=10), FS: Faecal slurry, R: Rutin, ISP: Ispaghula, ISP+R: Ispaghula+Rutin

Ispaghula did not inhibit the production of 3-HPAA or 4-HPAA over the time period of the experiment. Further analysis at 24 hours did not indicate any inhibitory effect of ispaghula.

Similar to these two phenolic acids, 3,4-DHPAA was not inhibited in the presence of ispaghula when all time points were considered together, however a further analysis showed an inhibitory effect of 27.6% by ispaghula at 24 hours post fermentation (p= 0.04, *Figure 3-11).*

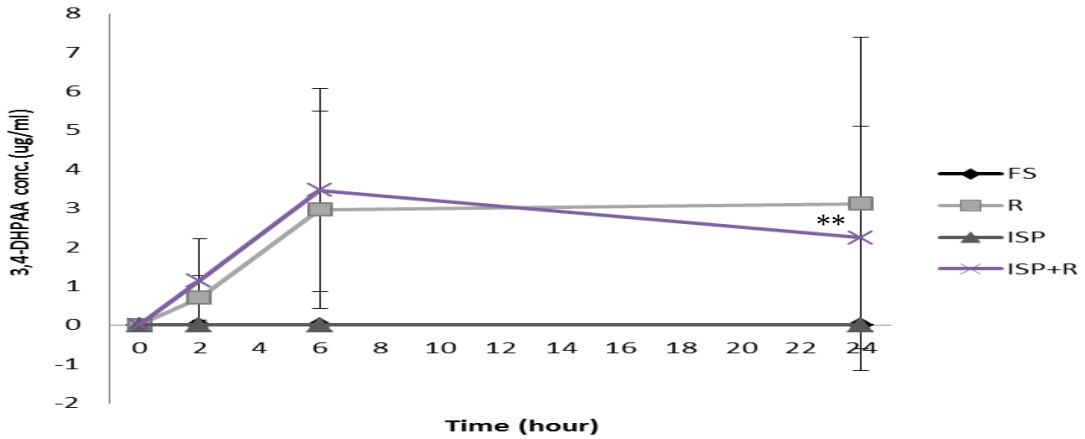


Figure 3-11 Impact of ispaghula on 3,4-DHPAA production from rutin incubation with human faecal bacteria
Values are displayed as mean (± SD) of 24 hour profile in 50ml faecal incubations having 28 umol/L rutin with 1g of ISP (n=10). FS: Faecal slurry, R: Rutin, ISP: Ispaghula, ISP+R: Ispaghula+Rutin
** at 24 h only p=0.04

As seen in *Figure 3-9* and *Figure 3-10*, 3-HPAA is produced only after 6 hours of fermentation, 4-HPAA is produced up to 2 hours post fermentation and plateaus thereafter, whereas 3,4-DHPAA is rapidly produced only up to 6 hours post fermentation. As seen for 3,4-DHPAA, ispaghula inhibited the production of total phenolic acids only at 24 hours post fermentation (p= 0.03 (42.3%), *Figure 3-12*) and not across the time period.

The total phenolic acid production for rutin with or without ispaghula is the same up to 6 hours post fermentation, after which ispaghula exhibited an inhibitory impact.

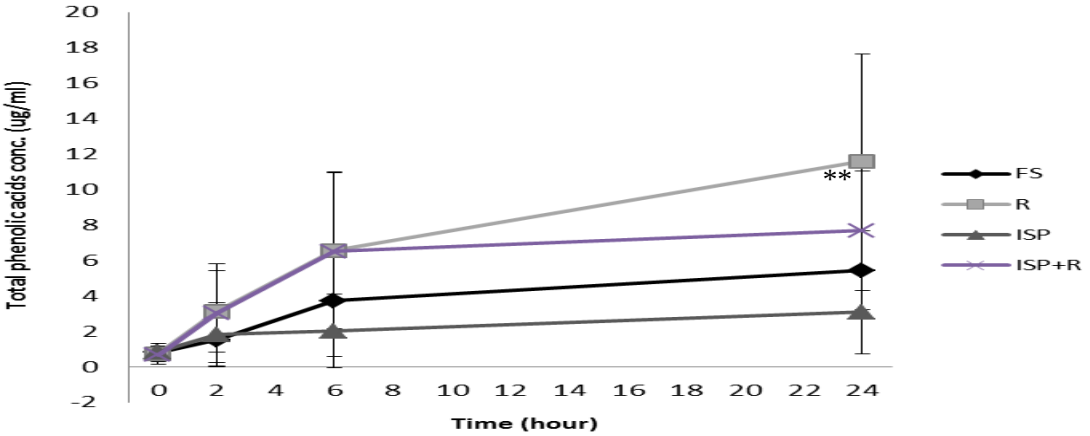


Figure 3-12 Impact of ispaghula on total phenolic acid production from rutin incubation with human faecal bacteria
Values are displayed as mean (± SD) of 24 hour profile in 50ml faecal incubations having 28 umol/L rutin with 1g of ISP (n=10), FS: Faecal slurry, R: Rutin, ISP: Ispaghula, ISP+R: Ispaghula+Rutin, ** at 24 h only p=0.03.

Ispaghula exhibited an inhibitory effect on PAA production across time (p= 0.0145), the same was not seen for 3-HPPA or 4-HPPA across time or at 24 hours post fermentation. In fact, an initial analysis demonstrated higher 3-HPPA production from rutin in the presence of ispaghula. This was due to the additive effect of high concentrations of 3-HPPA produced from both ispaghula and rutin. To determine the inhibitory impact of ispaghula on 3-HPPA production, a predicted value was calculated as the sum of 3-HPPA produced from ispaghula only fermentation and rutin only fermentation, this was then compared to the concentration obtained from when ispaghula and rutin were incubated together. The results did not show any difference between the two, hence we attributed the increased amount to the additive effect of 3-HPPA produced from both ispaghula and rutin.

Table 3-5 inhibitory effect of ispaghula on phenolic acid production from rutin

	Isp+R	R	P value
Substrate	Conc. at 24h (ug/ml)	Conc. at 24h (ug/ml)	
PAA	2.94 ± 2.6	5.60 ± 3.35	P < 0.01 (47.2%)
3-HPPA	0.69 ± 0.78	0.43 ± 0.28	P > 0.05
4-HPPA	0.48 ± 0.69	0.42 ± 0.59	P > 0.05
4-HBA	0	0.04 ± 0.02	P < 0.01

Results are shown at 24 hours in 50ml faecal incubations having 28 umol/L rutin with 1g of ISP (n=10), R: Rutin, ISP+R: Ispaghula+Rutin

3.3.3 Impact of pectin on phenolic acid production from rutin

Pectin fermentation resulted in the production of 3-HPAA in one volunteer, 4-HPAA in 8 volunteers and 3,4-DHPAA in none of the volunteers. Pectin had an inhibitory effect on the production of both of these phenolic acids from rutin fermentation (p < 0.01 (89.6%), *Figure 3-13 and (74.6%), Figure 3-14*). A similar inhibitory impact (p < 0.01 (66.8%), *Figure 3-15*) was seen on the production of 3,4-DHPAA.

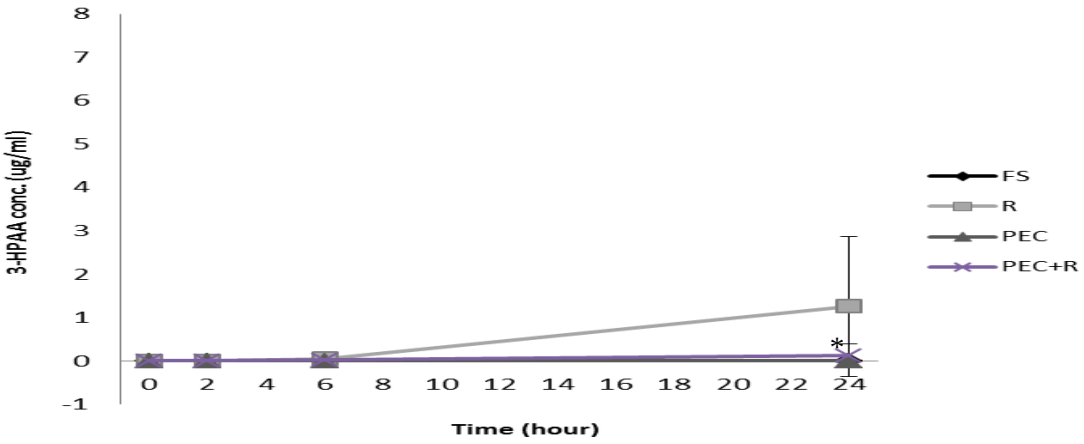


Figure 3-13 Impact of pectin on 3-HPAA production from rutin incubation with human faecal bacteria
Values are displayed as mean (± SD) of 24 hour profile in 50ml faecal incubations having 28 umol/L rutin with 1g of PEC (n=10). FS: Faecal slurry, R: Rutin, PEC: Pectin, PEC+R: Pectin+Rutin, *p < 0.01

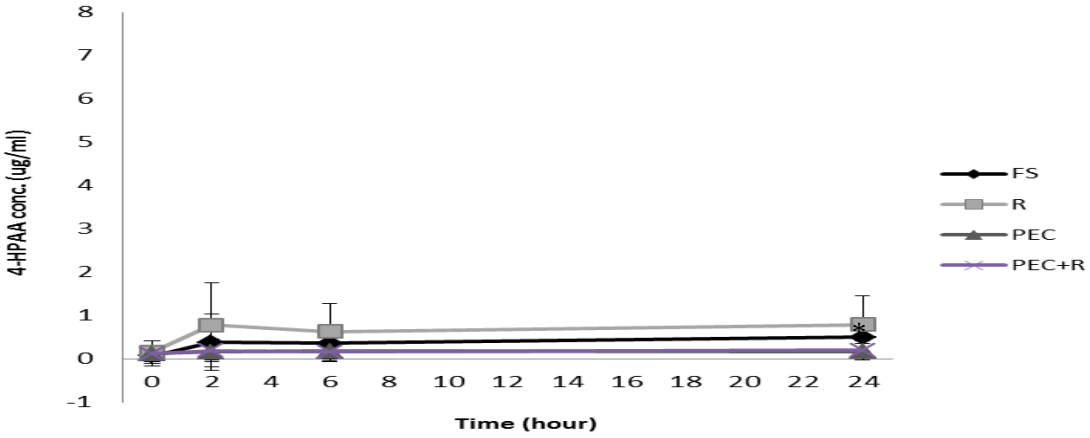


Figure 3-14 Impact of pectin on 4-HPAA production from rutin incubation with human faecal bacteria
Values are displayed as mean (± SD) of 24 hour profile in 50ml faecal incubations having 28 umol/L rutin with 1g of PEC (n=10), FS: Faecal slurry, R: Rutin, PEC: Pectin, PEC+R: Pectin+Rutin, *p < 0.01

The inhibitory effect of pectin on 4-HPAA and 3,4-DHPAA was seen as early as 2 hours post fermentation. The total sum of phenolic acid production from rutin was greatly inhibited by 78.1% in the presence of pectin ($p < 0.01$, *Figure 3-16*). Pectin also showed an inhibitory effect on the production of phenolic acids from polyphenolics present in the background diet of the volunteers, resulting in higher values for phenolic acids in the control as compared to that of pectin and rutin combined, or pectin alone .

Pectin inhibited the production of PAA, 3-HPAA, 4-HPAA, 3,4- DHPAA and total phenolic acids. This inhibitory effect was not seen for 3-HPPA and 4-HPPA.

The production of 4-HBA was not detected in the combination (pectin+rutin) fermentation.

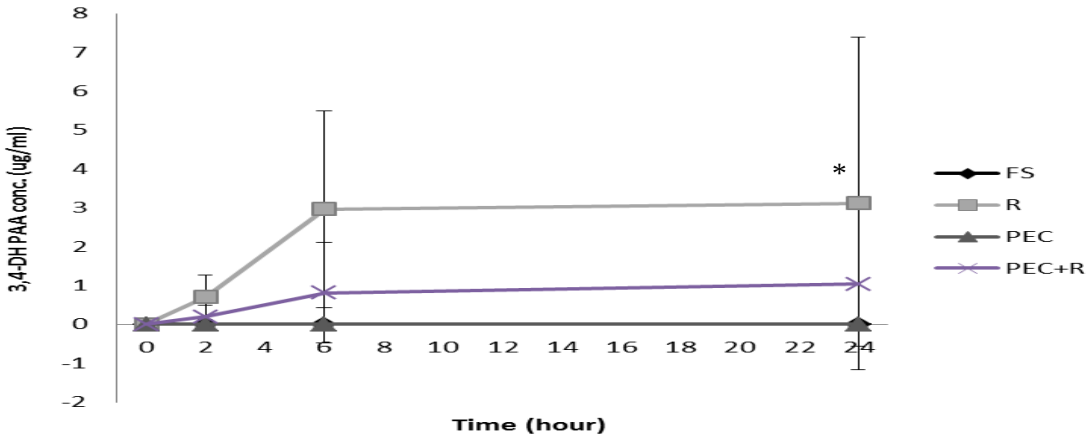


Figure 3-15 Impact of pectin on 3,4-DHPAA production from rutin incubation with human faecal bacteria
Values are displayed as mean (± SD) of 24 hour profile in 50ml faecal incubations having 28 umol/L rutin with 1g of PEC (n=10), FS: Faecal slurry, R: Rutin, PEC: Pectin, PEC+R: Pectin+Rutin, *p < 0.01

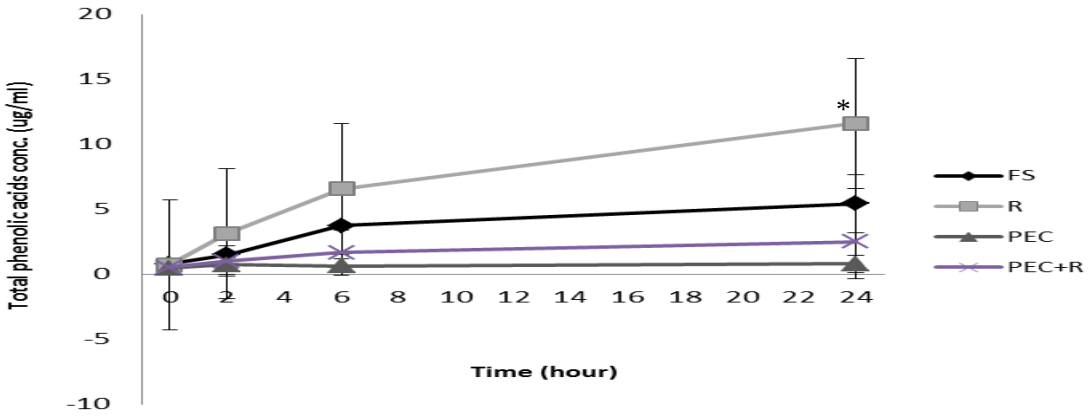


Figure 3-16 Impact of pectin on total phenolic acid production from rutin incubation with human faecal bacteria
Values are displayed as mean (± SD) of 24 hour profile in 50ml faecal incubations having 28 umol/L rutin with 1g of PEC (n=10) FS: Faecal slurry, R: Rutin, PEC: Pectin, PEC+R: Pectin+Rutin, *p < 0.01

Table 3-6 inhibitory effect of pectin on phenolic acid production from rutin incubation with human faecal bacteria

Substrate	Pec+R Conc. at 24h (ug/ml)	R Conc. at 24h (ug/ml)	P value
P AA	0.29 ± 0.54	5.60 ± 3.35	P < 0.01 (95%)
3-HPPA	0.40 ± 0.18	0.43 ± 0.28	P > 0.05
4-HPPA	0.10 ± 0.10	0.42 ± 0.59	P < 0.01
4-HBA	0	0.04 ± 0.02	P < 0.01(n=1)

Results are mean (± SD) shown at 24 hours in 50ml faecal incubations having 28 umol/L rutin with 1g of PEC (n=10), , R: Rutin, PEC+R: Pectin+Rutin

3.3.4 Concentration of rutin retrieved as phenolic acids

To estimate the efficiency in the bacterial metabolism and subsequent breakdown of rutin into phenolic acids, a percentage of total sum of phenolic acids produced from rutin catabolism was calculated (Table 3-3). The total sum of phenolic acids retrieved was estimated as 11.98 ± 6.0 ug/ml, accounting for 70% (*Figure 3-17*) of total rutin added to fermentation vessels (17.08 ug/ml).

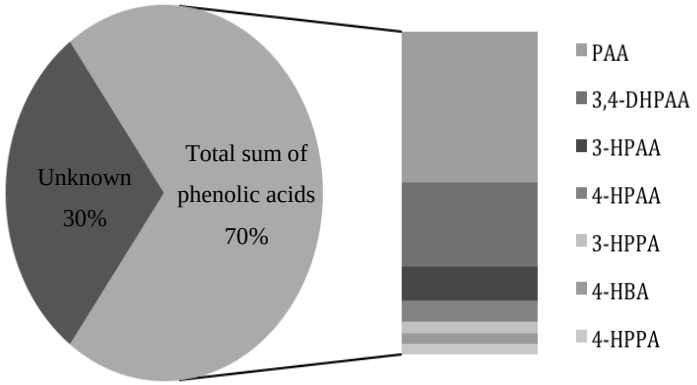


Figure 3-17 Concentration of 28 umol/ L of rutin retrieved as phenolic acid metabolites after faecal incubations with human colonic bacteria.

3.3.5 Impact of rutin and quercetin on fermentation of soluble fibres

The bacterial metabolism of rutin and quercetin was not expected to produce significant amounts of SCFA; hence the SCFA produced from the combination of fibre + rutin/quercetin was compared to that of fibre alone to determine if rutin or quercetin had any impact on SCFA production from soluble fibre fermentation. Analysis of the results focused mainly on acetic, propionic and butyric acid and the total production as the sum of acetic, propionic, butyric, valeric, caproic, enanthic, caprylic, isobutyrate, isovalerate and isocaproic acid).

Inter-individual variation in the gut microbiota of volunteers resulted in high standard deviations for SCFA production in these bacterial fermentations. Hence, a further analysis was conducted by separating high and low SCFA producers based on the average SCFA production of each fibre.

The polyphenols rutin and quercetin and their resulting metabolites from bacterial fermentation had no impact on the production of acetate, propionate, butyrate or total SCFA from raftiline fermentation.

The total sum of SCFA production demonstrated a rapid rate of increase up to 6 hours post fermentation for raftiline (64%) and pectin (65%) of total SCFA being produced between 0-6 hours; whereas majority of ispaghula fermentation took place between 6-24 hours (58%). The proportion of individual SCFA production from each fibre is displayed in table 3-7.

Table 3-7 Proportion of individual SCFA production from fibre fermentations with human faecal bacteria

Substrate	Acetate	Propionate	Butyrate
Raftiline	68.3%	9.6%	7%
Ispaghula	56.7%	27.7%	8.9%
Pectin	71%	15%	10.5%

The impact of rutin and quercetin on fermentation of fibres was further analysed by partitioning the volunteers into two categories of high and low producers of SCFA (*Table 3-8)* based on the average sum of all SCFA produced by the fermentation of fibres. The separate analysis of results for impact of rutin and quercetin on SCFA production of high producers and low producers did not demonstrate any impact of these polyphenols on the SCFA production from the fibres (*Table 3-8).*

Table 3-8 Total SCFA concentration for high producers of SCFA from raftiline fermentation with faecal bacteria

Substrate	Total SCFA concentration at 6 hours (mmol/l)	Total SCFA concentration at 24 hours (mmol/l)
Raftiline	49.6 ± 8.4	80.3 ± 8.2
Raftiline + Rutin	42.6 ± 11.3	76.6 ± 7
Raftiline + Quercetin	41.9 ± 17.8	80.6 ± 7.4
Ispaghula	28.5 ± 11.8	76.8 ± 7.8
Ispaghula + Rutin	29.1 ± 9.5	66.2 ± 4.5
Ispaghula + Quercetin	28.6 ± 9.7	63 ± 16.5
Pectin	39.9 ± 15.3	68.7 ± 47.6
Pectin + Rutin	38.8 ± 9.3	67.5 ± 21.8
Pectin + Quercetin	38 ± 13.6	59.8 ± 29.2

Values are mean (± SD) at 6 and 24 hours for 50ml faecal incubations having 28 umol/L rutin / quercetin with 1g of fibre (n=5).

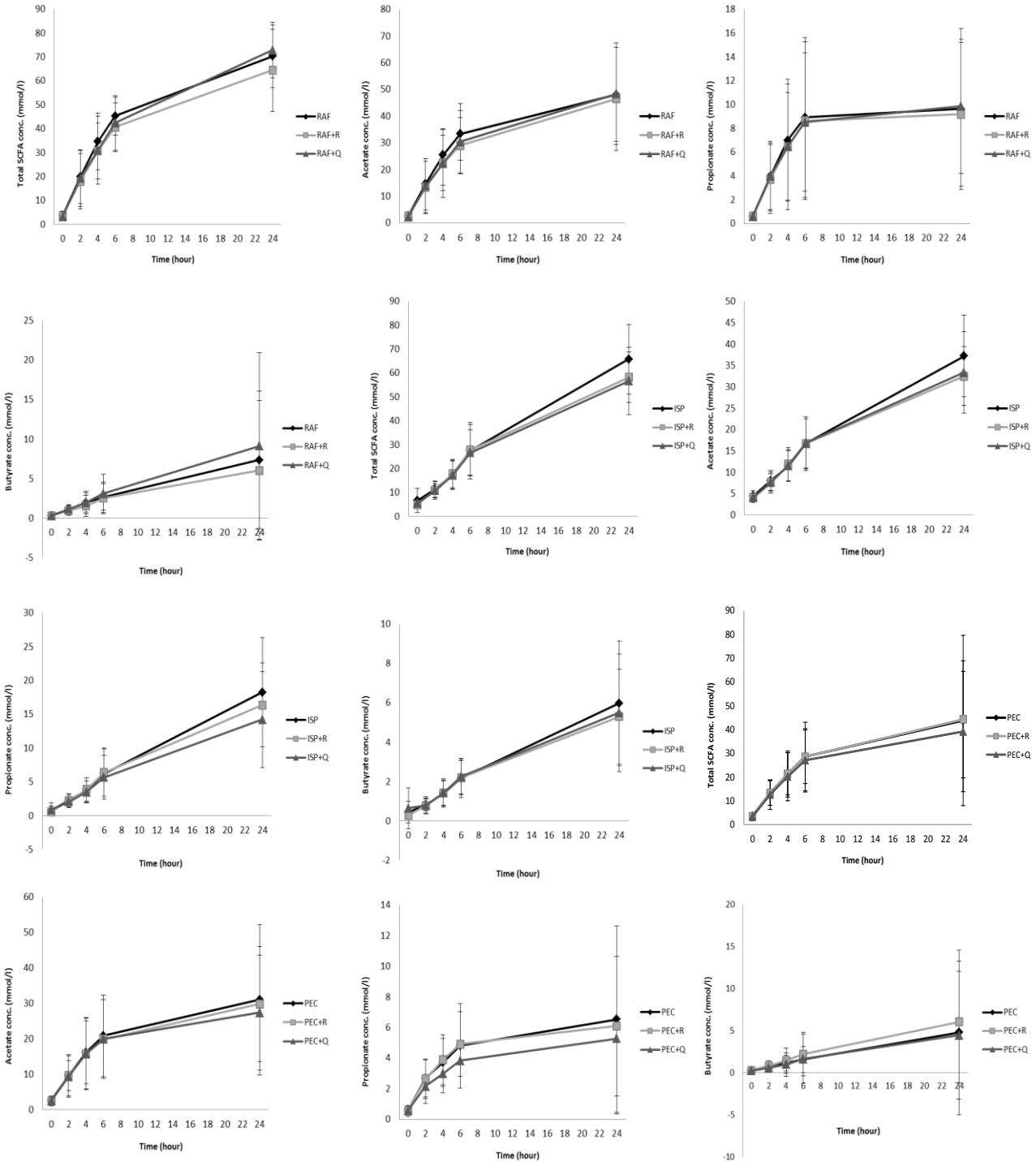


Figure 3-18 Impact of rutin and quercetin on SCFA production from fibre incubation with human faecal bacteria Values are displayed as mean (± SD) of 24 hour profile for 50ml faecal incubations having 28 umol/L rutin or quercetin with 1g of RAF / ISP /PEC (n=10), RAF: Raftiline, RAF+R: Raftiline + Rutin, RAF+Q: Raftiline+Quercetin,), ISP: Ispaghula, ISP+R: Ispaghula + Rutin, ISP+Q: Ispaghula+Quercetin, Pec: Pectin, Pec+R: Pectin + Rutin, Pec+Q: Pectin+Quercetin.

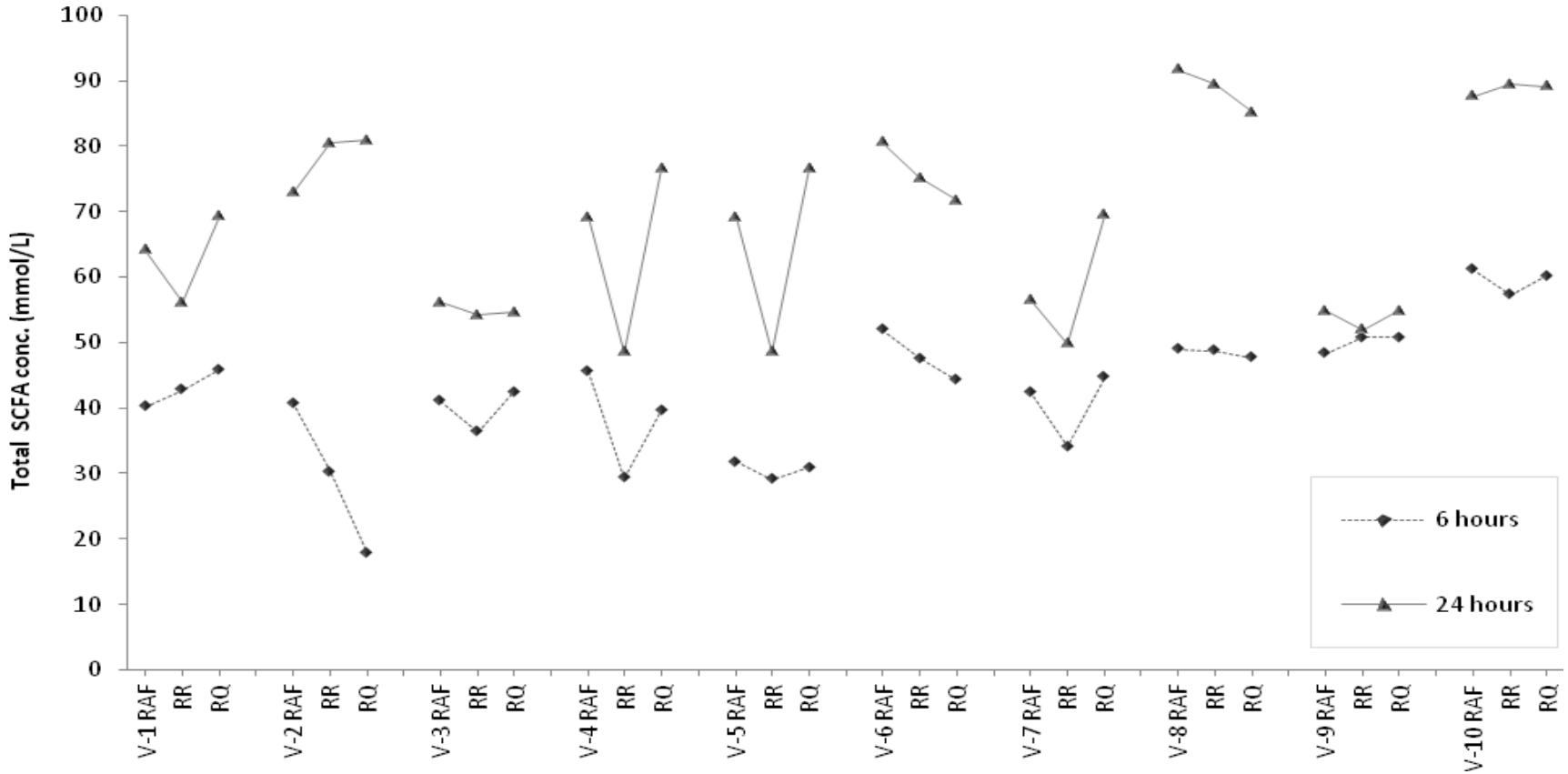


Figure 3-19 Individual volunteer plots of total SCFA production from raftiline incubation with human faecal bacteria with or without quercetin and rutin
Values are sum of total SCFA for each volunteer at 6 and 24 hours (n=10). RAF: Raftiline, RR: Raftiline+Rutin and RQ: Raftiline + Querceti

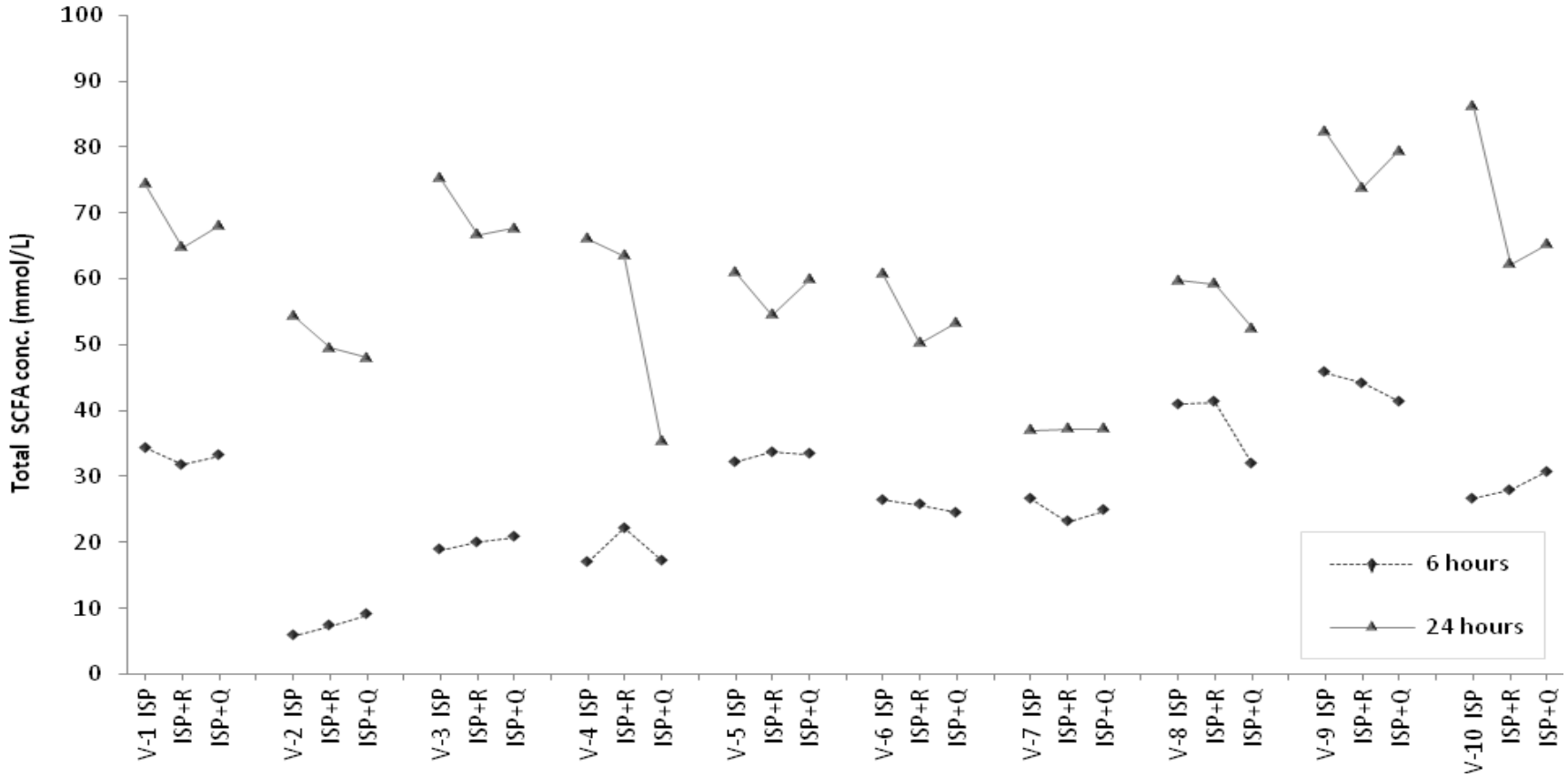


Figure 3-20 Individual volunteer plots of total SCFA production from ispaghula incubation with human faecal bacteria with and without quercetin and rutin
Values are sum of total SCFA for each volunteer at 6 and 24 hours (n=10). ISP: Ispaghula, ISP+R: Ispaghula+Rutin and ISP+Q: Ispaghula + Querceti

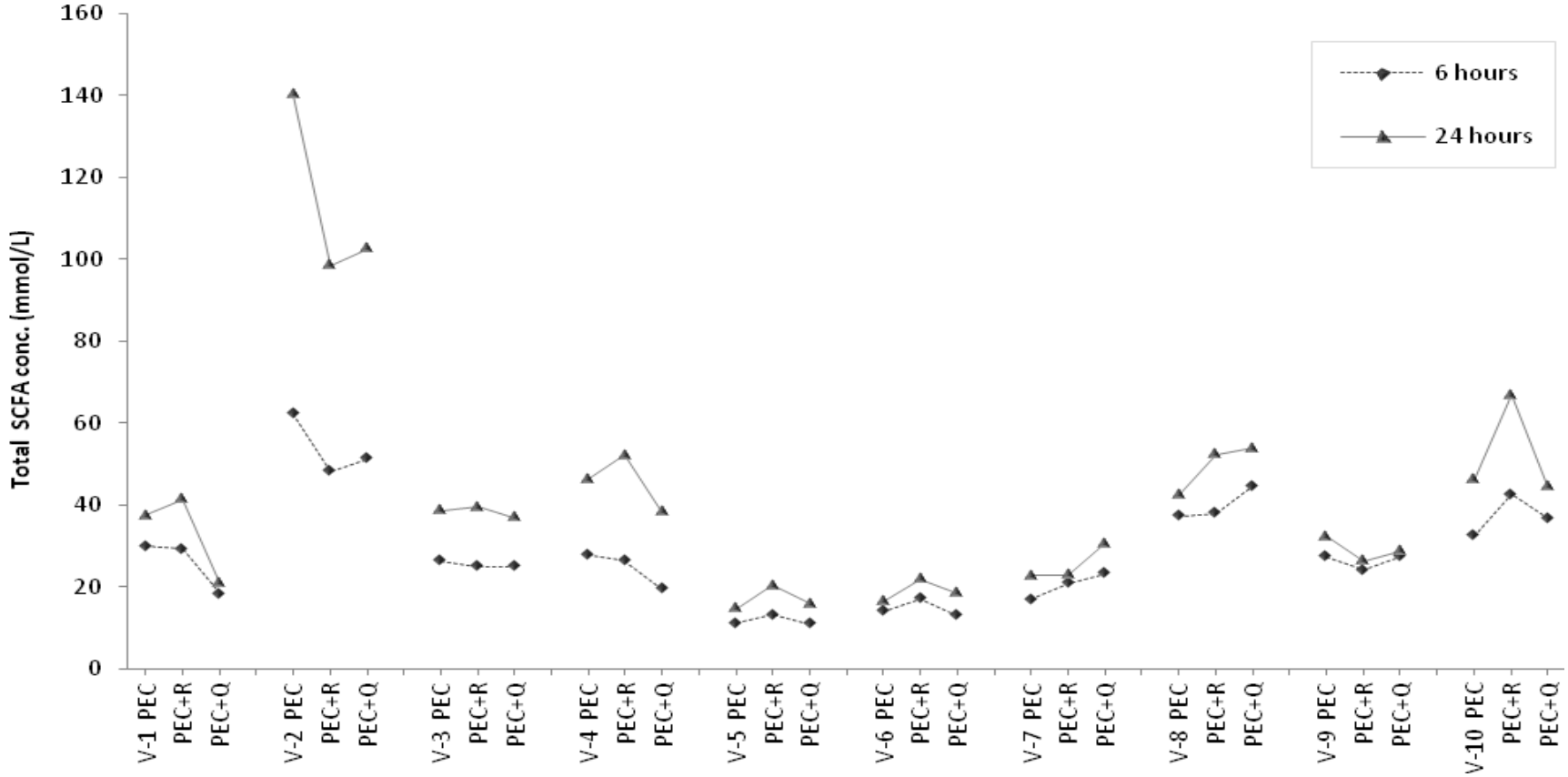


Figure 3-21 Individual volunteer plots of total SCFA production from pectin incubation with human faecal bacteria with or without quercetin and rutin
Values are sum of total SCFA for each volunteer at 6 and 24 hours (n=10). PEC: Pectin, PEC+R: Pectin+Rutin and PEC+Q: Pectin+ Quercetin

3.3.6 Impact of rutin and quercetin on pH within fermentation vessels

Change in pH can be used as an indication of bacterial activity and fermentation rate in the batch culture model

3.3.6.1 Measurement of pH in fermentation vessels

The measurement of pH using the pH meter demonstrated the same results as mentioned above. Rutin and quercetin did not impact the pH of raftiline, ispaghula or pectin as seen for all time points for 5 volunteers or for all volunteers at 6 and 24 hours.

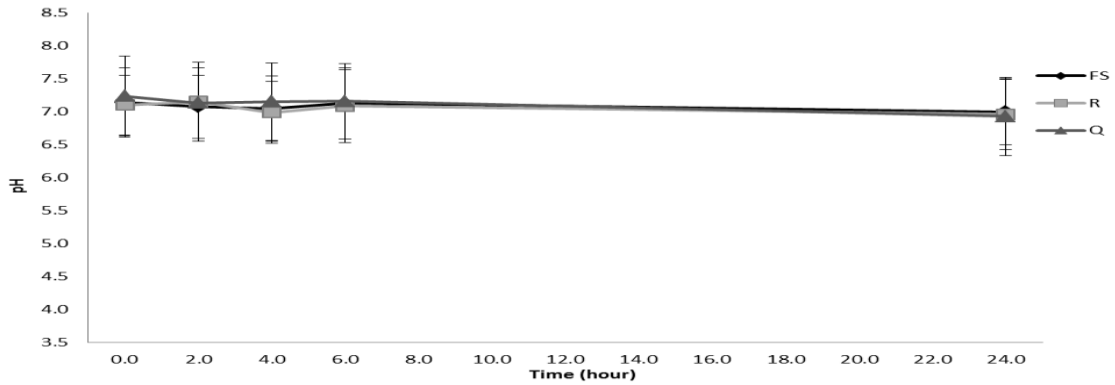


Figure 3-22 pH of control substrates
Values are displayed as mean (± SD) of 24 hour profile for 50ml faecal incubations having 28 umol/L rutin / quercetin (n=10)
FS: Faecal slurry, R: Rutin, Q: Quercetin

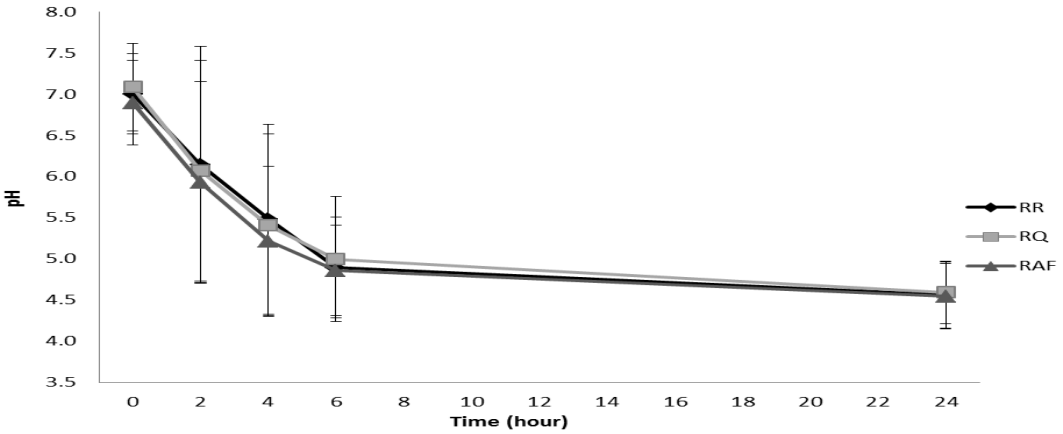


Figure 3-23 Impact of rutin and quercetin on the pH of raftiline vessels
Values are displayed as mean (± SD) of 24 hour profile for 50ml faecal incubations having 28 umol/L rutin / quercetin with 1g of RAF (n=10). RAF: Raftiline, RR: Raftiline+Rutin, RQ: Raftiline+Quercetin.

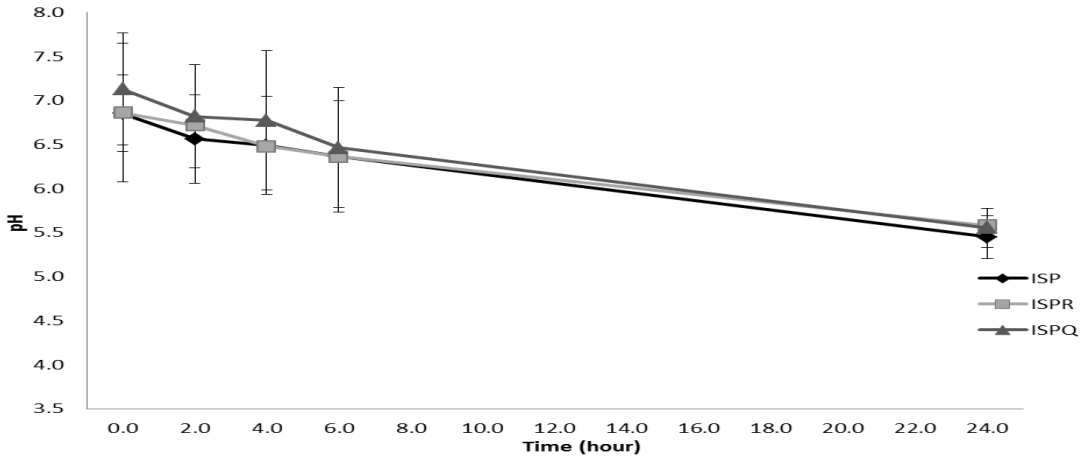


Figure 3-24 Impact of rutin and quercetin on the pH of ispaghula vessels
Values are displayed as mean (± SD) of 24 hour profile for 50ml faecal incubations having 28 umol/L rutin / quercetin with 1g of ISP (n=10), ISP: Ispaghula ISPR: Ispaghula+Rutin, ISPQ: Ispaghula+Quercetin

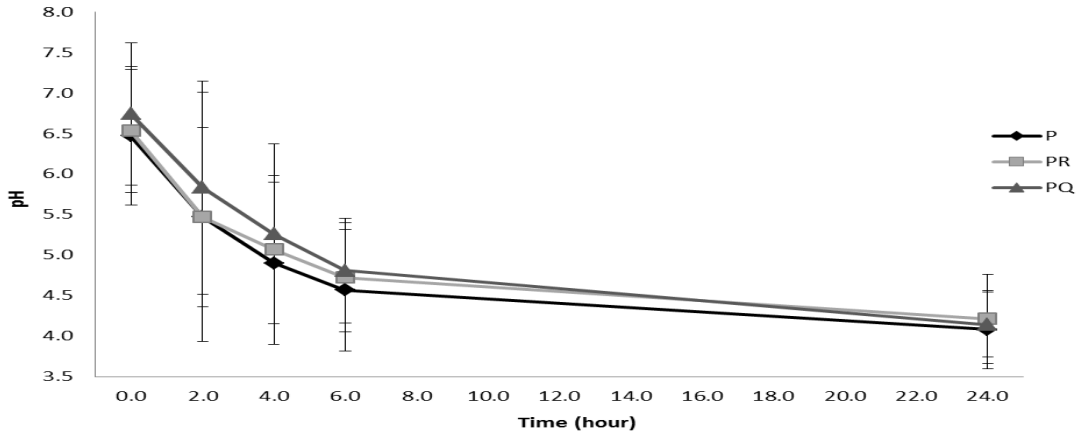


Figure 3-25 Impact of rutin and quercetin on the pH of pectin vessels
Values displayed as mean (± SD) of 24 hour profile for 50ml faecal incubations having 28 umol/L rutin / quercetin with 1g of PEC (n=10). PEC: Pectin, PR: Pectin+Rutin, PQ: Pectin+Quercetin.

3.3.7 Impact of rutin and quercetin on gas production

Gas in the head space of fermentation chambers was measured as an indication of bacterial activity and rate of fermentation. Rutin and quercetin did not affect the gas volume produced from the fermentation of raftiline, ispaghula or pectin. The data presented in this section is the mean cumulative gas production ± standard deviation over 24 hours post fermentation.

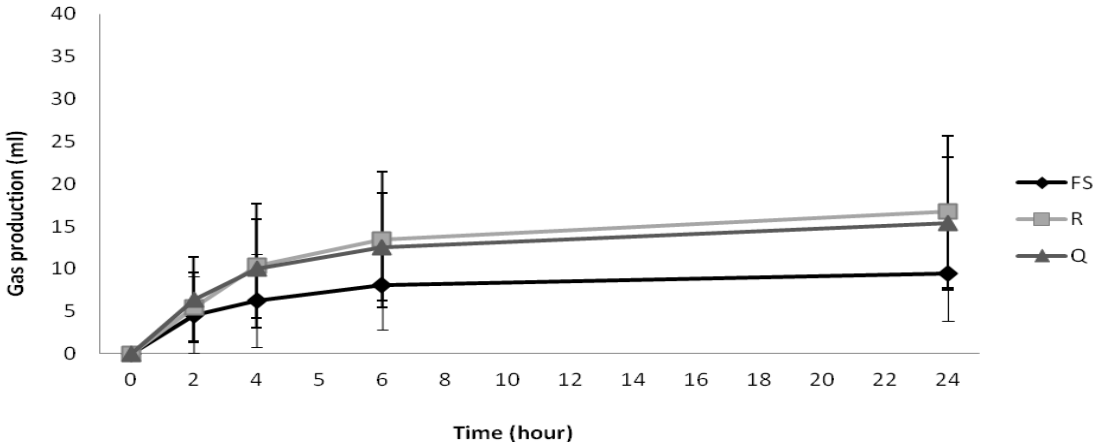


Figure 3-26 Gas productions in fermentation vessels of control substances
Values Values are displayed as mean (± SD) of 24 hour profile for 50ml faecal incubations having 28 umol/L rutin / quercetin (n=10) FS: Faecal slurry, R: Rutin, Q: Quercetin

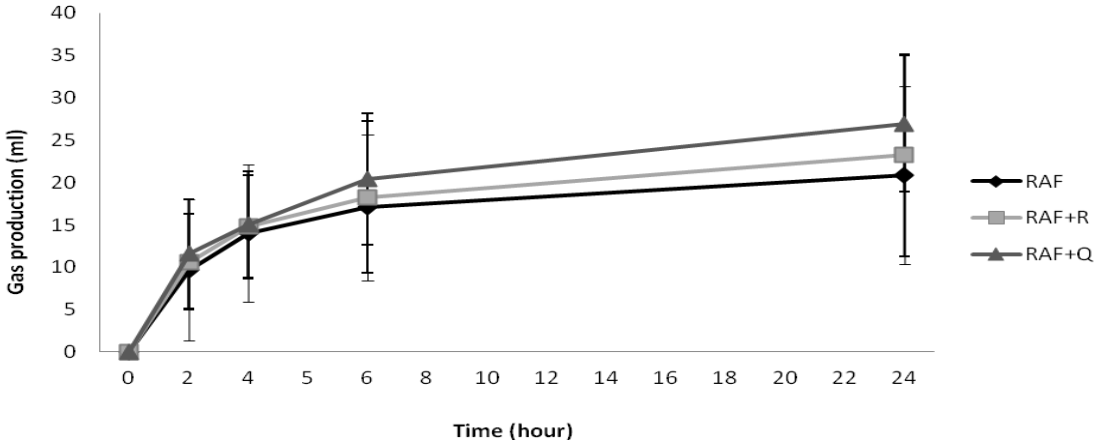


Figure 3-27 Impact of rutin and quercetin on gas production from raftiline fermentation
Values are displayed as mean (± SD) of 24 hour profile for 50ml faecal incubations having 28 umol/L rutin / quercetin with 1g of RAF (n=10). RAF: Raftiline, RR: Raftiline+Rutin, RQ: Raftiline+Quercetin.

