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Interaction of Cocoa Powder with Intestinal Microbiota

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INTERACTION OF COCOA POWDER WITH INTESTIAL MICROBIOTA

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ABSTRACT

Cocoa powder contains polyphenolic compounds, which can provide potential health benefits to humans. The human gastroinestinal tract hosts diverse microorganisms which are called the human gut microbiota. These microbes can utilize carbohydrates through fermentation resulting in the production of short chain fatty acids (SCFAs), which cause a reduction of pH in gastroinestinal tract. The cocoa processing can change the composition of the carbohydrates, which can affect the fermentation patterns.

In this study we established an *in vitro* model system which simulated gastric digestion and colon fermentation. Five different processed cocoa samples (alkalized cocoa samples: D-11-B and D-11-S; non-alkalized cocoa samples: natural cocoa, Lavado, shell) were digested, then they were fermented by the colonic bacteria from mixed pool fecal slurry under anaerobic conditions. The pH, SCFAs, procyanidin distribution, and polyphenolic compounds were measured. Then the Lavado was fermented with fecal slurries from six individuals. Changes in the pH, polyphenolic compounds, and SCFAs were determined.

The results of cocoa samples fermented with pooled fecal slurry demonstrated that pH of five cocoa samples significantly decreased after 24h of fermentation; meanwhile SCFAs concentration increased. Fermentation of the shell sample resulted in the highest production of SCFAs and the greatest reduction in pH. Polyphenolic compounds release was expressed both as gallic acid equivalent (GAE) and catechin

equivalent (CE). Increases in both values were observed in all cocoa samples. The final GAE of alkalized cocoa samples were higher than non-alkalized cocoa samples; while final CE of the shell sample was the highest among five cocoa samples. The pH and SCFAs results indicated that cocoa samples can be fermented by gut microbiota. The polyphenolic compounds results showed that polyphenol content of cocoa powder increased during fermentation.

The pH decrease, polyphenols increase, and SCFAs increase were observed in individual fecal fermentation groups. However, the change patterns of pH, polyphenols, and SCFAs in each treatment group were different. The cocoa fermentation by colonic bacteria led to an increase of phenolic compounds. The variations of human gut microbiota among individuals affected fermentation pattern, subsequently affected pH change pattern and polyphenols profile during fermentation.

CHAPTER 1. LITERATURE REVIEW

1.1 Cocoa and cocoa products

1.1.1 Cocoa

The cocoa tree (Theobroma cacao, L.) originated from the rainforests of Central and South America. There are three subspecies, Forastero, which is from Amazon area; Criollo, which is from Panama; and Trinitario, which is from Trinidad (Wilson, 1999). Cocoa was first introduced to Europesince 16th century, then into Asia and Africa. Today chocolate and cocoa products have become one of the most popular snack foods and confections consumed by children and adults. Cocoa tree is sensitive to climatic and environmental conditions, although there are some species which adapted and are cultivated under various agronomic conditions other than tropical conditions. Currently the main cocoa production areas are located in a narrow band referred as Cocoa Belt, which is between 20 degrees north latitudes to 20 degrees south latitudes (Crozier, Ashihara, and TomÃ 2011). In general, it takes 4 years for a cocoa tree to become productive which it lasts for 25-30 years (Wood and Lass 2008).

1.1.2 Production of cocoa

In 2013/2014, the worldwide cocoa production was 4.373 million tons, 73.1% of the production was from Africa, 16.6% was from Central and South America, 10.2% was from Asia & Oceania (ICCO 2016). The largest cocoa-producing countries were Côte d'Ivoire, Ghana, and Indonesia. Cocoa and cocoa products are consumed worldwide, according to the United States Department of Agriculture, the global retail

market for chocolate candy is valued at an estimated $107 billion in 2013 and is expected to grow to $143 billion by 2017 (USDA 2013).

1.1.3 Cocoa powder manufacture

Cocoa was once served as a divine drink for ancient Central American natives: the Olmecs and Mayans (Crozier, Ashihara, and TomÃ 2011). Cocoa is now processed through a series of steps that results in the production of cocoa powder and cocoa butter. The modern cocoa processing was first developed by Coenraad Johannes Van Houten in 1820 (Knight 1999). Van Houten's processing includes separation of cocoa butter and cocoa powder, which is still an important step in cocoa processing. In general cocoa processing, after harvesting the cocoa beans are subjected to fermentation, breaking, sterilization, alkalization, roasting, grinding, and pressing, and the final products are cocoa powder and cocoa butter (Garti and Widlak 2015). Usually, simple fermentation is the first step after cocoa beans are separated from broken cocoa pod. This processing step often takes 5 days, during this period carbohydrates are hydrolyzed and some are converted to acetic acid; proteins are also hydrolyzed, and the precursors of the cocoa flavor are developed (Schwan and Wheals 2004). After fermentation, the cocoa beans are dried, they then undergo breaking step to separate meat of bean (the "nib") from the shell (hull). Then alkalization is performed, the nibs are treated with alkaline reagent such as potassium carbonate (Miller et al. 2008). The alkaline processing step is to develop required color of cocoa powder. The degree of alkalizaton varies for different final products. Roasting is a

step which can be performed either before breaking step or after alkalization. The main purpose of roasting is improving flavor quality of cocoa powder. Formation of dark brown color and development of chocolate aroma occurrs during roasting. Depending on the various requirements of final cocoa products, roasting temperature and time are adjusted. Ramli et al. (2006) employed 6 increments of roasting temperatures ranging from 120°C to 170°C and 4 different roasting times ranging from 20 to 50 min. They found that there were significant differences in flavor which correlated with different conditions of roasting.

After alkalization and roasting, cocoa nibs are reduced to cocoa liquor by grinding. Cocoa liquor contains more than 50% fat and it can be directly used in chocolate manufacture, but usually the fat is separated from cocoa powder by pressing (Venter et al. 2007). This processing step results in cocoa powder and cocoa butter. Both cocoa powder and cocoa butter are basic ingredients and widely used in cocoa products such as chocolate, candy, ice cream, the manufacture of chocolate drinks (Minifie 2012).

1.1.4 Dietary fiber in cocoa

Dietary fibers are class of carbohydrates which are resistant to endogenous enzyme in small intestine and are not absorbed by GI tract, however, they can be fermented by microorganisms in human gastrointestinal tract. Dietary fibers include cellulose, hemicellulose, lignin, pectin, gums, inulin, beta-glucans, and resistant starch (Lattimer and Haub 2010). In dietary fiber the complex carbohydrates other than

starch or lignin are called non-starch polysaccharide (NSP). NSP can be further divided into two groups: soluble NSP and insoluble NSP. Soluble NSP include pectins, gums, inulin-type fructans, and some hemicellulose, these polysaccharides dissolve in water and be fermented by the human gut microbiota. Insoluble NSP include lignin, cellulose, and some hemicellulose. Insoluble NSP cannot dissolve in water and they are less fermentable than soluble NSP (Elleuch et al. 2011). Non-digestible and non- or partially fermentable dietary fiber are known by their laxative properties. They contribute to stool bulk, increase bowel movements, and prevent occurrence of constipation. Beside of these well-known effects, dietary fibers also benefit to human health. FDA (2014) states that dietary fibers may reduce some types of cancer, and they can also decrease risk of coronary heart disease. Cocoa is a dietary fiber-rich food source. The composition of dietary fiber in cocoa product depends on genetic variation and cocoa processing method. Sarriá et al. (2012) analyzed two different soluble cocoa products, one type of cocoa product contained 3.13g/100g soluble NSP and 15.09g/100g total NSP, polyphenol content was 3.404±0.228 mg equivalent gallic acid/100g. The second cocoa powder sample contained 1.68g/100g soluble NSP and 22g/100g total NSP, and polyphenol content was 1.575±0.067 mg equivalent gallic acid/100g. Lecumberri, Mateos, et al. (2007b) reported that a dietary-rich cocoa powder sample contained very high amount of total dietary fiber (over 60% dry matter); and dietary fiber mainly consist of insoluble dietary fiber (over 80% total dietary fiber), the soluble dietary fiber only account for less than 20% of

total fiber. The insoluble dietary fiber contained 33% of NSP; while uronic acids were the most abundant components in soluble NSP.