A non-significant trend was seen over time for higher gas production from the incubation of rutin and quercetin as compared to faecal slurry. This would in turn contribute to the higher gas production from the combination of raftiline with rutin and quercetin as compared to raftiline alone.

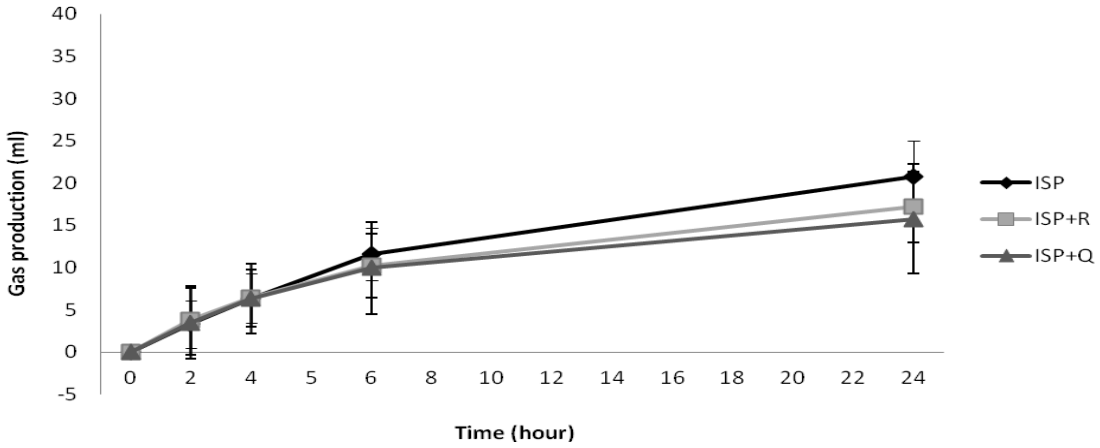


Figure 3-28 Impact of rutin and quercetin on gas production from ispaghula fermentation
Values are displayed as mean (± SD) of 24 hour profile for 50ml faecal incubations having 28 umol/L rutin / quercetin with 1g of ISP (n=10), ISP: Ispaghula ISPR: Ispaghula+Rutin, ISPQ: Ispaghula+Quercetin

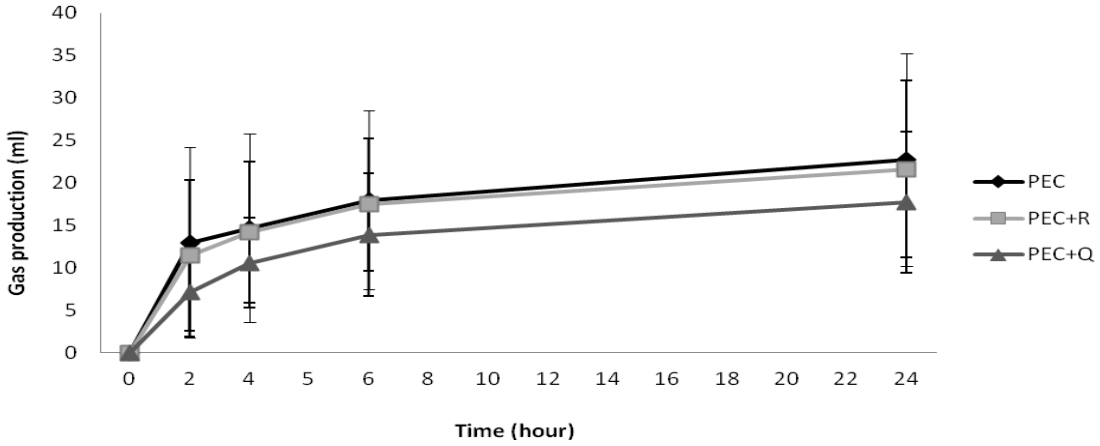


Figure 3-29 Impact of rutin and quercetin on gas production from pectin fermentation
Values displayed as mean (± SD) of 24 hour profile for 50ml faecal incubations having 28 umol/L rutin / quercetin with 1g of PEC (n=10). PEC: Pectin, PR: Pectin+Rutin, PQ: Pectin+Quercetin.

The reverse trend was seen for ispaghula and pectin compared to raftiline; with rutin and quercetin decreasing gas production at 24 hours for ispaghula and across time for pectin when combined with quercetin.

3.3.8 Summary of results

The highest produced phenolic acid identified in rutin incubations was found to be PAA, followed by 3,4-DHPAA and 3-HPAA, demonstrated in *Figure 3-2*.

Table 3-9 Summary of soluble fibre inhibition of phenolic acids production from rutin incubation with human faecal bacteria

Phenolic acids	Inhibition by raftiline	Inhibition by ispaghula	Inhibition by pectin
4-Hydroxy benzoic acid		↓	↓
Phenyl acetic acid	↓	↓	↓
3-Hydroxy phenyl acetic acid	↓		↓
4-hydroxy phenyl acetic acid	↓		↓
3,4- Dihydroxy phenyl acetic acid	↓	↓ *at 24 h only	↓
3-Hydroxy phenyl propionic acid			
4-Hydroxy phenyl propionic acid			
Sum total of phenolic acids	↓	↓ *at 24 h only	↓
Phenolic acids from background diet	↓	↓	↓

↓ Significant reduction in profuction of phenolic acid

All incubation groups demonstrated a rapid production of 3,4-DHPAA up to 6 hours and a plateu thereafter, as well as no production of 3-HPAA up to 6 hours and a rapid production thereafter. Suggesting that this phenolic acid is produced as a result of further degradation of 3,4-DHPAA.

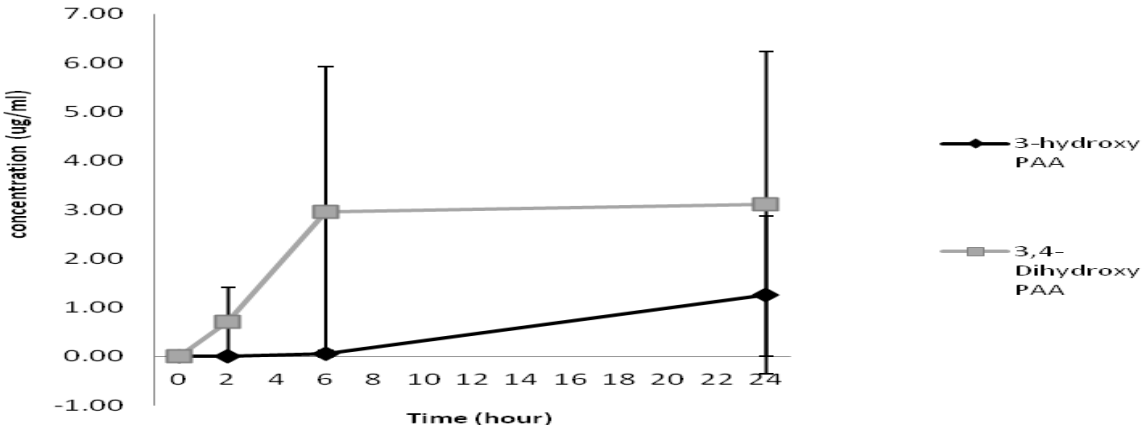


Figure 3-30 production of 3-HPAA from degradation of 3,4-DHPAA by human colonic microbiota
Values are displayed as mean (± SD) of 24 hour profile for 50ml faecal incubations having 28 umol/L rutin

3.4 Discussion

Seven phenolic acids produced in the incubation vessels using an *ex-vivo* batch fermentation model were identified and quantified: PAA, 3-HPAA, 4-HPAA, 3,4-DHPAA, 3-HPPA, 4-HPPA and 4-HBA. A comparison between phenolic acids identified in this chapter and previous studies is given Table 3-10. 3,4-DHPAA was a dominant phenolic acid produced in all seven studies from rutin/quercetin colonic degradation followed by 3-HPAA produced in six of the studies. The high standard deviations presented in the figures were expected due to varying inter-individual microbiota profile and metabolic potential. The high standard deviations in actual amounts of products are not relevant to the paired data presented in this chapter, where the change for each individual is the key outcome.

Table 3-10 phenolic acids identified from in-vitro fermentation of rutin/quercetin by human faecal bacterie

Phenolic acids	**Current study**	**Current study (GCMS)**	**Justesen et al., 2000 (LCMS)**	**Aura et al., 2002 (HPLCMS)**	**Rechner et al.,2004 (LCMS and GCMS)**	**Labib et al.,2004 (HPLC-DAD)**	**Jaganath et al.,2009 (HPLC-PDA-MS^2)**
	Main source	rutin/ quercetin	Rutin /quercetin	Rutin/ quercetin	rutin	quercetin	Rutin/ quercetin
PAA	FS, R	x					
3-HPAA	FS(n=1,)R	x	x	x	x		x
4-HPAA	FS, R	x	x				
3,4-DHPAA	R	**x**	x	**x**	x	x	**x**
4-HBA	R	x					x
3,4-DHBA							x
3-HPPA	FS, **R**	x			x		x
4-HPPA	FS, R	x					
3,4-DHPPA	R	x		x			
3,4-Dihydroxy tuluo			x			x	
3,4-Dihydroxy Benzaldehyde			x				
phloroglucinol						x	

FS: Faecal slurry, R: Rutin

Majority of PAA was produced from the background diet. The production of this phenolic acid was almost completely inhibited from both rutin and the background diet. Previous studies did not detect PAA in faecal incubations. This could be due to their analysis methods using LCMS compared to GCMS analysis conducted in this chapter. Considering PAA has a low molecular weight, and is non-polar in its non-conjugated form (Sandler et al., 1982), it is better suited for analysis by the GCMS as it may not ionise well in the LCMS. Previous studies have demonstrated low sensitivity of LCMS for

PAA and PBA detection, compared to improved sensitivity of GCMS (Hommes, 1999, Brusilow, 1991, Yu et al., 2001).

The production of 3,4-DHPAA was rapid from the degradation of rutin, reaching a plateau concomitant with the production of 3-HPAA at 6 hours; suggesting that 3-HPAA was produced from further degradation of 3,4-DHPAA. This is in accordance with previous data by Aura et al (2002) obtained from an *in-vitro* batch culture fermentation. They determined 3,4-DHPAA as the primary metabolite from rutin degradation. Unlike our study, they detected the presence of these phenolic acids in the background diet of the volunteers. This could be attributed to the volunteers ingesting a normal diet prior to faecal sample collection in the Aura study as compared to our volunteers, consuming a low polyphenol diet for three days prior to faecal sample collection.

In contrast to these two phenolic acids, majority of 4-HPAA was derived from the background diet. The production of 4-HPAA increased up to 2 hours post fermentation and remained steady up to 24 hours post fermentation, whereas a transient production was seen in the control incubations. The production of 3-HPAA, 4-HPAA and 3,4-DHPAA was inhibited across time by pectin and raftiline. Ispaghula only inhibited the production of 3,4-DHPAA at 24 hours post fermentation.

The production of 3-HPPA and 4-HPPA was not inhibited. In fact, an initial glance at the data suggested higher concentration of 3-HPPA in the presence of ispaghula. This was attributed to phenolic acid produced from ispaghula alone. To confirm this, a predicted value as the sum of 3-HPPA produced from ispaghula only and rutin only was compared to the combination incubation of ispaghula and rutin together for 3-HPPA and did not result in any difference between the two, confirming that ispaghula had no impact on increasing the production of this phenolic acid. Unlike the previous two phenolic acids, 4-HBA was inhibited by all the soluble fibres.

The presence of 3,4-DHBA in the study by Jaganath et al. (2009) and 3,4-DHPPA in the study by Aura et al. (2002) was not detected in our study. We speculate that this may be due to the rapid conversion of 3,4-DHPPA to 4-HPPA (Jaganath et al., 2009), and 3,4-DHPPA to 3-HPPA. Both of which were identified in our samples.

In the above mentioned study by Jaganath et al, It was proposed that quercetin was the first metabolite to be produced from degradation of rutin, followed by 3,4-DHPAA. They also found 3,4-DHPAA to be present in the highest concentration compared to other phenolic acids. These results were supportive of the observations made by Aura et al. (2002) demonstrating complete degradation of rutin within 60 minutes of incubation and concomitant production of quercetin which was initiated at 20 minutes, however transiently before further degradation to the primary metabolite 3.4-DHPAA. The degradation

of quercetin-glucuronides in their study occurred very rapidly with complete degradation of the compounds within 20 minutes, this was attributed to the high activity of β-glucosidases and β-glucuronidases, while the activity of α-rhamnosidases remained low, leading to the slower de-conjugation of rutin.

Unlike the above studies determining 3,4-DHPAA as the most produced phenolic acids, our study found PAA which was not detected in the above studies as the highest contributor to the total sum of phenolic acid production. Although we did not measure the release of quercetin from rutin our results are consistent with that of Jaganath et al. (2009) in terms of phenolic acids solely produced from rutin, showing 3,4-DHPAA being the highest produced phenolic acid. It is important to note the difference in analysis methods between the two studies, the GCMS analysis did not allow for the identification of smaller compounds such as PAA not detected in studies using LCMS analysis. The total phenolic acid production, calculated as the sum of all phenolic acids produced was greatly inhibited by pectin and raftiline across time and ispaghula at 24 hours.

The detection of phenolic acids in FS alone from the background diet although undesirable, did not affect the observations made in this study due to the paired study model. The dietary record of volunteers, used as a reference in case of abnormal values, demonstrated compliance to the low polyphenol diet. Hence, we speculate the phenolic acids detected in FS alone can either be due to inaccurate recording of diet or the release of insoluble phenolics bound to the cell-wall of cereals. Studies have shown that phenolic acids bound to the cell-wall of cereals such as brown rice, wheat, rye, barley and oats can be released *in-vivo* and result in a modest and transient increase in plasma phenolic acid concentrations. This modest increase is not sufficient to induce health benefits through antioxidant properties (Belobrajdic and Bird, 2013). The phenolic acids most associated with the cell-wall of cereals belong to the hydroxycinnamic acid group with the most prominent of them identified bound to cereal cell-walls being ferulic acid followed by diferulic acids, sinapic acid, *p*-coumaric acid, caffeic acid and benzoic acid derivatives (Vitaglione et al., 2008). These phenolic acids are covalently bound to the cell-wall polysaccharides through ester bonds. Ferulic acid is found bound to arabinoxylans by the acetylating acid group. Phenolic acids may also be found bound to cell wall pectins (Saura-Calixto and Díaz-Rubio, 2007, Hatfield et al., 1999). The amount of these bound phenolics is highly variable; they are not absorbed in the small intestine and reach the colon along with the fibres.

Figure 3-31 represents the speculated catabolic pathway of rutin. The compounds encircled in dashed red line were also identified in our study.

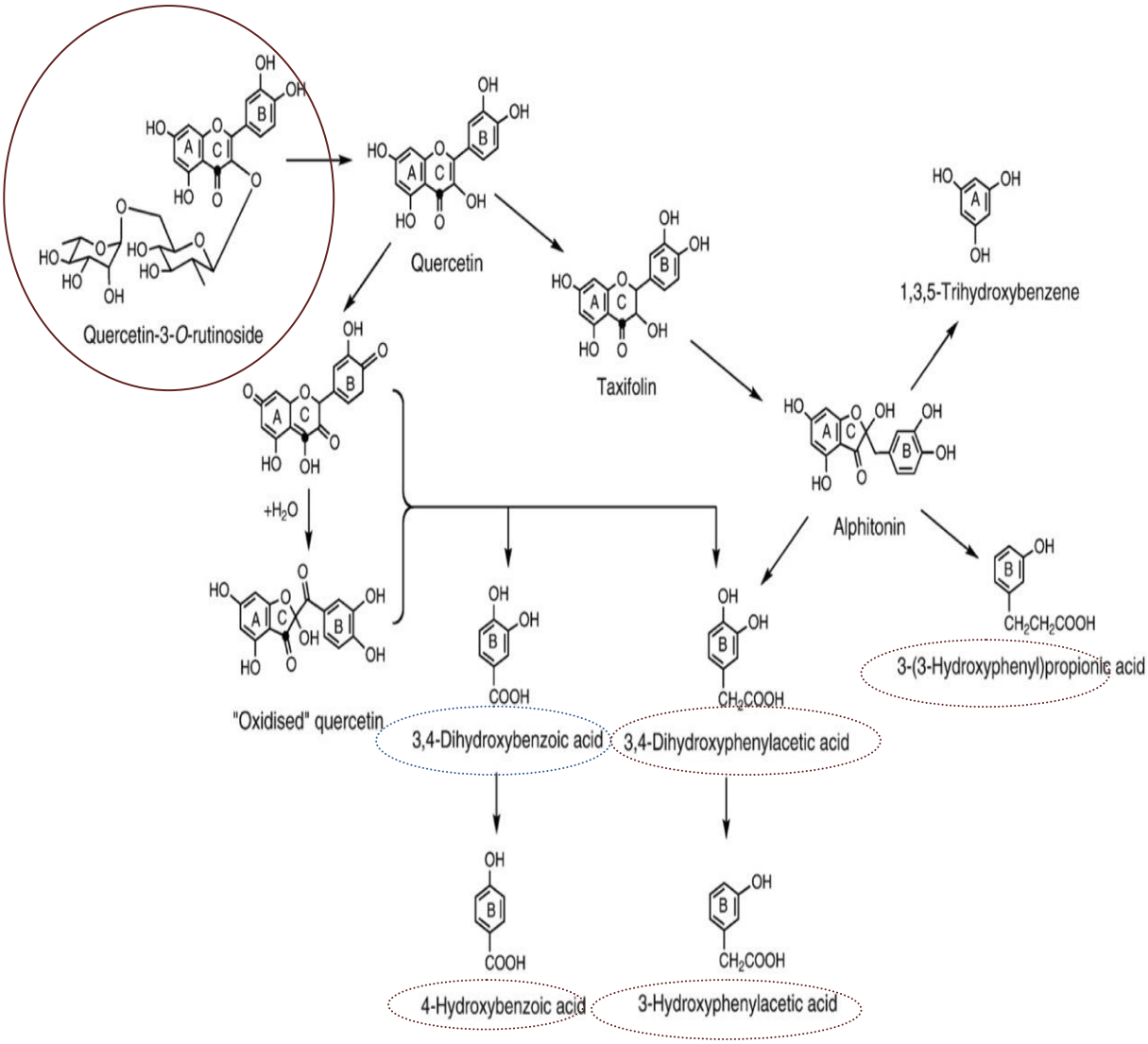


Figure 3-31 Speculated catabolic pathway of rutin in relation to the findings in this chapter. Adapted from Jaganath et al. (2008). Encircled in red line: parent compound, encircled in dashed red line: phenolic acids also identified in the results of this chapter from in-vitro catabolism of rutin

3,4-DHBA was encircled in blue even though it was not detected in our study, as the product of its degradation (4-HBA) was detected. It is possible that the presence of fermentable fibre in the fermentation vessels increased bacteria population, leading to increased activity and thus the early disappearance of the intermediate 3,4-DHBA. A similar observation was for the lack of quercetin intermediate when glucose was added to fermentation vessels in the Jaganath et al. (2008) study. In addition to the metabolites shown in Figure 4-20, we also detected 3,4-DHPPA, 4-HPPA, 4-HPAA and PAA. It can be speculated that PAA is derived from further dehydroxylation of some of the compounds

detected (eg: 3,4-DHPAA, 3-HPAA and 4-HPAA). 4-HPAA may also be produced from the dehydroxylation of 3,4-DHPAA at C-3. Most catabolic pathways of polyphenol compounds are speculative. This may be due to the complex family of microbiota with a range of metabolic capacity and the impact of other food components such as carbohydrates in the food matrix on the rate at which transitory phenolic are utilised.

It is not known exactly which bacteria are responsible for the catabolism of rutin and quercetin *in-vivo*, however some that have been found to have the ability to do so *in-vitro* consist of: *Eubacterium ramulus* (for rutin, quercetin-3-glucoside and quercetin aglycone), *Butyrivibrio sp* (rutin), *Enterococcus casseliflavus* (quercetin 3-glucoside) and *Flavonifractor plautii*, *Eubacterium oxidoreducens* and *Clostridium sp. (Blaut et al., 2003, Winter et al., 1989).* The impact of these bacteria on rutin and quercetin and subsequent phenolic acid production *in-vivo* is affected by factors such as their interaction with other bacteria, bacterial metabolites and food components such as carbohydrates in the colon.

Cheng et al. (1969), identified and extracted 15 anaerobic bacteria capable of degrading rutin from the bovine rumen contents. However the action of different bacteria resulted in different end products from rutin degradation. The bacterial species, *Butyrivibrio fibrisolvens D1* and *Selenomonas ruminantium GA192*, cleaved the glycosidic bond of rutin and fermented the released sugar moiety but failed to degrade the released insoluble aglycone; Unlike these two bacteria, *Peptostreptococcus sp*. *B178* degraded the substrate to soluble products. This was further demonstrated in an *in-vitro* study (Schneider et al., 1999) investigating the degradation of quercetin-glucosides by the two bacteria *Entero-coccus casseliflavus* and *Eubacterium ramulus*. While *Entero-coccus casseliflavus* only utilised the glucose moiety of the compound, *Eubacterium ramulus* also cleaved the A and C ring. Interestingly this bacteria was only capable of degrading quercetin in the presence of 10-20 mM of glucose and not in its absence. A review by Selma et al (Selma et al., 2009) has demonstrated that *Bacteroides uniformis* and *Bacteroides ovatus* are responsible for the conversion of rutin to quercetin while the conversion of quercetin to 3,4-DHPAA involves *Clostridium orbiscindens*, *Eubacterium oxidoreducens* and *Butyrivibrio spp.*

The impact of carbohydrates on colonic metabolism of rutin has not been investigated sufficiently. The study by Jaganath et al. (2009) demonstrated that the addition of 0.5g glucose to incubation vessels not only increases rate of rutin deconjugation leading to increased phenolic acids, but also alters the catabolic pathway possibly by causing a shift in microbiota composition. This was seen when the

conversion of 3,4-DHBA was directed toward the production of 4-HBA in the absence of glucose, and toward the production of HPAA derivatives in the presence of glucose.

The above mentioned studies have all investigated the impact of glucose, readily available as a substrate for bacteria on the metabolite production from rutin *in-vitro* as a model for fermentable fibres. While the *Eubacterium* and *Clostridium species* seem to be involved in the degradation of rutin and quercetin, the fermentation of raftiline, ispaghula and pectin is more dependent on the bacterial species *Bacteriodes*, representing one of the major species in the colon (Discussed in *Chapter-1*).

In our results we observed that the fibres having higher fermentability (raftiline) had more inhibitory impact on the phenolic acid production from rutin degradation than the fibre having higher viscosity and lower fermentability (ispaghula). We did not measure viscosity of fibres in this study and fermentability was estimated according to the concentration of SCFA production from fibre fermentation. It is possible that the fermentability of these fibres promotes the Bacteriodes species, thus reducing the activity of competing bacterial species responsible for rutin and quercetin degradation. This may also be induced through a reduction of pH due to SCFA accumulation. Additionally we did not detect an inhibitory impact on the higher molecular weight phenolic acids: 3-HPPA and 4-HPPA which were detected in incubation vessels in very low concentrations, possibly owing to their conversion to lower molecular weight phenolics. The low concentration of these two phenolic acids may have also resulted in a small effect size as a slight but non-significant trend for inhibition of these phenolic acids by raftiline and pectin was observed.

As expected the fermentation of the three different soluble fibres resulted in different proportions of SCFA produced by each fibre (Paturi et al., 2012, Chen et al., 2010). The pattern of SCFA production differed between the three fibres with raftiline having the highest relative molar ratio of butyrate, ispaghula having the highest molar ratio of propionate and pectin having the highest molar ratio of acetate. This difference in pattern of SCFA production relative to these fibres has been previously demonstrated in various studies (Timm et al., 2010, Chen et al., 2010) and summarised in *Table 1-1*. The total sum of SCFA production was highest for raftiline compared to ispaghula and pectin. This sum total of SCFA was mostly made of acetate, propionate and butyrate, which represented 92.3%, 93.6% and 96.7% of total SCFA production for raftiline, ispaghula and pectin respectively. Despite efforts to reduce inter-individual differences through diet and time of sample processed; there were factors beyond our control. Certain volunteers had a tendency to ferment pectin much more than the other fibres. An example of this was volunteer-4, exhibiting approximately double the concentration of all SCFA through the fermentation of pectin compared to the mean values obtained from the other nine

volunteers. Even though this does not affect the results of the study due to the samples being paired, it does result in very high standard deviations.

In this study we did not detect any impact of the polyphenols rutin and quercetin or their colonic metabolites on the pH, gas production or SCFA production from raftiline, ispaghula or pectin. The individual plot of outcomes did not demonstrate any pattern for the impact of rutin or quercetin on total SCFA production. The lack of impact of rutin and its metabolites on the pH in fermentation vessels was also demonstrated in an *in-vitro* fermentation study by Aura et al. (2002) over 24 hours of incubation.

There have been no previous studies on the interaction of rutin and quercetin with the fibres raftiline, ispaghula and pectin. Most studies have been developed to observe the antibacterial and antimicrobial properties of these polyphenols and not their impact on the colonic metabolite production of these bacteria. These studies can only provide an assumption that the possible antibacterial activity of these polyphenols will result in a different SCFA production from fibre. We did not detect this at a concentration of 28 umol/l for rutin and quercetin.

However in an *in-vitro* study, Rodriguez Vaquero et al. (2010) demonstrated antibacterial activity for both rutin and quercetin (summarised in table *Table 1-9),* they also demonstrated a synergistic impact when rutin and quercetin were mixed, whereas the addition of rutin to catechin resulted in an additive effect. The results are contradictory to many other studies not demonstrating any impact of rutin on bacterial growth (Arima et al., 2002, Parkar et al., 2008, Duda-Chodak, 2012). A possible explanation for this could be the use of 99.8% ethanol to dissolve phenolics used in this study, as well as the addition of ethanol to incubation plates to reach a concentration of 5% (v/v). Ethanol is toxic to bacteria at concentrations > 3% especially to more sensitive gram-negative bacteria such as E.coli. The use of 5% ethanol as a negative control may have corrected for this toxic effect to an extent. In studies using more standardised methods such as broth micro-dilution method (Parker et al., 2008) and using DMSO as a solvent (Arima et al.,2002), rutin did not demonstrate any inhibition on bacterial growth even at concentrations of 1000 ug/ml, while quercetin inhibited the growth at the lowest MIC (62.5 ug/ml and 250 ug/ml respectively). Interestingly even though rutin had no impact on its own, when it was mixed with quercetin, the MIC required for inhibition of bacteria reduced to 100 and 200 ug/ml respectively. The authors were not able to find an explanatory mechanism for this synergistic impact.

Most studies, looking into the antibacterial activity of rutin or quercetin have focused on specific bacterial species in an *in-vitro* model. The study undertaken in this chapter is more complex. This study introduces the food matrix interaction between fibre and polyphenols in an *ex-vivo* condition.

Hence, it is important to consider the prebiotic potential of the soluble fibres in conjunction to the antibacterial properties of the polyphenols. While rutin and quercetin might have had some antibacterial activity this could have been ameliorated by the prebiotic activity of the fibres. Interestingly, the above studies have demonstrated that the lactic acid bacteria such as lactobacillus are more resistant to the antibacterial activity of polyphenols (Parkar et al., 2008, Duda-Chodak, 2012, Lee et al., 2006), this information coupled with the ability of the soluble fibres to enhance the growth of probiotic bacteria such as Bifidobacteria and Lactobacillus, especially *in-vivo,* as the partial hydrolysis of ispaghula and pectin has shown to increase their prebiotic potential (Olano-Martin et al., 2002, Salyers et al., 1978, Kolida et al., 2002, Marteau et al., 1994). This may lead to a shift in bacterial composition, which may or may not reflect in their metabolite production. There are other factors that can play into the results obtained in this study, such as the reduction of the pH in the incubation bottles as a result of fermentation.

Additionally, the studies discussed above have used high concentrations of polyphenols mainly for their application in the pharmacology and food industry. However intake of these polyphenols at such high concentrations may prove toxic (Scalbert et al., 2002). Additionally many of the above studies claimed antibacterial activity at MIC > 100 ug/ml whereas the application of these antimicrobial agents is possible and of relevance only at MIC <100 ug/ml and of great interest at MIC <10 ug/ml (Rios and Recio, 2005). The concentration of rutin and quercetin used in our study (17.0 ug/ml rutin and 8.46 ug/ml quercetin) was much lower than the lowest MIC found for these compounds *in-vitro*. Additionally the *in-vitro* studies have not taken into consideration the bioavailability of these compounds, whereas the concentration of rutin and quercetin used in our study is based on their physiological bioavailability as recovered in the ileal fluid of patients 0-24 hours after consuming tomato juice supplemented with 17 5umol/L rutin (Jaganath et al., 2006a).

The *in-vitro* studies investigating the antibacterial impact of polyphenols on bacteria have based their observations on prolonged periods of incubation of these bacteria along with the polyphenols. Previous studies have shown that quercetin is almost completely degraded by the colonic microbiota within 6 hours (Jaganath et al., 2009), whereas in an *in-vitro* study the complete degradation of quercetin-glucuronides took place within 20 minutes and 60 minutes for rutin (Aura et al., 2002) thus yielding lower weight phenolic acids. Despite *in-vitro* studies demonstrating strong antibacterial impacts of these phenolic acids, these outcomes are highly dependent on the accumulation and prolonged interaction of these phenolic acids with gut microbiota. However *in-vivo* these phenolic acids are absorbed and enter circulation where they are further metabolised and utilised by various tissues preventing their accumulation in concentrations as high as those used in *in-vitro* studies.

It is difficult to draw an accurate comparison and conclusion from current studies relevant to this chapter, due to the use of various different strains of bacteria, concentrations and medium of polyphenols investigated and methods of analysis; all of which have shown to have an impact on the extent of antibacterial potential.

We have studied SCFA in this study as an indication of fibre fermentation and bacterial activity. However we cannot say with complete certainty that our results conclude that rutin and quercetin have had no antibacterial activity. These polyphenols might have had an inhibition effect on specific strains of bacteria as demonstrated and discussed above, which may have not reflected in the overall SCFA production. Our objective was to study the interaction of fibre and polyphenol and to observe the effect of polyphenols and soluble fibre on the metabolite production from the gut microbiota. Based on this we did not observe any impact of polyphenols on SCFA production from fibre, however there was a strong impact of soluble fibres on the phenolic acid production from the polyphenols.

3.5 Conclusion

Soluble fibres, especially highly fermented soluble fibres inhibit phenolic acid production from the degradation of rutin by human faecal bacteria. Whereas the polyphenols: rutin and quercetin have no impact on SCFA production from fibre fermentation.

CHAPTER 4

Matrix interaction between cocoa polyphenols and the soluble fibres raftiline, ispaghula and pectin, on their metabolism by colonic bacteria- *ex-vivo*

Cocoa is widely consumed worldwide. The health benefits associated with consumption of cocoa have been attributed to its high polyphenol content and their colonic metabolites. Thus it is important to determine the factors influencing the bioavailability of these polyphenols. Information on the impact of food matrix interaction between carbohydrates, especially fibre on the colonic phenolic acid production from cocoa is scarce. In this chapter we investigate the impact of cocoa polyphenols and phenolic acids on colonic metabolite production from soluble fibres, as well as the impact of soluble fibres, present in many cocoa products on the phenolic acid production from cocoa.

4.1 Introduction

Cocoa is widely consumed in the form of confectionary, chocolate products and beverages. It is a rich source of polyphenols (Detailed in *Chapter -1*), mainly the monomers (+)-catechin, (-)-epicatechin and their derived polymers known as procyanidins (Massot-Cladera et al., 2012). It is estimated that cocoa contributes towards 20% of total catechin consumption (Arts et al., 1999) also having a higher concentration of polyphenols per dose of consumption than tea or red wine (Lee et al., 2003) and higher procyanidin content than that of cranberries and blue berries on a dry weight basis (Gu et al., 2002). Dark chocolate (100g) contains on average, 42 mg (-)-epicatechin and 12 mg (+)-catechin (Tzounis et al., 2008a). The total polyphenol concentration in chocolate is dependent on the type of chocolate, and varies depending on the method of analysis. The total phenol content in dark chocolate, milk chocolate and cocoa powder may vary from 8.4, 5 and 20 mg/g respectively when measured as gallic acid equivalent (Waterhouse et al., 1996) to 36.5, 15 and 65 mg/g respectively when measured as catechin equivalent (Vinson et al., 1999).

Consumption of cocoa has been associated with increased levels of plasma antioxidants preventing LDL oxidation (Waterhouse et al., 1996, Salah et al., 1995, Pearson et al., 2001). Additionally cocoa flavanols have demonstrated the ability to increase nitric oxide synthesis, suppress platelet activation, positively modulate eicosanoid synthesis, inhibit the production of certain pro-inflammatory cytokines and stimulate the production of anti-inflammatory cytokines (Andújar et al., 2012, Zhu et al., 2002). All of these potent biological properties of cocoa polyphenols may contribute towards their health benefits; such as protective mechanism in heart disease and anti-carcinogenic properties. However, these beneficial properties exhibited by cocoa are largely dependent on its polyphenol content and their antioxidant properties. Hence, the bioavailability of these polyphenols is of great importance, as it would determine the extent of impact cocoa may exhibit on health.

However not all of these polyphenolic compounds are readily available for absorption. 20% of ingested epicatechin (Auger et al., 2008) as well as 90% of ingested procyanidins have been found in the ileostomy bags of patients (Kahle et al., 2007). The absorption of these compounds from the small intestine has been studied in ileostomy patients and summarised below (*Table 4-1*).

Table 4-1Type and amount of cocoa polyphenols available for colonic microbiota degradation

Author	Feed	Main component in feed	Concentration of component in feed	Recovery of component in ileal fluid	% of ingested dose
Kahle et al (2007)	Cloudy apple juice (1 L)	**Catechin**	10.3 umol	-	0%
		Epicatechin	51.7 umol	8.8 umol	17%
		Oligomeric procyanidins	97.9 umol	88.4 umol	90.2%
Auger et al (2008)	Polyphenol E (200mg)	Flavan-3-ol monomers:	452 umol:	194 umol	43%
		(–)-epicatechin	71 umol	15umol	21%
		(–)-Epigallocatechin-3-gallate	310 umol	160umol	52%
		(–)-epicatechin-3-gallate	32 umol	11umol	34%
		(−)-epigallocatechin	18 umol	-	-
		(+)-Gallocatechin gallate	-	7.8 umol	71%
Stalmach et al (2010)	Green tea (300ml)	Total flavan-3-ols:	634 umol:	207 umol	33%
		(+)-catechin	18 umol	1.2 umol	6.8%
		(-)-Epicatechin	69 umol	7.9 umol	11%
		(+)-Gallocatechin	50 umol	13 umol	27%
		(-)-Epigallocatechin	190 umol	35 umol	18%
		(-)-epigallocatechin-3-*O*-gallate	238 umol	116 umol	49%
		(+)-Gallocatechin-3-*O*-gallate	5.2 umol	4.6 umol	89%
		(-)-Epicatechin-*O*-gallate	64 umol	29 umol	45%
Hagl et al (2011)	Apple smoothie (0.7 L)	**Catechin**	1.1 mg	0.4mg	36.3%
		Epicatechin	1.3 mg	0.7mg	53.8%
		Procyanidins	1136.3 mg	705.6mg	62%

The least absorbed compounds are procyanidins followed by epicatechin and catechin. The recovered polyphenolic compounds in the ileal fluid would be available for colonic degradation by gut microbiota in healthy individuals. The colonic degradation products of cocoa or pure polyphenols present in cocoa have been summarised in table *Table 4-2.*

Table 4-2 Phenolic acid production from cocoa or cocoa polyphenols in *in-vitro* fermentation models

Author (year)	Study method	Phenolic acid production
(Déprez et al., 2000)	in-vitro fermentation (n=1) In-vitro fermentation (48h) with procyanidin or 150 or 100 mmol/L expressed	4-HPAA, , 3-HPAA, PPA, 4-HPPA, 3-HPPA, 3-hydroxy phenyl valeric acid
(Bazzocco et al., 2008)	Digestion followed by in-vitro fermentation apple – short PA chains and long PA chains	3,4-DHPPA, 3-HPPA, 3-PPA, BA, 3,4-DHPAA, 3-HPAA
Tzounis et al (2008)	In-vitro fermentation (n=3) 150mg/L or 1000 mg/L of catechin / epicatechin	Phenolic acids from (-)-epicatechin: 5-(3,4-DHP)—valerolactone, PPA, 5-phenyl—valerolactone Phenolic acids from (+)-catechin: (+)-epicatechin, 5-(3,4-DHP)—valerolactone, 5-phenyl—valerolactone
Appeldoorn et al (2009)	In-vitro fermentation (N=4 pooled) Mixture of procyanidins dimmers (5umol)	3,4-DHPAA, 3,4-DHPVA, 3-HPAA, 4-HPAA, 3-HPPA, PVA, Monohydroxylated phenylvalerolactone and 1-(3,4-dihydroxyphenyl)-3-(2,4,6-trihydroxyphenyl) propan-2-ol
Roowi et al 2009	In-vitro fermentation N=3 epicatechin	4-HPAA, 3-HPAA, 3-HPPA (54%) , 5-(3,4-dihydroxyphenyl)-γ-Valerolactone (38%) , 5-(3,4-dihydroxyphenyl)-valeric acid (32%).
Stoupi et al (2010)	In-vitro fermentation (N=1) 5mM (-)-epicatechin/ 5mM Dimer B_2	1-(3,4-DHP)-3-(2,4,6-THP)- propan-2-ol, 1-(HP)-3-(2,4,6-THP)-propan-2-ol, 5-(3,4-DHP)-γ-valerolactone, 3-HPPA
(Fogliano et al., 2011)	In-vitro fermentation (N=1) Insoluble-fraction of cocoa 20g of cocoa in 120ml of water digested, followed by dialysis and freeze drying A concentration of 1% w/v used.	3-HPAA, 3-HPPA, 3,4-DHBA -and an unknown compound with MRM fragmentation peak of *m/z* 289→245
Dall'Asta et al (2012)	In-vitro fermentation (N=3) (i)- FS+ 9% chocolate extract (90% chocolate)	5-(3,4-DHP)- γ-valerolactone, 3,4-DHPAA, protocatechuic, HBA

As shown in the above table, most research is based on degradation of pure polyphenolic compounds by the gut microbiota and very little information is available on the fate of these polyphenols as present in the cocoa matrix upon being subjected to the action of gut microbiota. Often these polyphenols are extracted from other food sources such as apple and green tea which may contain different length proanthocyanidin chains, which has shown to affect their colonic metabolites (Bazzocco et al., 2008).

As mentioned above, majority of cocoa polyphenols are not readily absorbed by the small intestine, thus reaching the colon where they are converted to phenolic acids. Hence it is possible that phenolic acids and not necessarily their parent compounds are responsible for the health benefits (*Chapter-1*) exhibited by cocoa. However information in this area is very limited, additionally these polyphenolic compounds are most often present in association with fibres, which are also subjected to colonic microbiota action.

Previously, the cocoa shell was considered to be a waste product of the bean. It was either used as fuel or garden mulch after being composted. However, recently the treated and ground cocoa shell is being sold as cocoa fibre. Depending on local regulations, these products can sometimes be incorporated into cocoa powder or chocolate to increase product fibre content. Similarly, some cocoa products such as cereal bars and breakfast cereals contain soluble fibre. The food matrix interaction between fibre and cocoa on the bioavailability of their metabolites is of importance, given that cocoa and fibre health benefits, associated with their metabolites, are increasingly being promoted. In this chapter we incubated the three soluble fibres ispaghula, raftiline and pectin having various physio-chemical properties such as fermentability and viscosity with cocoa in an *ex-vivo* faecal incubation model.

In a similar study to that conducted in this chapter, Tzounis et al. (2008) conducted a continuous *in-vitro* fermentation model (Summarised in Table 1-7) incubating 150 mg/L or 1000 mg/L of (+)-catechin and (-)-epicatechin in the presence or absence of sucrose, and / or fructo-oligosaccharides. Both catechin and epicatechin displayed higher bacterial metabolism in the presence of both sucrose and fructo-oligosaccharides. Unfortunately the impact of sucrose and FOS on the amount and kind of phenolic acid production was not described.

While simple sugars and prebiotics can enhance the deglycosilation of parent compounds reaching the colon and increase phenolic acid production, there is not enough evidence on the impact of more complex carbohydrates such as soluble fibres on phenolic acid production from polyphenols. In a study by Bazzocco et al. (2008) detailed in *Table 1-7*, briefly, long or short chain proanthocyanidn extract of apples were incubated with 100 mg of cell-wall preparation containing pectin. Cell-wall preparations delayed the maximal extent of conversion from 4 hours to 8 hours post incubation of proanthocyanidin, without affecting the total concentration of phenolic acid production. Additionally the maximum extent of conversion for apples was much later (6hours) than their proanthocyanidin extracts (2hours).

To our best knowledge, there have been no previous food matrix studies investigating the impact of soluble fibre on the phenolic acid production from cocoa. Most food matrix interaction studies on the

impact of carbohydrates on cocoa polyphenol bioavailability are conducted *in-vivo* and investigate the impact of simple carbohydrates such as glucose and bread (*Table 1-7*).

Carbohydrates such as fibre present in the colon are the preferred source of energy for the microbiota (Tzounis et al., 2008a), thus limiting the degradation of polyphenolic compounds and consequent phenolic acid production. Additionally the production and accumulation of SCFA through fibre fermentation may indirectly impact phenolic acid production by lowering the colonic pH, which has demonstrated an impact on colonic microbiota composition (*summarised in chapter-1*). Additionally SCFA may have a direct impact on specific bacterial species, as seen that the growth of Bifidobacteria was enhanced in the presence of propionate (Arora et al., 2011). Interestingly studies have demonstrated that cocoa polyphenols inhibit Bacteroides (Lee et al., 2006) growth and either increase or have no impact on the growth of Lactobacillus and Bifidobacteria.

Indeed, there is some evidence of a prebiotic effect of cocoa polyphenols. The impact of a cocoa drink on faecal microbiota was investigated in a long-term study (summarised in Table 1-8) by Tzounis et al. (2011), where volunteers were supplemented with either a high cocoa flavonol drink (494mg total cocoa flavanols) or a low cocoa flavonol drink (29mg total cocoa flavanols). The cocoa drinks were matched for macro / micro mineral and fibre content. This study demonstrated modification in bacterial composition with changes in *Bifidobacterium* spp, C. *histolyticum* group, E. *rectale*–C. *coccoides* group, *Lactobacillus* and *Enterococcus* spp for both interventions. However the high cocoa flavanol drink demonstrated a greater increase in Lactobacillus and Enterococcus spp than the low flavonoid intervention ($p < 0.01$), as well as an in increase in *Bifidobacterium* spp not observed for the low flavonoid group. Additionally the high flavonoid intervention resulted in a decrease in C. *histolyticum* as opposed to its increase in the low flavonoid group ($p < 0.01$). Interestingly the authors demonstrated a similar increase in L*actobacillus* spp. ($p < 0.01$) and *Bifidobacterium* spp ($p < 0.05$) and decrease in *C. histolyticum* group ($P < 0.01$) in an *in-vitro* faecal incubation model using a high-flavanol cocoa-powder extract (1 mg extract/mL; amounting to ≈0.4 mg/mL flavanols) containing less than 1% (10mg) of fibre.

A comparison of the recent Tzounis et al. (2011) study to a previous study by the same authors (2008a), demonstrates that the matrix in which cocoa polyphenols are introduced in, plays an important role on their pre/antibiotic properties. Contradicting their previous study the cocoa matrix did not result in the modification of the coccoides–E. rectale group. It was suggested that the *Lactobacillus* spp. uses the oligomers preferentially by catabolising them, whereas the same may be said in the case of the coccoides–E. rectale group and the monomers. The authors attributed the lack of increase in

coccoides–E. rectale to the presence of more complex phenolic compounds such as the oligomers, as these observations were replicated in their *in-vitro* study using a cocoa extract. However it is possible that the presence of fibre in the most recent study contributed to the observations made (= 3-4g in the in-vivo study and 10 mg/ml in the *in-vitro* study compared to 25mg/ml in our study). It is also interesting to note that their 2008 study mostly demonstrated a prebiotic impact at lower concentrations (150 ug/ml), whereas higher concentrations (1000 ug/ml) resulted in an inhibitory impact on bacteria. Thus both matrix in which polyphenols are presented and their concentration is of importance in such studies.

Similar observations were made by Massot- Cladera et al (2012) by enriching the diet of wistar rats with 10% (w/w) cocoa (10.6 mg/g polyphenols) compared to a standard diet (summarised in *Table 1-8*). The diets were isoenergetic, with similar levels of insoluble fibre, however the cocoa diet was higher in soluble fibre and polyphenols. They detected a reduction in Bacteroides, Clostridium and Staphylococcus ($p < 0.05$). It is possible that this resulted in an increase in other bacterial species, as the total number of bacterial count was not affected (Massot et al., 2012).