1.1.5 Health benefit of cocoa products

Since the early 2000's, research has focused on the potential health benefits of cocoa and its products. Buijsse et al. (2006) reported that cocoa intake was associated with significantly lower incidence of cardiovascular disease. Grassi et al. (2008) found that patients treated with dark chocolate for 15 days showed decreased insulin resistance and increased insulin sensitivity. Cocoa consuming patients exhibited decreases in systolic and diastolic blood pressure, and decreased total cholesterol level compared to the control group. Sies et al. (2005) reported cocoa beverages can increase the plasma nitric oxide (NO) concentration in patients with diminished endothelial function. Ramiro et al. (2005) studied effects of the cocoa extract on inflammation, and found that cocoa extract may reduce the production of nitric oxide (NO), tumor necrosis factor α (TNFα), and Interleukin (IL) 1α. Djoussé et al. (2011) found that individuals who consumed chocolate twice a week were 32% less likely to develop coronary artery calcification, and this risk was further reduced by 57% when chocolate consumption occurred over five times per week. Much of the evidence indicated that the health benefits of cocoa products are related to polyphenolic compounds in cocoa products, this will be discussed in detail in later section. However, the comprehensiveness and detail of the mechanism of the cocoa's health benefits is still not well understood.

1.2 Human gut microbiota

1.2.1 Composition of human gut microbiota

A microbiota is a community of microorganisms, which include bacteria, fungi, viruses, and archaea, that are present in the individual environment of the host (Biedermann and Rogler 2015). The human gastrointestinal tract (GI tract) hosts about 10 trillion microorganisms in the large intestine, and this complex microbial community is called the human gut microbiota (Gibson 2009). Microorganisms occur from the beginning to the end of human gut, but the density and the composition of the of microbial colonization of different sites of human gut is varies significantly. These differences depend on specific environmental condition of the individual host, the availability of substrates, transit rates, host secretions, and the organization of the gut wall (Graf et al. 2015). The sites with extreme pH environments, such as the stomach and the small intestine, support fewer microorganisms than the large intestine, and the microorganisms found in the stomach and small intestine are more tolerant of extreme pH and oxygen exposure. In contrast, the density of microorganisms in the large intestine is higher, and most of the microorganisms are obligate anaerobic bacteria (Louis and Flint 2009). More detailed composition of the microorganisms in the large intestine can be obtained by analysis of human fecal samples. With the developments of DNA sequencing technology and the improvements of the 16S rRNA database, phylogenetic analysis of the human gut microbiota is widely performed ((Kim et al. 2012) (Mardis 2011)). Eckburg et al.

(2005) adapted ecological statistical approaches to analyze more than 13 thousand human colonic mucosal and fecal samples. They found that there were significant differences of microorganism distribution among individual donors. A total of 395 bacterial phlotypes were identified, and most of microorganisms were members of the *Bacteroidetes* and *Firmicutes* phyla. Another study about carbohydrate-active enzymes in the human gut microbiota also supported this result. *Bacteroides* spp. from *Bacteroidetes* phylum and *Clostridium* spp., *Eubacterium rectale* and *Roseburia* spp. from *Firmicutes* phylum were identified. These microorganisms exhibited a broader carbohydrate substrate range than the other microorganisms (El Kaoutari et al. 2013). Walker et al. (2011) detected four most abundant phylotypes (*E. rectale*, *F. prausnitzii*, *C. aerofaciens*, and *B. vulgatus*) in stool samples from 14 male volunteers. The number of some bacterial groups in stool samples was increased when volunteers were treated with diets of resistant starch over 10 weeks. This result was supported by another study, the growth of *Ruminococcus bromii* was detected in human colon of volunteers who were treated with resistant starch (Ze et al. 2012). Although previous studies made significant progress toward the identification of microorganisms in the human gut and these results have expanded people's knowledge about the composition of the human gut microbiota, the understanding of the human gut microbiota, the substrate interactions and their impact on health is not yet well developed. This situation is because of the extremely complex of the human

gut microbiota, the very large diversity of microorganisms found in human individuals, and the limitation of anaerobic isolation and cultivation techniques.

1.2.2 Human gut microbiota and health

The human microbiota influences the health of the host, either beneficially or deleteriously. Most microorganisms in the GI tract are either harmless or beneficial to the host humans. Beneficial bacteria can directly inhibit foodborne pathogens and support human immune functions. Another very important role of the microbiota is to metabolize indigestible carbohydrates and provide energy and nutrients to the host (Clemente et al. 2012). When the equilibrium status between the host and the gut microbiota is disrupted, the "bad" microorganisms may cause metabolic disorder, obesity, malnutrition, or adverse pro-inflammation (Lozupone et al. 2012). Because the human gut microbiota can affect the human health in many aspects, it is necessary to further understand the characteristic of the human microbiota. The human gut microbiota varies with individuals age, population, culture, and lifestyles. However, as mentioned in 1.2.1, the composition and metabolism pattern of the human gut microbiota and how factors such as the environment and diet affect the human gut microbiota are not comprehensively understood. Italian children's microbiota were found to be different than the microbiota of children from rural Africa. Similarly, children in the United States, children from Malawi and Venezuela have been shown to have widely different microbiota. These differences may attribute to cultural effects, especially diet pattern difference (De Filippo et al. 2010).

1.2.3 Interaction of human gut microbiota with diet

Because diet is an important factor influencing the distribution of organisms in the human microbiota, studies of the human gut microbiota not only focused on characterization of community composition, but also revalued the interactions between microbiota and diet. Ley et al. (2006) reported that 12 obese people who consumed a carbohydrate or fat restricted low-calorie diet for year had statistically significant changes in the gut microbiota composition. They found that before diet treatment, there were fewer *Bacteroidetes* and more *Firmicutes* in human gut microbiota of obese people than lean controls. After 1-year diet therapy, the relative abundance of Bacteroidetes increased and the abundance of Firmicutes decreased in human gut microbiota of obese people. Wu et al. (2011) found that people who consumed a high fat, low fiber diet for 10 days had a significantly different microbiota from those who consumed a low fat, high fiber diet. Flint (2012) reported that the diet which has a high content of dietary fibers exhibits potential positive impact on human health, such as providing defense against pathogens, enhancement of the immune system, and promotes anti-inflammatory and anticarcinogenic effects. These positive effects of dietary fibers are attributed to bioactive compounds in dietary fibers, especially polyphenolic compounds. The bioavailability and effects of polyphenolic compounds rely on their biostransformation by microbiota in the lower GI tract (Laparra and Sanz 2010).

Humans do not have the enzymes that can metabolize glycosides, glucuronides, sulfates, amides, esters, and dietary fibers in the GI tract. When dietary fibers enter the GI tract and reach the large intestine, they are subjected to the microbial ecosystem and fermented under an anaerobic environment. The human colon serves as an incubator for microbiota, a major function is fermentation of indigestible carbohydrates which involves a series of complex reactions. The fermentation process produces energy which supports microbial growth. The microbiota produces fermentation products which can be utilized by the host. These products consist of a large range of low molecular weight metabolites, which include modified polyphenols and short chain fatty acids (Parkar, Trower, and Stevenson 2013), metabolism of polyphenols and short chain fatty acids will be discussed in section 1.3 and 1.4.