Although it is important to investigate the impact of cocoa as a whole matrix on the gut bacteria, it is also important to note that cocoa products especially cocoa powder contain dietary fibre which could be a contributing factor to the prebiotic effect observed in the Tzounis et al. (2011) study.

Thus for the better understanding of the impact of polyphenols on the faecal bacteria it is essential to investigate the impact of these polyphenols outside the cocoa matrix, eliminating the impact of any other components present in the cocoa matrix.

One such study was carried out by Smith and Mackie (2004) demonstrating that long term (3.5 weeks) consumption of 0.7% and 2% proanthocyanidins in rats significantly increased the number of tannin resistant bacteria ($p<0.05$), while gram-positive bacteria decreased in the presence of the proanthocyanidins. The post-proanthocyanidin diet analysis of faecal bacteria demonstrated a return of tannin resistant bacteria to pre-treatment numbers.

Most other studies investigating the impact of extracted polyphenols on the microbiota have been carried out *in-vitro* and summarised in *Table 1-8.* Duda-Chodak (2012) did not find any impact of catechin on the probiotics nor the pathogenic bacteria. A similar *in-vitro* study was conducted by Parkar et al (2008) using the more standardised broth microdilution technique according to the CLSI guidelines for analytical methods. Different concentrations of catechin and epicatechin were required to induce growth inhibition on different species.

The above mentioned *in-vitro* studies are mostly conducted for the use of polyphenolics as antimicrobial agents in the pharmaceutical industry. With many of these studies investigating the antibacterial effect of cocoa polyphenols on specific pathogenic bacteria, especially those threatening oral health (Bakri and Douglas, 2005, Choi et al., 2012, Petti and Scully, 2009). The concentrations used in these studies once translated into cocoa consumption are not physiological. As previously mentioned, 100g of cocoa (= 1 chocolate slab or > 4 cups of cocoa beverage) contains12 mg catechin and 42 mg epicatechin, keeping in mind that only 20% of these epi/catechins will reach the colon (~4 and 8.4 mg). The concentration of these polyphenols in the above studies vary from 20-1000 ug/ml (20, 100, 250, 500, 1000 ug/ml); *in-vivo* this translates to 6, 30, 75, 150 and 300 mg respectively in the colon (considering 300 ml of colonic content). These concentrations cannot be physiologically achieved through cocoa consumption. Moreover, these studies have not taken into consideration the fate of catechins *in-vivo*. The above results were obtained from continuous exposure of the bacteria to epi/catechin over 24 hours, whereas these compounds will be degraded to lower molecular weight phenolic acids by the microbiota. Hence, even though epi/catechins failed to have a strong antibacterial impact, it is possible that they may exhibit their antibacterial properties through their colonic metabolites.

Lee et al (2006) conducted a study investigating the impact of phenolics from catechin degradation on the faecal bacteria. They incubated 28 pathogenic and prebiotic strains (5% inoculum) in a broth with or without 1% epicatechin, catechin, 3-*O*-methyl gallic acid, gallic acid and caffeic acid, 3-PPA, 4-HPAA and 4-HPPA. The results of this study supported previous studies demonstrating a general inhibition of pathogenic bacteria (Clostridium perfrigens, Clostridium difficile and Bacteroides. spp) especially by the phenolic acid derivatives and a small to no impact on the prebiotic bacteria (such as Clostridium spp, Bifidobacterium spp and Lactobacillius). Thus demonstrating that epi/catechins and their metabolites and not only fibre can impart a prebiotic effect. However more research is required in this area.

These studies investigated only the impact of polyphenols on bacterial populations but not the impact of this antimicrobial activity on metabolite production from the colonic bacteria. Bazzocco et al (2008) investigated the impact of apple proanthocyanidins on the SCFA production from apple cell wall preparations (containing pectin). They found an inhibitory effect of apple PA on SCFA production with a correlation between PA chain length and inhibitory impact. Extracts with longer PA chains displayed a greater inhibitory impact on SCFA production than shorter chain PA. Unfortunately the extent of inhibition was not discussed. However this impact was not seen for apples when the PA was

present in the matrix (impact of apple). They found acetate to be the highest SCFA produced, followed by butyrate and propionate.

In another study Kemperman et al. (2013) investigated the impact of 1000 mg/day of tea extract polyphenols on the growth of gut microbiota and their metabolite production for 2 weeks in a Simulator of Human Intestinal Microbial Ecosystem (SHIM). In addition to the antimicrobial activity seen by the tea extract, a reduction in butyrate concentration in the proximal colon was demonstrated (from ~15mM to ~10mM), which was attributed to the inhibition of butyrate producing bacteria. Towards the end of the study a drastic reduction in total SCFA (data not provided) and acetate (from ~30mM to ~22mM) concentration was demonstrated. This reduction in SCFA production corresponded to reduction in bacterial numbers observed. Even though this *in-vitro* model is ideal for studying the longer-term impact of polyphenols on gut bacteria *in-vitro*, there is still the lack of other factors such as host-bacteria inter-relationship and presence of other influencing food matrix interactions.

To our knowledge there have been no previous studies investigating the matrix interaction of cocoa polyphenols and soluble fibres on their metabolites from gut microbiota

4.2 Hypothesis

To test the hypothesis that soluble fibres change phenolic acid production from cocoa polyphenols in the colon by fermentation related mechanisms, and that potential antibacterial properties of cocoa polyphenols and their colonic metabolites inhibit the production of SCFA from the microbial fermentation of soluble fibres.

4.3 Materials and methods

4.3.1 Study design

The study was an *ex-vivo* model of the gut, using faeces from ten human volunteers as a sample representation of the gut microbiota. Volunteers followed a three day low polyphenol diet; avoiding fruits, vegetables and beverages high in polyphenols such as tea, coffee and cocoa milk (*Appendix-1*). On the 4th day faeces was collected from volunteers according to the method described in *2.1 Faecal sample collection.*

4.3.2 Participants

For this *ex-vivo* fermentation, fresh faecal sample was collected from For this *ex-vivo* batch fermentation, fresh faecal sample was collected from healthy volunteers (n=10) with a mean (± SD) BMI of 22.4 ± 2.9 (range, 18.9 to 27.4) and a mean (± SD) of 23.6 ± 4.0 years (range, 19 to 33) Volunteers were non-smokers who had not received antibiotics, constipation remedies or probiotics for at least three months prior to start of the study.

Table 4-3 Participant characteristics

	gender	Age (years)	Height (cm)	Weight (kg)	BMI (Kg/m^2)	Waist circumference (cm)	Blood pressure (mmHg)
1	F	22	160	50.5	19.7	73.5	112/80
2	M	22	175.8	84	27.4	96	125/60
3	F	19	161	47.9	18.4	77	109/71
4	F	21	158	63.9	25.6	73	129/67
5	F	21	156	54	22.2	80	119/71
6	F	26	174	57.2	18.9	78	107/59
7	F	25	161	54.3	20.9	83.5	112/80
8	M	23	183	77.8	23.2	91	143/83
9	M	33	175	76	24.8	93	165/63
10	M	23	176	72.2	23.3	93	114/72
Mean ± STDEV		23.6 ±4.0	168.8 ± 9.8	65.3 ± 12.7	22.4 ± 2.9	84.9 ± 8.4	124.7 / 69.5 ± 18.8 / 8.3

4.3.3 Ex-vivo fermentation

Ten *ex-vivo* fermentations were carried out according to the method described in *2.2 Ex-vivo Modelling of anaerobic colonic fermentation*. The incubation bottles contained faecal slurry alone, or with one of seven possible combinations of fibre and/or cocoa. Incubation was allowed for 24 hours and samples for phenolic acid analysis were taken at 0, 2, 6 and 24 hours.

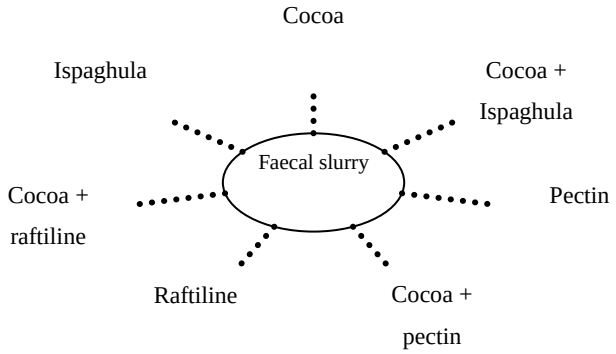


Figure 4-1 Substrate combinations for faecal fermentation.

4.3.4 Materials

1g of the soluble fibres: Ispaghula husk *(Whole ispaghula husk – myprotein.com)*, pectin *(pectin apple-250 grade, BDH laboratory supplies)* and raftiline *(Siber Hegner Ltd)* were incubated along with 0.5g cocoa *(extra brute Cocoa-Cacao Barry, Barry Callebaut, Hardricourt, France)*. Faecal samples were obtained from participating volunteers. The amount of fibre used was based on 6g of fibre consumed (moderate fibre supplementation) present in 300ml of colonic content, resulting in 1g of fibre present in 50ml incubation bottles. The amount of cocoa in the incubation bottles was based on an average of 2.5g of cocoa powder used in a 250ml cocoa drink. This translates to 0.5g in 50ml incubation bottles. The cocoa used in this study was a high grade 100% cacao powder *(extra brute Cocoa-Cacao Barry, Barry Callebaut, Hardricourt, France)*, used in the food industry, confectionary and patisserie, also available for purchase at specific outlets. The detailed composition of this cocoa is given in *Appendix-3*. A brief comparison of the composition of this cocoa powder to the more well-known Green & Black cocoa powder-16.3% powder *(Green and Black's Ltd, London, United Kingdom)* is given below.

Table 4-4 cocoa powder composition comparison

Nutritional data/100g	Extra brute Barry callebaut	Organic dark cocoa Green & Blacks
Energy	339 Kcal	350 Kcal
Available Carbohydrate	13.0g	13.6
• Sugars (mono+Disaccharides)	0.4g	0.4
• Starch	12.6g	Not specified
Dietary Fibre	27.7g	28.7
Total Protein	18.0g	23.6
Milk Protein	0.0g	Not specified
Total Fat	23.0g	22.3
Saturated Fat	14.5g	13
Vanilla extract	×	✓
Soya Lecithin-Emulsifier	×	✓

This information was kindly provided by the customer service and quality control administration unit of respective brands.

4.3.5 Phenolic acid analysis

Phenolic acid analysis was carried out on samples from the 0, 2, 6 and 24 hour time points using 23 phenolic acids (*Table 2-3*) as standard reference, according to the method described in *2.4 Phenolic acid analysis*

Data for all identified phenolic acids were quantified and analysed separately and a sum of all identified phenolic acids was considered as total sum of phenolic acid production.

4.3.6 SCFA analysis

SCFA were analysed in samples from the fermentation vessels at 0, 2, 4, 6 and 24 hours according to the method described in *2.3 SCFA analysis*. Analysis of the results focused mainly on acetic, propionic and butyric acid and the total sum production as the sum of acetate, propionate, butyrate, iso-butyric, iso-pentanoic, pentanoic, iso-hexanoic, hexanoic, heptanoic, octanoic).

4.3.7 pH measurement

pH was measured for all samples at 0, 2, 4, 6 and 24 hours according to the method described in 2.2.2 Measurement of pH.

4.3.8 Gas volume

Gas was measured for all samples at 0, 2, 4, 6 and 24 hours post incubation according to the method described in *2.2.1 Gas production*

4.3.9 Statistical analysis

To determine the impact of the soluble fibres raftiline, ispaghula and pectin on phenolic acid production from cocoa, as well as the impact of cocoa polyophenols on SCFA production rom the fibres, a general linear model Anova was performed using the software Minitab-15. This statistical model allowed for the analysis of paired data across time. In this analysis *Phenolic acid* was used as the response, with *volunteer, time, substrate and time*substrate* included in the model. *Volunteer* was included in the random factors.

Further analysis was conducted for the data at 24 hours separately, using a paired t-test model or 1-way Wilcoxon test after assessing for normality. This analysis was conducted as the general linear model considers change over time and ignores differences at specific time points.

Soluble fibres differ in their rate of fermentation; some slowly fermented fibres such as ispaghula demonstrate impact only at 24 hours and not before. Hence, it was important to investigate the 24 hour time point separately.

4.4 Results

The soluble fibres used in this study were plant extracts. Hence, some phenolic acid production from these soluble fibres was expected. The main outcome was the impact of fibre on the phenolic acid production from cocoa. Therefore we calculated a predicted value as the sum of phenolic acid produced from fibre only and phenolic acid produced from cocoa only. This is presented as Fibre-C and is compared to the true combination of fibre and cocoa incubated together, presented as Fibre+C.

A higher value for Fibre-C as compared to Fibre+C would be an indication of the fibre having an inhibitory effect on the production of phenolic acids from cocoa. The phenolic acids identified in the cocoa incubations are listed in *Table 4-5*. Phenolic acid production from soluble fibres is discussed in the relevant sections.

Table 4-5 Identified phenolic acids from Cocoa incubation with human faecal bacteria

Phenolic acid	Concentration at 24h (ug/ml)	Number of volunteers producing
Phenyl acetic acid	7.38 ± 3.80	10
3-Hydroxy phenyl acetic acid	0.64 ± 0.93	8
4-hydroxy phenyl acetic acid	0.38 ± 0.41	10
3,4- Dihydroxy phenyl acetic acid	0.27 ± 0.49	10
3-Hydroxy phenyl propionic acid	8.73 ± 16.0	10
4-Hydroxy phenyl propionic acid	1.74 ± 2.51	9
4-Hydroxy benzoic acid	0.02 ± 0.04	4
3,4-Dihydroxy benzoic acid	0.08 ± 0.09	10
3,4-Dihydroxy phenyl propionic acid	0.08 ± 0.12	6
Hippuric acid	0.01 ± 0.04	2
Vanillic acid	0.009 ± 0.02	1

4.4.1 Impact of Raftiline on phenolic acid production from cocoa

3-HPAA was detected in the fermentation vessels of raftiline in 1 volunteer; mainly derived from the background diet as there was no difference in the concentration of 3-HPAA between FS and raftiline fermentations. Whereas 3,4-DHPAA was detected in raftiline only fermentations. Raftiline did not have an inhibitory effect on the production of these phenolic acids over time. However the analysis at 24 hours demonstrated an inhibition of 3-HPAA production (*Figure 4-2*, $p < 0.01$).

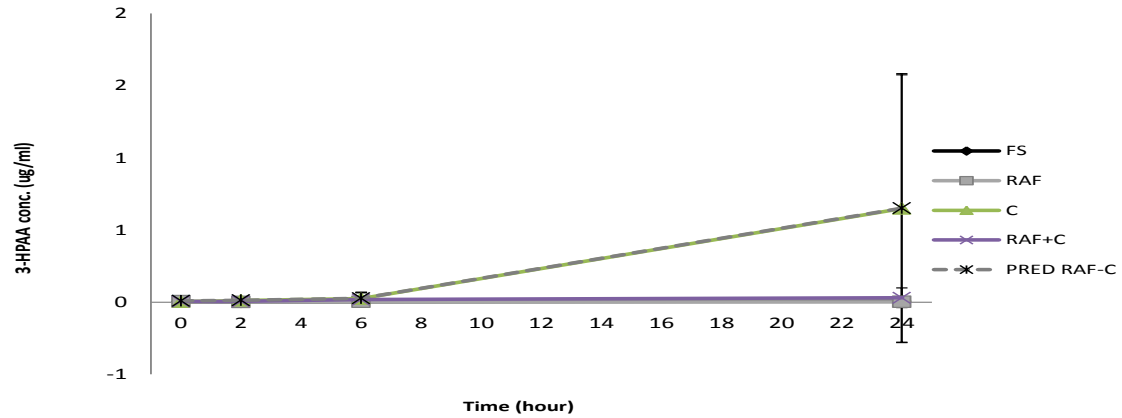


Figure 4-2 Impact of raftiline on 3-HPAA production rom cocoa incubations with human faecal bacteria
Values are displayed as mean (± SD) of 24 hour profile for faecal incubations having 0.5g cocoa powder with 1g of RAF (n=10), FS: Faecal slurry, RAF: Raftiline, C: Cocoa, RAF+C: Raftiline+Cocoa. PRED RAF-C: predicted value for Raftiline and cocoa combination, *only at 24h $p < 0.01$

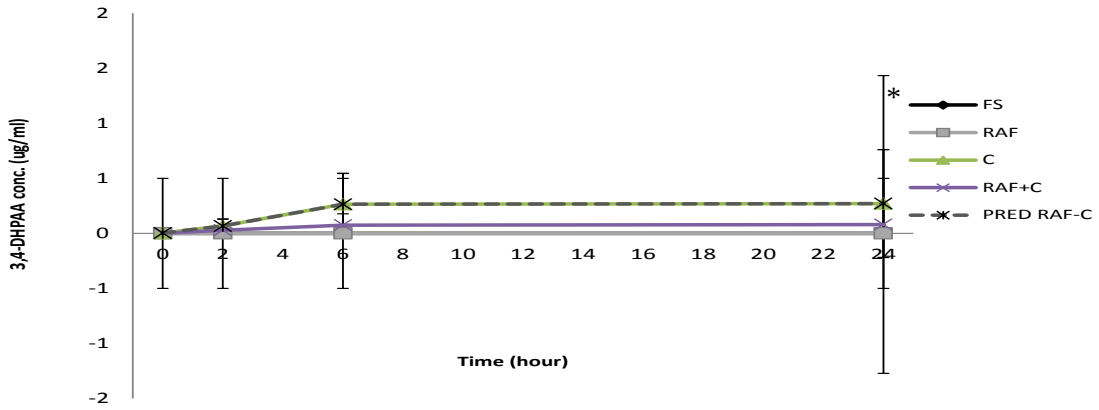


Figure 4-3 Impact of raftiline on 3,4- DHPAA production from cocoa with human faecal bacteria
Values are displayed as mean (± SD) of 24 hour profile for faecal incubations having 0.5g cocoa powder with 1g of RAF (n=10), FS: Faecal slurry, RAF: Raftiline, C: Cocoa, RAF+C: Raftiline+Cocoa. PRED RAF-C: predicted value for Raftiline and cocoa combination.

Similarly, 4-HPAA was not inhibited by raftiline. This phenolic acid was detected as part of the background diet in all 10 volunteers and the concentration present in FS alone was no different from that of cocoa incubations. However the comparison of predicted to true combination value demonstrated an inhibitory impact on the production of this phenolic acid by raftiline ($p < 0.01$).

59% of PAA concentration detected in cocoa incubations was derived from the background diet and there was no difference in concentration over time between FS and cocoa incubations. However, the presence of raftiline in the cocoa incubations reduced the production of PAA from cocoa ($p < 0.01$, *Figure 4-4*), a comparison of predicted to true combination values demonstrated an even greater inhibition ($p < 0.01$, *Figure 4-4*). Whereas there was no impact on the PAA production from the background diet ($p > 0.05$).

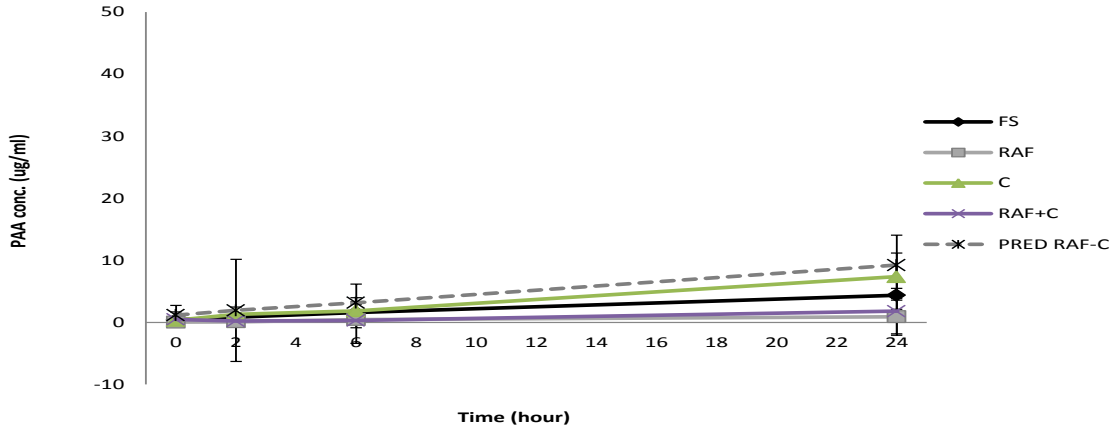


Figure 4-4 Impact of raftiline on PAA production from cocoa with human faecal bacteria
Values are displayed as mean (± SD) of 24 hour profile for faecal incubations having 0.5g cocoa powder with 1g of RAF (n=10), FS: Faecal slurry, RAF: Raftiline, C: Cocoa, RAF+C: Raftiline+Cocoa. PRED RAF-C: predicted value for Raftiline and cocoa combination. *P < 0.01 for RAF+C vs. C and RAF+C vs. PRED RAF-C.

4-HBA and 3,4-DHBA were produced in very low concentrations (0.03, 0.08 ug/ml respectively), neither of which were affected by the presence of raftiline. These two phenolic acids were not detected in the background diet or raftiline only incubations. 3,4-DHBA increased rapidly in cocoa incubations up to 6 hours post fermentation, after which a great decline was seen. However, in the Raf+C incubations, the production did not decline after 6 hours, but reached a plateau. 4-HBA concentration remained constant over time, suggesting that this phenolic acid was not produced but present in cocoa.

3,4-DHPPA was detected in incubation bottles containing cocoa only; demonstrating rapid increase up to 6 hours post fermentation after which it reached a plateau. Concomitantly 3-HPPA was produced only after 6 hours of incubation in both control (n=1) and cocoa incubations, suggesting its production from 3,4-DHPPA degradation. 4-HPPA increased gradually over time in both control and cocoa

incubations. Neither of these phenolic acids were affected by the presence of raftiline in the fermentation bottles. This could be due to the low concentration of these phenolic acids resulting in a smaller effect size.

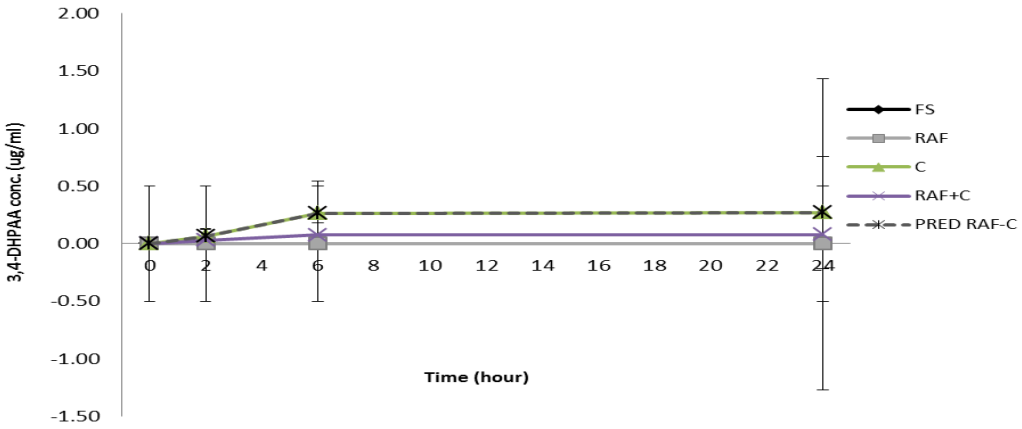


Figure 4-5 Impact of raftiline on 3,4- DHPPA production from cocoa with human faecal bacteria
Values are displayed as mean (± SD) of 24 hour profile for faecal incubations having 0.5g cocoa powder with 1g of RAF (n=10), FS: Faecal slurry, RAF: Raftiline, C: Cocoa, RAF+C: Raftiline+Cocoa. PRED RAF-C: predicted value for Raftiline and cocoa combination.

The production of hippuric acid (produced in 2 volunteers: volunteer-5 and 6) and vanillic acid (produced in 1 volunteer: volunteer-5) were not affected by the presence of Raftiline in the incubation bottles.

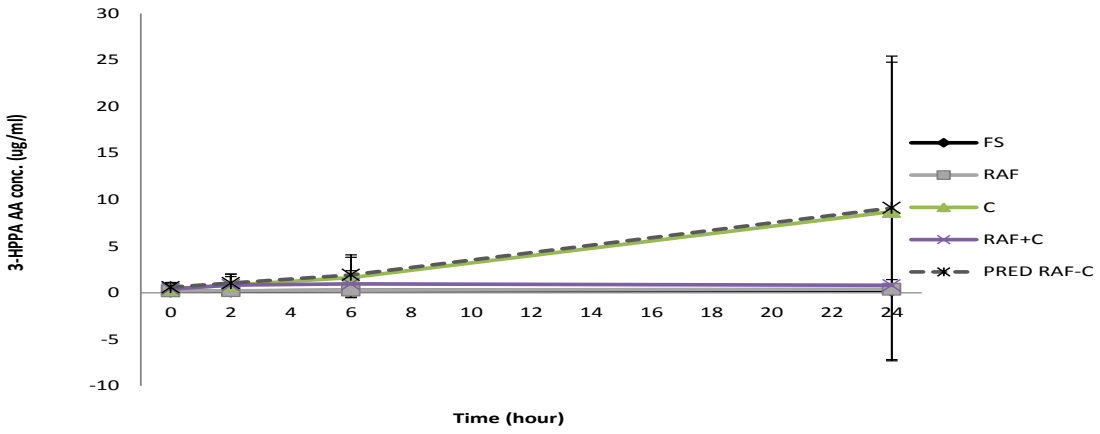


Figure 4-6 Impact of raftiline on 3- HPPA production from cocoa with human faecal bacteria
Values are displayed as mean (± SD) of 24 hour profile for faecal incubations having 0.5g cocoa powder with 1g of RAF (n=10), FS: Faecal slurry, RAF: Raftiline, C: Cocoa, RAF+C: Raftiline+Cocoa. PRED RAF-C: predicted value for Raftiline and cocoa combination.

The total sum of phenolic acid production was greatly inhibited by the presence of raftiline in the incubation bottles ($p < 0.01$, *Figure 4-7)*. As seen for PAA, the total sum of phenolic acid concentration in Raf+C incubations was reduced to the same concentration seen from raftiline incubation alone.

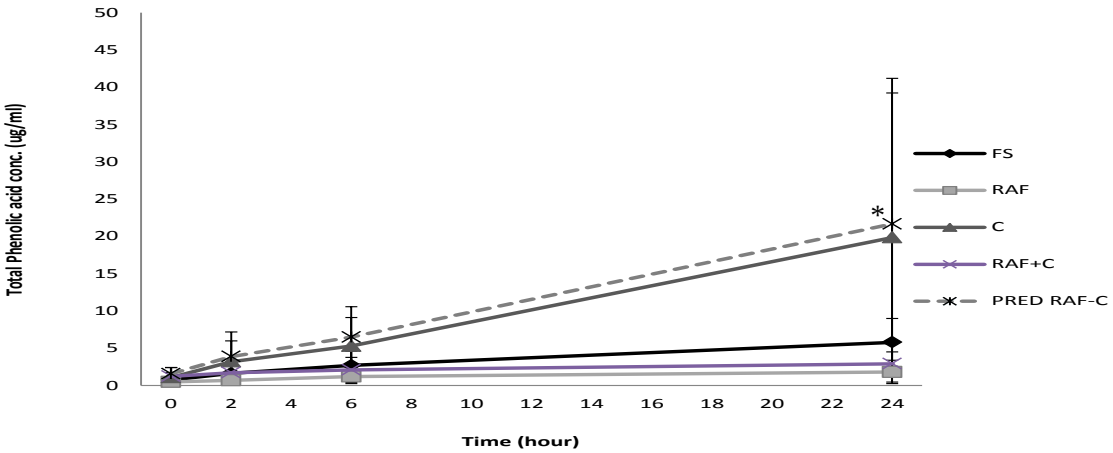


Figure 4-7 Impact of raftiline on total sum of phenolic acid production from cocoa with human faecal bacteria
Values are displayed as mean (± SD) of 24 hour profile for faecal incubations having 0.5g cocoa powder with 1g of RAF (n=10), FS: Faecal slurry, RAF: Raftiline, C: Cocoa, RAF+C: Raftiline+Cocoa. PRED RAF-C: predicted value for Raftiline and cocoa combination. *p < 0.01for RAF+C vs. C and RAF+C vs. PRED RAF-C.

Table 4-6 Impact of raftiline on phenolic acid production from cocoa with human faecal bacteria

Substrate	Raf+C At 24h (ug/ml)	C At 24h (ug/ml)	P value
4-HBA	0.02 ± 0.03	0.02 ± 0.04	P > 0.05
3,4-DHBA	0.14 ± 0.13	0.08 ± 0.09	P > 0.05
3-HPPA	0.80 ± 0.62	8.73 ± 0.16	P > 0.05
4-HPPA	0.41 ± 0.62	1.57 ± 2.55	P > 0.05
3,4-DHPPA	0.01 ± 0.03	0.08 ± 0.11	P > 0.05
Hippuric acid	0.02 ± 0.04	0.01 ± 0.04	P > 0.05
Vanillic acid	0.009 ± 0.02	0.009 ± 0.02	P > 0.05

Values are mean (± SD) at 24 hours (n=10) , C: Cocoa, RAF+C: Raftiline+Cocoa, p-value is presented for the comparison of Raftiline+Cocoa to cocoa alone.

4.4.2 Impact of ispaghula on phenolic acid production from cocoa

Ispaghula is a slowly fermented fibre, thus any impact induced through fermentation products would be demonstrated at 24 hours post fermentation. 3-HPAA was produced in ispaghula fermentation vessels of only one volunteer (mainly derived from the background diet). 3,4-DHPAA was not detected in ispaghula only fermentations. Ispaghula did not have an inhibitory effect on the production of these phenolic acids from cocoa over time ($p > 0.5$, $p > 0.05$) or at 24 hours.

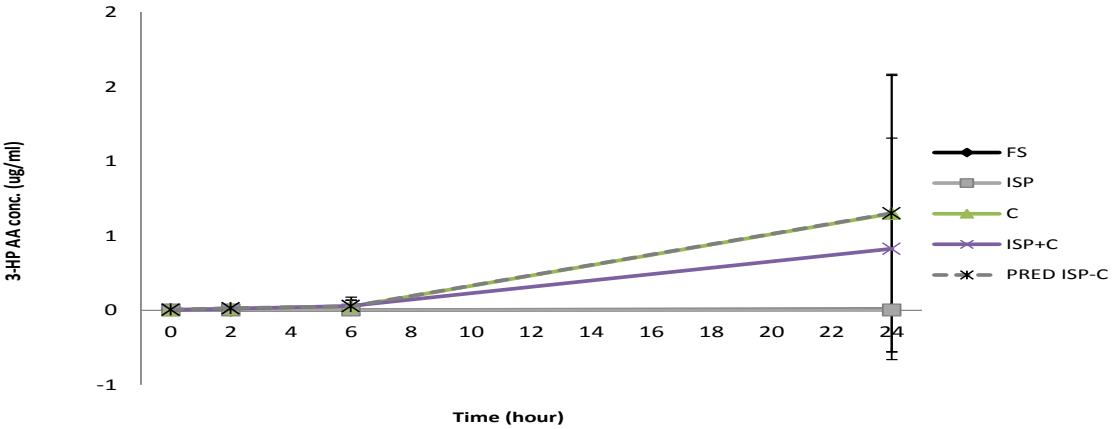


Figure 4-8 Impact of ispaghula on 3-HPAA production from cocoa with human faecal bacteria
Values are displayed as mean (± SD) of 24 hour profile for faecal incubations having 0.5g cocoa powder with 1g of ISP (n=10), FS: Faecal slurry, ISP: Ispaghula, C: Cocoa, ISP+C: Ispaghula+Cocoa. PRED ISP-C: predicted value for Ispaghula and cocoa combination.

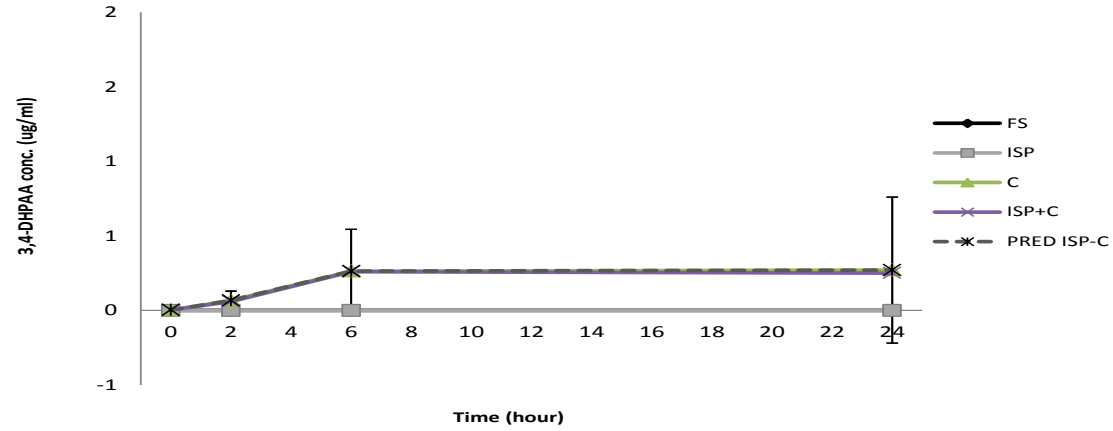


Figure 4-9 Impact of ispaghula on 3,4-DHPAA production from cocoa with human faecal bacteria
Values are displayed as mean ± STDEV (n=10), FS: Faecal slurry, ISP: Ispaghula, C: Cocoa, ISP+C: Ispaghula+Cocoa. PRED ISP-C: predicted value for Ispaghula and cocoa combination.

Similarly, 4-HPAA production from cocoa was not inhibited by ispaghula ($p > 0.05$). The presence of ispaghula in the cocoa incubations reduced the production of PAA at 24 hours post fermentation ($p < 0.01$, *Figure 4-10*). Initial analysis did not demonstrate the same impact on PAA across time ($p > 0.05$). However, the concentration of PAA detected in ispaghula only incubations was higher than the other two soluble fibres (2.27 ug/ml) resulting in a statistically significant difference over time between predicted value and the true combination ($p < 0.01$), indicating an inhibitory impact of ispaghula on PAA production over time ($p < 0.01$). However, the higher concentration of PAA in ispaghula vessels may not necessarily be due to its production from ispaghula, as the concentration of this phenolic acid is much higher in FS (4.39 ug/ml) than raftiline (0.90 ug/ml), pectin (0.50 ug/ml) and ispaghula (2.27 ug/ml). Thus it may be that raftiline and pectin inhibited the production of this phenolic acid from the background diet more successfully than ispaghula. 75% of PAA production was seen after 6 hours of incubation, indicating that some of this phenolic acid might be produced from further de-hydroxylation of other phenolic acids such as 4-HPAA.

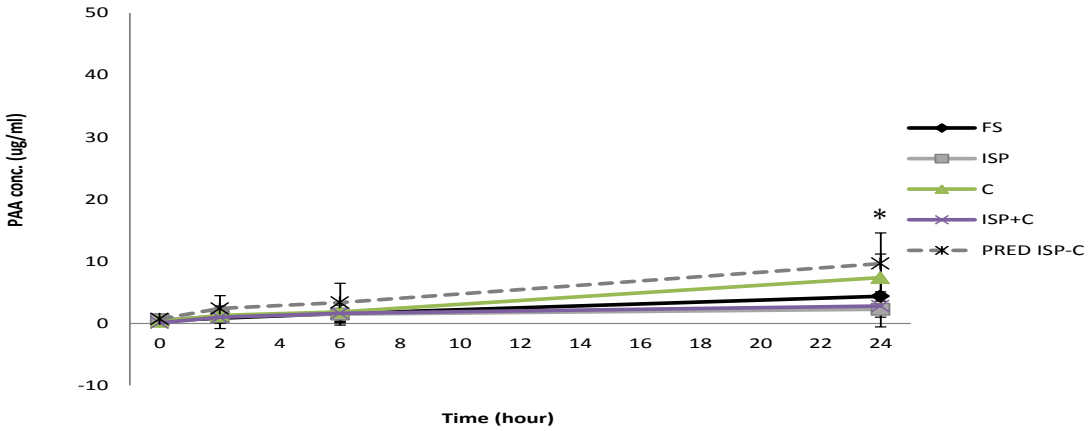


Figure 4-10 Impact of ispaghula on PAA production from cocoa with human faecal bacteria
Values are displayed as mean (± SD) of 24 hour profile for faecal incubations having 0.5g cocoa powder with 1g of ISP (n=10), FS: Faecal slurry, ISP: Ispaghula, C: Cocoa, ISP+C: Ispaghula+Cocoa. PRED ISP-C: predicted value for Ispaghula and cocoa combination. *$p < 0.01$

4-HBA and 3,4-DHBA were not detected in ispaghula only incubations and neither of the two phenolic acids were affected by the presence of ispaghula in cocoa incubation bottles. The combination of cocoa and ispaghula followed the same trend in 3,4-DHBA production as cocoa only incubations; increasing rapidly up to 6 hours post fermentation and declining thereafter.

3,4-DHPPA was not detected in ispaghula incubations; 3-HPPA and 4-HPPA were produced in both ispaghula and cocoa incubations. None of these three phenolic acids were affected by the presence of ispaghula in the fermentation bottles.

Similarly, hippuric acid and vanillic acid were not detected in ispaghula incubations and their production from cocoa was not affected in the presence of ispaghula.

The results seen for PAA were reflected in the sum of phenolic acid results as PAA was one of the highest contributors to the makeup of the total phenolic acids. Therefore, ispaghula had the highest concentration of total sum of phenolic acids compared to raftiline and pectin. This led to a significant difference across time between the predicted value and the true combination ($p < 0.01$, *Figure 4-11*). The comparison of Isp+C to cocoa alone did not show an inhibitory impact of ispaghula on total phenolic acid production from cocoa across time ($p > 0.05$). However a separate analysis at 24 hours for the same comparison groups resulted in a lower concentration in the combination group than cocoa alone ($p= 0.03$, *Figure 4-11*).

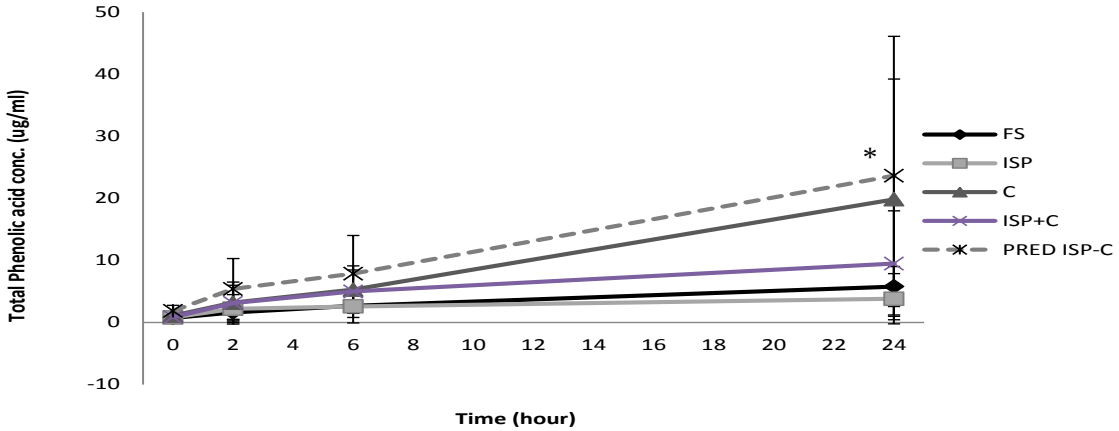


Figure 4-11 Impact of ispaghula on total sum of phenolic acid production from cocoa with human faecal bacteria
Values are displayed as mean (± SD) of 24 hour profile for faecal incubations having 0.5g cocoa powder with 1g of ISP (n=10), FS: Faecal slurry, ISP: Ispaghula, C: Cocoa, ISP+C: Ispaghula+Cocoa. PRED ISP-C: predicted value for Ispaghula and cocoa combination. *P= 0.031

Table 4-7 Impact of ispaghula on phenolic acid production from cocoa with human faecal bacteria

Substrate	ISP+C At 24h (ug/ml)	C At 24h (ug/ml)	P value
4-HBA	0.03 ± 0.08	0.02 ± 0.04	P > 0.05
3,4-DHBA	0.11 ± 0.15	0.08 ± 0.09	P > 0.05
3-HPPA	3.48 ± 6.64	8.73 ± 16.03	P > 0.05
4-HPPA	1.31 ± 1.71	1.74 ± 2.51	P > > 0.05
3,4-DHPPA	0.27 ± 0.44	0.08 ± 0.11	P > 0.05
Hippuric acid	0.01 ± 0.03	0.01 ± 0.04	P > 0.05
Vanillic acid	0.007 ± 0.02	0.009 ± 0.02	P > 0.05

Values are mean (± SD) at 24 hours (n=10) at 24 hours, C: Cocoa, ISP+C: Ispaghula+Cocoa, p-value is presented for the comparison of Ispaghula+Cocoa to cocoa alone.

4.4.3 Impact of pectin on phenolic acid production from cocoa

Pectin fermentation resulted in the production of 3-HPAA in 1 volunteer, however 3,4-DHPAA was not produced from pectin only incubations. Pectin did not have an inhibitory effect on the production of these phenolic acids over time. However, as seen for raftiline, the analysis at 24 hours demonstrates an inhibition of 3-HPAA production (p = 0.02, *Figure 4-12*).

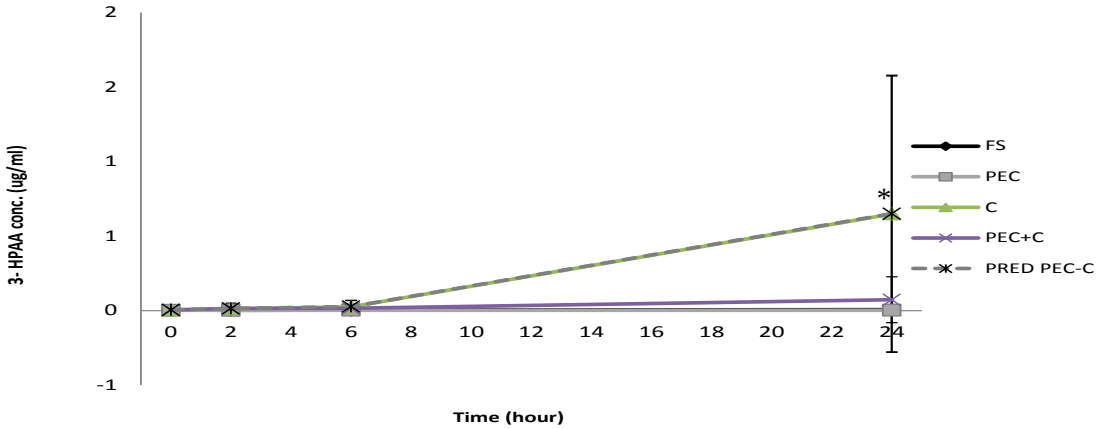


Figure 4-12 Impact of pectin on 3-HPAA production from cocoa with human faecal bacteria
Values are displayed as mean (± SD) of 24 hour profile for faecal incubations having 0.5g cocoa powder with 1g of PEC (n=10), FS: Faecal slurry, PEC: Pectin, C: Cocoa, PEC+C: Pectin+Cocoa. PRED PEC-C: predicted value for pectin and cocoa combination. *at 24h only p=0.02.

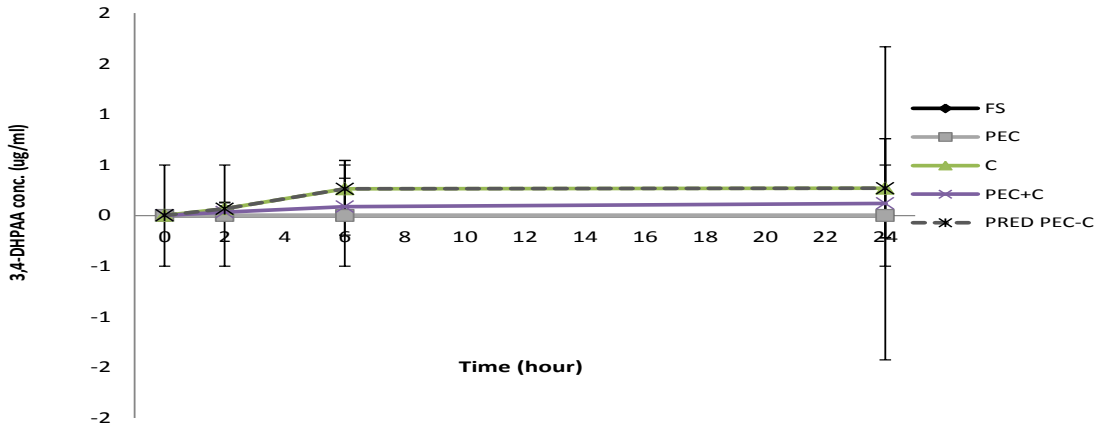


Figure 4-13 Impact of pectin on 3,4-DHPAA production from cocoa with human faecal bacteria
Values are displayed as mean (± SD) of 24 hour profile for faecal incubations having 0.5g cocoa powder with 1g of PEC (n=10), FS: Faecal slurry, PEC: Pectin, C: Cocoa, PEC+C: Pectin+Cocoa. PRED PEC-C: predicted value for pectin and cocoa combination

4-HPAA was detected in pectin only incubations as well as cocoa incubations, initial analysis did not demonstrate an inhibited of 4-HPPA production by pectin, however the comparison of predicted to true

value did demonstrate an inhibitory impact ($p < 0.01$). The presence of Pectin in the cocoa incubations reduced the production of PAA ($p=0.03$, *Figure 4-14*), the comparison of predicted to true combination group demonstrated an even greater inhibition ($p < 0.01$, *Figure 4-14*). The impact of pectin on the PAA production from the background diet was investigated as the background diet contributed largely to total PAA concentration. There was no impact from pectin on the PAA production from the background diet.