1.3 Polyphenolic compounds

1.3.1 Classification of phenolic compounds

Phenolic compounds are moderately water-soluble organic compounds with molecular weight ranging from 400 to 5000 Da (Haslam and Cai 1994). Phenolic compounds possess at least one aromatic ring (6 carbons) and multiple hydroxyl (-OH) groups, thus they are stable electron acceptors, natural reducing agents, free-radical terminators, and good antioxidant with strong antioxidation capacity (Tsao, 2010). Phenolic compounds are widely distributed in plants, they can act as protective agents and inhibitors against invading fungal and insects, they can prevent infection of

pathogens of plant, they also contribute to plant pigmentation (Bhattacharya, Sood, and Citovsky 2010).

Polyphenolic compounds, also known as polyphenols, are usually referred to a class of phenolic compounds that with multiple aromatic rings and phenol structure, however phenolic acids often included (Figure 1.1) (Ignat, Volf, and Popa 2011). Polyphenols are classified as nonflavonoids and flavonoids. Nonflavonoids includes stilbenes, lignan, and phenolic acids; while flavonoids include flavonols, flavanols, flavones, and anthocyanidins (Etxeberria et al. 2013).

Phenolic acids are nonflavonoids with an aromatic ring and more than one hydroxyl groups. Based on the chemical structure, they can be divided into two groups: hydroxybenzoic acids witch C6-C1 structure, such as gallic acid, syringic acid, phloroglucinol carboxylic acid, etc.; and hydroxycinnamic acids with C6-C3 structure, such as caffeic acid, cinnamic acid, ferulic acid, etc. (Figure 1.1). There are two forms of phenolic acids naturally existing in plants: the free form and bound form which link to the cell wall (Yoshida et al. 2010). They can be found in tea, cocoa beans, and soybeans (Scalbert and Williamson 2000). Stilbenes have 2 aromatic rings which are connected through ethene double bond, thus there are *trans* and *cis* stereoisomers. They can be found in grapes, soybeans, and traditional herbal medicine (Burns et al. 2002). Lignans consist of two C6-C3 building blocks; they exist in fiber-rich foods like sesame seed and many grains (Adolphe et al. 2010).