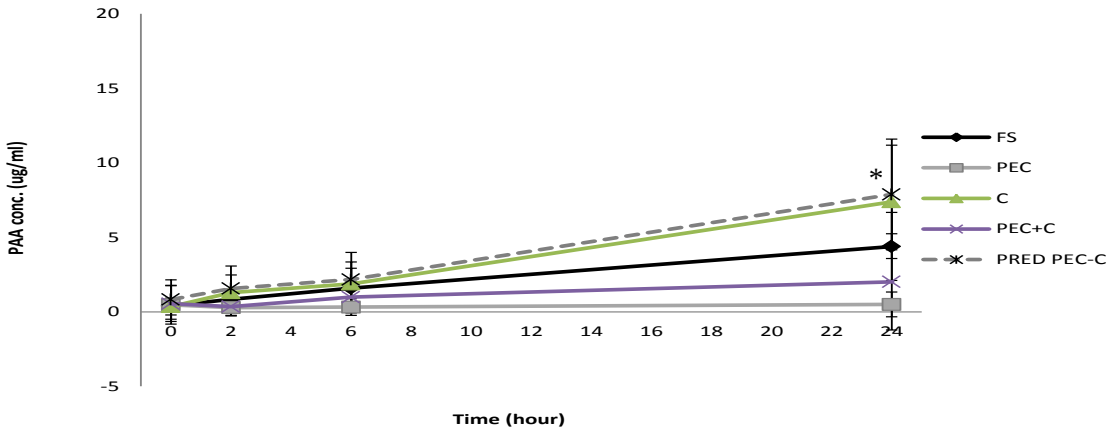


Figure 4-14 Impact of pectin on PAA production from cocoa with human faecal bacteria
Values are displayed as mean (± SD) of 24 hour profile for faecal incubations having 0.5g cocoa powder with 1g of PEC (n=10), FS: Faecal slurry, PEC: Pectin, C: Cocoa, PEC+C: Pectin+Cocoa. PRED PEC-C: predicted value for pectin and cocoa combination.*P= 0.03 (RAF+C vs. C) and $p < 0.01$ (RAF+C vs. PRED RAF-C).

4-HBA and 3,4-DHBA were not detected in pectin only incubations. Interestingly the presence of pectin in cocoa incubations resulted in a significantly higher production of 4-HBA across time as compared to cocoa only incubations ($p <0.01$). This increase was seen prominently 2 hours post fermentation. The concentration of 3,4-DHBA for the combination group was seen to increase up to 2 hours reaching a plateau thereafter, resulting in a significant difference between the PEC+C and C only at 6 hours post fermentation ($p=0.01$). This would suggest that pectin acts as a facilitating factor for the production of 4-HBA from 3,4-DHBA.

3,4-DHPPA was not detected in pectin only incubation bottles. In cocoa fermentation vessels it demonstrated a rapid increase up to 6 hours post fermentation after which it reached a plateau. 3-HPPA was produced only after 6 hours of incubation in both pectin and cocoa incubations. 4-HPPA was produced gradually over time in both pectin and cocoa incubations. Neither of these phenolic acids was affected by the presence of pectin in the fermentation bottles. Similarly the production of hippuric acid and vanillic acid were not affected by the presence of pectin. These phenolic acids were not detected in pectin only incubations.

The sum of total phenolic acid production was greatly inhibited by the presence of pectin in the incubation bottles across time (p = 0.01, *Figure 4-15*). A comparison of predicted to true combination values demonstrated an even greater inhibition (P < 0.01, *Figure 4-15*).

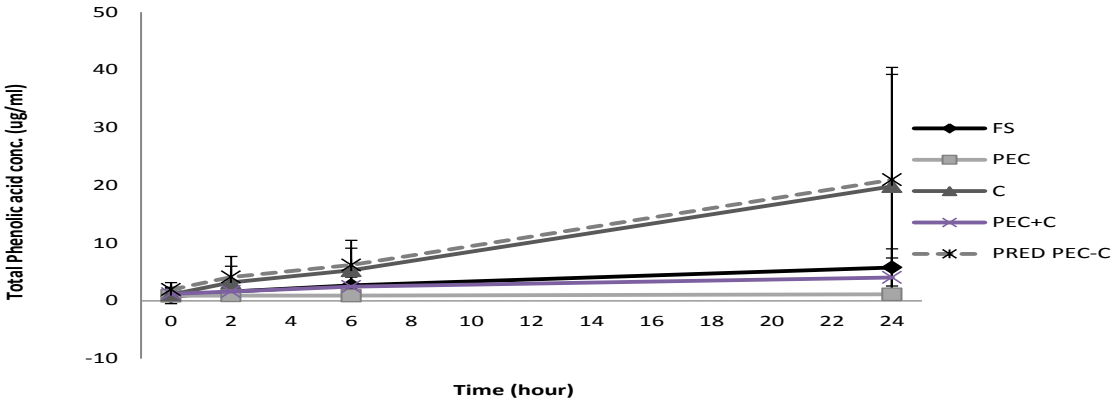


Figure 4-15 Impact of pectin on total sum of phenolic acid production from cocoa with human faecal bacteria
Values are displayed as mean (± SD) of 24 hour profile for faecal incubations having 0.5g cocoa powder with 1g of PEC (n=10), FS: Faecal slurry, PEC: Pectin, C: Cocoa, PEC+C: Pectin+Cocoa. PRED PEC-C: predicted value for pectin and cocoa combination*P= 0.01 (RAF+C vs. C) and p < 0.01 (RAF+C vs. PRED RAF-C).

Table 4-8 Impact of pectin on phenolic acid production from cocoa with human faecal bacteria

Substrate	Pec+C At 24h (ug/ml)	C At 24h (ug/ml)	P value
4-Hydroxy BA	0.07 ± 0.10	0.02 ± 0.04	P < 0.01*
3,4-Dihydroxy BA	0.08 ± 0.15	0.08 ± 0.09	P > 0.05
3-Hydroxy PPA	0.99 ± 0.95	8.73 ± 16.03	P > 0.05
4-Hydroxy PPA	0.39 ± 0.53	1.74 ± 2.51	P > 0.05
3,4-Dihydroxy PPA	0.00 ± 0.01	0.08 ± 0.11	P > 0.05
Hippuric acid	0.02 ± 0.04	0.01 ± 0.04	P > 0.05
Vanillic acid	0.01 ± 0.04	0.009 ± 0.02	P > 0.05

Values are mean (± SD) at 24 hours (n=10), C: Cocoa, PEC+C: Pectin+Cocoa, p-value is presented for the comparison of Pectin+Cocoa to cocoa alone.

4.4.4 Concentration of cocoa monomers retrieved as phenolic acids

To estimate the efficiency in the bacterial catabolism of cocoa polyphenols into phenolic acids, a percentage of total sum of phenolic acids produced was calculated as 3.1% of cocoa monomers added to fermentation vessels. This recovery is very low compared to that of rutin phenolic acids (70%- chapter 3); further indicating matrix interaction when polyphenols are present in carbohydrate containing foods

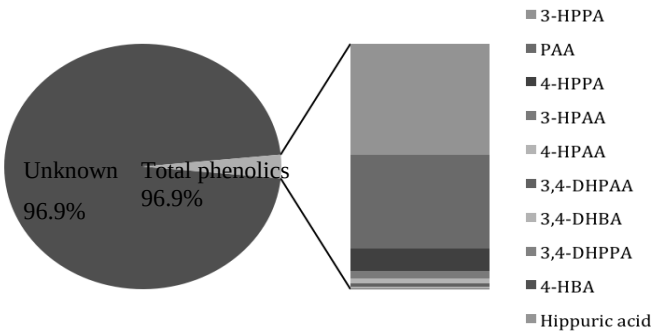


Figure 4-16 Concentration of 28 umol/ L of rutin retrieved as phenolic acid metabolites after faecal incubations with human colonic bacteria

4.4.5 SCFA production from cocoa

The carbohydrate and fibre content of the cocoa is considerably high. As this is an *ex-vivo* fermentation model, the starch and carbohydrate present in the cocoa was not subjected to digestion and was available for fermentation by the gut bacteria. Substantial amounts of SCFA were produced through the fermentation of these carbohydrates in cocoa. Acetate was produced as the highest percentage (50.1 %) to total sum of SCFA followed by propionate (18.3%) and butyrate (16.3%). However, this was lower than the SCFA production from the soluble fibre fermentation. The difference between SCFA production from fibre and cocoa was the most pronounced in the case of raftiline, followed by ispaghula and pectin.

Based on the composition of the cocoa powder, 0.5g of cocoa powder added to the incubation bottles contains 0.138g dietary fibre and 0.065g carbohydrates made up of 0.063g starch and 0.002g sugars compared to 1g of soluble fibre added to the incubation vessels. To correct for the fermentation of carbohydrates in cocoa, a predicted SCFA production was calculated as the sum of SCFA produced in cocoa alone to SCFA produced in fibre alone. This was then compared to the actual SCFA production of the mixture incubation. This allowed for the investigation of impact of cocoa polyphenols on the SCFA production from fibre. A difference between predicted SCFA and true SCFA value was considered as the inhibitory effect of the polyphenols on SCFA production.

Figure 4-17 and *Figure 4-18* present the contribution of each SCFA to total sum of SCFA production before and after deduction of FS values from samples. As seen in the figures, total sum of SCFA did not increase for cocoa after 6 hours of fermentation when FS values were deducted from the samples.

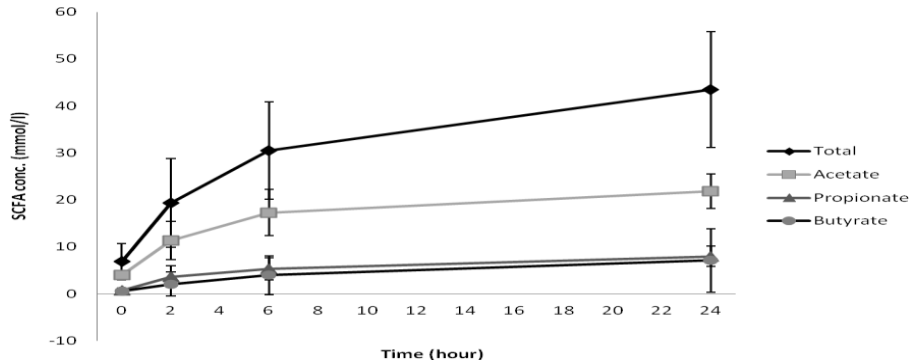


Figure 4-17 SCFA production from cocoa incubation with human faecal bacteria
Values are displayed as mean (± SD) of 24 hour profile for faecal incubations having 0.5g cocoa powder (n=10).

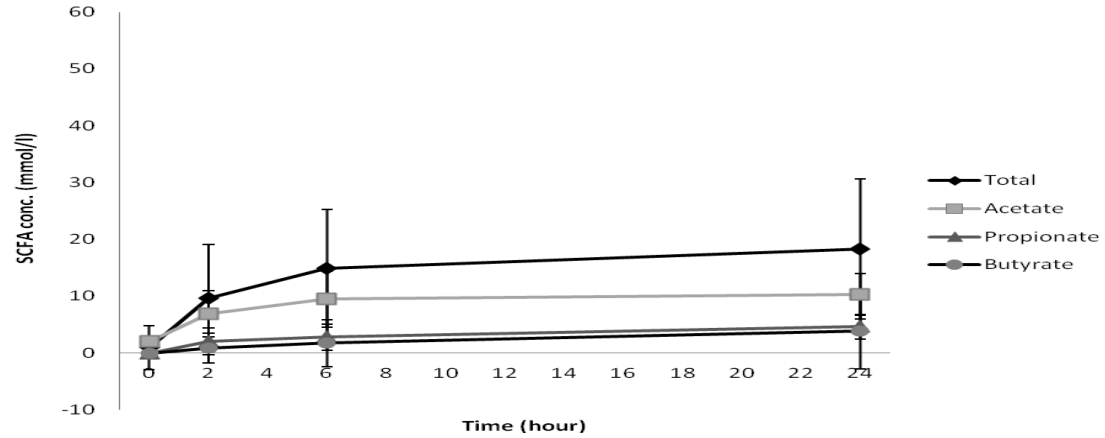


Figure 4-18 SCFA production from cocoa incubation with human faecal bacteria after deduction of FS
Values are displayed as mean (± SD) of 24 hour profile for faecal incubations having 0.5g cocoa powder (n=10).

4.4.6 Impact of cocoa polyphenols on the SCFA production from raftiline

An initial comparison of the predicted value to the true combination value for total SCFA concentration demonstrated an inhibitory effect from the cocoa polyphenols on the production of total SCFA ($p < 0.01$, *Figure 4-19*) However, this was due the additive effect of faecal slurry present in both cocoa and ispaghula, resulting in a higher value for the predicted value [(FS+C) + (FS+FIBRE)] as compared to the true combination value (FS+C+RAF).

The concentration of total sum of SCFA produced in faecal slurry was deducted from all comparison groups to correct for the additive effect of faecal slurry in the predicted value. This analysis was carried

out for all following results described in this chapter. After deduction of total sum of SCFA produced in faecal slurry from comparison groups, there no longer was an inhibitory effect of cocoa polyphenols on the total sum of SCFA production from raftiline.

The total sum of SCFA production demonstrated a rapid rate of increase up to 6 hours post fermentation for raftiline (68.5%) and pectin (62%); whereas majority of ispaghula fermentation took place between 6-24 hours (57%). The proportion of individual SCFA production from each fibre is displayed in *Table 4-9.*

Table 4-9 Proportion of each SCFA produced to total SCFA production for different fibres

Substrate	Acetate	Propionate	Butyrate
Raftiline	64.5%	18.1%	13.2%
Ispaghula	64.7%	27.2%	12.3%
Pectin	57.3%	15.6%	21.8%

The impact of cocoa on fermentation of fibres was further analysed by partitioning the volunteers into two categories of high and low producers of SCFA (*Table 4-10*) based on the average sum of all SCFA produced by the fermentation of fibres. The separate analysis of results for impact of cocoa on SCFA production of high producers and low producers did not demonstrate any impact of these polyphenols on the SCFA production from the fibres (*Table 4-10).*

Table 4-10 Total SCFA concentration for high producers of SCFA from raftiline fermentation with faecal bacteria

Substrate	Total SCFA concentration at 6 hours (mmol/l)	Total SCFA concentration at 24 hours (mmol/l)
Raftiline*	51.3 ± 17.4	77.7 ± 16.4
Raftiline + Cocoa	59.3± 15.8	88.2 ± 16.9
Predicted Raftiline + Cocoa	96.7 ± 14.6	70.1 ± 17.2
Ispaghula**	14.0 ± 6.2	50.1 ± 8.9
Ispaghula + Cocoa	24.3 ± 3.44	60.6 ± 21.6
Predicted Ispaghula + Cocoa	27.7 ± 11.3	69.4 ± 9.9
Pectin**	47.8 ± 8.6	84 ± 11.2
Pectin+ Cocoa	57.03 ± 14.6	89.8 ± 37.17
Predicted Pectin + Cocoa	70 ± 11.1	104.5 ± 10.7

Values are displayed as mean (± SD), at 6 and 24 hours for 50ml faecal incubations having 0.5g cocoa powder with 1g of fibre *(n=3), ** (n=5)

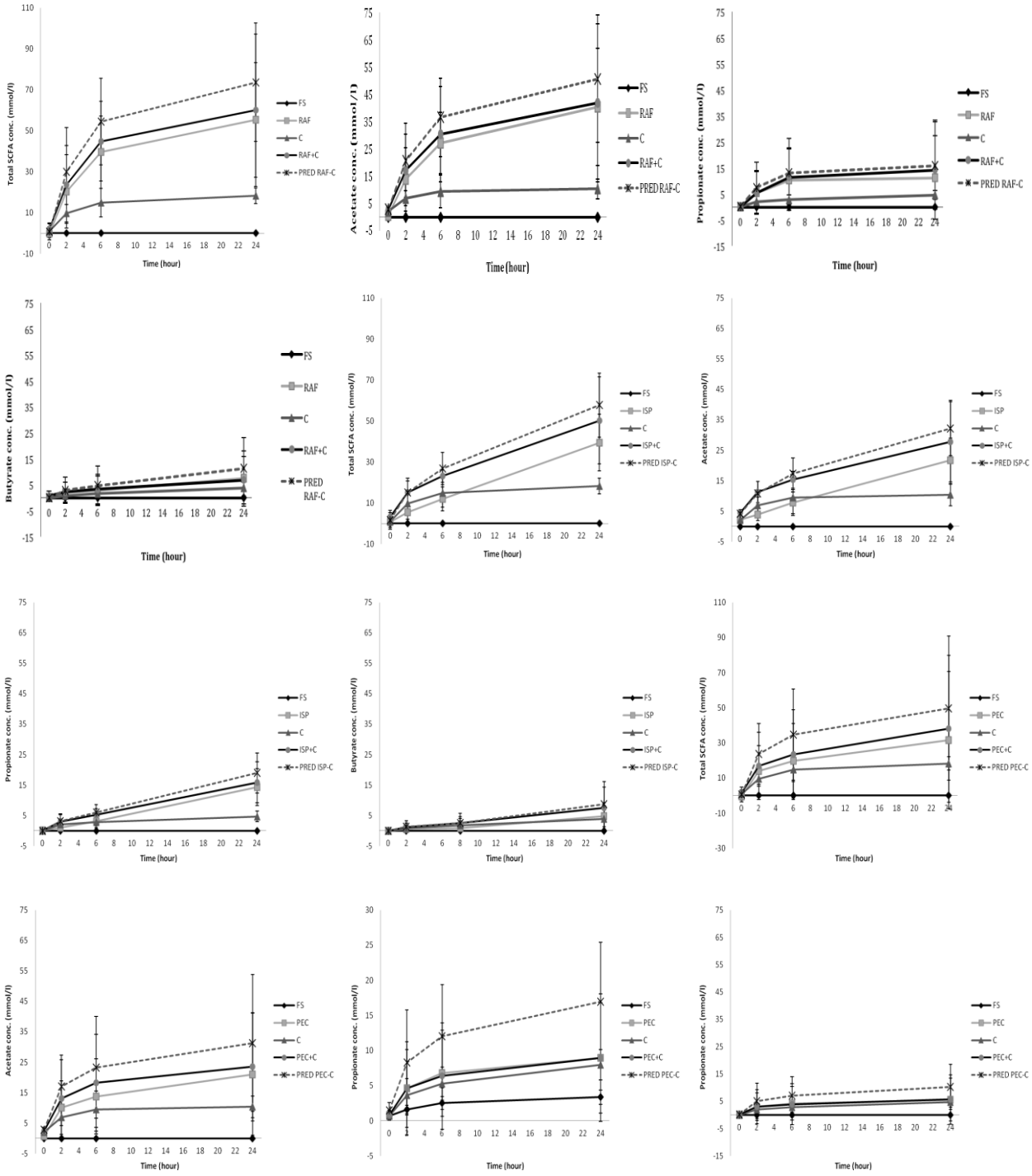


Figure 4-19 Impact of cocoa on SCFA production from fibre incubation with human faecal bacteria after deduction of FS. Values are displayed as mean (± SD) of 24 hour profile for 50ml faecal incubations having 0.5g cocoa powder with 1g of RAF / ISP /PEC (n=10), FS: Faecal slurry, C: Cocoa, RAF: Raftiline, RAF+C: Raftiline+Cocoa. PRED RAF-C: predicted value for Raftiline and cocoa combination, ISP: Ispaghula, ISP+C: Ispaghula+Cocoa. PRED ISP-C: predicted value for ispaghula and cocoa combination, PEC: Pectin, PEC+C: Pectin+Cocoa. PRED PEC-C: predicted value for pectin and cocoa combination.

4.4.7 Impact of cocoa on pH within fermentation vessels

Change in pH can be used as an indication of bacterial activity and fermentation rate in the batch culture model.

The pH of fermentation vessels was measured for all volunteers at all time points using a pH measuring paper according to the method mentioned in *2.2.2 Measurement of pH*. In addition to this, pH was measured using a pH meter according to the method in section *3.2.7* for all time points in 5 volunteers and for all volunteers at 6 and 24 hours.

4.4.7.1 Measurement of pH in fermentation vessels

Cocoa did not affect the pH in the fermentation vessels of raftiline or pectin, however the combination of cocoa and ispaghula resulted in a lower pH than ispaghula alone ($p < 0.01$). Additionally cocoa incubations resulted in a lower pH than FS alone ($p < 0.01$) incubations. Raftiline and pectin resulted in a lower pH than cocoa ($p < 0.01$), however there was no difference in pH between ispaghula and cocoa incubations.

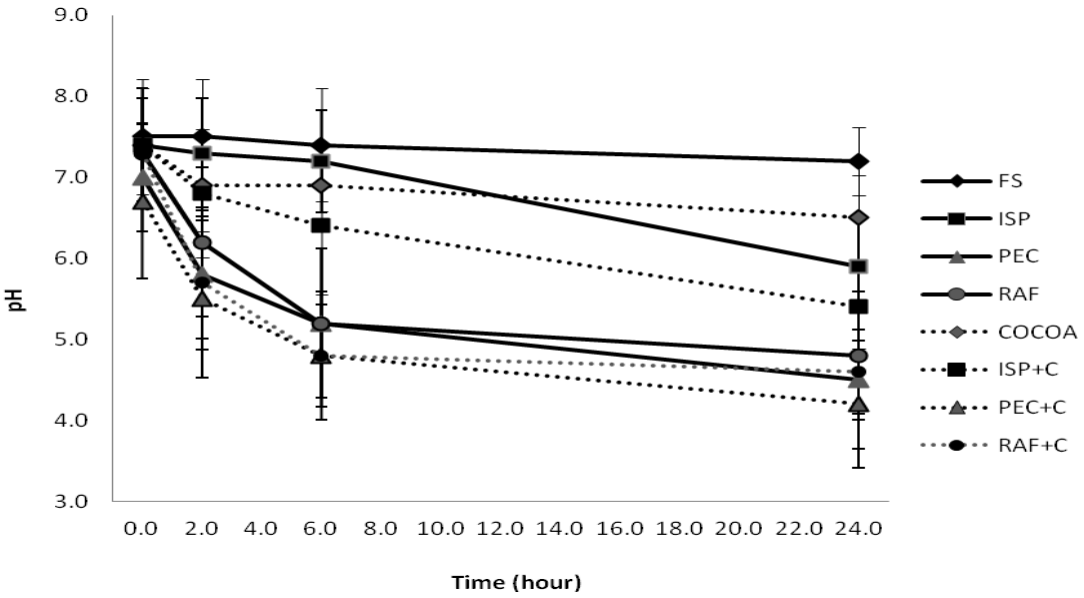


Figure 4-20 pH in batch culture vessels at all time points using pH paper
Values displayed as mean (± SD) of 24 hour profile for 50ml faecal incubations having 0.5 g cocoa powder with 1g of RAF/ ISP / PEC (n=10). FS: Faecal slurry ISP: Ispaghula, PEC: Pectin, RAF: Raftiline, C: Cocoa, ISP+C: Ispaghula+Cococa, PEC+C: Pectin+Cocoa, RAF+C: Raftiline+Cocoa

The pH of cocoa is seen to reduce between 0-2 hours, whereas this is seen after 6 hours of fermentation for ispaghula. Both raftiline and pectin caused a rapid reduction of pH up to 6 hours after fermentation after which little change was seen.

4.4.8 Impact cocoa on gas production

Gas in the head space of fermentation vessels was measured as an indication of bacterial activity and rate of fermentation. Cocoa did not impact gas production over time, however the combination of cocoa and pectin resulted in significantly higher gas at 24 hours than pectin alone ($p=0.03$). While pectin and raftiline resulted in higher gas production than cocoa ($p=0.02$ and $p < 0.01$respectively), there was no difference in gas production between cocoa and FS alone or ispaghula.

The data presented in this section is the mean cumulative gas production ± standard deviation over 24 hours post fermentation.

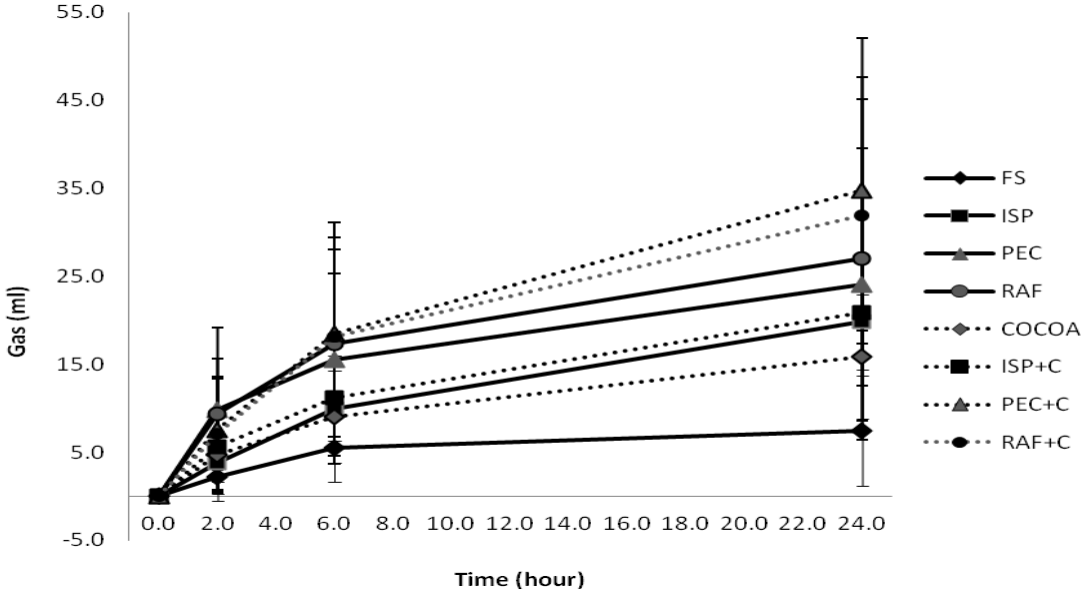


Figure 4-21 Cumulative gas productions in fermentation vessels
Values are displayed as mean (± SD) of 24 hour profile for 50ml faecal incubations having 0.5 g cocoa powder with 1g of RAF/ ISP / PEC (n=10), FS: Faecal slurry ISP: Ispaghula, PEC: Pectin, RAF: Raftiline, C: Cocoa, ISP+C: Ispaghula+Cococa, PEC+C: Pectin+Cocoa, RAF+C: Raftiline+Cocoa.

4.4.9 Summary of results

The comparison of the predicted value to the true combination value, demonstrated lower concentrations of acetate and total SCFA across time for all fibres, before deducting FS values from the samples. This was attributed to the additive effect of FS present in both cocoa and fibre when calculating the predicted value:

- Predicted value = [(**FS** + Cocoa) + (**FS** + Fibre)]
- True combination = (**FS** + Cocoa + Ispaghula)

Hence, FS values were deducted from the samples, allowing for the correct comparison of the combination group to the predicted value:

- Predicted value = (Cocoa) + (Fibre)
- True combination = (Cocoa + Ispaghula)

Cocoa polyphenols did not inhibit the SCFA production from the soluble fibre incubations with faecal bacteria.

3,4-DHPAA, 3,4-DHPPA and 3,4-DHBA were produced in cocoa only incubations and not in controls. They all increased up to 6 hours post fermentation, the former 2 reached a plateau thereafter, whereas 3,4-DHBA declined greatly after 6h. Concomitantly, 3-HPAA and 3-HPPA were produced only after 6 hours of incubation, suggesting that they were produced from further degradation of 3,4-DHPAA, 3,4-DHPPA respectively. An example of this can be seen in *Figure 4-22*.

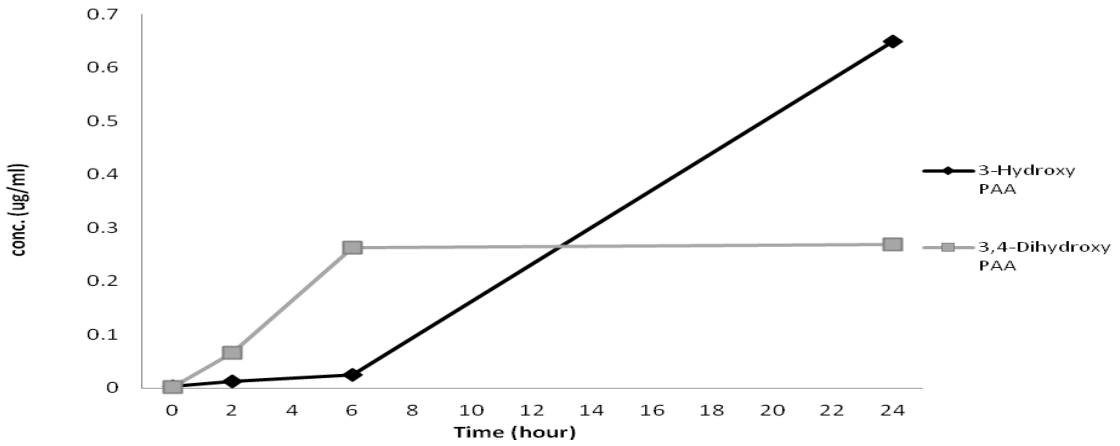


Figure 4-22 production of 3-HPAA from degradation of 3,4-DHPAA by human colonic microbiota
Values are displayed as mean (± SD) of 24 hour profile for 50ml faecal incubations having 0.5g cocoa powder.

Raftiline and pectin, both had an inhibitory impact on the production of 3-HPAA at 24 hours only and PAA and total phenolic acids across time. Pectin displayed a possible role in the conversion of 3,4-DHBA to 4-HBA. Ispaghula alone had the highest concentration of PAA and consequently the highest concentration of total phenolic acids, inhibiting the production of both when present in cocoa incubation bottles.

Table 4-11 Summary of soluble fibre inhibition of phenolic acids production from cocoa incubation with human faecal bacteria

Phenolic acids	Inhibition by raftiline	Inhibition by ispaghula	Inhibition by pectin
Phenyl acetic acid	x	x	x
3-Hydroxy phenyl acetic acid	x * @24 hours	-	x * @24 hours
4-hydroxy phenyl acetic acid	x	x	x
3,4- Dihydroxy phenyl acetic acid	-	-	-
3-Hydroxy phenyl propionic acid	-	-	-
4-Hydroxy phenyl propionic acid	-	-	-
3,4-Dihydroxy phenyl propionic acid	-	-	-
4-Hydroxy benzoic acid	-	-	x
3,4-Dihydroxy benzoic acid	-	-	-
Hippuric acid	-	-	-
Vanillic acid	-	-	-
Sum total of phenolic acids	x	x	x
Phenolic acids from background diet	x	x	x

4.5 Discussion

The *in-vitro* incubation of cocoa resulted in the production of PAA, 3-HPAA, 4-HPAA, 3,4-DHPAA, 3-HPPA, 4-HPPA, 3,4-DHPPA, 4-HBA, 3,4-DHBA, Hippuric acid and vanillic acid. Due to inter-individual variation in gut microbiota as previously presented by Gross et al (2010), the large standard deviations seen in metabolite production were expected. The paired model of the study design makes these large standard deviations irrelevant to the data presented in this chapter.

The recovery of phenolic acids as a product of cocoa polyphenol catabolism was found to be very low (3.1%) when compared to the phenolic acids recovered from rutin catabolism (70%, chapter-3). This may be attributed to the presence of carbohydrates and fibre in the cocoa powder, further indicating a matrix interaction when carbohydrates, especially fibres are present along with polyphenols.

To our best knowledge there have been no previous studies investigating the *ex-vivo* catabolism of cocoa polyphenols as present in the intact cocoa matrix. A study similar to that conducted in this chapter was the *in-vitro* fermentation of the insoluble fraction of cocoa by Fogliano et al. (2011). This study contained an additional digestion step following the centrifugation of cocoa powder along with water and subsequent disposal of the supernatant containing soluble fractions of the cocoa. All metabolites identified in this study (summarised in *Table 4-2*) were also detected in the current study. A more recent study (Dall'Asta et al., 2012) was conducted by adding 9% chocolate extract obtained from 90% dark chocolate to fermentation vessels. The two main metabolites identified in this study (3,4-DHPAA, 3,4-DHBA) were also detected in the current study. The above two mentioned studies used different analytical methods: LCMS and LC/MS/MS respectively compared to GCMS used in the current study. The three main metabolites present in at least two out of three above mentioned studies were found to be: 3,4-DHPAA, 3,4-DHBA (present in cocoa only incubations) and 3-HPPA (present in both control and cocoa incubations). In addition to these metabolites we identified eight more metabolites (*Table 4-2*).

Similarly to the study by Fogliano et al. (2011), we did not detect HBA which was identified in the Dall'Asta et al. (2012) study; likely produced from further dehydroxylation of 4-HBA (Fairley et al., 2002). The current study and the Fogliano et al. (2011) study were conducted using cocoa powder containing more carbohydrates than the chocolate extract used in the latter study. We speculate that the absence of HBA in the former two studies may be due to the high amount of carbohydrates present in the cocoa matrix delaying the production of this phenolic acid. As shown in the matrix interaction study by Bazzocco et al, the addition of apple cell-wall (made up of pectin) delayed the maximal

conversion extent from 4h to 8h. The mechanism behind this delay *in-vitro* has not been clearly understood, however a putative explanation could be the preferential use of carbohydrates as a source of energy by the microbiota (Tzounis et al., 2008a). It is possible that the fewer number of metabolites detected in the above two mentioned studies could be due to the loss of total flavan-3-ols and short chain flavan-3-ols through the process of digestion and water rinsing. Previous studies have demonstrated that shorter chain proanthocyanidins demonstrate significant loss of total and short chain flavan-3-ol content through water extraction and enzymatic digestions (Bazzocco et al., 2008).

However, the presence of hippuric acid and vanillic acid in the fermentation vessels was not expected as they are both hepatic metabolites derived from 3-HBA and 3,4-DHBA respectively (Monagas et al., 2010, Urpi-Sarda et al., 2010). Hippuric acid is mostly produced through the glycination of benzoic acid in the liver, whereas vanillic acid can be derived from the oxidation of vanillin often added to cocoa products by the aldehyde oxidase of the liver or arise from the methylation of protocatechuic acid (Urpi-Sarda et al., 2010). Some studies have demonstrated that specific bacteria, such as the *Pseudomonas fluorescens* AN1 03 may be able to oxidise vanillin to vanillic acid *in-vitro* (Narbad and Gasson, 1998). However, the cocoa powder used in our study did not contain any vanilla extract. In the current study, hippuric acid was seen at a higher level at 0 hours than at 24 hours and in very low concentrations. On the other hand vanillic acid was produced only after 2 hours of fermentation up to 6 hours and remained constant thereafter. Hippuric acid (volunteer 5 and 6) and vanillic acid (volunteer 5) were detected at a maximum concentration of 0.27 ug/ml 0.01 ug/ml respectively. These concentrations are extremely low compared to total sum of phenolic acids produced (19.84 ug/ml). Both phenolic acids were only detected in cocoa incubations. The lack of their presence in the FS alone and fibre alone incubations eliminates the background diet as the source. Unfortunately we do not have a concrete explanation for the presence of these metabolites in the cocoa faecal incubations.

The production of 3,4-DHPAA increased rapidly reaching a plateau concomitant with the production of 3-HPAA at 6 hours; suggesting that 3-HPAA was produced from further degradation of 3,4-DHPAA. As discussed in *Chapter-3,* this pattern was also observed in the *in-vitro* catabolism of rutin in both our study and a study conducted by (Aura et al., 2002).

The concentration of 4-HBA was constant from 0-24 hours, indicating that 4-HBA was not produced but present in the cocoa product. The concentration of 3,4-DHBA was lower at 6 hours for pectin and cocoa combination than cocoa alone ($p= 0.014$) reaching a plateau thereafter. The same combination demonstrated a higher concentration of 4-HBA after 6 hours, suggesting that pectin plays a role in the conversion of 3,4-DHBA to 4-HBA. This was not seen for ispaghula or raftiline.

Table 4-12 Phenolic acid production from in-vitro fermentation of cocoa or cocoa polyphenols by faecal bacteria

Phenolic acids	**Current study**	**Current study (GCMS)**	**Fogliano et al., 2011 (LCMS-**	**Dall'Asta et al., 2012 (LCMS)**	**Deprez et al 2000 (GCMS)**	**Bazzocco et al., 2008 (GCMS)**	**Appeldoorn et al 2009 (HPLC)**	**Tzounis et al., 2008 (HPLC)**	**Roowi et al., 2009 (GCMS)**	**Stoupi et al.,2010 (LCMS)**
	Main source of phenolic acid	0.5 g cocoa powder	20g insoluble cocoa fraction (pre-	90% dark chocolate extract	100-150 mmol procyanidins	Apple long/short proanthocyanidin chains	1% / 5% (w/v) procyanidin	150-1000 ug/ml catechin / epicatechin	50 umol epicatechin	5 mmol/l Epicatechin
PAA	FS, C	x								
3-HPAA	C	x	x		x	x	x		x	
4-HPAA	FS	x			x		x		x	
3,4-DHPAA	C	x		x		x	x			
4-HBA	C	x								
3,4-DHBA	C	x	x	x						
BA				x (HBA)		x				
Vanillic	C	x								
Hippuric	C	x								
3-PPA						x				
PPA					x			x		
3-HPPA	FS, **C**	x	x		x	x	x		x	x
4-HPPA	FS, C	x			x					
3,4-DHPPA	C	x				x				
1-(3,4-DHPl)-3-(2,4,6-THPl) propan-2-ol							x			x
1-(HP)-3-(2,4,6-THP)-propan-2-ol										x
Monohydroxylated phenylvalerolactone							x			
PVA							x			
5-P-valerolactone								x		
5-(3,4-DHP)-valeric acid							x		x	
5-(3,4-DHP)-valerolactone				x				x	x	x
3-HPValericA					x					

3-HPPA was only produced after 6 hours of incubation. As seen in the PAA derivatives, it can be postulated that the 3-HPPA is produced as a result of further degradation and de-hydroxylation of 3,4-DHPPA reaching a plateau at this time point.

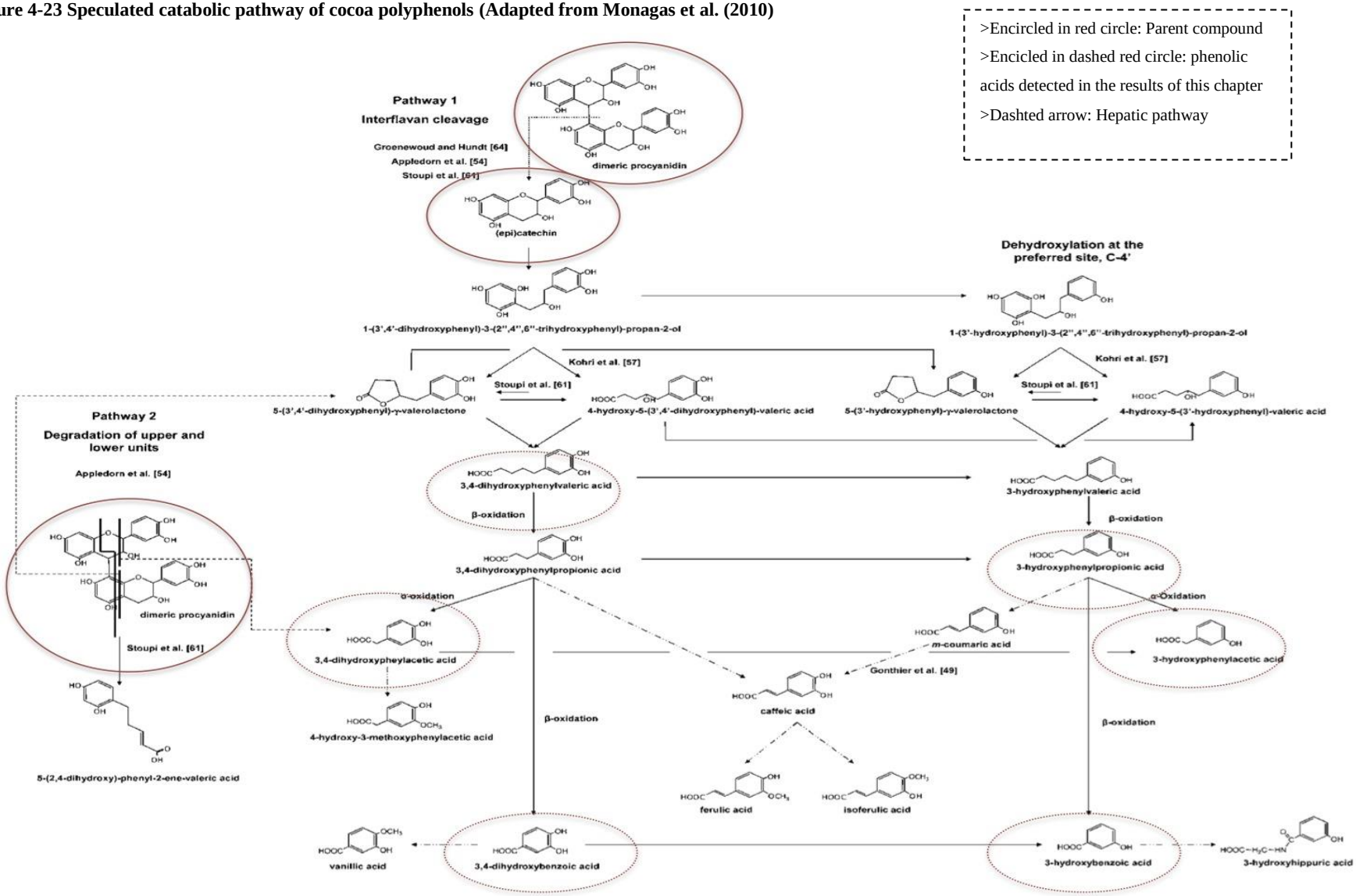
PAA was the second highest phenolic acid detected in cocoa incubations. The absence of this phenolic acid in the incubations of Fogliano. (2011) or Dall'Asta et al. (2012) has been discussed in *Chapter-3*. 59% of total PAA quantified in the cocoa samples was derived from the background diet and there was no difference across time. While raftiline and pectin inhibited the production of PAA across time from cocoa, they had no impact on the PAA production from the background diet. Ispaghula demonstrated a lower inhibitory impact than the other two fibres. A similar trend was seen for 4-HPPA, which was detected mainly due to its presence in the background diet. The total phenolic acid production was greatly inhibited by raftiline, ispaghula and pectin across time.

Our data demonstrated more colonic metabolite production than the previous studies. The lack of 5-(3,4-DHP)- γ-valerolactone was in agreement with the Fogliano et al. (2011) study, using insoluble fractions of cocoa powder and the studies using procyanidins in faecal incubations (Bazzocco et al., 2008, Déprez et al., 2000, Appeldoorn et al., 2009). Interestingly all three studies involving incubation of epi/catechins demonstrated 5-(3,4-DHP)- γ-valerolactone production. However it is important to note that compared to procyanidins, the percentage of ingested epi/catechins reaching the colon *in-vivo* is smaller. As suggested by Stoupi et al. (2010) and Fogliano et al. (2011), the longer incubation times used in their studies may have contributed to the consumption of 5-(3,4-DHP)- γ-valerolactone resulting in lower molecular weight phenolic acids. However this may not necessarily be valid as both studies have taken samples at different intervals from the baseline to the end point of incubation.

The difference in metabolite production between the different studies may be attributed to the various food matrices used: cocoa containing dietary fibre vs. cocoa extracts or pure polyphenolic compounds. The pre-treatments of cocoa such as digestion and water rinsing also contribute greatly to these observed differences. Despite the various *in-vitro* studies carried out, the exact catabolic pathway of proanthocyanidins and the monomers has not been well elucidated.

Figure 6-17 elucidates a compilation of speculated catabolic pathways of cocoa polyphenols by colonic microbiota, derived from various *in-vitro* studies and reviewed/compiled by Monagas et al. (2010). The compounds encircled in a red dash are common with the phenolic acids identified from *in-vitro* faecal incubation of cocoa in this chapter. However we did detect other phenolic acids, which are not displayed in this figure but discussed above.

Figure 4-23 Speculated catabolic pathway of cocoa polyphenols (Adapted from Monagas et al. (2010)



The limitation in understanding the catabolic pathway of these flavan-3-ol metabolites (summarised in *1.3.6.6 Flavan-3-ols*) may perhaps be due to the use of different analytical methods, the complexity of the colonic bacterial composition, and their interactions with colonic content including other species. Depending on the catabolic capacity of these microbiota it is possible that different pathways exist, with some more predominant than others. Unfortunately there is not sufficient information on the bacteria involved in the degradation of these flavan-3-ols. The understanding of this subject is limited by the antibacterial growth inhibitory properties of these phenolic compounds. The bacteria involved in the degradation of other flavonoid subclasses such as flavonols and flavones have been shown to belong to the Clostridium and Eubacterium groups (Monagas et al., 2010). However it has been demonstrated that the same bacterial groups may also be responsible for the degradation of epi/catechin compounds and the ring fission of flavan-3-ols (Selma et al., 2009, Vitaglione et al., 2007).

Some *in-vivo* work has demonstrated that *Eggerthella lenta* and *Flavonifractor plautii* are able to catalyse the degradation of both catechin and epicatechin (Kutschera et al., 2011). Others have also reported *Eubacterium sp. SDG-2* for the catabolism of (+) and (-)-catechins; *Clostridium cocoides - Eubacterium rectale group* for (-) and (+)-epicatechins and *Bifidobacterium spp* and *Escherichia coli* for (-)-epicatechin ring fission and catabolism of catechin and epicatechin (Wang et al., 2001, Tzounis et al., 2008b).

As previously discussed the composition of the microbiota on the catabolic pathway and the type of metabolites produced is crucial. However there have been no previous studies investigating the presence of soluble fibres and cocoa polyphenols on their subsequent impact on gut microbiota metabolite production. The study most relevant to this food matrix interaction was conducted by (Bazzocco et al., 2008) in an *in-vitro* faecal fermentation model, summarised in *Table 1-7* following enzymatic digestion of apple Proanthocyanidins with or without apple cell-walls (containing pectin). They demonstrated that when apple cell-wall was present in the incubation vessels the maximal extent of conversion was delayed from 4 hours to 8 hours. This delay did not result in an enhanced conversion efficiency of the proanthocyanidins compounds. Unlike the Bazzocco study (2008), we did not find any difference in time of maximal conversion for cocoa phenolics. However we did demonstrate an inhibitory impact of soluble fibres on phenolic acid production from cocoa.

This impact was more prominent in raftiline, followed by pectin and ispaghula. We did not measure viscosity in the incubation vessels of this study, thus we cannot say with conviction that this may be related to the viscosity of the fibres. However it is well known that raftiline is not viscous and ispaghula has great viscosity forming a gel upon mixture with water, which is maintained throughout the fermentation. We did measure the fermentability of these soluble fibres by measuring SCFA production from their fermentation. In this regard, raftiline demonstrated the highest fermentability,

followed by pectin and ispaghula. Thus it can be speculated that the inhibitory impact of the soluble fibres on the phenolic acid production from cocoa may be dependent on fermentability rate.