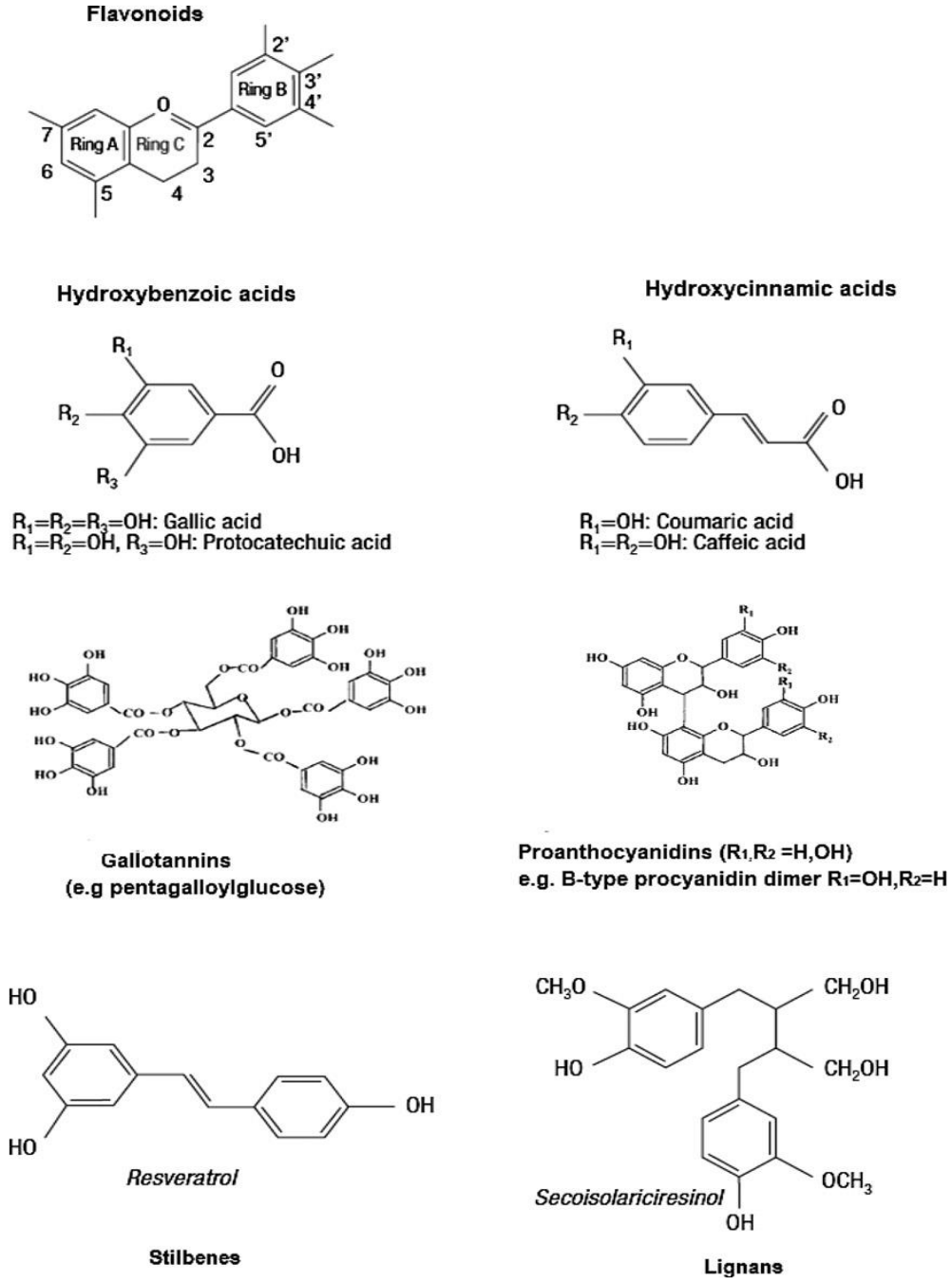


Figure 1.1. Classification of polyphenols. (Ignat, Volf, and Popa 2011)

Basic chemical structure of flavonoids consists of 3 rings with 15 carbons, arranged in a C6-C3-C6 configuration, the ring A and ring B are aromatic rings. At least 4000 flavonoids are identified; they are mainly synthesized through acetate/ malonate or polyketide pathway in plant and can be found in most of higher plant

(Bhattacharya, Sood, and Citovsky 2010). Based on different substitution patterns of functional groups on ring C, flavonoids are classified as flavones, flavonols, flavanones, flavanonols, isoflavones, flavan-3-ols (flavanols or catechins), and anthocyanidins (Figure 1.2). Within each class of flavonoids, different substitution patterns of functional groups on ring A and ring B result in various flavonoids compounds. Flavones have a double bond between C2 and C3, with a carbonyl group (-C=O) on C4. Flavones include apigenin, luteolin, and tangeritin etc., they are most commonly found in parsley and citrus fruits (Zhang et al. 2010). Flavonols have similar chemical structures to flavones, but they have a hydroxyl group (-OH) on C3, they include quercetin, myricetin, and kaempferol etc., and they are present in high concentration in apples, cherries and tea (Dilis, Vasilopoulou, and Trichopoulou 2007). Flavanones also have similar structures to flavones, but they have a saturated ring C. Flavanones include naringenin and hesperedin, they only abundantly present in citrus fruits (Ignat, Volf, and Popa 2011). Flavanonols are characterized by presence of a saturated ring C with hydroxyl group (-OH) on C3 and a carbonyl group (-C=O) on C4, which is similar to flavonols. Taxifolin is a typical flavanonol, it can be found in conifer cone (Wang et al. 2016). Isoflavones chemical structures are different from previous mentioned flavonoids, the ring B connects to ring C at C3 rather than C2. Isoflavones include genistein and daizein, soybeans are one of the famous food sources rich in isoflavones (Xiao 2008).