As discussed in *Chapter-1* these soluble fibres and their fermentation products have demonstrated bacterial growth enhancing properties. It is possible that the preferential use of raftiline, followed by pectin and ispaghula as a carbon source, resulting in relative SCFA production may be a factor to be considered in their inhibitory impact. In this regard we measured the pH in the incubation vessels, as higher concentrations of SCFA and their accumulation is associated with lower pH in incubation vessels. However the lowest pH was found to be in pectin incubation vessels followed by raftiline and ispaghula. The difference in pH between pectin and raftiline was not significant and the lower pH in pectin incubation vessels may be due to the high concentrations of galacturonic acid present in the pectin structure. The change in pH from baseline was found to be greater for raftiline than pectin.

Thus, the inhibitory impact of soluble fibres on the phenolic acid production from cocoa may be due to their SCFA production, which in turn may alter microbiota composition directly or indirectly through the reduction of pH. It is also possible that the higher fermentability may be related to the preferential use of the specific fibre as a source of energy resulting in a delay of phenolic acid production as seen in the Bazzocco study or inhibition of phenolic acid production in our study.

However as demonstrated by Bazzocco et al. (2008) pure Proanthocyanidins reached maximal extent of conversion at 2 hours compared to 6-8 hours when present in the apple matrix. In our study the cocoa contained substantial amounts of carbohydrates and fibre possibly further delaying the degradation of the phenolic compounds. As seen by total phenolic acid production in our findings, the production of these phenolic acids is continuously increasing, thus it would be of interest in future studies to analyse later time points beyond 24 hours until maximal conversion is reached, in order to investigate if the production of these phenolic acids is truly inhibited or delayed.

For the analysis of the impact of cocoa on SCFA production from fibres, we corrected for the carbohydrate content of cocoa and the additive effect of SCFA produced from FS (summarised in *4.4 Results*). The SCFA production from cocoa alone resulted in acetate as the highest SCFA followed by propionate and butyrate, produced almost in equal amounts. As expected, acetate was the dominating SCFA as a percentage of total SCFA production for all fibres, cocoa and FS. This is a contributing factor to the initial results seen for a higher acetate concentration in the predicted value as compared to the true combination value, also reflected in total SCFA concentration; which was not seen for propionate or butyrate.

These results are in line with the results seen in *Chapter-3*, demonstrating no impact of the polyphenols on SCFA production from the same soluble fibres. The majority of the volunteers (8 out of 10) in the cocoa fermentation study were common with that of the rutin fermentation study, resulting in the same faecal sample being used for both fermentation sets.

The fermentation products are an indication of bacterial activity and viability. However, studies investigating the impact of cocoa polyphenols (Tzounis et al., 2011, Lee et al., 2006) have demonstrated that these polyphenols can impact certain species of bacteria more than others.

As mentioned previously (*4.1 Introduction*), the cross-over study by (Tzounis et al., 2011) demonstrated a change in bacterial composition. However this modification appears to be transitory as there was no difference in bacterial composition between the baselines of the two arms in this crossover model. The return of bacterial composition to the baseline profile was also demonstrated in a long-term study by Smith and Mackie (2004).

It is also possible that the impact of cocoa on inhibition of pathogenic bacteria is indirectly related to the increase of *Lactobacillus* spp. and *Bifidobacterium*, as the increase in both species have previously demonstrated to inhibit the growth of pathogenic bacteria (Saulnier et al., 2009, O'Sullivan et al., 2002).

This change in bacterial composition may not necessarily be reflected in SCFA concentrations, as both Tzounis et al. (2011) and Massot- Cladera et al (2012) demonstrated that even though certain species of bacteria were promoted or inhibited, the total bacterial count was not affected by the cocoa polyphenols. Hence, it is not possible to derive from the results in this chapter that cocoa polyphenols did not exhibit any antibacterial activity.

However an inhibitory impact of apple PA extracts on SCFA production from apple cell walls was demonstrated by Bazzocco et al. (2008). This impact was no longer seen when PAs were present in the food matrix (whole apple). Apples having shorter chain PAs, exhibited an inhibitory impact on SCFA production from the cell walls both in the form of PA extract and when present in the food matrix. It was also shown that the longer chain PA extract had a greater inhibitory impact on the production of SCFA from the cell wall than shorter chain PA extract. It is difficult to conclude the extent of impact on SCFA production due to the lack of statistics on the above mentioned data. As previously emphasised, the type of polyphenolic compound and its constituting matrix play a key role on the ability of polyphephenols to produce pre/antibiotic properties.

Apple cell wall has a high level of pectin. The fermentation of this cell wall resulted in the same proportions of SCFA seen in our study for pectin fermentation. Similarly to the long chain proanthocyanidins present in the apple matrix, we did not see any inhibitory impact of the polyphenols present in the cocoa matrix on SCFA production from the fibres.

A more specific result of a similar study model (SHIM) was provided by Kemperman et al. (2013) investigating the impact of 1000 mg/day of tea extract polyphenols on the growth of gut microbiota. In addition to the antimicrobial activity seen by the tea extract, a reduction in butyrate concentration in the proximal colon was demonstrated (from ~15mM to ~10mM), which was attributed to the inhibition of butyrate producing bacteria. Towards the end of the study a drastic reduction in total SCFA (data not provided) and acetate (from ~30mM to ~22mM) concentration was demonstrated. This reduction in SCFA production corresponded to the reduction in bacterial numbers observed. Even though this *in-vitro* model is ideal for studying the longer-term impact of polyphenols on gut bacteria *in-vitro*, there is still the lack of other factors such as host-bacteria inter-relationship and presence of other influencing food matrix interactions.

Neither of the above studies investigating the impact of polyphenols on the colonic metabolite production have demonstrated the impact of these polyphenols while present in their constituting food matrix on the colonic metabolite production or taken into consideration the fate of the polyphenols such as their degradation to phenolic acids *in-vivo.* This is of great importance as previous studies have shown that phenolic acids produced from cocoa polyphenols may exert a stronger antibacterial impact than the parent compound itself (Lee et al., 2006).

Conclusion

Soluble fibres can inhibit phenolic acid production from polyphenols present in the cocoa matrix. This inhibitory impact may be related to their fermentability rate. However cocoa polyphenols and their metabolites have no impact on SCFA production from the soluble fibres.

CHAPTER 5

Food matrix interaction between ispaghula and cocoa *in-vivo*

In the previous chapter an inhibitory impact of soluble fibres on phenolic acid production from cocoa was demonstrated *in-vitro*. However the *in-vitro* study was designed to mimic the matrix interaction of soluble fibres on cocoa polyphenol bioavailability and metabolism only in the colon and did not provide any information on the impact of soluble fibres on the absorption of polyphenols in the small intestine. Such effects would alter the amount of polyphenolics absorbed intact but also the amount reaching the colon and available for bacterial catabolism. In this chapter, the impact of ispaghula on the appearance of phenolic acids in urine *in-vivo* was investigated to better understand the food matrix interaction of cocoa and fibre in both the upper and lower digestive tract. The phenolic acids in urine are influenced by the amount of polyphenolic compounds escaping the small intestine as well as the metabolic activity of the colonic bacteria both of which could be affected by the presence of a soluble dietary fibre.

5.1 Introduction

The polyphenolic content of cocoa and related possible health benefits were discussed in *chapter-1* and 6. It was noted that 20% of monomers and 90% of the polyphenolic oligomers from cocoa and foods with similar polyphenol profiles, escape absorption in the small intestine and enter the colon. The main products of polyphenol metabolism by the colonic microbiota are phenolic acids. The phenolic acids produced in vitro from the Cocoa used in this human study were presented and discussed in chapter 4.

The phenolic acids, produced in the colon *in-vivo* may be absorbed into the body, reaching tissues where they may exhibit beneficial effects (but there is very little data on any health effects of phenolic acids rather than their parent polyphenolic compound), be further metabolised by the liver and kidneys, through phase II metabolism, before being excreted in urine. Studies which have measured urinary phenolic acids from cocoa *in-vivo* are summarised in *Table 5-1*. Strong correlations have been reported between polyphenol consumption and urinary phenolic acid excretion (Mennen et al., 2006, Mennen et al., 2008).

The potential biological activity of the flavan-3-ol compounds in cocoa depend on their bioavailability in the body. Most publications investigating the bioavailability of cocoa polyphenols have focused on catechin, epicatechin and procyanidins. However, it is becoming evident that the health benefits associated with polyphenol rich foods is not due to the native polyphenol compounds present in foods but their metabolites produced through the microbiota (Del Rio et al., 2010).

Table 5-1 Phenolic acid production from cocoa polyphenols *in-vivo*

Author (year)	Study method	Phenolic acid production
(Rios et al., 2003)	N=11 healthy (7men, 4 women) cross over 80g of chocolate (439 mg proanthocyanidins and 147 mg catechin)	3-HPAA, 3,4-DHPAA, 3-HPPA, 3-HBA, Ferulic acid, Vanillic acid
(Mullen et., al 2009)	N=9 (6 male and 3 female) cross over 250ml of a cocoa [45 umol (-)epicatechin and (-) catechin]drink with hot water	3,4-DHPPA, 3,4-DHPAA, 3-Methoxy-4-hydroxy PAA, 3,HPAA, PAA, protocatechuic, 4-HBA, 3-HBA, 4-HPA, Hippuric, Vanillic, Caffeic, Ferulic, p-coumaric, m-coumaric
(Sarda et al., 2009)	N=42 (19 male, 23 female) cross over Long term study 20g cocoa *2 day +250ml skimmed milk for 4 weeks	3,4-DHPAA, 3-HPAA, 5-(3,4-DDHP)-γ – valerolactone, 3,4-DHPPA, 3-HPPA, PAA, Homo-vanillic acid, m-coumaric, p-coumaric, caffeic, ferulic, protocatechuic, Vanillic, 4-HBA, 3-HBA, 4-HHA, 3-HHA
(Llorach et al.,2009)	Acute- in-vivo randomised control crossover N=10 (5male, 5 female) 40g cocoa [0.71 ± 0.09 mg/g of (−)-epicatechin, 0.21 ± 0.01 mg/g (+)-catechin, 0.64 ± 0.06 mg/g of procyanidin B2] + 250ml water	5-(3,4-dihydroxyphenyl)- γ-Valerolactone sulphate, 5-(3,4-dihydroxyphenyl)- γ-Valerolactone glucuronide, *O*-Methylepicatechin, Epicatechin-O-Sulphate, 3-Methoxy-4-hydroxyphenylvalerolactone, 3-Methoxy-4-hydroxyphenylvalerolactone glucuronide, 4-hydroxy-5-(3,4-dihydroxyphenyl)-valeric acid, Vanillic acid

Production of phenolic acids from polyphenols increases their bioavailability as previously large molecular weight polyphenols not suitable for absorption can be absorbed as their lower molecular weight metabolites (Scalbert and Williamson, 2000). Thus it is important to consider factors influencing the gut microbiota such as antibiotics, prebiotics and other food components in the colon when assessing the bioavailability of polyphenols.

Polyphenol metabolite production *in-vivo* can be measured by comparing urinary excretion of these metabolites when the parent compound is consumed alone or in combination with dietary ingredients that influence the microbiota, such as fibre. Fibre may alter the gastric emptying, mouth-caecum transit time and colonic transit time; subsequently changing time of phenolic acid appearance in urine. Fibre may also act as a preferential source of energy for the bacteria thus delaying or inhibiting the catabolism of the polyphenols. The fermentation of fibres also results in the production of SCFA and subsequent fall in pH, which as previously mentioned may impact bacterial composition and their metabolites. Additionally viscous fibres may entrap the polyphenols thus reducing their absorption in the small intestine, and ultimately making more of these polyphenols available to the distal colon and possibly increasing their excretion (Detailed in *section 1.2.2* and *section 1.2.4*). In the *in-vitro* study in *Chapter 4* ispaghula had the least effect on phenolic acid production from Cocoa. However, *in-vivo*

ispaghula, which may increase the viscosity of the intestinal contents, may have a significant impact on the amount of polyphenolics entering the colon and also a prolonged effect on colonic metabolism potentially over several days rather than the 24 hours of the in vitro study.

Most studies investigating the food matrix interaction on the bioavailability of cocoa polyphenols have focused on milk, commonly consumed along with cocoa powder or present along with cocoa in the form of chocolate. One of the factors that can impact polyphenol bioavailability *in-vivo* is gastric emptying and mouth-caecum transit time which could be affected by the fat content of milk or cream, but there also may be some physic chemical interactions between the constituents of milk eg minerals or proteins and cocoa.

The results of these studies are contradictory and largely dependent on the amount of cocoa ingested and the polyphenol concentration of the cocoa used. A detailed description of these studies is provided in section 1.4 *Food matrix* on *page 74*, Briefly:

Mullen et al (2009) investigated the impact of milk on bioavailability of cocoa flavan-3-ols. They demonstrated an inhibitory effect of milk on the bioavailability of these flavan-3-ols using the commercially available Green and Blacks cocoa having 45 umol flavan-3-ol monomers. The previous studies by Roura et al. (2007) and Keogh et al. (2007) did not show any inhibitory impact of milk or milk proteins on the flavan-3-ol bioavailability of cocoa. These studies used cocoa containing 128 umol and 2374 umol flavan-3-ol monomers respectively, 3 and 25 fold the flavan-3-ols used in the Mullen et al. (2009) study.

The potential impact of changing gastric emptying and small bowel transit was seen in the study of Mullen et al. (2008b) who investigated the impact of cream on polyphenol bioavailability from strawberries. The cream delayed gastric emptying and small bowel transit time resulting in more than a one hour delay in T_{max} for pelargonidin-3-O-glucuronide without affecting total absorption of this compound and its urinary excretion. This was reflected in delay in urinary excretion of pelargonidin-3-O-glucuronide when strawberry was consumed with cream. Interestingly yoghurt consumption with orange juice had little impact on polyphenolic absorption, with no impact in transit times; Mullen et al (2008), however severely reduced urinary phenolic acid excretion by some unknown mechanism (Roowi et al 2009).

Although there are no published studies of the impact of fibre on urinary phenolic acid excretion it is well established that viscous fibres can influence upper gut transit times. Meal viscosity was related to delayed gastric emptying (Di Lorenzo et al., 1988, Marciani et al., 2000) and subsequent decrease in appetite (Di Lorenzo et al., 1988, Bergmann et al., 1992, Marciani et al., 2000). However there is great

inconsistency in the literature, which may be due to the use of different fibres and methods of fibre administration i.e. as a liquid, mixed with water or as solids such as fibre in cookies. Viscous fibres may slow gastric emptying when consumed along with a liquid meal but accelerate emptying when eaten with a solid meal (Mcrorie and Fahey, 2013). Of course fibres may influence phenolic acid production mostly by affecting colonic bacterial metabolisms as in our in vitro studies.

Despite this important potential impact of fibres on polyphenol bioavailability, most studies have only investigated the impact of simple carbohydrates such as glucose on their bioavailability *in-vivo.* Such studies (Detailed in section *1.4.1 on page 81*) have demonstrated that the presence of simple carbohydrates such as sucrose and bread can enhance the absorption of cocoa polyphenols from the small intestine and consequently increase the C_{max} and AUC of these compounds in plasma ($p<0.05$), also resulting in a faster rate of their elimination ($p<0.05$) (Schramm et al., 2003). There are several hypotheses explaining the increased polyphenolic absorption aided by simple carbohydrates such as sugar *in-vivo*. Some of these include controlled cellular flavanol absorption by SGLT1 transporters and lactase phlorizin hydrolase enzymes (Gee et al., 2000).

While simple sugars and prebiotics can enhance the deglycosylation of parent compounds reaching the colon and increase phenolic acid production, there is not enough evidence on the impact of more complex carbohydrates such as soluble fibres on phenolic acid production from polyphenols in the colon. These interactions have also been investigated *in-vitro* demonstrating enhanced catabolism of cocoa polyphenols and consequent increase in phenolic products by the gut microbiota in the presence of sucrose or fructo-oligosaccharides (Tzounis et al., 2008a).

In a study by Bazzocco et al. (2008), previously described in detail (chapter-1), cell-wall preparation containing pectin delayed the maximal extent of conversion of proanthocyanidins from 4 to 8 hours post incubation without affecting the total concentration of phenolic acid production. Additionally the maximum extent of conversion for apples was much later (6hours) than their proanthocyanidin extracts (2hours) with maximum conversion of proanthocyanidins occurring in cider samples having the shortest proanthocyanidin chains. However this study is based on an *in-vitro* fermentation model and does not provide any information on the impact of the cell-walls on the absorption and excretion of polyphenols *in-vivo.*

In this regard, the soluble fibre ispaghula, commercially available for purchase as a constipation remedy was desirable due to its high viscosity when mixed with water; maintained throughout the colon and the subsequent potential alteration of gastric emptying, mouth-caecum transit time, colonic transit time and other benefits in line with those seen from cocoa consumption. These properties can

potentially impact the absorption of polyphenols in the small intestine resulting in higher concentrations of the polyphenols reaching the colon and possibly higher amounts of polyphenols bound to the viscous mass being excreted through faeces.

Similar to all fibres, the data on the impact of ispaghula on gastric emptying is inconsistent and in need of standardisation of analytical method and placebo used. As demonstrated in *Table 5-2* ispaghula had no impact on gastric emptying or mouth-caecum transit time when consumed along with solid meals. When consumption of ispaghula added to a liquid lactulose meal was compared to lactulose only meal, a delay in gastric emptying attributed to ispaghula was demonstrated ($p<0.05$) with no impact on small bowel transit time.

Table 5-2 Impact of ispaghula on gatsric emptying and mouth-caecum transit time in humans

Author (year)	Study design	Analytical method	Phenolic acid production
(Bianchi and Capurso, 2002)	Standard meal (white bread,70 g egg the yolk of which was mixed with 100 mg of 13C octanoic acid and fried) with or without 5g of microcrystalline cellulose, guar gum or ispaghula	Breath $^{13}CO2$ measurements with IRMS	Ispaghula, microcrystalline cellulose and guar gum did not impact gastric emptying time or mouth-caecum transit time
(Rigaud et al., 1998)	doubly labelled solid±liquid meal (omelet which contain two eggs, 70 g white bread,10 g butter, 220 mL water) with 7.4g ispaghula or 6.9g placebo (gelatin)	gamma scintigraphy	Ispaghula had no impact on gastric emptying Ispaghula caused higher satiety and lower energy intake than placebo
(Frost et al., 2003)	50g of pasta was consumed with or without 1.7g ispaghula	Paracetamol absorption test	Ispaghula had no impact on gastric emptying
(Washington et al., 1998)	20ml radiolabeled lactulose (13.4g lactulose) x 3 times daily with or without 3.5g ispaghula Measurements were taken on the 5th day.	gamma scintigraphy and Breath hydrogen measurement	Ispaghula significantly delayed gastric emptying time ($p<0.05$) Ispaghula had no impact on mouth-caecum transit time Ispaghula delayed the rise in breath hydrogen
(McIntyre et al., 1997)	Radio labelled rice pudding was consumed with either 15g wheat bran or 7g of Fybrogel containing 3.5g of ispaghula or 15g plastic particles	gamma scintigraphy	Ispaghula, wheat bran and plastic particles had no impact on gastric emptying. Small bowel transit time was reduced by wheat bran and plastic particles but no impact from ispaghula.

Unlike rapidly fermented soluble fibres, the benefits of slowly fermented fibres such as ispaghula can be extended to the distal colon. Rapidly fermented fibres are predominantly fermented in the proximal colon and the health benefits derived through their fermentation might not extend to the distal colon. It

is more likely for all of the colon epithelium to be exposed to fermentation products when a slowly fermented fibre is used. This was demonstrated in a study by Edwards et al. (1992) where rats were fed a basal diet supplemented with either 5% ispaghula or 10% wheat bran for 28 days. The measurement of SCFA concentration in different parts of the colon demonstrated a higher propionate production than wheat bran which was maintained throughout the colon, whereas wheat bran resulted in higher butyrate production in the caecum; however this concentration was lower in the distal colon and faeces compared to ispaghula, indicating that ispaghula pushes fermentation and production of SCFA towards the distal colon. Furthermore the presence of ispaghula can alter the fermentation rate of other carbohydrates, shifting their fermentation site in the direction of the distal colon. This was demonstrated when rats were fed a diet of low-amylose or high-amylose (50 g/kg) with or without 15 g/kg of ispaghula. The high butyrate concentration seen from the high amylose diet reduced along the length of the colon reaching a third of its concentration in the caecum. When ispaghula was added to the diet butyrate concentrations were maintained along the large bowel with higher faecal butyrate excretions than the sum of low amylose + ispaghula and high amylose only diets (Morita et al., 1999). This is highly relevant as most disease occurs in the distal colon. In a similar manner ispaghula may lead to higher concentrations of polyphenolic compounds in the distal colon where they may have anti-carcinogenic properties.

Additionally the slower rate of fermentation of ispaghula results in less gas production and less discomfort in the abdomen as demonstrated by Bianchi and Capurso (2002) which may increase compliance in long-term interventions.

Ispaghula can also increase satiety (Karhunen et al., 2010) which may reduce food consumption and thus polyphenol consumption in long-term interventions. To assess the impact of ispaghula on satiety we measured subjective appetite using VAS. In this human study, we did not investigate SCFA production as the *in-vitro* study (Chapter 4) did not show any impact of cocoa on SCFA production from ispaghula.

5.1.1 Hypothesis

This study aimed to test the hypothesis that the soluble fibre ispaghula delays or inhibits phenolic acid production from cocoa polyphenols through viscosity and fermentation related mechanisms.

5.2 Subjects and Methods

5.2.1 Participants

No previous studies have investigated the combination of ispaghula and cocoa, thus power calculations suitable for these specific outcomes cannot be performed, however based on a study by Mullen et al (2009a) investigating the food matrix interactions of milk and cocoa on polyphenol bioavailability; nine healthy volunteers were sufficient to observe a statistically significant reduction in 24h phenolic acid (flavan-3-ol) urinary excretion between the two food matrixes (3300 nmol/d) and SD (2000 nmol/d).

Healthy European male volunteers (n=12) with a mean (± SD) BMI of 23 ± 2.5 (range, 19.8 to 27.4) and a mean (± SD) of 23 ± 3.8 years (range, 21 to 31) were recruited through poster advertisements (*Appendix-4*) and word of mouth to allow for a 33% drop out rate. Volunteers with a BMI below 18 or over 29.9 and aged less than 20 years or above 40 years were excluded. Volunteers were given study information (*Appendix-6*), dietary record sheets (*Appendix-2*), appointment cards and urine collection kits at the first meeting. The study protocol was approved by the University of Glasgow, Faculty of Medicine Ethics Committee for Non Clinical Research Involving Human Subjects (Project No: FM08119). All subjects gave written informed consent.

5.2.2 Study design

The study design was adapted from Mullen et al (2009a). The study design was one that reduced the diversity in the study population and allowed for better comparison of outcomes. The cross-over model eliminated the impact of inter-individual variation in microbiota composition and subsequent metabolite production and a homogenous population was included to reduce inter-individual variations. The study was an acute randomised control cross over study using three meals:

- 15g of ispaghula (whole ispaghula husk by MYProtein).
- 20g of cocoa (*extra brute Cocoa-Cacao Barry, Barry Callebaut, Hardricourt, France*).
- 15g of ispaghula and 20g of cocoa powder.

The composition of the Barry Callebaut cocoa powder used in this study and a comparison to the more well-known Green and Blacks Cocoa powder *(Green and Black's Ltd, London, United Kingdom)* is given in *Table 5-3.*

Table 5-3 Composition of cocoa powder used with comparison to well-known *Green and Blacks* cocoa powder

Nutritional data/100g	Extra brute Barry callebaut	Organic dark cocoa Green & Blacks
Typical Composition	100% cocoa powder	16.3%
Energy	339 Kcal	350 Kcal
Available Carbohydrate	13.0g	13.6
• Sugars (mono+Disaccharides)	0.4g	0.4
• Starch	12.6g	Not specified
Dietary Fibre	27.7g	28.7
Total Protein	18.0g	23.6
Milk Protein	0.0g	Not specified
Total Fat	23.0g	22.3
Saturated Fat	14.5g	13
Vanilla extract	×	✓
Soya Lecithin-Emulsifier	×	✓

This information was kindly provided by the customer service and quality control administration unit of respective brands.

volunteers were randomised to one of six randomisation patterns: 1- ispaghula+cocoa, ispaghula, cocoa. 2- ispaghula+cocoa, cocoa, ispaghula. 3-ispaghula, cocoa, ispaghula+cocoa. 4- ispaghula, ispaghula+cocoa, cocoa. 5- cocoa, ispaghula, ispaghula+cocoa. 6- cocoa , ispaghula+cocoa, ispaghula.

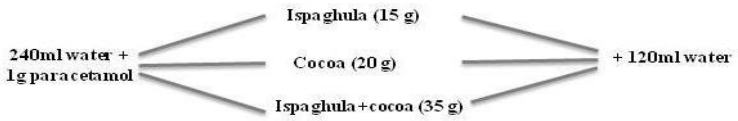


Figure 5-1 Intervention substrates in cocoa in-vivo study

All volunteers were asked to follow a low polyphenol diet, which excluded fruits, vegetable and polyphenol containing beverages such as fruit drinks, tea, coffee, cocoa drinks, and wine (Appendix-1) for 3 days prior to the study to reduce baseline urinary phenolic acid concentration. Volunteers maintained a food diary to determine compliance to the diet. They visited the Human Nutrition unit on

the 4th day after a 10 hour fast with no restrictions on water consumption.

Upon arrival at the unit, anthropometric measurements were taken *(height, weight, waist/ hip circumference and body composition)* followed by fasting blood, urine and breath hydrogen samples. Volunteers completed a visual analogue scale (VAS) for the subjective assessment of appetite at baseline. They then consumed the supplement along with 240ml of water followed by 120ml of water to rinse any remaining supplement from the glass and 1g of paracetamol for estimation of gastric emptying rate. Urine (***5.2.5***), blood (***5.2.4***), breath hydrogen (***5.2.13***) and completed VAS questionnaires (***5.2.12***) were collected (*Figure 5-2*). Four hours after the test meal, volunteers were fed a low-polyphenol and low-fibre meal of chicken sandwich made of white bun, 2 layers of thin sliced chicken and 1 slice of low fat cheddar cheese. Collection of VAS questionnaires, blood and breath hydrogen ceased at 6 hours and volunteers left the unit with urine collection kits to collect to 24 hours. Volunteers remained on the low polyphenol diet until the completion of the 24-hour urine sample collection.

There was a two week washout period between different arms of the study, and volunteers were asked to return to the unit and the study was carried out under identical circumstances for all arms.

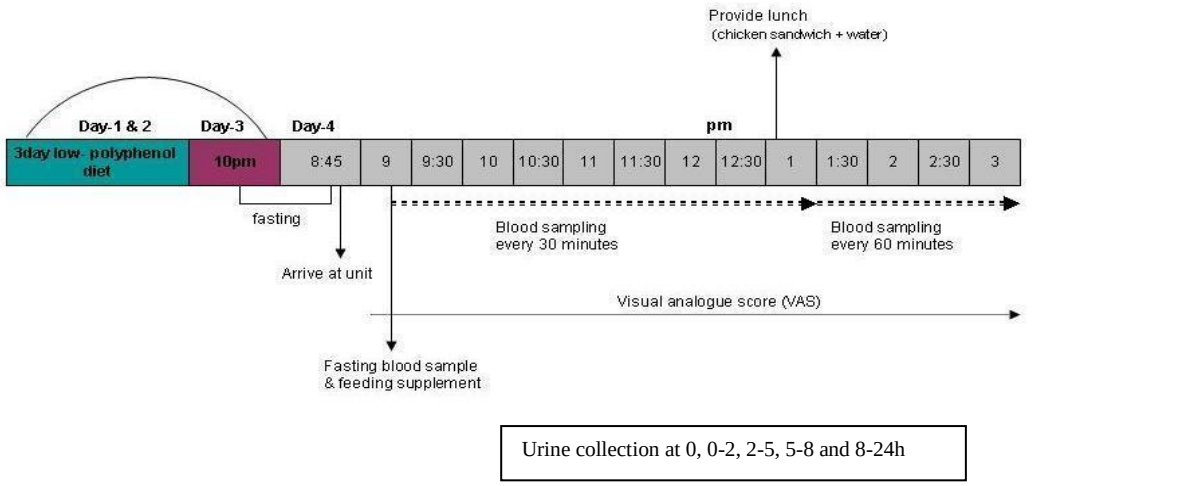


Figure 5-2 *In-vivo* study design for the matrix interaction of cocoa polyphenols and ispaghula.

5.2.3 Anthropometric measurements:

Anthropometric measurements were taken at every visit (total of 3 times) to ensure no significant change in weight. Volunteers were asked to remove shoes, socks and jewellery and to empty their pockets prior to measurements. Height was measured in centimetres using a stadiometer with volunteers standing straight having their back to the stadiometer, their head positioned to look straight forward and hands to their side. Body composition measurements were done using bioimpedance in duplicate for weight, body fat %, total fat mass, fat-free mass and total body water with the volunteers using a *TBF-300, TANITA, Cranlea UK*. Water consumption was restricted (no more than 240ml of water) before body composition measurement. Waist circumference was measured midway between the lowest point of the costal margin and highest point of the iliac crest in the standing position and hip circumference was measured at the largest portion of the hip using a non-stretch tape measure *(Waistwatcher, Kunzte Design, Germany)*.

5.2.4 Blood samples collection:

After completion of anthropometric measurement, volunteers were asked to rest on a bed. A venous cannula was inserted in the non-domimant arm. 12ml of blood was collected into EDTA tubes at baseline and every half hour for 4 hours and at the 6th hour. Saline was used to flush the cannula every half hour or more frequently when necessary to prevent any clots from forming.

For the measurement of paracetamol, insulin, glucose and polyphenols; blood was centrifuged at 3000g at 4°C for 15 minutes, plasma was separated and aliquoted. For the analysis of CCK/PYY; Aprotonin (80 ul) was added to 1ml of blood, vortexed and centrifuged at 1400g for 4 minutes. All aliquots were stored at -80°C.

5.2.5 Urine sample collection:

Urine samples were collected at baseline and for 24 hours after test meal ingestion. The 24 hour urine sample was collected in 4 batches of 0-2h, 2-5h, 5-8h, 8-24h. Urine samples collection took place in the unit up to 6 hours post supplement ingestion, after which volunteers were issued with multiple ice packs in cool bags and spare collection containers to take home. 5-8h and 8-24h urine samples were returned to the unit by 9:30am the following morning. Collected urine samples were weighed immediately after collection, mixed well and aliquoted (2ml × 8) and stored at -80°C for later analysis of phenolic acids, total phenol and total antioxidant capacity.

5.2.6 Four day food records:

Volunteers maintained a food diary to check for compliance and for better replication of the same diet for all arms of the study (Appendix-2).

5.2.7 Phenolic acid measurement

Phenolic acids were measured in duplicate in urine samples using the Phenolic acid analysis method detailed in *CHAPTER 2.*

5.2.8 Total phenol

The Folin & Ciocateau method (Singleton et al., 1999) was used to determine total phenols and other easily oxidised substances in the urine and plasma samples.

For the preparation of the Folins reagent (1:10), 100 ml Folin & Ciocateau reagent was added to 900ml of distilled water. A standard calibration curve ranging from 50 – 500 ug/ml in methanol was prepared using 50ug/ ml gallic acid stock (0, 25, 50, 100, 200, 300, 400, 500 ug/ml). 100ul of the prepared Folin reagent was added to 20ul of sample or standard in a 96 well plate, followed by 70ul of distilled water using a multi-channel pipette. After 5 minutes of incubation 70ul of sodium carbonate solution prepared by dissolving 115g of sodium carbonate in 1L of distilled water was added to the plate. The plate was sealed with the Mylar plate sealer *(Fisher scientific)* and left to incubate for 2 hours. The plate was read at an absorbance of 765nm using a spectrometer *(Thermo scientific multiskan spectrum)*

5.2.8.1 Total phenol analysis of free and bound phenolics (method1)

This method was adapted from Holtekjølen et al (2008). Ten ml cold (4 °C) 60% acetone was added to 200mg of cocoa or ispaghula. The mixture was shaken for 10 minutes at room temperature, followed by centrifugation (2800 rpm) for 10 minutes. The supernatant was used for measuring free phenolic acids. 2M NaOH (10ml) was added to the pellet and left overnight. This allowed the release of esterified (bound) compounds. The mixture was then acidified to pH 1.45-1.55 using 6M HCL. The released phenolic compounds were extracted with 10ml ethyl acetate ×4. The pooled extractions were

then concentrated to dryness and resolved in 10ml of DMSO. This was then analysed for bound phenolics quantification using the total phenol method.

5.2.8.2 Total phenol analysis for free phenolics (method2)

This method was adapted from Thondre et al. (2011) and is similar to the first step of method 1. Four ml of 70% acetone was added to 200mg of cocoa or ispaghula and shaken (2h) at room temperature. The extracts were centrifuged at 3000g for 10minutes. The supernatant was used in accordance to the method detailed in section *5.2.8* for the determination of free phenolic acids in cocoa and ispaghula.

5.2.9 FRAP

The ferric ion reducing antioxidant power assay was conducted for the measurement of total antioxidant capacity of both urine and plasma. This assay is based on the ability of the antioxidant to reduce the oxidant. in this context antioxidant are considered as reductants in a redox-linked colorimetric method. This assay measures the ferric reducing ability of plasma/ urine. The reduction of the ferric-tripyridyltriazine (Fe III-TPTZ) to ferrous (Fe II) at a low pH results in an intense blue colour with a maximum absorption at 593 nm (Benzie and Strain, 1996).

For the preparation of the FRAP reagent, 10ml of 20mM Ferric chloride hexahydrate (0.54g ferrous chloride in 100ml distilled water) was added to 10ml of 10mM 2,4,6-tripyridyl-s-triazine (TPTZ) solution (0.03 TPTZ in 10ml 0f 40mM hydrochloric acid). This was then added to 100ml of acetate buffer, PH 3.6 (3.1g sodium acetate and 16ml acetic acid in 1 litre distilled water).

The standard calibration curve was prepared by serial dilutions of Fe^{2+} (0.0278g ferrous sulphate heptahydrate, in 100ml distilled water). A range of 0.1mM – 1.0 mM Fe^{2+} was used for preparing the calibration curve (0, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 mM Fe^{2+}). 25ul of standard or sample was added to the 96 well plate in triplicate, Followed by the addition of 225ul FRAP reagent. Four minutes of incubation was allowed before reading the plate at 593nm using a spectrometer *(Thermo scientific multiskan spectrum).* This assay was repeated with different dilutions of urine in order to achieve more accurate results. This ensured that the concentrations obtained from urine samples were below that of the most concentrated standard. For the purpose of this assay, a 1:4 dilution (5ul urine, 15ul distilled water) was used for the urine samples.

5.2.10 Analysis of cocoa

To quantify the concentration of catechin/epicatechin and procyanidins in the cocoa used in our study we adapted a method by Robbins et al (2009). Fat was extracted in triplicates, by adding 30ml n-hexane to 1g of cocoa powder. This was vortexed for 1 minute and sonicated for 10 minutes at 38°C, followed by centrifugation at 3000 g. The supernatant was discarded and the pellet was dried with nitrogen and left overnight to ensure complete drying. 50mg of dried sample was extracted in triplicate with 2.5ml of acetone/water/acetic acid (70:28:2), vortexed for 1 minute, sonicated for 10 minutes at 38°C and centrifuged at 3000 g. The supernatants were pooled, diluted in the following solvents (1:3) and filtered. Solvent A: acetonitrile/acetic acid (98:2) and solvent B: methanol/ water/ acetic acid (95:3:2). The linear gradient for the determination of compounds was [mobile phase B, time (min)]: (7%, 3), (37%, 3-57), (100%, 57-60), (100%, 60-67), (7%, 67-73), (7%, 73-83) in a Surveyor HPLC system comprising a HPLC pump, a PDA detector (*Thermo Fisher Scientific*).

The extraction of fat with n-hexene resulted in a high loss of catechin/epicatechin giving very low concentrations recovered from cocoa. Hence, we repeated the extraction of cocoa catechin/epicatechin and procyanidins adapting the method by Mullen et al.,(2009b). For this method 5ml of methanol or water was added to 1g of cocoa. This was vortexed for 2 minutes and centrifuged at 3000g for 10 minutes in triplicate. The supernatants were pooled. 1 ml of the supernatant was further centrifuged at 3000g to ensure clean injection. Standards for calibration curve were prepared as 50, 25, 12.5, 2.5, 0.5 ng/ul for catechin and 800, 400, 200, 40, 8 and 0 ng/ul for epicatechin. Dimers, trimers and tetramers were quantified as catechin equivalents.

5ul of standards or cocoa extracts were analysed on a Surveyor HPLC system comprising a HPLC pump, a PDA detector, scanning from 250 to 600 nm, and an autosampler cooled to 6 °C (Thermo Fisher Scientific). Analysis were carried out at 40 °C using a 100 × 2.1 mm i.d., 4 μmm Hypersil Gold column (Thermo Scientific) eluted with a 20 min gradient of 5-90% acetonitrile (solvent A), in 0.1% aqueous formic acid (solvent B), at a flow rate of 300 μl/min. The linear gradient for the determination of compounds was [% mobile phase B, time (min)]: (95%, 0-1), (70%, 1-7), (50%, 7-10), (10% 10-10.10), (10%, 10.10-15.0), (95%, 15-15.10), (95% 15.10-20). After passing through the flow cell of the PDA detector, the column eluate was directed to an LCQ Advantage ion trap mass spectrometer fitted with an electrospray interface (Thermo Fisher Scientific). Analyses utilised the negative ion mode. Samples were analysed in the mass spectrometer using selected reaction monitoring (SRM) scanning for fragments of the *m*/*z* 289 parent ion from *m*/*z* 150 to *m*/*z* 449. Capillary temperature was 300 °C, sheath gas and auxiliary gas were 30 and 10 units, respectively, the source voltage was 3 kV, and SRM

maximum cycle time was 200 ms. Both HPLC-MS systems were controlled by Xcalibur software (Thermo Fisher Scientific). This analysis was carried out with thanks to Dr. William Mullen (*Senior Research Fellow-Institute of Cardiovascular and Medical Sciences- University of Glasgow*) for his guidance and allowing the use of his lab.

5.2.11 Paracetamol measurement

Paracetamol was measured as an estimation of gastric emptying. Given that gastric emptying is the limiting factor in paracetamol reaching its site of absorption, the time of paracetamol appearance in plasma is reflective of time of gastric emptying. Additionally there is a good correlation between gastric emptying measured by scintigraphy and paracetamol absorption (Willems et al., 2001).

Paracetamol levels were measured in plasma using an acetaminophen assay kit (*Cambridge Life Sciences, Cambridge, United Kingdom*). The standard calibration curve was prepared by serial dilutions of paracetamol standard in distilled water and ranged from 125-0.24 mmol/L (125, 62.5, 31.25, 15.62, 7.8, 3.9, 1.95, 0.97, 0.48, 0.24, 0). The standard concentrations were determined during test-runs of the assay.

After the preparation of the calibration curve, 50ul of either standard or sample was added to a 96 well plate in duplicate, followed by 0.5ml of reconstituted enzyme reagent. This was mixed well and incubated at room temperature for 5 minutes. Reagent A (1ml) and Reagent B (1ml) were added to all wells. The plate was left to incubate at room temperature for 30minutes after which it was read at 615nm using a spectrometer *(Thermo scientific multiskan spectrum).* An index of gastric emptying time was obtained by applying curve fittings to the data from paraceramol concentrations calculated with subsequent calculation of T1/2 and paracetamol AUC. (Willems et al., 2001). The analysis of this data was carried out with thanks to Dr. Douglas Morrison (*Senior Lecturer, Isotope Biochemistry, University of Glasgow*).

5.2.12 Subjective appetite assessment:

Visual analogue questionnaires were used to assess subjective appetite and hunger levels after the test meals (Appendix-7). They were completed by the volunteers at baseline and every 30 minutes for 6 hours. A validated VAS questionnaire (Flint et al., 2000) was used. Volunteers were asked to mark their sensation of hunger, fullness, satiety and prospective food consumption on a 100mm non-scaled VAS anchored at both sides with the most extreme response for each sensation, using the following 4

questions:

- How hungry do you feel?
- How satisfied do you feel?
- How full do you feel?
- How much do you think you can eat?

A questionnaire was obtained before introduction of supplement and displayed as BL (baseline), and another immediately after introduction of supplement being the 0h time point.

5.2.13 Breath hydrogen:

Breath hydrogen (ppm) was measured as an estimation of oro-caecal transit time. Expiratory breath hydrogen concentrations were initially to be measured using a portable breath hydrogen monitor (Bedfont Scientific Ltd, Rochester, Kent, United Kingdom) allowing volunteers to measure breath hydrogen after leaving the unit for 9 hours post supplement ingestion.

However due to a fault with equipment, we had to use a lab based machine and volunteers collected their breath very 30 mins for 6 hours into 20ml syringes and capped them immediately. Collected samples were injected into a breath hydrogen monitor (GMI, Medical Ltd, Renfrew) calibrated using Bedfont calibration gas (100ppm hydrogen, 100ppm methane and 20.9% air). The time of first sustained rise in breath hydrogen was taken as the mouth-to-cecum transit time (Mullen et al., 2009a).

5.2.14 Statistical analysis

To determine the impact of the ispaghula on phenolic acid production from cocoa, a general linear model Anova was performed using the software Minitab-15. This statistical model allowed for the analysis of paired data across time. In this analysis *Phenolic acid* was used as the response, with *volunteer, time, substrate and time*substrate* included in the model. *Volunteer* was included in the random factors. Total phenol and FRAP results were analysed either by paired t-test model or 1-way Wilcoxon Test after assessing for Normality. AUC was calculated for plasma paracetamol, breath hydrogen and VAS questionnaires and analysed either by paired t-test model or Mann-Whitney Test after assessing for Normality. Correlations were determined using Minitab-15.

5.3 Results

5.3.1 Anthropometric measurements

Table 5-4 shows the anthropometric measurements for volunteers meeting the study criteria.

Table 5-4 *In-vivo* cocoa study - participant information

Volunteer no.	Age (years)	Height (cm)	Weight (kg)	BMI	Waist circumference (cm)	Waist/Hip Ratio (cm)	Body Fat%
1	31	181	81	24.7	89.5	0.88	14.5
2	23	182	68.8	20.8	80	0.88	11.5
3	28	196	76.2	19.8	80	0.82	9.7
4	21	200	88.6	22.2	86	0.88	11.9
5	21	180	68.8	21.2	73	0.84	10.2
6	21	189	74.6	20.9	90	0.98	15.3
7	23	170	62.6	21.7	80.5	0.91	12.7
8	24	176	86.2	27.8	98	0.91	21.7
9	21	174	78.6	26	89.2	0.93	18.9
10	23	176	78.4	25.3	80.5	0.89	15.4
11	22	179	70.4	22	86	0.87	12.3
12	21	186	79.6	23	88	0.84	14.4
Mean ±	23.3	182.4	76.2	23	85.1	0.89	14.0
STDEV	± 3.2	± 8.9	± 7.6	± 2.5	± 6.6	± 0.04	±3.5

Values are means (± SD), BMI: body mass index

5.3.2 Quantification of Cocoa Flavan-3-ols

There have been no previous published studies quantifying the flavan-3-ol concentration of Barry Callebaut extra brute cocoa powder. This analysis of this cocoa powder was carried out with thanks to Dr. William Mullen for his guidance and allowing the use of his lab.

The flavan-3-ol monomers were quantified using catechin/ epicatechin calibration curves. Dimmers, trimer and tetramers were quantified as epicatechin equivalents. Some traces of pentomers were also detected. A compound having a target ion of 305.0729 was putatively identified as gallocatechin having an extra hydroxyl group than catechin (accounting for the +16 mass of the identified compound). The chromatograph presented in

Figure 5-3 was obtained from a prior extraction. However, due to lack of mass-spec and standards at the given time, quantification was not possible. Hence, this analysis was used solely for the presentation of compound elution.

Table 5-5 Flavan-3-ol quantification of extrabrute (Barry Callebaut) cocoa

	MeOH extraction (ug/g)	H_2O extraction (ug/g)
Catechin	1724.5	1757.7
Epicatechin	9479.5	7390.5
Gallocatechin	43992.7	238877.1
Dimers	9225.86	10584.1
Trimers	1798.76	1856.6
Tetramers	187.8	7 109.16

values are presented as mean concentration.

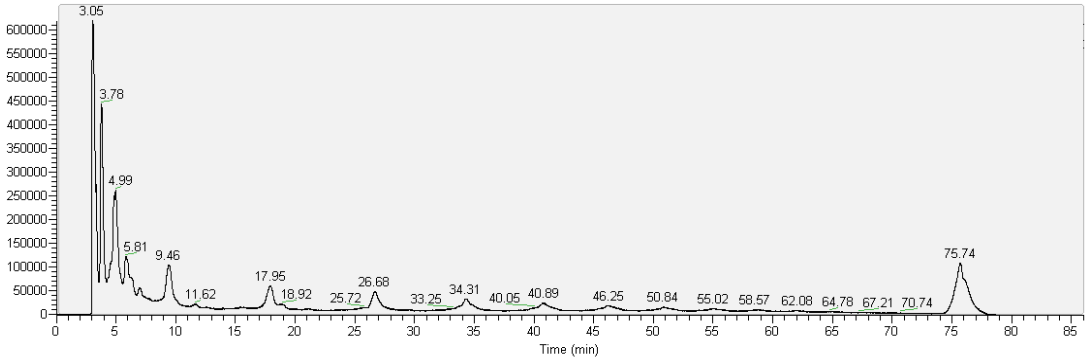


Figure 5-3 Flavan-3-ol chromatograph of extra brute (Barry Callebaut) cocoa

The chromatograph was obtained with thanks to Dr. Gina Borges (*Research Associate, Life-course Nutrition and Health, University of Glasgow*) for her guidance and instructions.

5.3.3 Phenolic acids

Ispaghula husk is a plant based product and may contain polyphenolic compounds, which are released during fermentation. Hence, for better understanding of the impact of ispaghula on the phenolic acid production from cocoa we calculated a predicted value as the sum of phenolic acid produced from ispaghula only and phenolic acid produced from cocoa only. The predicted value is presented as ISP-C and is compared to the true combination of ISP+C ingested. A higher value for ISP-C as compared to

ISP+C is an indication of food matrix interaction having an inhibitory effect on the production of phenolic acids from cocoa.

Due to the high inter-individual variations observed in the results, volunteers were further divided into high producers and low producers of phenolic acids based on mean sum of total phenolic production. Volunteers producting total sum of phenolics above the mean were considered as high producers and those below the mean as low producer. Analysis of data was conducted for each group separately.