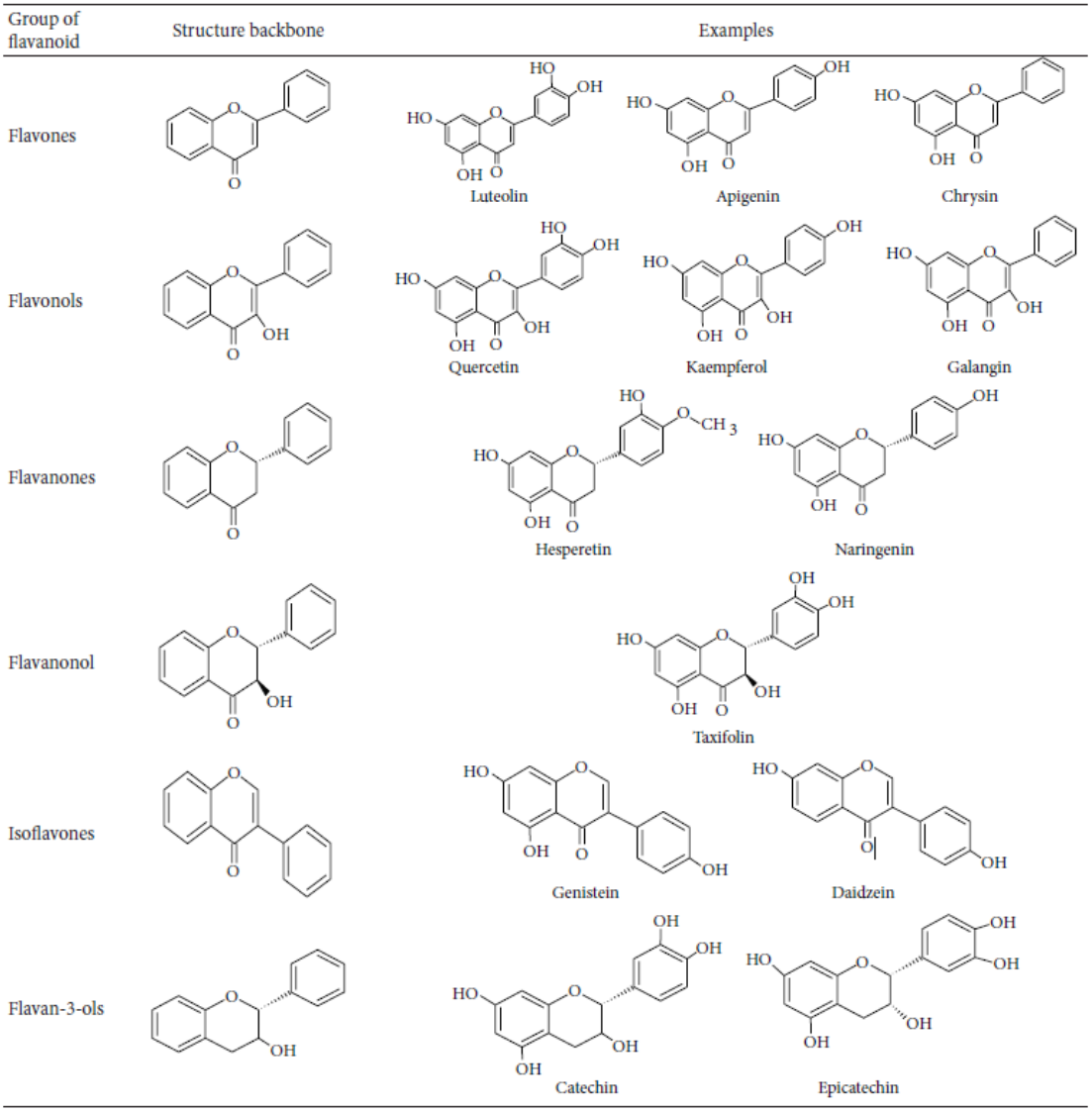


Figure 1.2. Structure of flavonoids. (Kumar and Pandey 2013)