The maximum concentration reached is defined as C_{max} and the time in hours at which Cmax was reached is defined as (T_{max}).The phenolic acids identified in the cocoa incubations are listed in *table 5-6.*

5-6 Identified phenolic acids from cocoa consumption

Phenolic acid (PA)	Cumulative concentration at 24h (mg/ time point)	No. of volunteers producing PA from cocoa	No. of volunteers producing PA from Ispaghula	No. of volunteers producing PA from Ispaghula+Cocoa
3-Hydroxy phenyl acetic acid	0.12 ± 0.07	12	10	11
4-hydroxy phenyl acetic acid	1.21 ± 0.86	12	12	12
3,4- Dihydroxy phenyl acetic acid	0.02 ± 0.02	9	5	6
Hippuric acid	11.7 ± 7.1	12	12	12
4-Hydroxy hippuric acid	0.09 ± 0.14	5	5	4
4-Hydroxy benzoic acid	0.09 ± 0.04	12	7	11
3,4-Dihydroxy benzoic acid	0.03 ± 0.05	9	3	6
Vanillic acid	0.16 ± 0.07	12	8	11
Homo-vanillic acid	0.17 ± 0.08	12	12	12
Mandelic acid	0.02 ± 0.04	5	2	2
4-Hydroxy mandelic acid	0.30 ± 0.15	12	11	11
Total sum of phenolic acids	14.0 ± 7.26	12	12	12

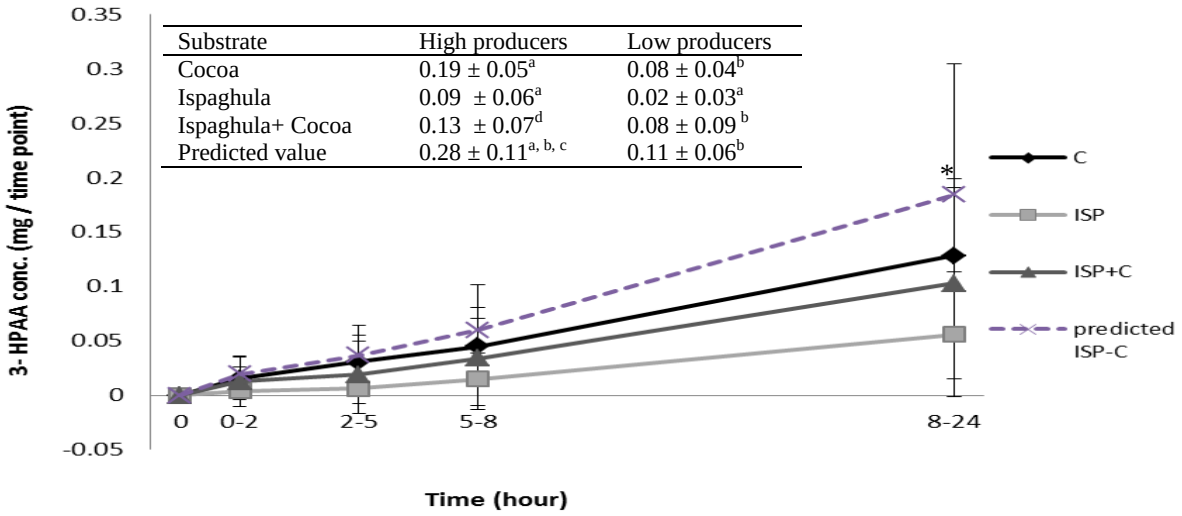
Values are presented as means (± SD) at 24 hours after 20g of cocoa in water (360ml) consumption (n=12).

5.3.3.1 Impact of ispaghula on phenolic acid production from cocoa

Ispaghula was slowly fermented and hence most of the phenolic acid production from this fibre was seen at 24 hours after ingestion. Data was analysed for time points over 24 hours and cumulative data (calculated as the sum of 0-2, 2-5, 5-8 and 8-24 hour samples) separately.

5.3.3.1.1 Phenolic acids present in higher concentration after cocoa consumption than ispaghula

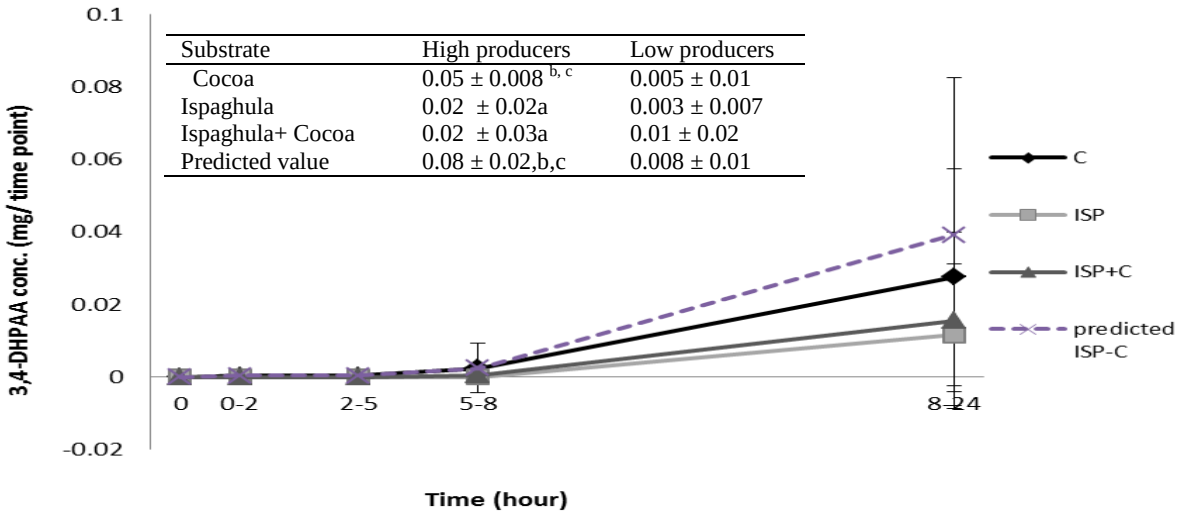
- 3-HPAA*:* Ispaghula inhibited the production for the cumulative data *(Figure 7-4, p < 0.01)* but not for non-cumulative data. Cocoa produced 3-HPAA at 8 hours after ingestion compared with 5 hours after ingestion of ispaghula. The same observation was made for both high and low producers ($p < 0.01$ and $p = 0.01$ respectively *Figure 5-4)*. Ispaghula inhibited 3-HPAA for high producers ($p < 0.01$) but not low producers.



Substrate	High producers	Low producers
Cocoa	0.19 ± 0.05^{a}	0.08 ± 0.04^{b}
Ispaghula	0.09 ± 0.06^{a}	0.02 ± 0.03^{a}
Ispaghula+ Cocoa	0.13 ± 0.07^{d}	0.08 ± 0.09^{b}
Predicted value	$0.28 \pm 0.11^{a, b, c}$	0.11 ± 0.06^{b}

Figure 5-4 Impact of matrix interaction between ispaghula and cocoa on cumulative excretion of 3-HPAA in urine
Values are displayed as cumulative mean (± SD) across 24 h after consumption of 20g cocoa and /or15g ispaghula in water (n=12), ISP: Ispaghula, C: Cocoa, ISP+C: Ispaghula+Cocoa. Predicted ISP-C: predicted value for Ispaghula and cocoa combination. *$p < 0.01$. a: $p < 0.05$ vs. cocoa, b: $p < 0.05$ vs. ispaghula, c: $p < 0.05$ vs. Ispaghula+cocoa, d: $p < 0.05$ vs. predicted value

- 3,4-DHPAA: Ispaghula did not inhibit the production when all volunteers were considered together (*Figure 5-5*). However it did inhibit production for high producers ($p < 0.01$). The higher concentration of 3,4-DHPAA for cocoa than ispaghula was only observed for high producers but not when all volunteers were considered together.

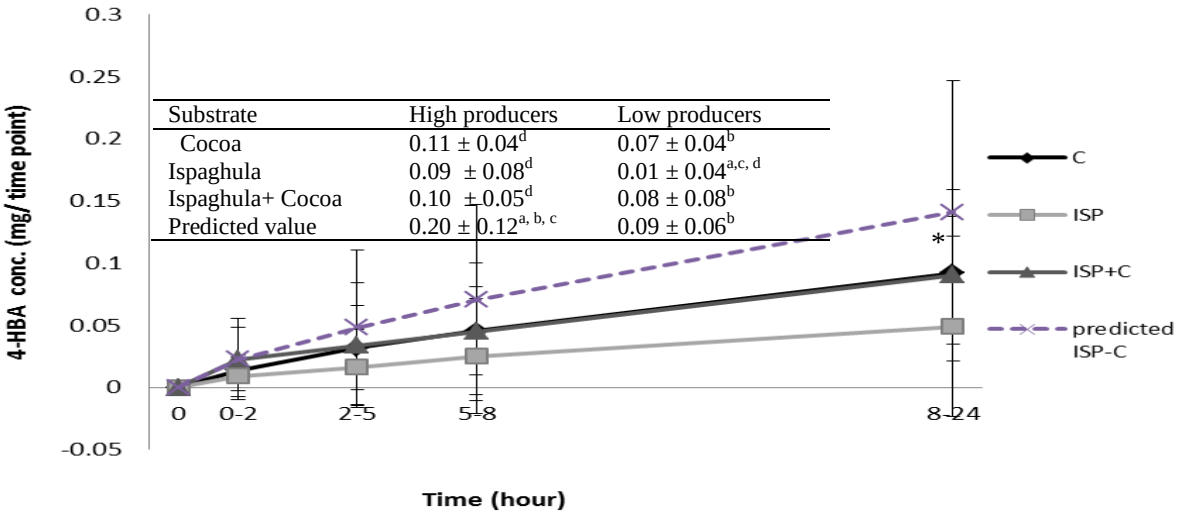


Substrate	High producers	Low producers
Cocoa	0.05 ± 0.008 [b, c]	0.005 ± 0.01
Ispaghula	0.02 ± 0.02a	0.003 ± 0.007
Ispaghula+ Cocoa	0.02 ± 0.03a	0.01 ± 0.02
Predicted value	0.08 ± 0.02,b,c	0.008 ± 0.01

Figure 5-5 Impact of matrix interaction between ispaghula and cocoa on cumulative excretion of 3,4-DHPAA in urine
Values are displayed as cumulative mean (± SD) across 24 h after consumption of 20g cocoa and /or15g ispaghula in water (n=12), ISP: Ispaghula, C: Cocoa, ISP+C: Ispaghula+Cocoa. Predicted ISP-C: predicted value for Ispaghula and cocoa combination. *ISP+C vs. cocoa, ispaghula and predicted (p= 0.01, p=0.01 & p=0.04). a: $p < 0.05$ vs. cocoa, b: $p < 0.05$ vs. ispaghula, c: $p < 0.05$ vs. Ispaghula+cocoa, d: $p < 0.05$ vs. predicted value

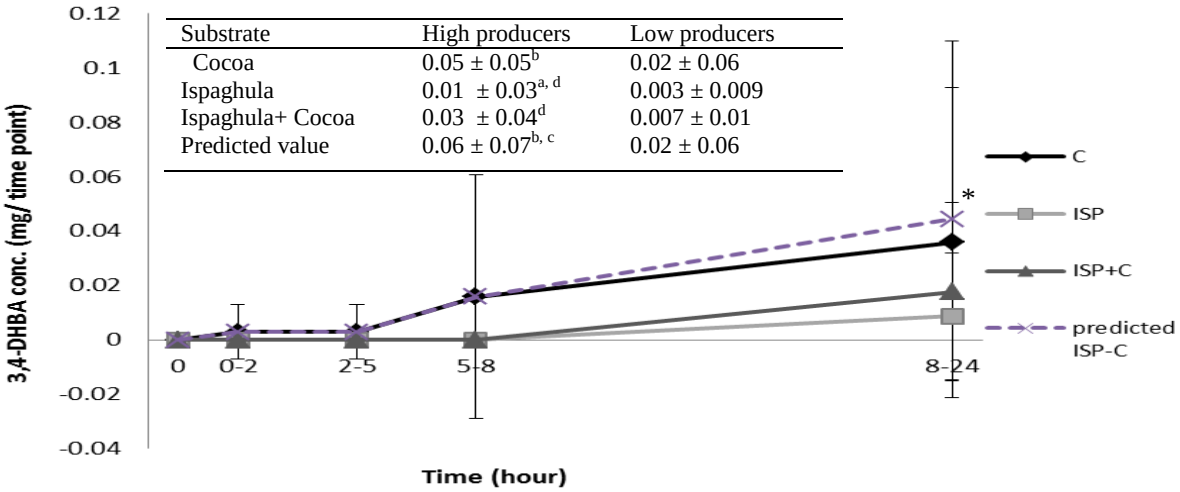
- 4-HBA: Ispaghula inhibited production for both cumulative (*Figure 5-6*) and non-cumulative data (p=0.02 and p=0.01 respectively). The production from cocoa began between 2-5 hours, followed by a decline at 5-8 hours and a rapid increase thereafter. The production began at 5 hours after ispaghula consumption. When ispaghula and cocoa were ingested together, the production was delayed to 8 hours after ingestion. Ispaghula inhibited the production of 4-HBA for high producers ($p < 0.01$) but not low producers.

- 3,4-DHBA: Ispaghula inhibited production for cumulative data only (p=0.01). Ispaghula also delayed the production (8 hours after ingestion) as compared with cocoa alone (5 hours after ingestion). Ispaghula inhibited production for high producers only (predicted vs. true value $p < 0.01$, true value vs. cocoa p=0.02).



Substrate	High producers	Low producers
Cocoa	0.11 ± 0.04[d]	0.07 ± 0.04[b]
Ispaghula	0.09 ± 0.08[d]	0.01 ± 0.04[a, c, d]
Ispaghula+ Cocoa	0.10 ± 0.05[d]	0.08 ± 0.08[b]
Predicted value	0.20 ± 0.12[a, b, c]	0.09 ± 0.06[b]

Figure 5-6 Impact of matrix interaction between ispaghula and cocoa on cumulative excretion of 4-HBA in urine
Values are displayed as cumulative mean (± SD) across 24 h after consumption of 20g cocoa and /or15g ispaghula in water (n=12), ISP: Ispaghula, C: Cocoa, ISP+C: Ispaghula+Cocoa. Predicted ISP-C: predicted value for Ispaghula and cocoa combination. *p=0.02 a: p < 0.05 vs. cocoa, b: p < 0.05 vs. ispaghula, c: p < 0.05 vs. Ispaghula+cocoa, d: p < 0.05 vs. predicted value.



Substrate	High producers	Low producers
Cocoa	0.05 ± 0.05[b]	0.02 ± 0.06
Ispaghula	0.01 ± 0.03[a, d]	0.003 ± 0.009
Ispaghula+ Cocoa	0.03 ± 0.04[d]	0.007 ± 0.01
Predicted value	0.06 ± 0.07[b, c]	0.02 ± 0.06

Figure 5-7 Impact of matrix interaction between ispaghula and cocoa on cumulative excretion of 3,4-DHBA in urine
Values are displayed as cumulative mean (± SD) across 24 h after consumption of 20g cocoa and /or15g ispaghula in water (n=12), ISP: Ispaghula, C: Cocoa, ISP+C: Ispaghula+Cocoa. Predicted ISP-C: predicted value for Ispaghula and cocoa combination. *p=0.01. a: p < 0.05 vs. cocoa, b: p < 0.05 vs. ispaghula, c: p < 0.05 vs. Ispaghula+cocoa, d: p < 0.05 vs. predicted value.

- Mandelic acid: Ispaghula inhibited production for both cumulative and non-cumulative data (p=0.01 and p < 0.01 respectively). The production was seen after 2 hours of cocoa, 8 hours of

ispaghula and 2 hours after the combination (reaching a plateau after 8 hours) ingestion. Similar results were observed for high producers; there was no inhibition in production for high producers.

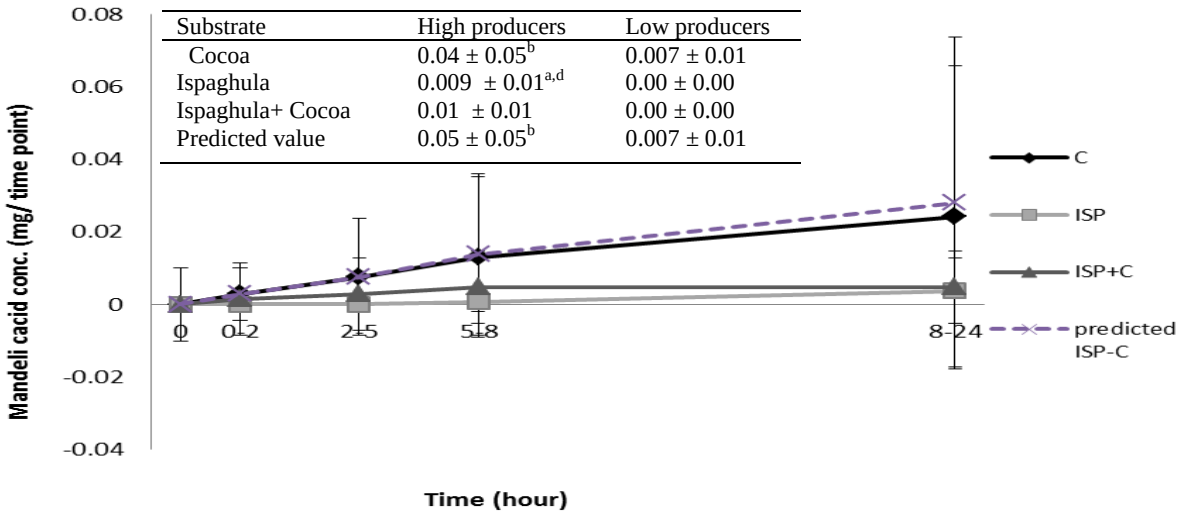
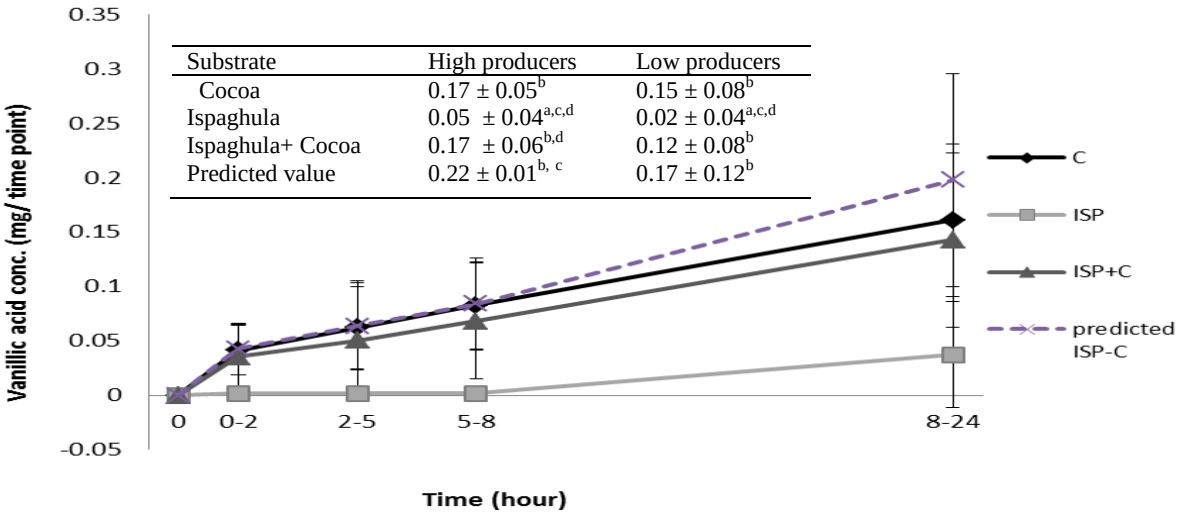


Figure 5-8 Impact of matrix interaction between ispaghula and cocoa on cumulative excretion of mandelic acid in urine. Values are displayed as cumulative mean (± SD) across 24 h after consumption of 20g cocoa and /or15g ispaghula in water (n=12), ISP: Ispaghula, C: Cocoa, ISP+C: Ispaghula+Cocoa. Predicted ISP-C: predicted value for Ispaghula and cocoa combination. *$p < 0.01$. a: $p < 0.05$ vs. cocoa, b: $p < 0.05$ vs. ispaghula, c: $p < 0.05$ vs. Ispaghula+cocoa, d: $p < 0.05$ vs. predicted value.

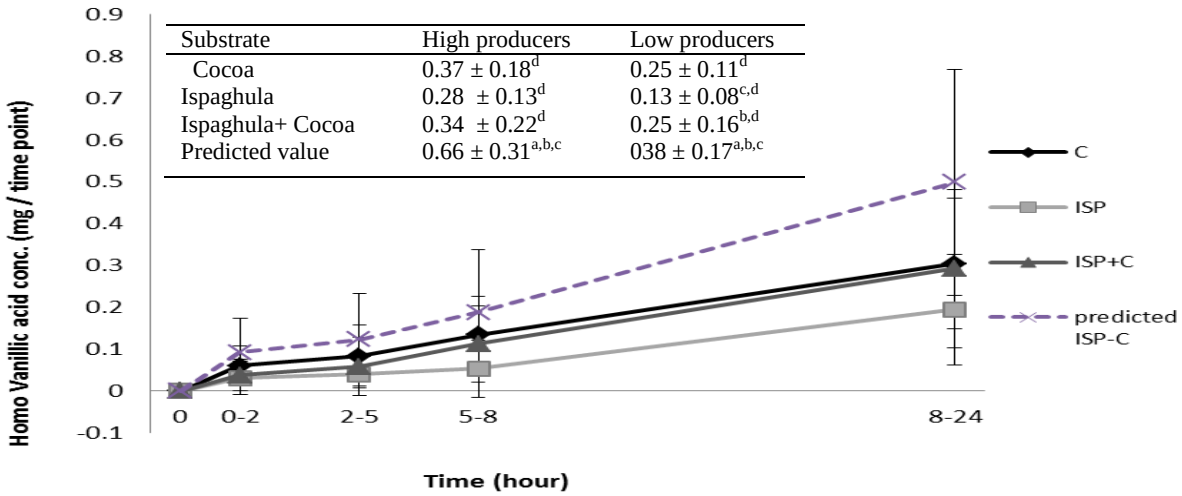
- Vanillic acid: The production was not inhibited by ispaghula. The production began between 0-2 hours of cocoa ingestion, declining thereafter and increased again at 8 hours; this increase was only seen at 8 hours after ispaghula ingestion and 5-8 hours after combination ingestion The higher production of vanillic acid from cocoa than ispaghula was observed in both high and low producers ($p < 0.01$). Ispaghula inhibited vanillic acid production in high producers only ($p=0.04$).

- Homo-vanillic acid: ispaghula inhibited production for both cumulative and non-cumulative data ($p < 0.01$). The higher production of homo-vanillic acid from cocoa than ispaghula was observed in both high and low producers ($p < 0.01$). The concentration of this phenolic acid increased 5 hours after cocoa and combination group ingestion, but 8 hours after ispaghula ingestion.



Substrate	High producers	Low producers
Cocoa	0.17 ± 0.05^{b}	0.15 ± 0.08^{b}
Ispaghula	$0.05 \pm 0.04^{a,c,d}$	$0.02 \pm 0.04^{a,c,d}$
Ispaghula+ Cocoa	$0.17 \pm 0.06^{b,d}$	0.12 ± 0.08^{b}
Predicted value	$0.22 \pm 0.01^{b,c}$	0.17 ± 0.12^{b}

Figure 5-9 Impact of matrix interaction between ispaghula and cocoa on cumulative excretion of vanillic acid in urine
Values are displayed as cumulative mean (± SD) across 24 h after consumption of 20g cocoa and /or15g ispaghula in water (n=12), ISP: Ispaghula, C: Cocoa, ISP+C: Ispaghula+Cocoa. Predicted ISP-C: predicted value for Ispaghula and cocoa combination. a: $p < 0.05$ vs. cocoa, b: $p < 0.05$ vs. ispaghula, c: $p < 0.05$ vs. Ispaghula+cocoa, d: $p < 0.05$ vs. predicted value.

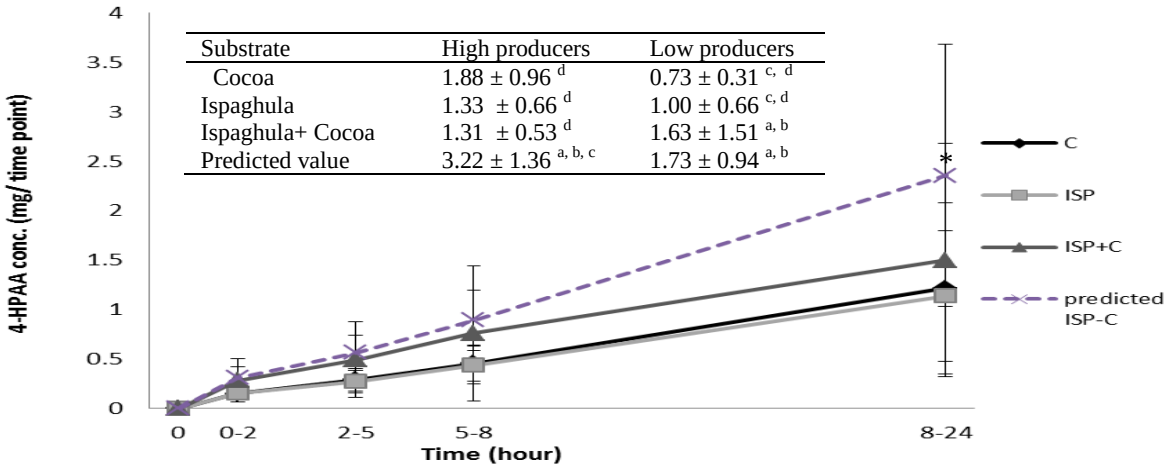


Substrate	High producers	Low producers
Cocoa	0.37 ± 0.18^{d}	0.25 ± 0.11^{d}
Ispaghula	0.28 ± 0.13^{d}	$0.13 \pm 0.08^{c,d}$
Ispaghula+ Cocoa	0.34 ± 0.22^{d}	$0.25 \pm 0.16^{b,d}$
Predicted value	$0.66 \pm 0.31^{a,b,c}$	$038 \pm 0.17^{a,b,c}$

Figure 5-10 Impact of matrix interaction between ispaghula and cocoa on cumulative excretion of homo-vanillic acid in urine. Values are displayed as cumulative mean (± SD) across 24 h after consumption of 20g cocoa and /or15g ispaghula in water (n=12), ISP: Ispaghula, C: Cocoa, ISP+C: Ispaghula+Cocoa. Predicted ISP-C: predicted value for Ispaghula and cocoa combination. *$p < 0.01$. a: $p < 0.05$ vs. cocoa, b: $p < 0.05$ vs. ispaghula, c: $p < 0.05$ vs. Ispaghula+cocoa, d: $p < 0.05$ vs. predicted value.

5.3.3.1.2 Phenolic acids present in high concentration after both cocoa and ispaghula consumption:

- 4-HPAA: The matrix interaction of ispaghula and cocoa inhibited production for cumulative data (*Figure 5-11*, p = 0.04) and non-cumulative data (p = 0.04). This inhibition was also seen for high producers low producers (p < 0.01).

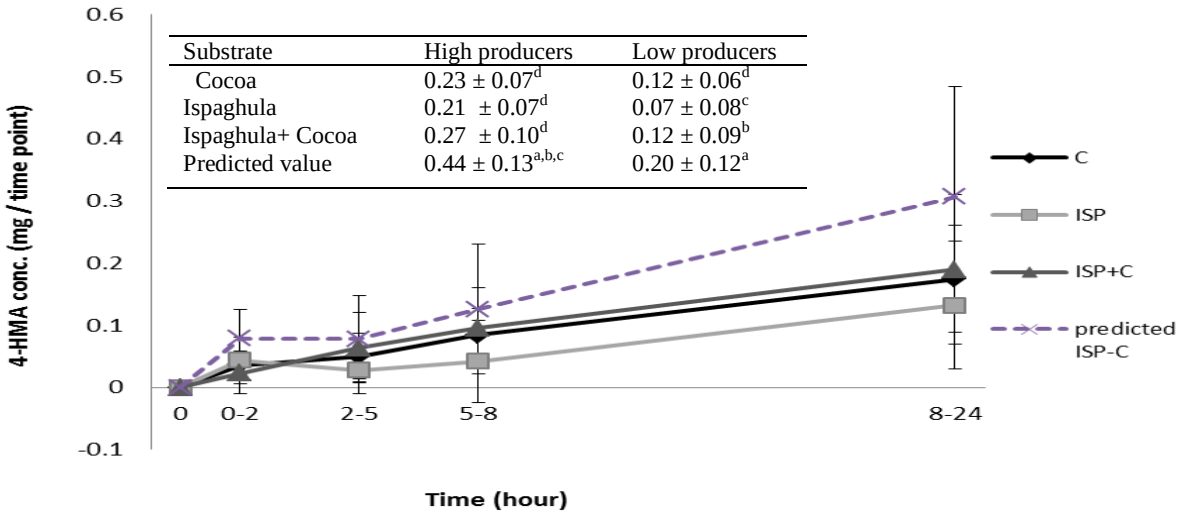


Substrate	High producers	Low producers
Cocoa	1.88 ± 0.96 [d]	0.73 ± 0.31 [c, d]
Ispaghula	1.33 ± 0.66 [d]	1.00 ± 0.66 [c, d]
Ispaghula+ Cocoa	1.31 ± 0.53 [d]	1.63 ± 1.51 [a, b]
Predicted value	3.22 ± 1.36 [a, b, c]	1.73 ± 0.94 [a, b]

Figure 5-11 Impact of matrix interaction between ispaghula and cocoa on cumulative excretion of 4-HPAA in urine
Values are displayed as cumulative mean (± SD) across 24 h after consumption of 20g cocoa and /or15g ispaghula in water (n=12), ISP: Ispaghula, C: Cocoa, ISP+C: Ispaghula+Cocoa. Predicted ISP-C: predicted value for Ispaghula and cocoa combination. *ISP+C vs. cocoa, ispaghula and predicted (p= 0.01, p=0.01 & p=0.04). a: p < 0.05 vs. cocoa, b: p < 0.05 vs. ispaghula, c: p < 0.05 vs. Ispaghula+cocoa, d: p < 0.05 vs. predicted value

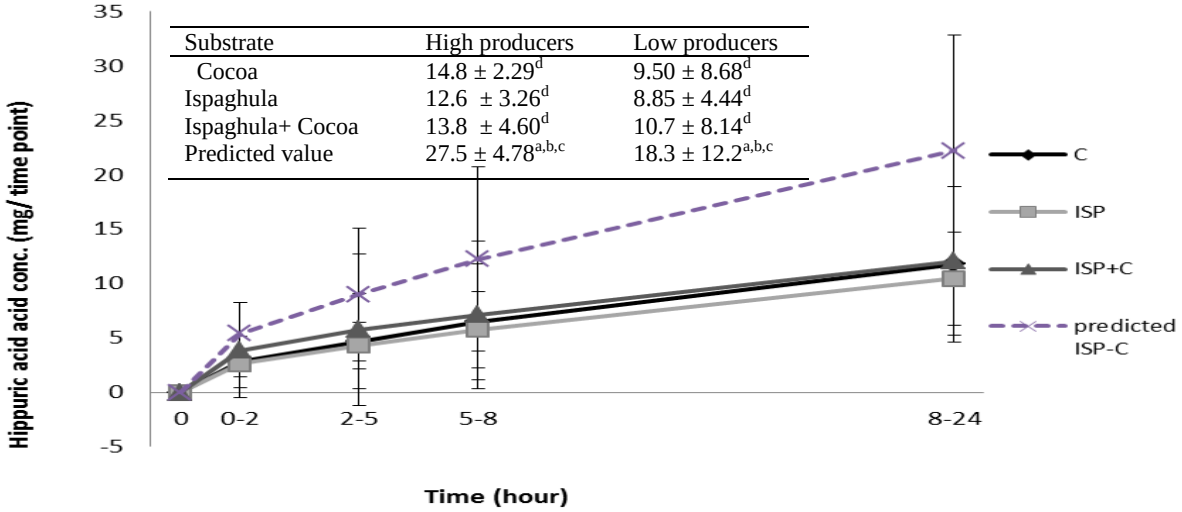
- 4-HMA: The matrix interaction of ispaghula and cocoa inhibited production for both cumulative and non-cumulative data (p < 0.01). This inhibitory impact was only observed for the high producer group (p < 0.01) and not low producers.

- Hippuric acid was the highest produced phenolic acid from both cocoa and ispaghula. Hippuric acid is produced through various metabolic pathways in the body, which may explain its high concentration at baseline. The production of hippuric acid was seen between 8-24 hours after ingestion and was inhibited by the matrix interaction of cocoa and ispaghula for both cumulative and non-cumulative data (p < 0.01). This inhibitory impact was observed for both high and low producers as well (p < 0.01).



Substrate	High producers	Low producers
Cocoa	0.23 ± 0.07^{d}	0.12 ± 0.06^{d}
Ispaghula	0.21 ± 0.07^{d}	0.07 ± 0.08^{c}
Ispaghula+ Cocoa	0.27 ± 0.10^{d}	0.12 ± 0.09^{b}
Predicted value	$0.44 \pm 0.13^{a,b,c}$	0.20 ± 0.12^{a}

Figure 5-12 Impact of matrix interaction between ispaghula and cocoa on cumulative excretion of 4-HMA in urine
Values are displayed as cumulative mean (± SD) across 24 h after consumption of 20g cocoa and /or15g ispaghula in water (n=12), ISP: Ispaghula, C: Cocoa, ISP+C: Ispaghula+Cocoa. Predicted ISP-C: predicted value for Ispaghula and cocoa combination. *p < 0.01. a: p < 0.05 vs. cocoa, b: p < 0.05 vs. ispaghula, c: p< 0.05 vs. Ispaghula+cocoa, d: p < 0.05 vs. predicted value.



Substrate	High producers	Low producers
Cocoa	14.8 ± 2.29^{d}	9.50 ± 8.68^{d}
Ispaghula	12.6 ± 3.26^{d}	8.85 ± 4.44^{d}
Ispaghula+ Cocoa	13.8 ± 4.60^{d}	10.7 ± 8.14^{d}
Predicted value	$27.5 \pm 4.78^{a,b,c}$	$18.3 \pm 12.2^{a,b,c}$

Figure 5-13 Impact of matrix interaction between ispaghula and cocoa on cumulative excretion of hippuric acid in urine. Values are displayed as cumulative mean (± SD) across 24 h after consumption of 20g cocoa and /or15g ispaghula in water (n=12), ISP: Ispaghula, C: Cocoa, ISP+C: Ispaghula+Cocoa. Predicted ISP-C: predicted value for Ispaghula and cocoa combination. a: p < 0.05 vs. cocoa, b: p < 0.05 vs. ispaghula, c: p < 0.05 vs. Ispaghula+cocoa, d: p < 0.05 vs. predicted value

- 4-HHA: The matrix interaction of cocoa and ispaghula inhibited the cumulative concentration of ($p = 0.01$). Contrary to the observations made for hippuric acid, this inhibition was only seen for low producers ($p = 0.02$). The production of 4-HPA was seen earlier for ispaghula and ispaghula+cocoa (5 hours after ingestion) than cocoa *8 hours after ingestion).

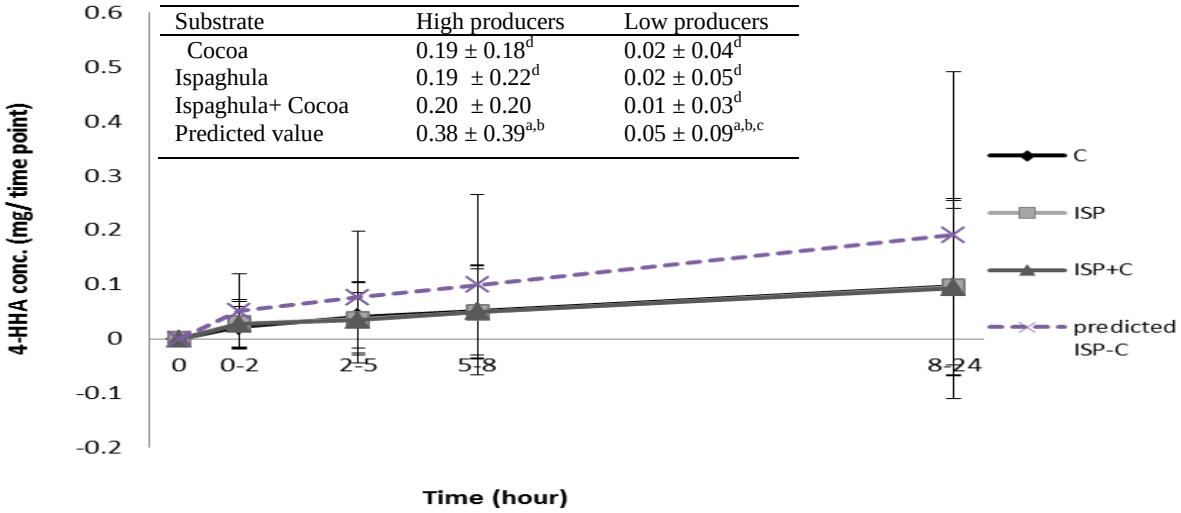


Figure 5-14 Impact of matrix interaction between ispaghula and cocoa on cumulative excretion of 4-HHA acid in urine. Values are displayed as cumulative mean (± SD) across 24 h after consumption of 20g cocoa and /or15g ispaghula in water (n=12), ISP: Ispaghula, C: Cocoa, ISP+C: Ispaghula+Cocoa. Predicted ISP-C: predicted value for Ispaghula and cocoa combination. *p= 0.01. a: $p < 0.05$ vs. cocoa, b: $p < 0.05$ vs. ispaghula, c: $p < 0.05$ vs. Ispaghula+cocoa, d: $p < 0.05$ vs. predicted value.

5.3.3.1.3 Impact of matrix interaction between ispaghula and cocoa on total sum of phenolic acid production:

Hippuric acid was the highest contributor to the sum of total phenolic acids. The similar concentration of hippuric acid for both ispaghula and cocoa was reflected in sum of total phenolic acids; resulting in no difference between the two. However, the food matrix interaction between cocoa and ispaghula resulted in an inhibition of total phenolic acid production for both cumulative and non-cumulative concentrations ($p < 0.01$, *Figure 5-15*).

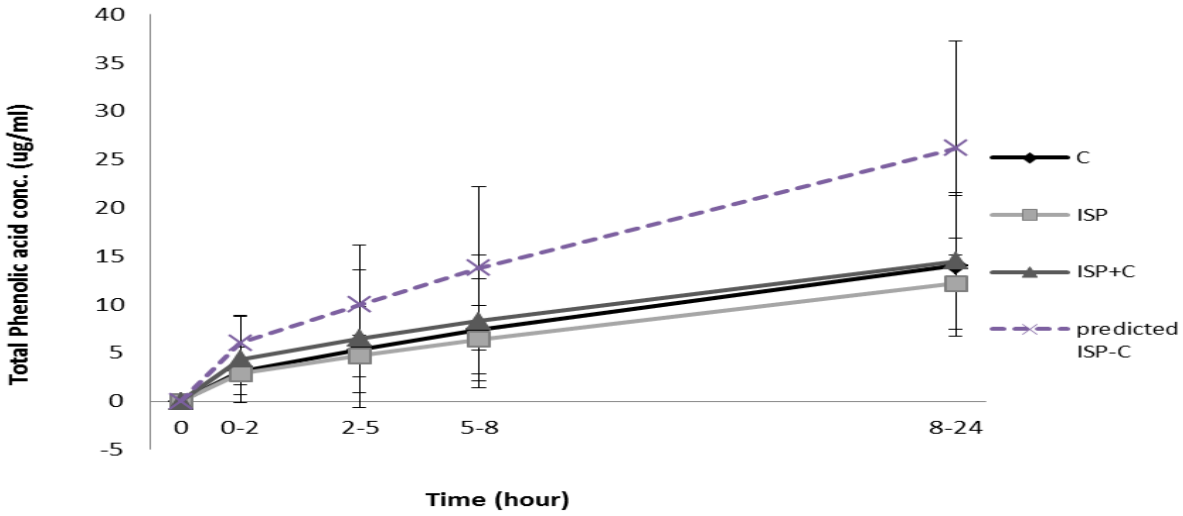


Figure 5-15 Impact of matrix interaction between ispaghula and cocoa on cumulative sum of total phenolis in urine
Values are displayed as cumulative mean (± SD) across 24 h after consumption of 20g cocoa and /or15g ispaghula in water (n=12), ISP: Ispaghula, C: Cocoa, ISP+C: Ispaghula+Cocoa. Predicted ISP-C: predicted value for Ispaghula and cocoa combination. *p < 0.01. a: p< 0.05 vs. cocoa, b: p < 0.05 vs. ispaghula, c: p < 0.05 vs. Ispaghula+cocoa, d: p < 0.05 vs. predicted value.

This inhibition was also seen for high and low producer and low producers (p < 0.01, Table 5-7 and *Figure 5-16*)

Substrate	High producers	Low producers
Cocoa	18.2 ± 1.6	11.0 ± 8.3
Ispaghula	14.9 ± 3.6	10.1 ± 4.4
Ispaghula+ Cocoa	16.4 ± 4.4	13.0 ± 8.5
Predicted value	33.1 ± 4.7	21.1 ± 11.7

Table 5-7 Impact of matrix interaction between ispaghula and cocoa on cumulative sum of total phenolics in urine for high and low producers.

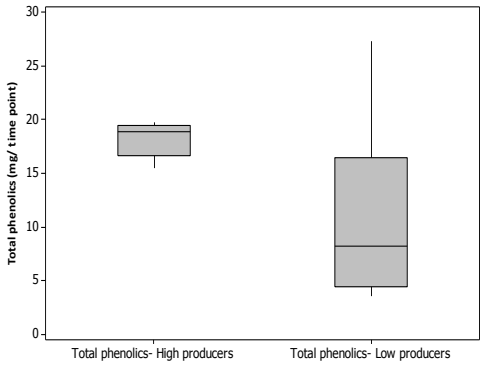


Figure 5-16 cumulative sum of total phenolics in urine of high and low producers at 24 h

Hippuric acid was the highest contributor in the makeup of the total phenolic acids. Followed by 4-HBA > Vanillic acid > 4-HMA > Homo-vanillic acid and 3-HPAA (*Figure 5-17*).

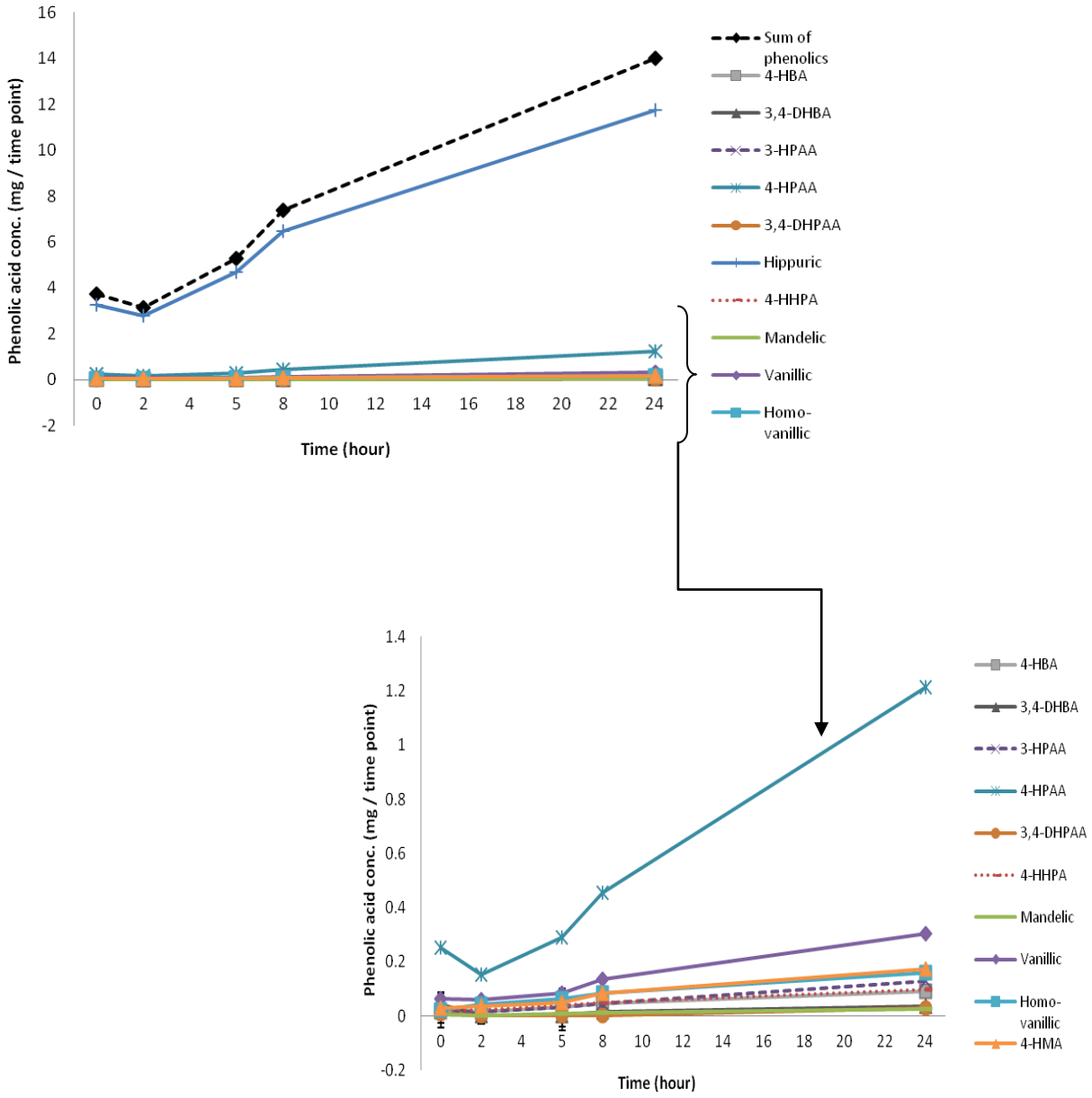


Figure 5-17 contribution of individual phenolic acids to sum of phenolics from cocoa ingestion.

5.3.4 Total phenol

5.3.4.1 Total phenol analysis (TP assay) of cocoa and ispaghula

As phenolics can be found in both free and bound form, we conducted this analysis for both. The free phenolics are the highest in concentration; hence, we used two different methods of free phenolic measurements for comparison purposes. The free phenolics were measured with 3 levels of dilution (with distilled H_2O), to control for any impact of acetone. Extraction of phenolics for Method-1 was conducted with 10ml of 60% acetone and allowed to shake for 10 minutes, followed by centrifugation. Method-2 extraction was conducted with 4ml of 70% acetone and was allowed to shake for 2 hours prior to centrifugation.

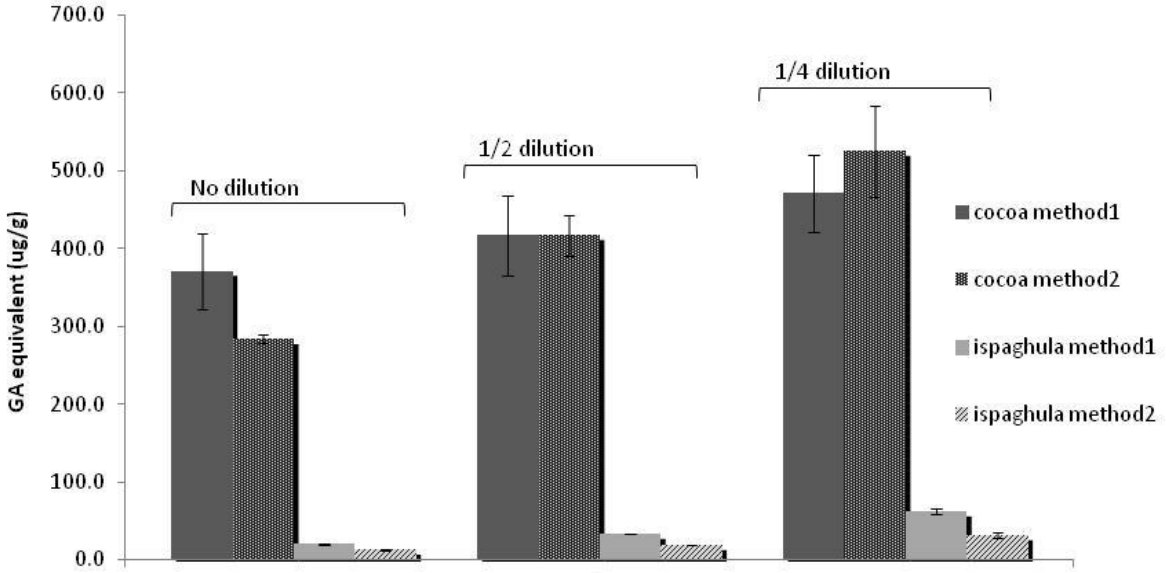


Figure 5-18 Free phenolics present in cocoa and ispaghula as measured by the Holtekjølen et al (2008)- method 1 and Thondre et al. (2011) – method 2.

As seen in *Figure 5-18* the higher concentration of acetone could be interfering with absorbance reading, resulting in lower calculated concentration for cocoa and ispaghula in method-2. When diluted to 50% this interference is reduced, resulting in equal concentration of free phenolics from cocoa for both methods. Further dilution to 75% resulted in higher concentration from cocoa using method-2 compared to method-1. Even though the concentration is higher when further diluted to 75% for method-2, higher volumes of acetone at lower concentration appears to better extract these free phenolics, resulting in constant higher values for free phenolics in ispaghula for method-1 despite the dilution factor. We also observed that the relative proportion of ispaghula to cocoa increases with further dilution for method-1 (5.39%, 8.03% and 21.2%). This was not seen for method-2 (4.69 %,

4.60% and 6.04%). This demonstrates that the data on free phenolics obtained from method-2 are more reliable due to proportionate increase in concentration for both cocoa and ispaghula.

Table 5-8 concentration of free phenolics from cocoa and ispaghula

Substrate	Cocoa	Ispaghula	Cocoa 1/2	Ispaghula 1/2	Cocoa 1/4	Ispaghula 1/4
Method 1	371.0 ± 48.5	20.0 ± 0.31	416.8 ± 51.3	33.54 ± 0.34	470.66 ± 50.1	62.30 ± 3.6
Method 2	283.5 ± 5.3	13.3 ± 0.78	417.0 ± 25.98	19.2 ± 0.53	**524.8 ± 59.0**	**31.7 ± 2.8**

Values are presented as mean GA equivalent (ug/g) ± STDEV

To better determine any impact of acetone on absorbance, a standard curve prepared with acetone was compared to the standard curve prepared with methanol. We did not identify any impact of acetone on the standard curve slope.

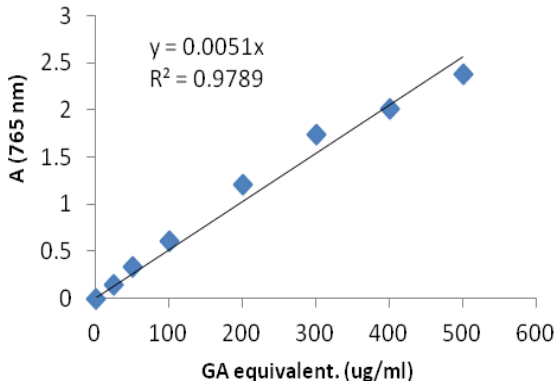


Figure 5-19 Standard curve with acetone

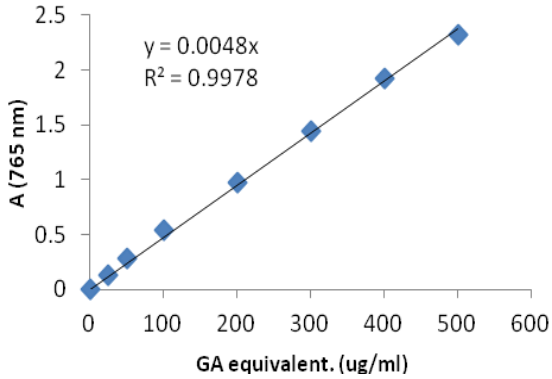


Figure 5-20 Standard curve with methanol

The bound phenolics for cocoa and ispaghula were extracted using method-1 as described in *5.2.8.1*. These results demonstrated relatively but not significantly higher proportions of bound phenolics for ispaghula to cocoa (*Figure 5-21*).

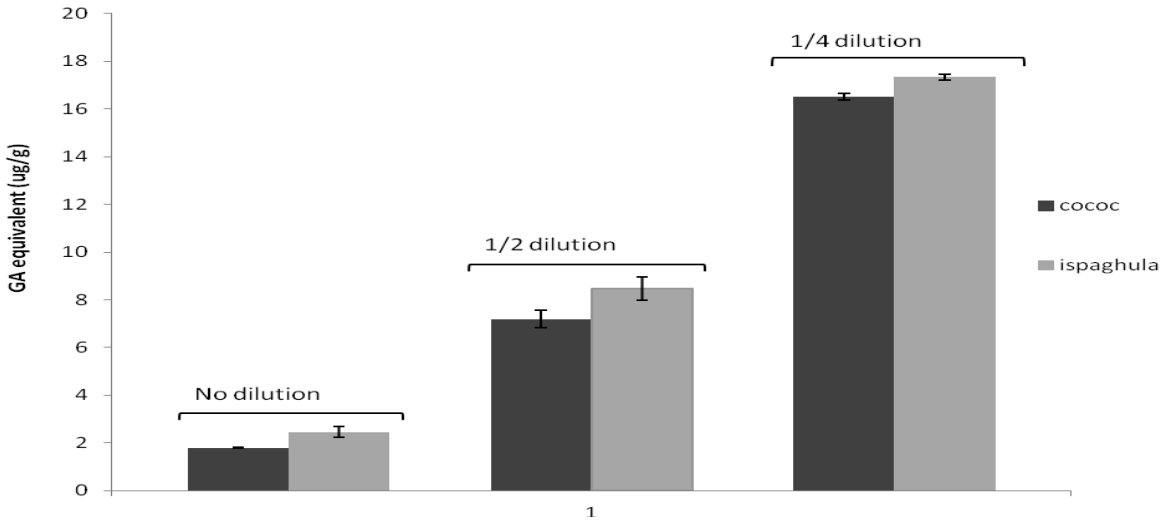


Figure 5-21 Bound phenolics concentration in cocoa and ispaghula as measured by the Holtekjølen et al (2008)-method 1 and Thondre et al. (2011) – method 2.

The absorbance was read at 765nm rather than 450nm used to read the absorbance of brown solutions. To ascertain that the brown colour of the cocoa extract had no impact on the read absorbance, we used cocoa extract at different dilutions (50% and 75%) to spike the GA standards. The obtained standard curve from these spiked standards was compared to that of GA standards in methanol only. The cocoa extract was not used for spiking standards without dilution, as the combination of cocoa extract and GA would result in concentrations above that of 500 ug/ml standard. Any concentration above this level would result in a plateau of the standard curve (*Figure 5-22, 5-23 and 5-24*).

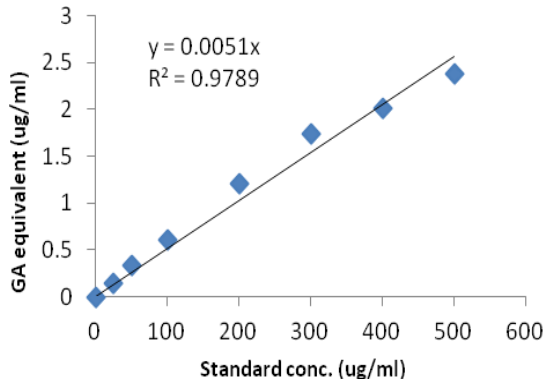


Figure 5-22 GA/Methanol standard curve + 0% cocoa extract
Values are displayed as the mean of triplicate measurements. GA: Gallic acid

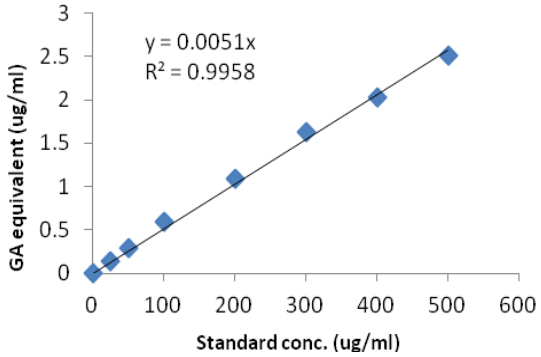


Figure 5-24 Standard curve + 50%cocoa extract
Values are displayed as the mean of triplicate measurements.
GA: Gallic acid

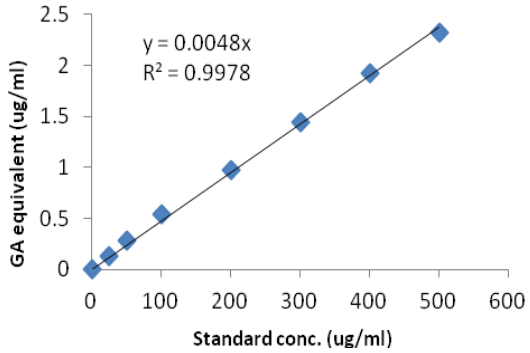


Figure 5-23 Standard curve+ 25% cocoa extract
Values are displayed as the mean of triplicate measurements.
GA: Gallic acid

As seen in the above figures, the presence of cocoa extract at different concentrations had no impact on the standard curve slope, and hence, had no impact on the read absorbance.

5.3.4.2 Total phenol concentration of urine samples

Due to the high concentration of total phenols in urine, a 50% dilution in distilled water was used. Similar to the results shown for phenolic acids, ispaghula consumption resulted in high urine total phenol concentrations. This could be attributed to the high concentrations of hippuric acid present in urine samples. The total phenol concentration in urine was not different after cocoa was ingested alone to when ispaghula was ingested alone for cumulative or non-cumulative analysis. However, the concentration of total phenols for the true combination was significantly lower than the predicted value ($p < 0.01$); demonstrating that interaction of ispaghula and cocoa results in a lower total phenol concentration. The concentration of total phenols began to increase at 2 hours after cocoa ingestion, whereas this increase was seen after 8 hours of ispaghula ingestion (*Figure 5-25).*

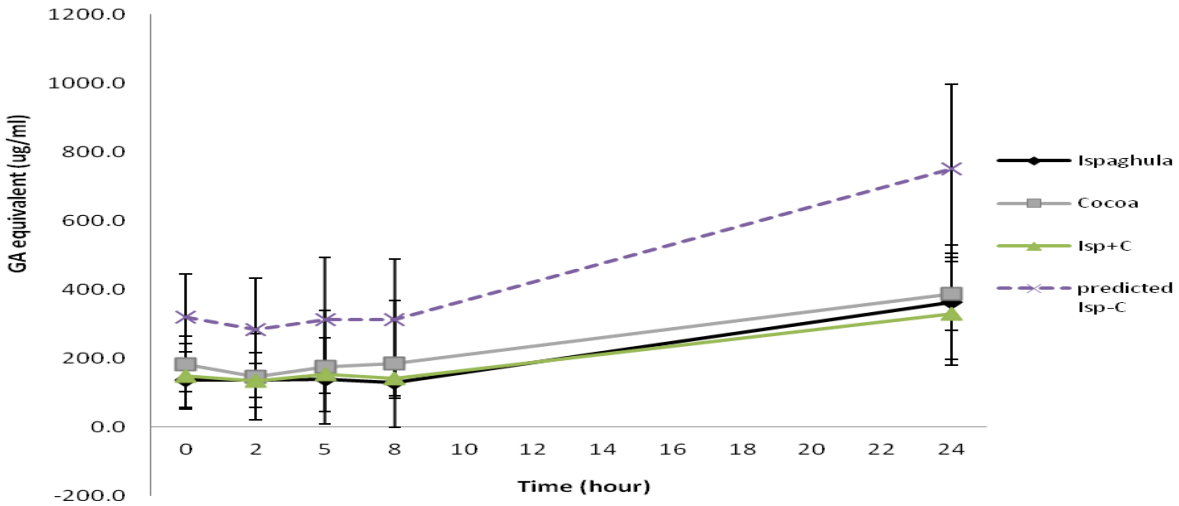


Figure 5-25 Non-cumulative concentration of total phenols in urine
Values are displayed as non-cumulative mean (± SD) across 24 h after consumption of 20g cocoa and /or15g ispaghula in water (n=12), ISP: Ispaghula, C: Cocoa, ISP+C: Ispaghula+Cocoa. Predicted ISP-C: predicted value for Ispaghula and cocoa combination.

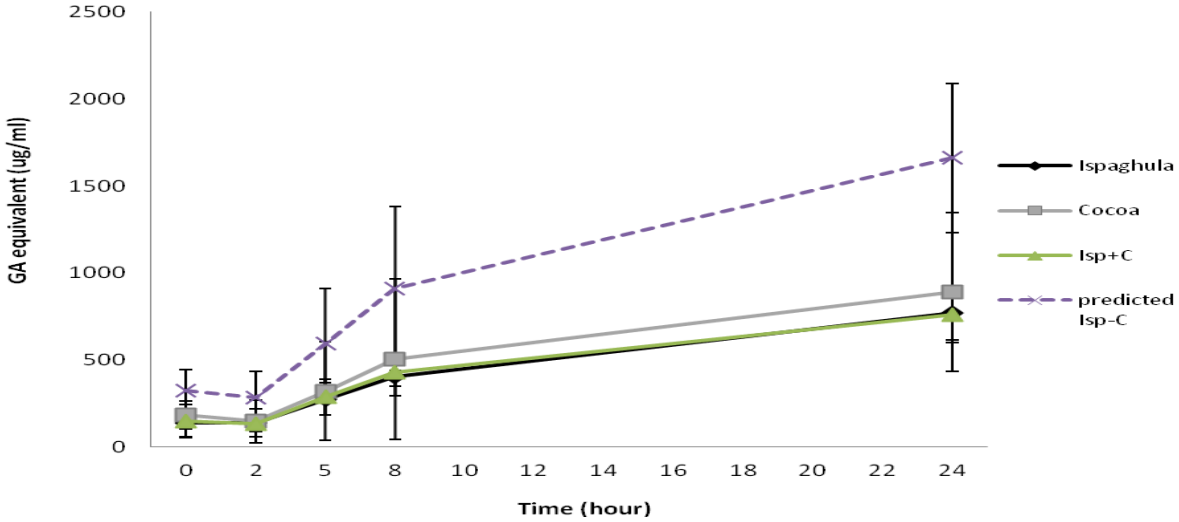


Figure 5-26 Cumulative concentration of total phenols in urine
Values are displayed as cumulative mean (± SD) across 24 h after consumption of 20g cocoa and /or15g ispaghula in water (n=12), ISP: Ispaghula, C: Cocoa, ISP+C: Ispaghula+Cocoa. Predicted ISP-C: predicted value for Ispaghula and cocoa combination.

TP concentration in urine increased significantly in 24 urine samples compared to baseline for both cocoa and ispaghula ($p < 0.01$)

5.3.4.3 Total phenol concentration in plasma

Plasma samples for total phenol analysis did not require dilution. Plasma total phenols did not differ between cocoa alone and ispaghula alone consumption. When they were consumed together, the total phenol plasma concentration was lower than predicted ($p < 0.01$).

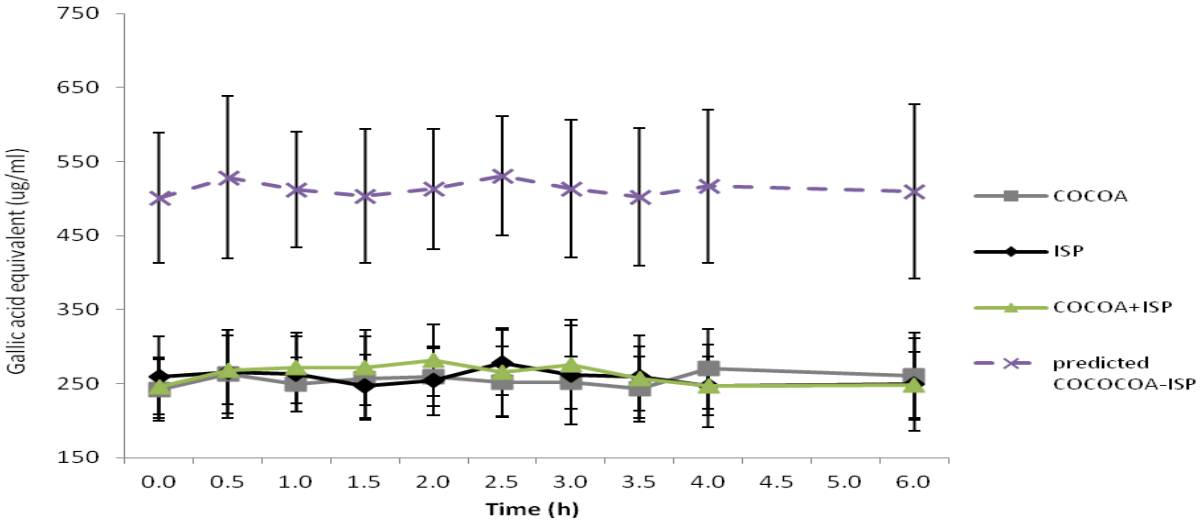


Figure 5-27 Total phenol from cocoa and ispaghula in plasma
Values are displayed as mean (± SD) across 24 h after consumption of 20g cocoa and /or15g ispaghula in water (n=12), ISP: Ispaghula, C: Cocoa, ISP+C: Ispaghula+Cocoa. Predicted ISP-C: predicted value for Ispaghula and cocoa combination.

Despite similar overall plasma concentrations of total phenol for cocoa, ispaghula and cocoa+ispaghula, there was a difference in T_{max} for the three groups. The earliest T_{max} was seen for the combination group at 2.0 hours, followed by ispaghula at 2.5 hours and cocoa at 4.0 hours after ingestion. 6h plasma TP did not increase significantly after the consumption of cocoa or ispaghula ($p=226$ and $p= 394$ respectively).

5.3.5 Antioxidant capacity in urine

Urine samples were diluted 1:4 for the analysis of antioxidant capacity after the consumption of cocoa, ispaghula or the combination of both. Similar to the results seen for total phenol-urine, the ingestion of

cocoa and ispaghula did not result in different antioxidant capacity in urine, for cumulative or non-cumulative analysis. However, both analyses demonstrated an inhibitory impact on antioxidant capacity of cocoa and ispaghula when ingested together, when predicted and true combination values were compared ($p < 0.01$).

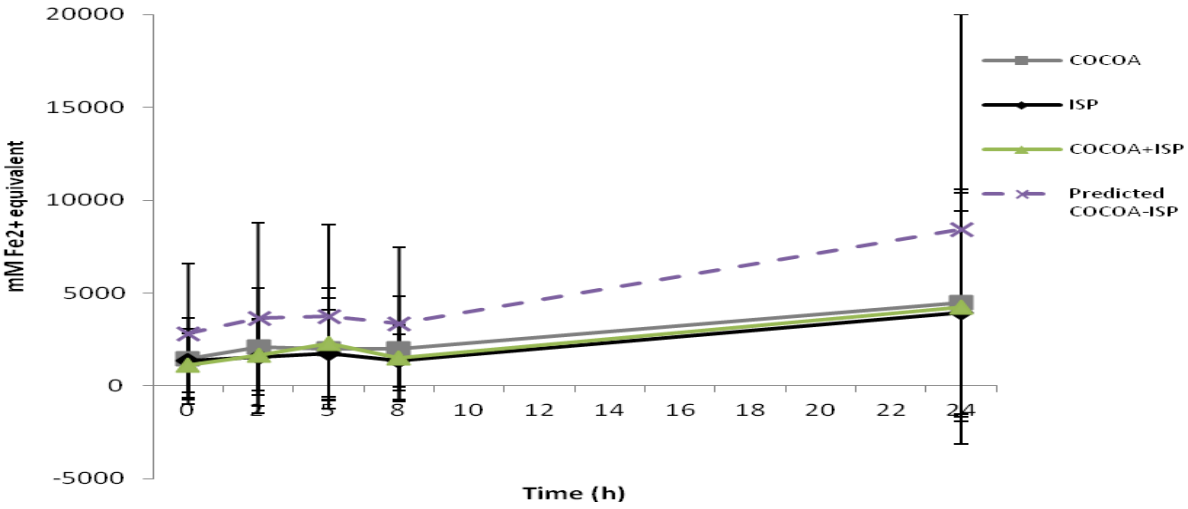


Figure 5-28 Cumulative antioxidant capacity in urine
Values are displayed as cumulative mean (± SD) across 24 h after consumption of 20g cocoa and /or15g ispaghula in water (n=12), ISP: Ispaghula, C: Cocoa, ISP+C: Ispaghula+Cocoa. Predicted ISP-C: predicted value for Ispaghula and cocoa combination.

5.3.6 Gastric emptying

The cocoa meal had the highest $t_{1/2}$ for gastric emptying compared to combination group and ispaghula alone (p=0.01 and p < 0.01 respectively, *Figure 5-29*). There was no difference between the combination group and ispaghula alone. Similar results were seen for $T_{max.}$ The combination group and ispaghula did not differ in their impact on paracetamol T_{max} (p=0.679); however, they both had lower T_{max} compared to cocoa (p=0.01 and p < 0.01 respectively). There was a correlation in the results by volunteer across trial arms (*figures 7-32 to 7-35*).

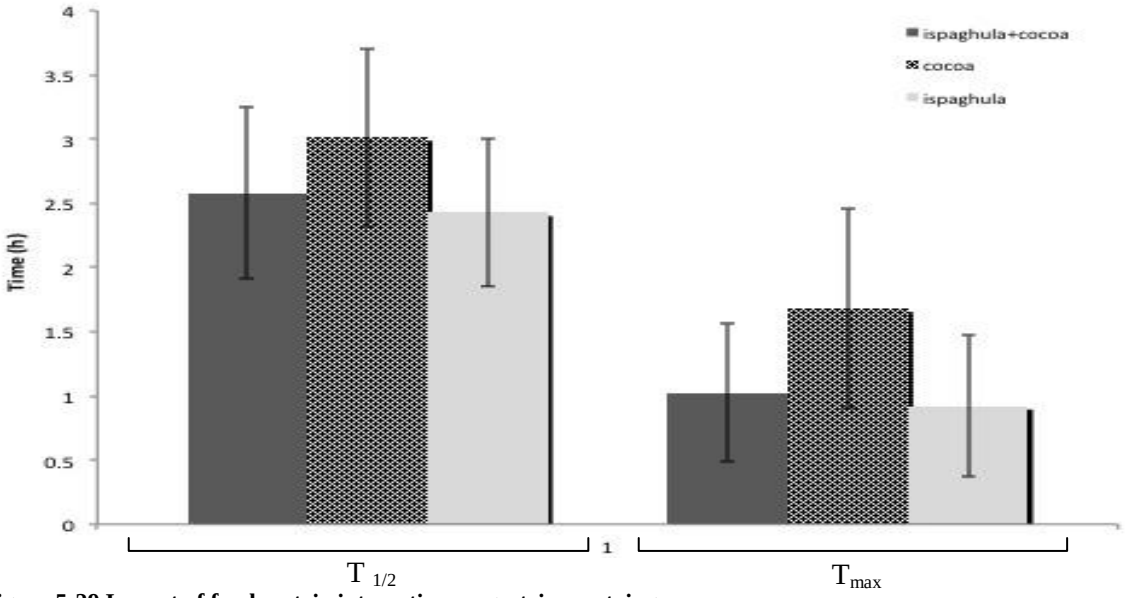


Figure 5-29 Impact of food matrix interaction on gastric emptying
Values are displayed as mean (± SD) across 24 h after consumption of 20g cocoa and /or15g ispaghula in water (n=12) measured as plasma concentration of paracetamol (1g). $T_{1/2}$ and T_{max}: p= 0.01 (C vs. C+ISP), <0.01 (C vs. ISP).

There were no significant differences in AUC for the different arms of the study. However, the area under the curve from highest to low would be in the following order cocoa > ispaghula+cocoa > ispaghula order.

Figure 5-32 C vs. I+C t1/2 correlation

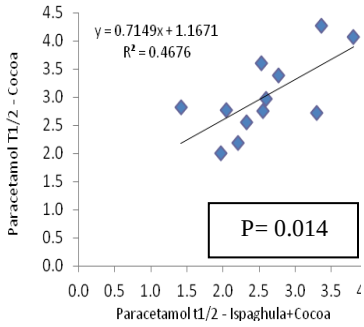


Figure 5-32 C vs. I t1/2 correlation

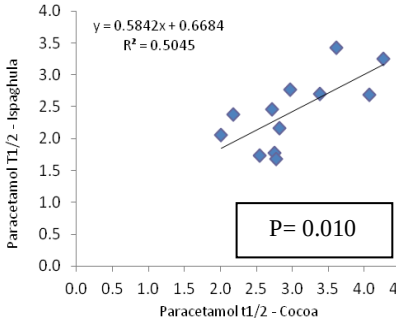


Figure 5-32 I vs. I+C t1/2 correlation

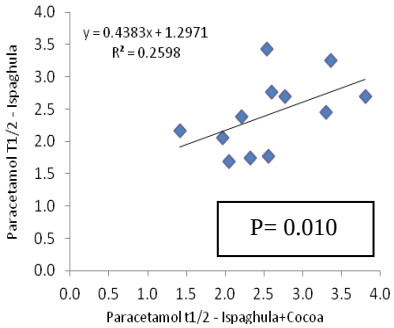


Figure 5-35C vs. I+C AUC correlation

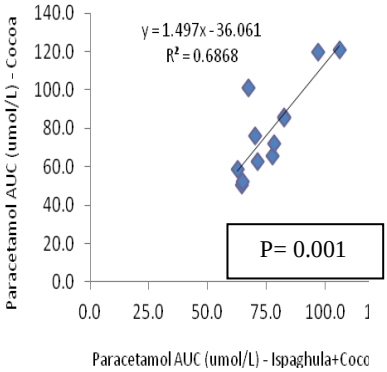


Figure 5-35 C vs. I AUC correlation

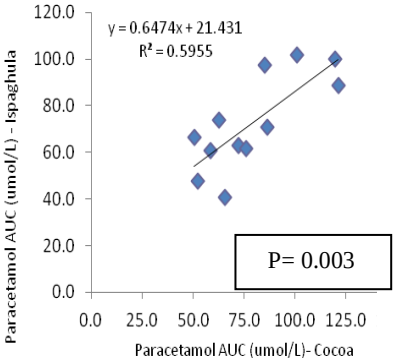
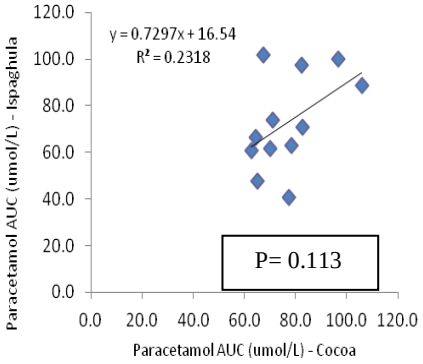


Figure 5-35 I vs. I+C t1/2 correlation



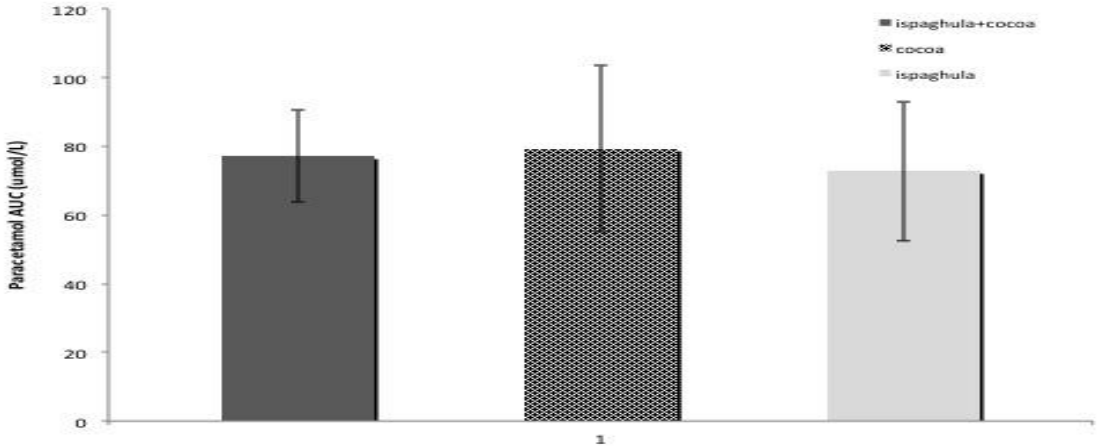


Figure 5-36 Paracetamol AUC

Values are displayed as mean (± SD) across 24 h after consumption of 20g cocoa and /or15g ispaghula in water (n=12) measured as plasma concentration of paracetamol (1g). AUC: area under the curve

5.3.7 Mouth to caecum transit time

Breath hydrogen was measured as an estimation of transit time and reported for each volunteer. >6 hours was assigned when the head of meal did not reach the caecum before 6 hours of meal consumption. There was no difference in MCTT between the meals.

Table 5-9 Mouth to caecum transit time measured as exhaled breath hydrogen

	Time for head of meal reaching caecum (hours)		
volunteer	Ispaghula	Cocoa	Ispaghula+Cocoa
2	>6.0	>6. 0	4.5
3	4.5	4.5	4.5
4	4.5	5.0	4.5
5	5.0	5.5	4.5
6	2.5	4.5	3.5
7	5.0	4.5	4.0
8	>6.0	>6.0	>6.0
9	5.5	>6.0	5.5
10	5.5	4.5	4.5
11	5.0	4.5	5.5
12	4.5	4.0	4.5
13	4.5	>6.0	5.5
Mean ± STDEV	4.0 ± 1.0	5.25 ± 0.9	4.7 ± 0.8

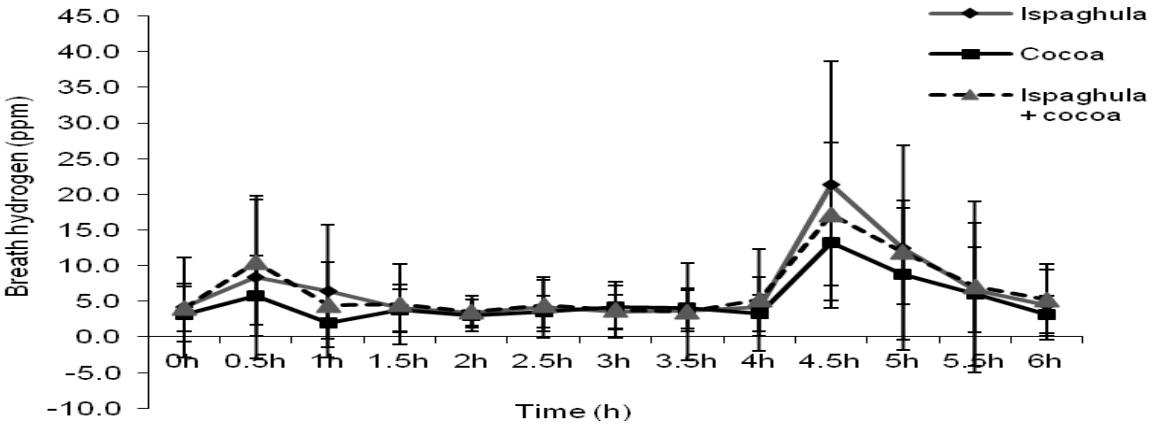


Figure 5-37 mouth to caecum transit time
Values are displayed as mean (± SD) across 6 h after consumption of 20g cocoa and /or15g ispaghula in water (n=12) measured as exhaled breath hydrogen.

Breath hydrogen is also an indication of fermentation by the gut microbiota, ispaghula had the highest AUC for breath hydrogen (12.9) followed by ispaghula+cocoa (11.8) and cocoa (8.7).

5.3.8 Subjective assessment of appetite

The results were analysed for first meal (feed-1: ispaghula, cocoa or ispaghula+cocoa) and second meal (feed-2: lunch) separately. The area under the curve was used as an indication of subjective assessment of appetite.

5.3.8.1 Q1- How hungry do you feel?

Hunger ratings for cocoa were higher than the combination group for feeding-1 (p=0.037), however there were no differences between cocoa/ispaghula and ispaghula/ ispaghula+cocoa (p=0.175 and p=0.06 respectively). There were no differences between groups for feeding-2.

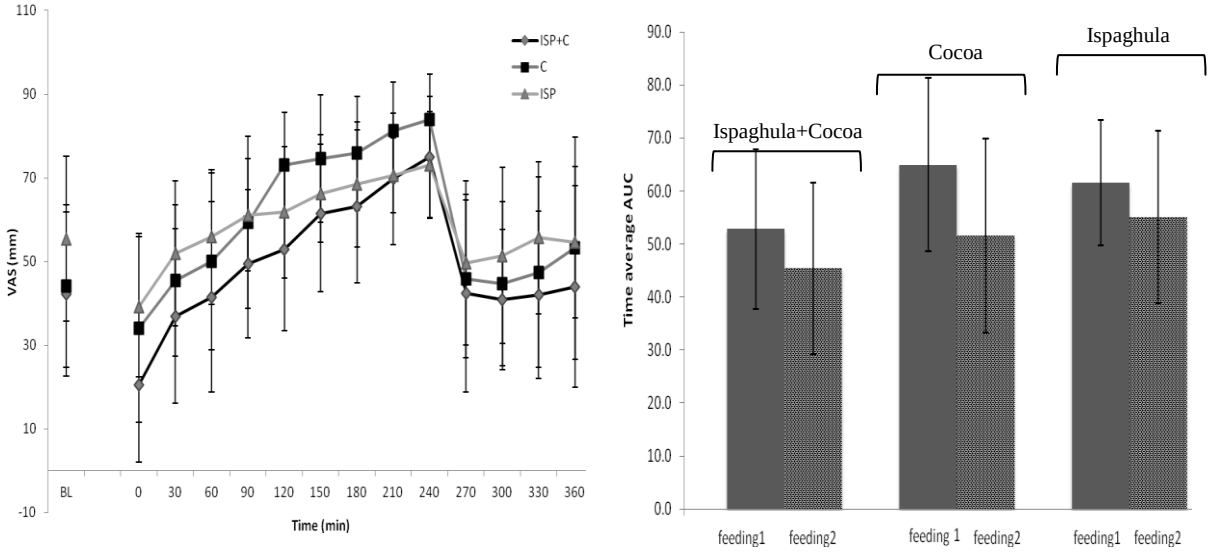


Figure 5-38 VAS rating and AUC for Q1
Q1: How hungry do you feel? Values are displayed as mean (± SD) across 6 h after consumption of 20g cocoa and /or15g ispaghula in water (n=12). C: cocoa, ISP: ispaghula, ISP+C: ispaghula+cocoa, BL: baseline, feeding-1 (pre-lunch) <4h, feeding-2 (post-lunch) >4h

The change in hunger levels was the highest for the combination group from BL to 0h (21.7mm) followed by ispaghula (16.4mm) and cocoa (10 mm). Hence, the immediate satiating effect was most for the combination group and least for cocoa. This pattern was extended up to 270 minutes, after which lunch meal was introduced.

5.3.8.2 Q2-How satisfied do you feel?

The combination group had the highest rating compared to cocoa (p=0.03) but there were no differences for ispaghula with the combination group or cocoa for feeding-1. Similarly to Q1, there were no differences between groups for feeding-2.

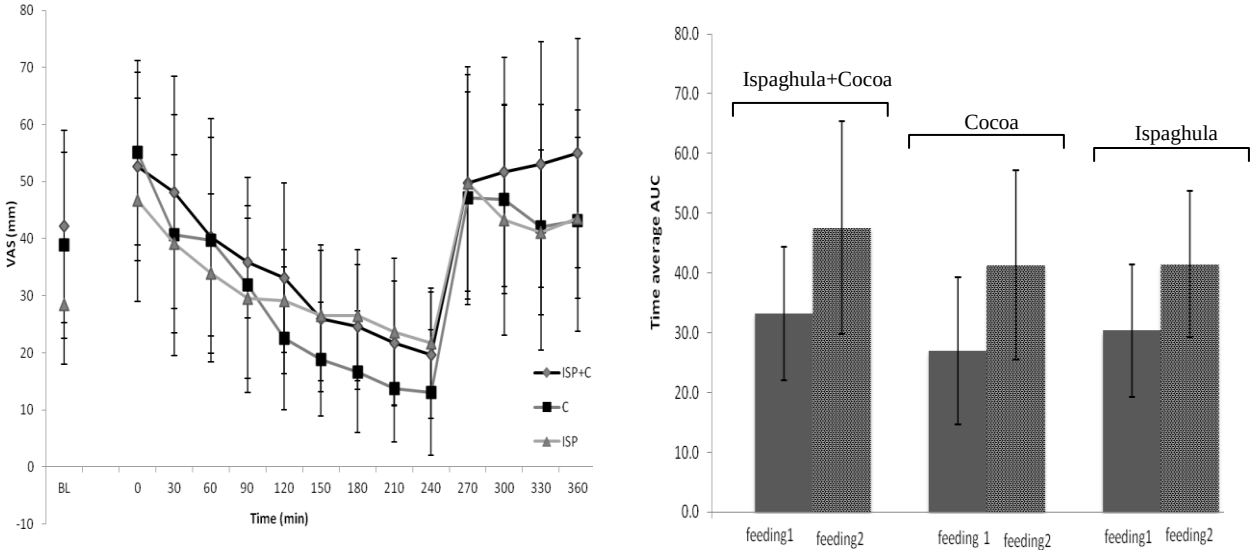


Figure 5-39 VAS rating and AUC for Q2
Q2: How satisfied do you feel? Values are displayed as mean (± SD) across 6 h after consumption of 20g cocoa and /or15g ispaghula in water (n=12). C: cocoa, ISP: ispaghula, ISP+C: ispaghula+cocoa, BL: baseline, feeding-1 (pre-lunch) <4h, feeding-2 (post-lunch) >4h

5.3.8.3 Q3-How full do you feel?

Similarly to Q2 the combination group had the highest rating compared to cocoa (p=0.02) but there were no differences for ispaghula with the combination group or cocoa for feeding-1. There were no differences between groups for feeding-2.

5.3.8.4 Q4-How much more do you think you can eat?

There were no differences in VAS ratings between groups for feeding-1 or feeding-2. A correlation was found between feeding-1 and feeding-2 for Ispaghula+cocoa (p=0.02), cocoa ($p < 0.01$) and ispaghula ($p < 0.01$). This could be an indication that the impact of the supplement on appetite is extended to after secondary meal consumption.

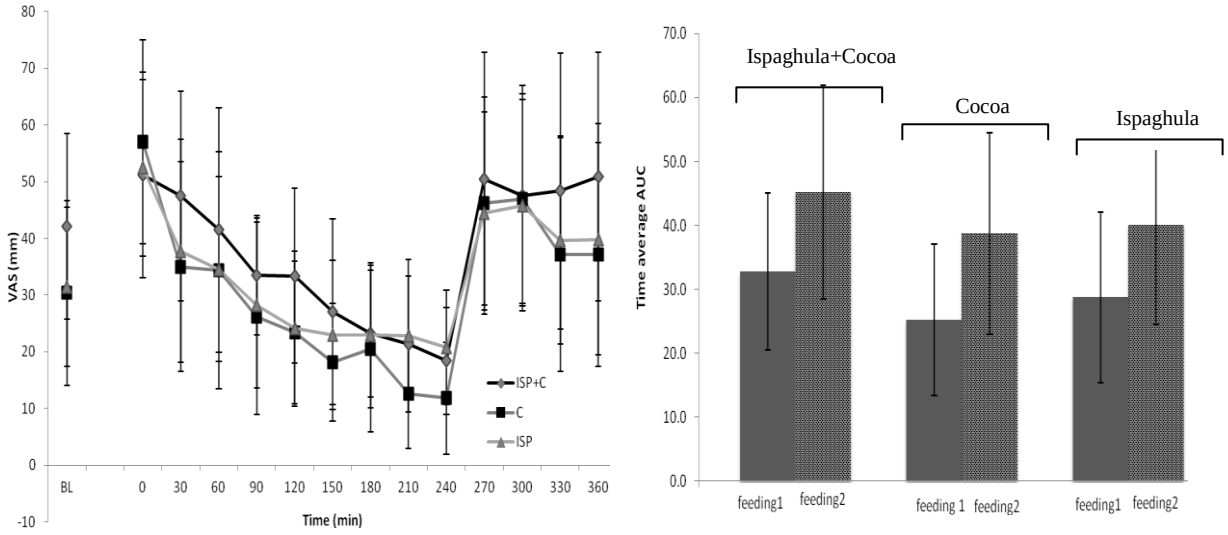


Figure 5-40 VAS rating and AUC for Q3
Q2: How full do you feel? Values are displayed as mean (± SD) across 6 h after consumption of 20g cocoa and /or15g ispaghula in water (n=12). C: cocoa, ISP: ispaghula, ISP+C: ispaghula+cocoa, BL: baseline, feeding-1 (pre-lunch) <4h, feeding-2 (post-lunch) >4h

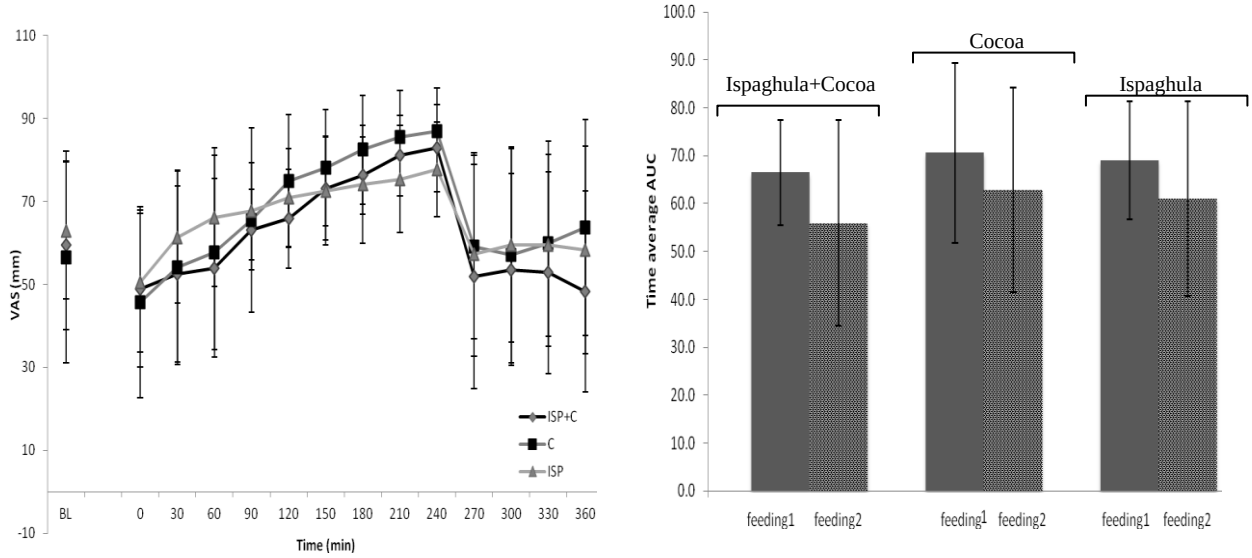


Figure 5-41 VAS rating and AUC for Q4
Q2: How much more do you think you can eat? Values are displayed as mean (± SD) across 6 h after consumption of 20g cocoa and /or15g ispaghula in water (n=12). C: cocoa, ISP: ispaghula, ISP+C: ispaghula+cocoa, BL: baseline, feeding-1 (pre-lunch) <4h, feeding-2 (post-lunch) >4h.

An expected high variation is seen in the subjective assessment of appetite among volunteers. Due to the paired natured of the study, the standard deviations presented in the above figures are not relevant to the statistical analysis of the results.

5.4 Discussion

The ingestion of 20g cocoa increased the urinary excretion of eleven phenolic acids, nine of which were significantly reduced when cocoa was consumed along with ispaghula (*Table 5-10*). Consumption of 15g ispaghula also resulted in the increase in concentration of four phenolic acids in urine as well as an acceleration in gastric emptying rate. Total phenol and antioxidant capacity were not different between cocoa and ispaghula in urine or plasma, however the consumption of cocoa and ispaghula together significantly reduced the total phenol (TP assay) and antioxidant capacity. While free phenolics were found to be significantly higher in cocoa, the reverse was seen for bound phenolics. The overall sum of bound and free phenolics remained higher for cocoa than ispaghula.

The study model was optimised by administering ispaghula and cocoa with water rather than milk. This allowed for the better understanding of the impact of ispaghula on gastric emptying, eliminating fat and proteins present in milk as a co-founding factor. Additionally, studies investigating matrix interactions between cocoa consumed with milk, demonstrated a lower bioavailability of cocoa flavan-3-ols when cocoa was consumed with milk (Mullen et al., 2009a). Epicatechin levels have been shown to return to baseline measurements within 6 hours after the consumption of cocoa in both chocolate and beverage form (Schramm et al., 2003, Serafini et al., 2003, Neilson et al., 2009). Based on this evidence we collected plasma samples over 6 hours.

The phenolic acids produced from cocoa consumption *in-vivo* were 3-HPAA, 4-HPAA, 3,4-DHPAA, Hippuric, 4-HPA, 4-HBA, 3,4-DHBA, Vanillic, 4-HVA, Mandelic and 4-HMA. A summary of urinary phenolic acid excretion after cocoa consumption, comparison to previous *in-vitro* study (*Chapter-4*) and previously published studies has been summarised in *Table 5-101*.

It is important to note that in the previously published papers, even though these phenolic acids were detected in urine, not all were significantly higher from baseline after the consumption of cocoa. An example of this is the identification of 17 colonic metabolites after long-term cocoa consumption in the study by Urpi-Sarda et al., (2009). Out of the 17 metabolites only DHPV, vanillic acid , 3-HPAA, 3,4-DHPAA and 3-HHA increased significantly from baseline with DHPV being the highest produced and 3-HPAA the lowest produced phenolic acid. As vanillic acid and 3-HPAA were produced in all four studies and at significantly higher concentration than baseline after cocoa consumption, they could be considered as good biomarkers of cocoa polyphenol bioavailability. Ispaghula, being a plant extract produced relatively high concentrations of phenolic acids. However, in our study vanillic acid and 3-HPAA, along with 3,4-DHBA, mandelic acid and homo-vanillic acid were significantly higher after

cocoa consumption than ispaghula consumption. This further supports the use of these two phenolic acids as biomarkers of cocoa polyphenols.

Table 5-10 Impact of ispaghula on phenolic acid production in-vitro and in-vivo

Phenolic acids	**Current study (GCMS)**	**Ex-vivo study (Chapter-4) GCMS**
	20g cocoa powder	0.5g cocoa in 50ml incubation vessel
PAA		↓ X
3-HPAA	↓ X	X
4-HPAA	↓ X	↓ X
3,4-DHPAA	X	X
4-HBA	↓ X	X
3,4-DHBA	↓ X	X
Vanillic	X	X*
Homo-vanillic	↓ X	
Hippuric	↓ X	X**
4-HHA	↓ X	
3-HPPA		X
4-HPPA		X
3,4-DHPPA		X
Mandelic	↓ X	
4-HMA	↓ X	
Total sum of phenolic acids	↓	↓

↓ inhibition in production either by ispaghula (*ex-vivo* study) and/or matrix interaction between ispaghula and cocoa (*in-vivo* study), *produced only in one volunteer **produced in only two volunteers.

Incidentally the two phenolic acids (vanillic acid and 3,4-DHPAA) not inhibited by ispaghula were also found in significantly higher concentration after cocoa consumption than ispaghula. Despite there being no inhibitory impact of ispaghula on production of these two phenolic acids when all subjects were considered, an inhibitory impact of ispaghula on the production of these phenolic acids were demonstrated in the high producer group but not in the low producer group. The production of the remaining nine phenolic acids was inhibited which was determined by the comparison of the predicted value to the true combination value. Additionally 4-HPAA exhibited a lower concentration for the combination group than cocoa.

An inhibitory impact of food matrix interaction was seen in high producers of phenolic acids for all phenolic acids except 4-HHA. Only three of the phenolic acids (HVA, Hippuric acid and 4-HHA) were inhibited in the low producers of phenolic acids. It makes sense that if an individual does not produce much of a phenolic acid that it will be less easy to deomstrate an inhibitory effect of a fibre than if a larger amount is produced.

In addition to an inhibitory impact, the food matrix interaction resulted in a delay of phenolic acid appearance in excreted urine. Examples of this delay observed in this study were 3,4-DHBA detected in urine 5h after consumption of cocoa and 8h after consumption of cocoa+ispaghula, 4-HBA 2h for cocoa and 5h for cocoa+ispaghula, HVA acid was detected at 2h for cocoa, 8h for ispaghula and 5h for cocoa+ispaghula.

We may have expected ispaghula to slow gastric emptying but it speeded up paracetamol accumulation in the plasma. Viscous have been shown to both slow and speed gastric emptying depending on whether the meal was liquid or solid. Amidon (1985) hypothesised that the relation between the movement of food particles and their suspending fluid is dependent on how fast these particles sink or float out of the fast moving central stream of water in the lumen. Thus based on this hypothesis increased viscosity can result in faster gastric emptying and small bowel transit of solid particles. Meyer et al. (1986) investigated the impact of 200-800ml of saline or saline+guar on the gastric emptying of the solid meal 75 g of steak plus 25 g of 99mTc-labeled chicken liver. They demonstrated that guar accelerated the gastric emptying of the solids and delayed the gastric emptying of the fluids. Thus it is important for studies interested on the impact of viscosity on gastric emptying and MMCT to consider both liquid and solid meals.

The mean time required for the head of the meal to reach the caecum was not affected in previous studies regardless of physical state of food or analytical method i.e the gold standard scintigraphy or breath ^{13}C measurement (Washington et al., 1998, Bianchi and Capurso, 2002, McIntyre et al., 1997) as summarised in *Table 5-2*. However ispaghula delayed the gastric emptying of a liquid meal in a study by Washington et al but had no impact on the gastric emptying of solid meals (McIntyre et al., 1997, Bianchi and Capurso, 2002, Rigaud et al., 1998, Frost et al., 2003).

In our study the MCTT was found to be the slightly but not significantly higher for ispaghula alone, followed by the combination of cocoa+ispaghula and cocoa alone. This could possibly explain the delay in phenolic acid excretion in urine from ispaghula and when ispaghula was combined with cocoa. The breath hydrogen method measures the transit time of the head of the meal only and it is possible that the transit of the rest of the meal was delayed. Additionally, the AUC for breath hydrogen was found to be highest for ispaghula, followed by the combination group and cocoa alone; indicating that cocoa has a slight inhibiting impact on ispaghula fermentation *in-vivo*. However, this claim was not supported by statistical analysis.

Another factor playing a key role in matrix interaction studies is the flavanol concentration of the cocoa used. Mullen et al (2009) investigated the impact of milk on bioavailability of cocoa flavan-3-

ols. They demonstrated an inhibitory effect of milk on the bioavailability of these flavan-3-ols using a cocoa having 45 umol flavan-3-ol monomers. The previous studies by Roura et al (2007) and Keogh et al (2007) did not show any inhibitory impact of milk or milk proteins on the flavan-3-ol bioavailability of cocoa. These studies used cocoa containing 128 umol and 2374 umol flavan-3-ol monomers respectively, amounting to 3 and 25 fold of the flavan-3-ols used in the Mullen et al (2009) study. The flavanol monomer concentration of our study was 39 umol, which was similar to that of the Mullen et al (2009). Hence, it is possible that cocoa phenolic production could be inhibited in the presence of viscous fibre and other food components such as milk when commercially available cocoa is used in amounts feasible for daily consumption.

There have been no previous studies investigating the impact of viscous fibre on cocoa flavanol bioavailability and there is certainly a need for more investigation of different kinds of carbohydrates and their matrix interaction on cocoa flavanols.

Due to the high concentration of phenolic acids produced by ispaghula we conducted a total phenol assay investigating both free and bound phenolics in cocoa and ispaghula. There was not time for a full polyphenolics analysis. While cocoa had significantly higher free phenolics, the reverse was seen for bound phenolics; with ispaghula having relatively high concentrations of bound phenolics. The consumption of both cocoa and ispaghula resulted in an increase in urine Total phenol and antioxidant capacity at 24 hours compared to baseline. This increase was not seen in plasma samples.

We aimed to investigate the satiating effect of ispaghula on appetite using 3 questions after the test meal (meal 1) and after introducing lunch (meal 2). We observed the following pattern for the questions on hunger levels (Q1) and the ability to eat more (Q4) for meal 1: Cocoa > Ispaghula >Ispaghula+cocoa. The reverse order (Ispaghula+cocoa > Ispaghula> cocoa) was seen for level of satisfaction and fullness. There was a significant difference between the combination group and cocoa for Q1,Q2 and Q3. Even though there were no significant differences for the second meal, a correlation was found between meal 1 and meal 2 patterns, indicating that the satiating impact of the supplements can be extended to after secondary meal consumption. Understandably, the results of these subjective appetite assessments could have been influenced by the caloric content of the test meal. As the main outcome of this study was the impact of food matrix interaction on the bioavailability of cocoa phenolic acids, we did not introduce a further matrix interaction such as sugar/fat or protein, previously seen to influence cocoa flavan-3-ol bioavailability to produce 3 isocaloric supplements. Furthermore, most of the satiating effects seen in the study can be attributed to ispaghula as we did not find any differences between ispaghula+cocoa (having higher caloric content) and ispaghula alone. The only

differences observed were between ispaghula+cocoa and cocoa alone. The results observed for subjective assessment of appetite are in accordance with the observations made for mouth-caecum transit time.

Table 5-11 Phenolic acid excretion in urine after cocoa consumption

Phenolic acids	**Current study (GCMS)**	**Mullen et al., 2009 (LCMS)**	**Rios et al., 2003 (GCMS)**	**Sarda et al., 2009 (LCMS-MS)**
	20g cocoa powder	10g cocoa powder	80g Chocolate	(20g cocoa powder)* 2 /day for 4 weeks
PAA		X		X
3-HPAA	↓ X	X	X	X
4-HPAA	↓ X			
3,4-DHPAA	X		X	X
4-HBA	↓ X	X		X
3-HBA		X	X	X
3,4-DHBA	↓ X			
Vanillic	X	X	X	X
Homo-vanillic	↓ X	X		X
Hippuric	↓ X	X	X	
4-HHA	↓ X	X		X
3-HHA				X
3-HPPA			X	X
3,4-DHPPA		X		X
Mandelic	↓ X			
4-HMA	↓ X			
Ferulic		X	X	X
Caffeic		X		X
P-coumaric		X		X
m-coumaric		X		X
protocatechuic		X		X
DHPV				X
Total sum of phenolic acids	↓			

↓ inhibition in production either by ispaghula (*ex-vivo* study) and/or matrix interaction between ispaghula and cocoa (*in-vivo* study),

The exact nature of the mechanism resulting in the reduction of urinary phenolic acids after ispaghula is unclear as it may be due to the viscosity inhibiting mixing of the bacteria and substrates and this could push more of the phenolics to the distal colon. However, it may be that the bacteria are diverted to other metabolic activities such as ispaghula degradation or that the bacterial composition has been altered by the fibre although there is not much time for this in the acute study. The viscosity of the ispaghula may also have brought more of other compounds such as lipids to the colon and these may have some impact too. There was less inhibition in the in vitro model for ispaghula than the *in-vivo*

study but this may have been due to the impact of viscosity in the upper gut and the impact of gut motility and other *in-vivo* factors not present in the *in-vitro* model. It wsa not possible to explore these further.

5.5 Conclusion

The consumption of both ispaghula and cocoa resulted in increased urinary Total phenol, antioxidant capacity and phenolic acid concentration. The food matrix interaction between the two, inhibited and delayed the production of phenolic acids from cocoa, reduced total phenol and antioxidant capacity. The consumption of ispaghula and cocoa separately could induce beneficial health effects by increasing total phenol and phenolic acid production, however the consumption of both simultaneously does not correspond to the sum of individual (cocoa / ispaghula) total phenol and phenolic acid production. More studies are required to better understand the impact of different kind of carbohydrates on phenolic acid production from cocoa *in-vivo*.

CHAPTER 6

Discussion

6.1 Research questions:

The research questions addressed in this PhD build on previously established evidence on the role of the colonic microbiota in the fermentation of fibre, and degradation of polyphenols and, prebiotic and antibiotic impact as well as the low absorption of both of these compounds in the small intestine, making the colon their main site of metabolism.

After consideration of the relevant literature, it was clear that the metabolism of fibre and polyphenolics by colonic bacteria will involve some element of interaction. It was therefore important to ask whether the interaction of these compounds and their metabolites results in synergistic, additive or modification of their metabolism and how this may impact on their potential health benefits. This led to two main research questions:

Q1- Do fermentable fibres affect phenolic acid production from polyphenols?
Q2- Do polyphenols affect the SCFA production from fermentable fibres?

By using the model demonstrated in *Figure 6-1*, we addressed the research questions and demonstrated that there is indeed an impact of the matrix interaction between fibres and polyphenols.

A series of studies were conducted to achieve the aims and objectives of this PhD. The studies were designed to feed into a final long-term intervention study (*Figure 6-1*). However due to limitations discussed below, the long-term study was omitted, thus changing the direction of the PhD yet maintaining the same research questions.

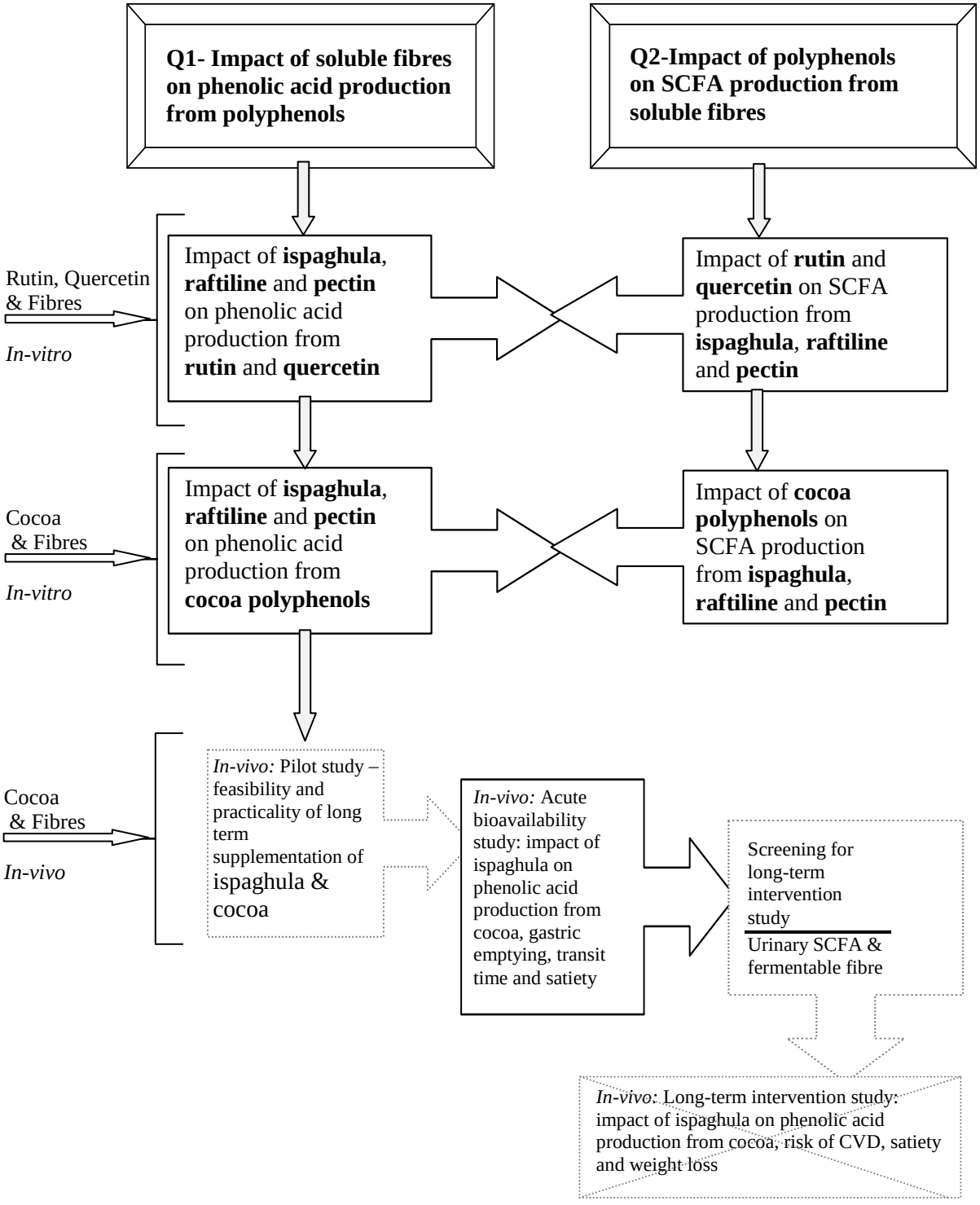


Figure 6-1 Overview of the studies in this PhD.

6.2 Do fermentable fibres affect phenolic acid production from polyphenols?

The impact of soluble fibres on the production of phenolic acids was investigated both *in-vitro* and *in-vivo.* The soluble fibres ispaghula, raftiline and pectin were chosen based on their different physio-chemical properties such as fermentability and viscosity.

We began by exploring the impact of these soluble fibres on the colonic metabolism of the pure polyphenolic compounds; rutin and quercetin, which are present in abundance in the diet. This allowed the potential observed impact to be directly attributed to the action of the soluble fibres. In previous studies, the addition of glucose to fermentation vessels containing rutin by (Jaganath et al., 2006b) to simulate the presence of fibres such as cellulose, beta glucan and other such fibres with glucose as their common constituent, resulted in an increased deglycosilation of rutin and subsequent increase in phenolic acid production. Given that ~77-86% of ingested rutin and quercetin escape absorption in the small intestine (Hollman et al., 1995, Walle et al., 2000, Kahle et al., 2005, Jaganath et al., 2006); their bioavailability is greatly dependent on their colonic metabolite production.

The studies in this thesis provided evidence that fermentable carbohydrates inhibit the phenolic acid production from polyphenols like rutin *in-vitro*, thus reducing their bioavailability.

However, polyphenols are usually consumed as part of the diet integrated in plant based foods. Thus we further explored the impact of soluble fibres on polyphenols present within their food matrix. Cocoa is a rich source of polyphenols (Total polyphenols: 512 mg/100g, phenolic acids: 37.06 mg/100g), especially compared with commonly consumed foods containing rutin or quercetin (Onion: 167 mg/100g total polyphenols, 2.0 mg/100g phenolic acids, Tomato: 3.87 mg/100g total polyphenols, 0.29 mg/100 g phenolic acids). However, the bioavailability of cocoa polyphenols may be affected by its food matrix, such as its reduction when consumed with milk (Mullen et al., 2009a). Most importantly cocoa is a popular food choice and commonly consumed, making it a suitable meal rich in polyphenols for long-term interventions. This is of importance as one of the limitations of long-term interventions is the compliance of volunteers to the food/diet. Cocoa is also incorporated in commonly consumed foods along with soluble fibres such as the Kellogg's-All Bran-Bran Buds Cereal containing ispaghula husk, wheat bran and oat bran in the USA.

Using this information we conducted another in-vitro study incubating cocoa with or without ispaghula, raftiline or pectin. Similar to the results seen for rutin and quercetin, phenolic acid production from cocoa was inhibited by the soluble fibres.

Such *in-vitro* studies provide information on the potential interaction of fibres with polyphenols in the colon but are limited by the accumulation of fermentation products in the vessels due to the lack of absorptive mechanisms; not providing any information on the potential impact of these fibres on polyphenol absorption in the small intestine. Thus we followed up the above study with an *in-vivo* study model.

In this regard the high viscosity of ispaghula was ideal for investigating the impact of gastric emptying and transit time modification on the subsequent reduction or delay of the cocoa phenolic acid excretion in the urine.

Initially I planned to do a long-term feeding trial in over-weight individuals with increased risk of CVD. The plan of studies comprised of three parts: Pilot study, acute in-vivo cross-over study, followed by long-term intervention (4 weeks/arm, Appendix-5) cross-over study. I obtained the ethics and began with the pilot study and screening individuals for the CVD risk factors including hyperlidemia > 2.6 mmol/l LDL-C.

The pilot study was conducted to assess the feasibility and practicality of long-term supplementation/ consumption of ispaghula and cocoa. Thus we tried to find a good dietary vehicle for the cocoa and ispaghula. 30 volunteers received ispaghula alone or in combination with cocoa in three different supplementation forms for two days each: biscuits, milk or water based beverage. An estimated food diary was kept to monitor changes in energy intake (E_i). VAS questionnaires were used for appetite ratings as well as taste and convenience of supplement form. The biscuit meal was the most preferred whereas the water beverage was hardly tolerable. VAS questionnaires were not easy to be completed in everyday life. It was concluded that acceptability of the cocoa/ispaghula supplement was good in the correct administration form such as biscuits.

Upon completion of the pilot study we began the screening of volunteers for the long-term study. Inclusion criteria included over the age of 30, having a waist circumference over 94 cm for men, and over 80 cm for women and LDL levels > 2.6 mmol/L. Participants were excluded for one of the following reasons: participants younger than 30 years of age, smoking, females on menopause, medication e.g. statins. Over 40 individuals who met all criteria were screened for plasma LDL levels. None of the individuals displayed LDL > 2.6 mmol/L. Additionally there was no association between BMI (over weight vs. obese) and plasma cholesterol levels. Thus the long-term intervention was deemed non-feasible in the time frame of this PHD.

We then proceeded with the acute bioavailability study, which has been described in *Chapter-5*. Given that the long-term study was omitted, the risk of CVD was no longer relevant. Hence the inclusion

criteria of the bioavailability study were modified to represent a homogenous healthy population, more suitable for assessing the impact of ispaghula on cocoa polyphenol bioavailability and minimising inter-individual variation.

Our initial observation was the high urinary phenolic acid excretion for four of eleven phenolic acids after ispaghula ingestion. This was not surprising as ispaghula is a plant extract and some phenolic acid production may be expected. The test meals in this study were ispaghula, ispaghula+cocoa and cocoa, thus there was no point of reference for the urinary excretion in the absence of these compounds and we could not determine if these phenolic acids were produced from ispaghula or through other metabolic pathways in the body. We addressed this limitation in the analysis for the impact of ispaghula on the phenolic acid production of cocoa by comparing the data obtained from simultaneous ingestion of cocoa and ispaghula to the expected/ predicted value for the urinary excretion of phenolics after consumption of cocoa+ispaghula (sum of urinary phenolic acid excretion after ispaghula consumption to after cocoa consumption).

The results obtained from this study were supportive of the *in-vitro* study results, providing further evidence of the inhibitory impact of soluble fibres on phenolic acid production from polyphenols. However it may be that the phenolic acid production from cocoa is delayed rather than completely inhibited as the total sum of phenolic acid demonstrated a linear increase in concentration of total phenolics up to 24 hours. This can be determined in future studies by measuring phenolic acids until maximal conversion of polyphenols is achieved. The comparison of the *in-vitro* study and *in-vivo* study clearly demonstrated a stronger inhibitory impact of ispaghula *in-vivo* than *in-vitro*. It would be important to explore the impact of pectin and raftiline on cocoa phenolic acids *in-vivo* to determine if their impact is magnified as in with ispaghula or reduced.

Some of the impact of ispaghula *in-vivo* may be due to its viscosity and the potential modification of gastric emptying rate and MCTT. The high viscosity of ispaghula may entrap the polyphenols, preventing their absorption in the small intestine, thus delivering higher amounts of the polyphenols to the colon. Within the colon the high viscosity of ispaghula may reduce the accessibility of the polyphenols to the bacteria and it may also deliver higher amounts of the polyphenols to the distal colon and subsequent result in higher amounts of the polyphenol being excreted through faeces (*Figure 6-2).*

Additionally fermentable fibres may be used as a preferential source of energy for the bacteria thus the hydrolysis of the polyphenols may be delayed in the presence of fermentable fibres. The fermentation of the fibre by the colonic bacteria results in SCFA production and the accumulation of these

metabolites decrease the colonic pH, which may affect the composition of the bacteria. Very little is known on how this change in colonic pH may affect the metabolism of polyphenols. Next Generation DNA sequencing would provide information on bacterial diversity and the mechanism behind the impact of fermentable fibres on phenolic acid production from polyphenols, especially for *in-vitro* models. It would be difficult to get meaningful information in vivo as the only samples available would be faecal and the metabolism of phenolics mainly happens in the proximal colon. We did not have sufficient funds to carry out this analysis but it should be included in future studies, especially now the costs have substantially reduced.

Another measurement which would be key to the understanding interactions with fibre, especially *in-vivo* is the measurement of parent polyphenolic compounds, their appearance in plasma and urine and their disappearance in in vitro models. However due to limited funding, access to equipment and the time available, this analysis was not possible during the period of this PhD.

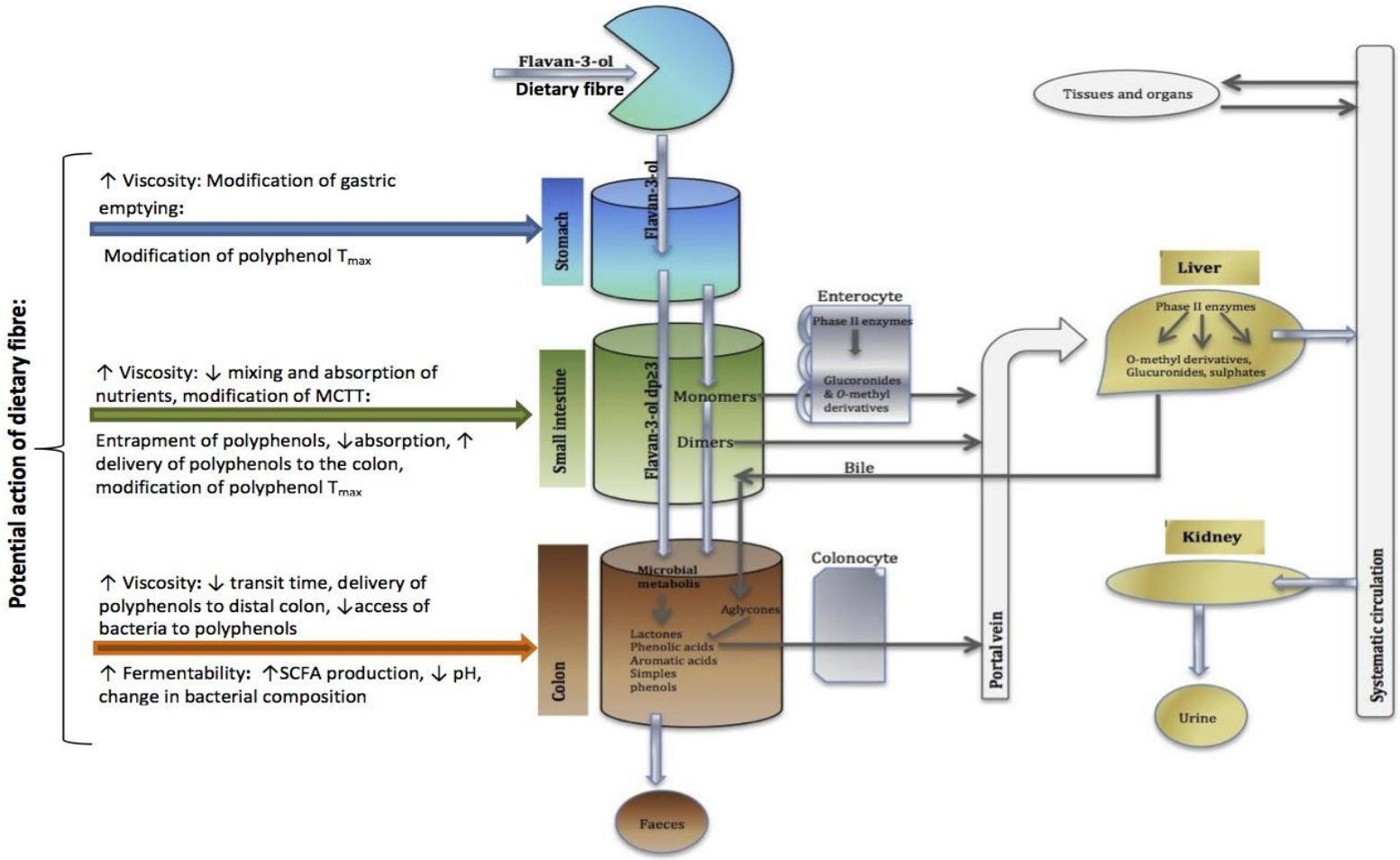


Figure 6-2 Impact of dietary fibre on bioavailability of polyphenols
The figure illustrates the impact of fibre on bioavailability of polyphenols using flavan-3-ols as an example. Adapted from Monagas et al. (2010)

6.3 Do polyphenols affect the SCFA production from soluble fibres?

The impact of polyphenols on soluble fibres was investigated based on previous evidence demonstrating the potential antibiotic and prebiotic impact of some polyphenolic compounds and that this possible modification of colonic microbiota may impact their fermentation action on fibre and subsequent SCFA production. The review of literature on the antibiotic impact of polyphenols clearly demonstrated great inconsistency in findings and poor understanding of the impact of these polyphenols in relation to their physiological concentration in the diet; as majority of the methods have used non-physiological concentrations of polyphenols applicable to the pharmaceutical industry but not achievable through the diet. Additionally the majority of studies were conducted using *in-vitro* pure batch cultures of selected bacterial species, which is not representative of the complex microbiota colony.

An overview of these studies may suggest a stronger impact of phenolic acids and aglycones than glycosidic compounds on the inhibition of bacterial growth, with little to no change in total bacterial count (Arima et al., 2002, Lee et al., 2008, Parker et al.,2008). Many studies have also demonstrated that the beneficial bacteria Lactobacilli are more resistant to this antimicrobial action (Parker et al.,2008, Tzounis et al.,2008). Although there is some evidence on the antibacterial properties of polyphenols, there is no information available on how this may impact the production of metabolites such as SCFA from the fermentation of fibre, which is one of the mechanisms through which the microbiota benefit the host.

Quercetin has been demonstrated to have one of the strongest antibiotic effects compared to other tested polyphenolic compounds (Vaquero et al., 2007, Parker et al., 2008). Its glycosidic form rutin has however shown little if no impact on the colonic microbiota population in-vitro (pure culture growth), which may not necessarily relate to *in-vivo* conditions; where rutin is degraded by the colonic microbiota to quercetin and smaller molecular weight phenolics which may in turn exhibit antimicrobial properties (Lee et al., 2008). Thus we investigated the impact of both rutin and quercetin on the metabolite production from fibres using the highly fermented non-viscous raftiline, highly fermented viscous pectin and incompletely fermented, highly viscous ispaghula. There was no evidence on the impact of these polyphenols on metabolic action of the microbiota. The polyphenols did not affect the pH or gas production from fermentations. It may be that the promotion of growth by the fibres overcomes any antibiotic action but also as we did not measure directly any bacterial populations there may be some inhibition of bacteria but no impact on metabolism.

We then continued to investigate the impact of the polyphenols present in the cocoa matrix on the SCFA production from the same fermentable fibres. Unlike the pure polyphenolic compounds, cocoa

contains high carbohydrate and fibre content, which resulted in substantial SCFA production. The digestion of cocoa before it is added to fermentation vessels in future studies may reduce SCFA production from the simple carbohydrates, which would normally be absorbed in the small intestine. However some starch may escape digestion *in-vivo* and be fermented in the colon. We applied the same analytical method for the correction of SCFA production from cocoa as that described for the impact of ispaghula on cocoa phenolic acids *in-vivo* (expected/predicted value vs. true combination value). The outcomes of this study were in line with the results of rutin/quercetin impact on SCFA production; not demonstrating any impact of cocoa polyphenols on SCFA production from the soluble fibres.

Rutin, quercetin and cocoa polyphenols did not affect the pH or gas production from the fermentation vessels, In fact there was no difference in pH of rutin or quercetin to that of faecal slurry alone. Due to the lack of evidence on the impact of the polyphenols on SCFA production from soluble fibres *in-vitro* and the relatively low concentration of urinary SCFAs, we did not proceed with the investigation of this interaction *in-vivo*. The use of faecal sample for measurement of SCFAs is not a good measure due to the continuous absorption of SCFAs throughout the colon, thus faecal SCFAs may not be representative of metabolism in the proximal colon.

There was a high level of biological variation in the bioactive molecules produced in the models used in this PhD supporting previous statements on high inter-individual variation of colonic microbiota profile and their metabolic products. The overall design of the PhD was meant to facilitate the reduction in this inter-individual variation. The faecal samples used for both rutin and cocoa *in-vitro* studies were common with the exception of two volunteers. The age, BMI and ethnicity of participants did not differ between the two studies. Additionally the high standard deviations were not relevant to the statistical analysis of the paired model of the in-vitro studies or the cross-over model used for the in-vivo studies.

6.4 Relevance and applications of findings:

To our best knowledge there have been no previous studies investigating the matrix interaction of the fermentable fibres and polyphenols.

The microbiota and their role in health and disease is currently a subject of much interest (Turnbaugh et al., 2007, Greenhill, 2014). Fibre and polyphenols have demonstrated potential to modify these microbiota and they are both largely dependent on the colonic microbiota for their metabolism in the gut. The health benefits of fibres and polyphenols are to a great extent dependent on this colonic metabolism.

Previous evidence reporting health benefits from polyphenols or fibre consumption have not considered the interaction of these two components, most often than not present in the colon together; where their potential interaction with the microbiota may impact the production of their bio-active metabolites. We have demonstrated that fibre inhibits or delays the phenolic acid production from polyphenols, this must be considered in future studies assessing bioavailability and health impact of polyphenols both in-vitro and in-vivo.

The health claims for fibre has led the food industry to incorporate fibre in many products which are naturally high in polyphenols. Cocoa bean shell which was previously discarded as waste, used as fuel or garden mulch is now being incorporated into cocoa products, which may lead to the reduced bioavailability of their polyphenols, and phenolic acids and subsequent benefits on health.

The considerable phenolic acid production detected in our in-vivo study from ispaghula adds to previous findings which showed polyphenols are found in association with dietary fibre in plant based foods and may be considered as an important constituent of dietary fibre (Goñi et al., 2009). Thus when considering the health benefits and the bioavailability of polyphenols the impact of fibre both as a delivery vehicle of the bound phenolics and inhibition of their metabolism in the colon must be considered.

6.5 Research questions arising from the findings of this PhD and future work:

- Is the impact of soluble fibre on phenolic acid production inhibitory or retardation?

  (*Assessed by measuring phenolic acids until maximal extent of conversion has been achieved*)

  Our findings demonstrated a reduction in urinary excretion of phenolic acids and their concentration in fermentation vessels. However the total sum of phenolic acids demonstrated a linear increase suggesting that is more time were available the final total amount could have been much higher. To confirm if the effect of fermentable fibre on polyphenols is inhibitory or retardation, a longer period of incubation and urine collection is required; until all parent compounds have been degraded to their maximum extent.

- Do viscous fibres have a stronger impact on phenolic acid production from polyphenols *in-vivo* than *in-vitro*?

  (*Replication of the acute in-vivo study and substituting ispaghula with pectin and raftiline*)

  Ispaghula demonstrated a much stronger impact on reducing cocoa phenolic acid production *in-vivo* (*Chapter-5*) than *in-vitro* (*Chapter-4*). This may possibly be due to the viscosity of ispaghula, which is maintained in the colon and the reduction in bio-accessibility of the polyphenolic compounds, modification of transit time and increased excretion of the compound (*Figure 6-2*). Using fibres with different viscosities, which may be lost or maintained throughout the digestive system in a similar model to the acute bioavailability study (*Chpater-5*) will aid the understanding of impact of viscosity on phenolic acid production from the polyphenols.

- How does the composition of the bacteria change when they are subjected to both prebiotic fibre and antibacterial polyphenols and how may this impact the metabolite production?

  (*Analysis of bacterial profile in an in-vitro setting or direct faecal sample after a long-term intervention*).

In both *in-vitro* studies (Chpater-3 and Chapter-4), the fermentable fibres having the highest fermentability and the lowest pH had a stronger impact on reducing phenolic acid production. However the mechanism behind this impact is not clear. Two mechanisms may be postulated. The first being that the highly fermentable fibres were a preferential source of energy for the bacteria and the uptake of these compounds delayed the hydrolysis of the polyphenols. It is also possible that high fermentability and subsequent high amounts of SCFA resulted in lower colonic pH, which may have altered bacterial composition involving the bacteria responsible for the degradation of polyphenols. In this regard next generation DNA sequencing and metabolomics may provide information on the change in bacterial composition when they are exposed to fermentable fibre and polyphenols at the same time and possible association between this change and the profile of metabolites produced. Due to limitation of funding, resources and time this measurement was not possible during the time of this PhD.

- Correlation between fermentability, viscosity and inhibitory impact of phenolic acid production observed?

  (*Viscosity of fibres to be measured*)

  In all *in-vitro* studies conducted in this PhD, fibres with different physio-chemical properties were used. Even though we observed that the fibres with higher fermentability had more impact in reducing phenolic acid production than fibres with high viscosity, an association could not be made. However using fibres with different known viscosity may allow the understanding of the association between fermentability, viscosity and inhibition of phenolic acid production.

- How do the soluble fibres impact the polyphenol absorption and subsequent excretion? (*Measurement of parent compounds in urine/plasma to determine the extent of their absorption and measurement of the polyphenols in faecal sample to determine the percentage of ingested concentration being excreted*).

  Due to limitation of funding, resources and time this measurement was not possible during the time of this PhD. The measurement of the parent compounds may demonstrate the

impact of fibre on the delay or reduction in absorption of these compounds, which may then be associated with the concomitant phenolic acid production.

- What is the catabolic pathway of the parent compounds and what is the role of the phenolic acid produced on health?

  (*Use of radio or stable isotope labelled parent compounds to determine the catabolic pathway and site of accumulation and action of metabolites*).

  It is becoming more evident that very small amounts of the polyphenols are absorbed with the majority reaching the colon where they are subjected to the bacterial action, resulting in the production of phenolic acids. However, the catabolic pathway responsible for phenolic acid production is not very well understood and many of the pathways given are speculative. In the same manner, the fate of these phenolic acids and their bioavailability is not understood. The use of isotopically labelled polyphenol compounds may aid the understanding of the catabolic pathways and target tissues of phenolic acids in human and animal models in vivo. Additionally there is a big gap in the literature on the health benefits of these phenolic acids. Given that they contribute greatly to the bioavailability of the polyphenols, it is essential to understand their role in health.

6.6 Conclusion

Fermentable fibres inhibit or delay the phenolic acid production of polyphenols in-vitro and in-vivo, reduce gastric emptying time and do not affect small bowel transit time. Polyphenols present as isolated compounds or within their food matrix have no impact on the fermentability and SCFA production from fermentable fibres. It is essential for studies investigating the bioavailability of polyphenols and their impact on health to take into consideration such matrix interactions between fermentable fibres and polyphenols, which may impact the bioavailability of the polyphenols.

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Appendix-1: Dietary guidelines for low polyphenol diet

Dietary guidelines

Baseline diet:

Avoid all fruits, vegetables, onions, coffee, tea, chocolate, vanilla and similar flavourings, wholemeal products, spices, **wine and other alcoholic drinks**. Avoid all dietary supplements (vitamins, mineral, and herbal products).

Examples of suitable breakfast include:

Eggs
Cheeses
Sausage
Beacon
Fish
Toast
Bread
Croissant (NO chocolate)
Waffles, pancakes with butter and sugar (NO jam)
Butter
Milk
Biscuits (NOT wholemeal)
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 and vinegar (NO ketchup)
Sardines on white toast (no tomato sauce)
Chicken fried rice with eggs and oyster sauce (NO soya products or vegetables
Roast Chicken/ prawns with white rice
Noodle
Chicken nuggets and chips
Roast Beef Sandwich with crisps
Salmon with white rice
Steak and mashed potatoes

Examples of suitable snacks include:

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

Examples of suitable drinks include: Water and milk

Appendix-2: Dietary record forms

University of Glasgow | Section of Human Nutrition

Dietary record form
Praticipant study number:
Diet:
Date:

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

Appendix-3: Barry Callebaut cocoa specification

CACAO BARRY — Since 1842

Product Specification

Article : DCP-22SP-760
Legal denomination : Cocoa powder alkalized
Description : Cacao Barry Brut 1KG/UC 6UC/BOX 70BOX/PAL
Commodity code : 1805.0000

Typical composition (Unsuitable data for refund purposes)
Cocoa powder 100.0 %

Additional article information
Appearance : red
Colour : deep dark red
Smell and taste : typical for an alkalized cocoa powder without off-taste

Chemical limits

MOISTURE	max 5.00 %	IOCCC1(1952)
TOT. FAT CONTENT ON DRY MATTER	22.0 - 24.0 %	IOCCC14(1972)
pH	7.8 - 8.2 -	IOCCC15(1972)
SHELLS ON ALKALI FREE NIBS	max 1.75 %	winnower control
ASHES (F.F.D.M)	max 14.0 %	IOCCC16(1973)

Physical limits
Particle size : 99.5 % min < 75 µm, 200 mesh sieve — IOCCC 38(1990)

Microbiological limits		**Ref.Method**
AEROBIC MESOPHILIC COUNT	<=5,000/g	ISO4833
YEASTS	<=50/g	ISO7954
MOULDS	<=50/g	ISO7954
ENTEROBACTERIACEAE	<=10/g	ISO21528-2
COLIFORMS	<=10.0/g	ISO4832
E.COLI	0/g	ISO16649-2
SALMONELLAE	0/25g	ISO6579

Barry Callebaut works with the latest version of the ISO standard of the reference methods as can be found on the International Standard Organization website http://www.iso.org
Alternative methods are also used place of the reference ISO standard after validation through the ISO 16140 scheme by an international recognized reference body (AOAC, Microval, AFNOR e.g.) and complimentary internal validation study on cocoa and chocolate matrices.

Typical dimensions
Not specified

Shelf life and recommended storage conditions
24 month(s) after production date
Storage Temperature : 12 - 20 °C
Store the product in a clean, dry (relative humidity max.70 %) and odourless environment.

Nutritional data for 100g (by calculation based on literature data)

ENERGY VALUE	383 kcal	VITAMIN D (IU)	40.0
ENERGY VALUE	1,602 kJ	VITAMIN E ALPHA-TOCOPHEROL	1.6 mg
TOTAL PROTEIN	18.8 g	VITAMIN E RDA	19.2 %
MILK PROTEIN	0.0 g	VITAMIN E (IU)	2.4

Article : DCP-22SP-760 — Customer :
Barry Callebaut Cocoa AG - Pfingstweidstrasse 60
8005 ZURICH - SWITZERLAND -
Tel. : 41 43 204 04 04 Fax. : 41 43 204 04 00
Type : EU - Extended nutri -PR
13.01.2014 p. 1 / 2

CACAO BARRY
Since 1842

Product Specification

AVAILABLE CARBOHYDRATES	8.8 g
SUGARS (MONO+DISACCHARIDES)	0.4 g
POLYOLS	0.0 g
STARCH	8.4 g
TOTAL FAT	23.0 g
SATURATED FAT	14.5 g
MONO UNSATURATED FAT	7.8 g
POLY UNSATURATED FAT	0.7 g
TRANS FATTY ACID (TFA) TOTAL	0.0 g
TFA (PLANT ORIGIN)	0.0 g
CHOLESTEROL	0.0 mg
ORGANIC ACIDS	2.60 g
DIETARY FIBRE	28.8 g
TOTAL ALKALOIDS	2.4 g
POLY HYDROXYPHENOLS	2.6 g
CAFFEINE	0.4 g
THEOBROMINE	2.0 g
ALCOHOL	0.0 g
VITAMIN A RETINOL	8 µg
VITAMIN A RDA	1.0 %
VITAMIN A (IU)	27
PROVITAMIN A BETA-CAROTENE	0 µg
VITAMIN B1 THIAMIN	0.3 mg
VITAMIN B1 RDA	27.3 %
VITAMIN B2 RIBOFLAVIN	0.3 mg
VITAMIN B2 RDA	21.4 %
VITAMIN B3/PP NIACIN/NICOTIN	2.4 mg
VITAMIN B3 RDA	15.0 %
VITAMIN B5 PANTOIC ACID	1.4 mg
VITAMIN B5 RDA	23.3 %
VITAMIN B6 PYRIDOXIN	0.2 mg
VITAMIN B6 RDA	14.3 %
VITAMIN B12 CYANO-COBALAMINE	0.0 µg
VITAMIN B12 RDA	0.0 %
VITAMIN C L-ASCORBIC ACID	0.0 mg
VITAMIN C RDA	0.0 %
VITAMIN D CALCIFEROL	1.0 µg
VITAMIN D RDA	20.0 %
VITAMIN H BIOTIN	0.0 mg
VITAMIN H RDA	0.0 %
VITAMIN M FOLIC ACID	35.5 µg
VITAMIN M RDA	17.8 %
VITAMIN K - PHYLLOQUINONES	0.0 µg
VITAMIN K RDA	0 %
SODIUM	16.2 mg
PHOSPHORUS	620.1 mg
PHOSPHORUS RDA	77.5 %
IRON	42.0 mg
IRON RDA	300.0 %
MAGNESIUM	391.3 mg
MAGNESIUM RDA	104.3 %
ZINC	5.5 mg
ZINC RDA	55.0 %
IODINE	0 µg
IODINE RDA	0.0 %
CALCIUM	107.8 mg
CALCIUM RDA	13.5 %
CHLORIDE	30.2 mg
CHLORIDE RDA	4 %
POTASSIUM	3,323.0 mg
POTASSIUM RDA	166 %
COPPER	3.8 mg
COPPER RDA	381 %
MANGANESE	0.0 mg
MANGANESE RDA	0 %
FLUORIDE	0.1 mg
FLUORIDE RDA	3 %
SELENIUM	4.6 µg
SELENIUM RDA	8 %
CHROMIUM	60.0 µg
CHROMIUM RDA	150 %
MOLYBDENUM	73.0 µg
MOLYBDENUM RDA	146 %
ASH CONTENT	9.5 g

Kosher certification :
Kosher Pareve
OK Kosher certificate available on request. Kosher status is confirmed on packaging (only for solid products).

Issued on 13.01.2014 for

Philippe Goujon

Appendix-4: Recruitment poster for long-term cocoa study

Nutrition study

ARE YOU ABOVE 30 AND INTERESTED IN WEIGHT LOSS ?

We are looking for men and women above 30 years old, in good health and interested in enhancing weight loss and reducing the risk of cardiovascular disease. If you are interested we will ask you to take 2dietary supplements of fibre and cocoa, 1 month each and to provide fasting blood sample and urine sample at the start of each stage. You would also need to take part in one study day which will require you to spend 6 hours in the unit. Expenses will be compensated. For more information please contact :

Fibre study- B.Mansoorian 07599723838 nutrition.glasgow@gmail.com	Fibre study- B.Mansoorian 07599723838 nutrition.glasgow@gmail.com	Fibre study-B.Mansoorian 07599723838 nutrition.glasgow@gmail.com	Fibre study – B.Mansoorian 07599723838 nutrition.glasgow@gmail.com	Fibre study- B.Mansoorian 07599723838 nutrition.glasgow@gmail.com	Fibre study- B.Mansoorian 0141 201 0768 nutrition.glasgow@gmail.com	Fibre study- - B.Mansoorian 07599723838 nutrition.glasgow@gmail.com	Fibre study- B.Mansoorian 07599723838 nutrition.glasgow@gmail.com	Fibre study- B.Mansoorian 07599723838 nutrition.glasgow@gmail.com	Fibre study- B.Mansoorian 0141 201 0768 nutrition.glasgow@gmail.com

Appendix-5: Long-term human intervention trial design