Flavan-3-ols or flavanols are commonly called catechins, they have unique chemical structure: ring C is saturated, and without carbonyl group on C4. This structure results in 2 chiral carbonson C2 and C3 in ring C, and totally 4 diastereomers exist (Figure 1.3). Taking simplest substitution for an example. When R_1 and R_2 both are hydrogen, the diastereomer with *trans* configuration is catechin, and the one with *cis* configuration is epicatechin. Catechin and epicatechin both have 2 stereoisomers, they are (+)-catechin, (-)-catechin, (+)-epicatechin, and (-)-

epicatechin. In these 4stereoisomers, only (+)-catechin and (-)-epicatechin are widely found in plants, such as cocoa, grape, and tea (Tsao 2010).

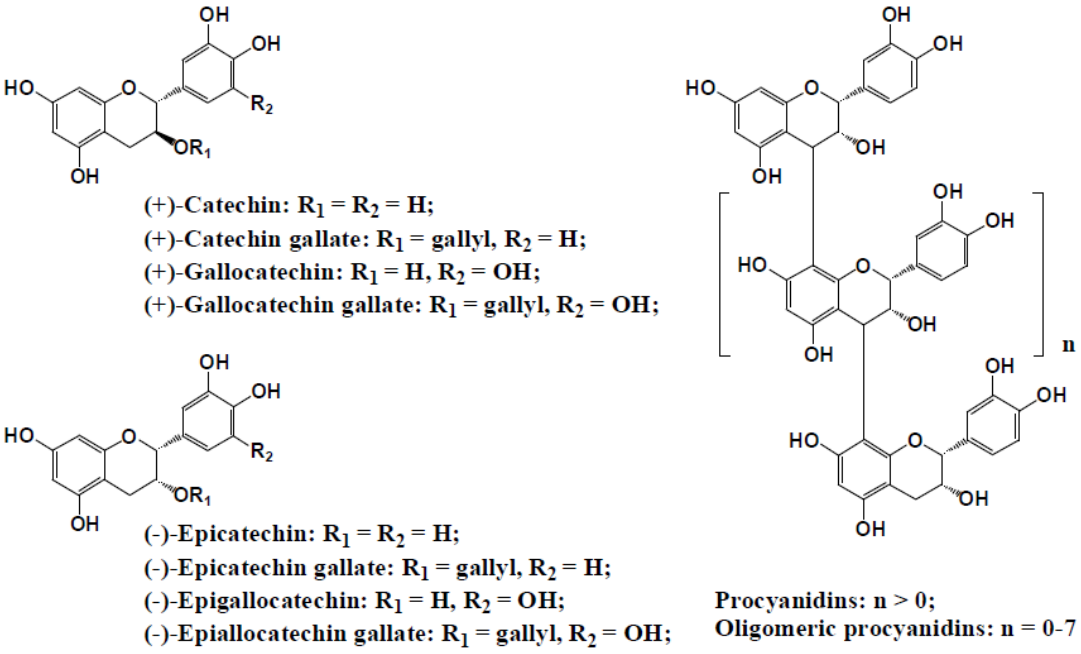


Figure 1.3. Flavanols and procyanidins. (Tsao 2010)

Procyanidins belong to proanthocyanidins, they are oligomers and polymers of flavonoids with catechin and epicatechin as building blocks. Oligomeric procyanidins have monomeric building blocks range from 1 to 9, and polymeric procyanidins have more than 10 monomeric building blocks. Procyanidins can be divided into two sub-groups based on the linkage bond between monomeric building blocks (Figure 1.4). For type A procyanind, monomers are linked through C2-O-C5 or C2-O-C7 ether bond; while in type B procyanidin, monomers are linked through C4-C8 or C4-C6 bond (Heim, Tagliaferro, and Bobilya 2002). Procyanidins can be found in cocoa

beans and a lot of fruits like apple, grape, and blueberry, etc. (Hammerstone et al. 1999).

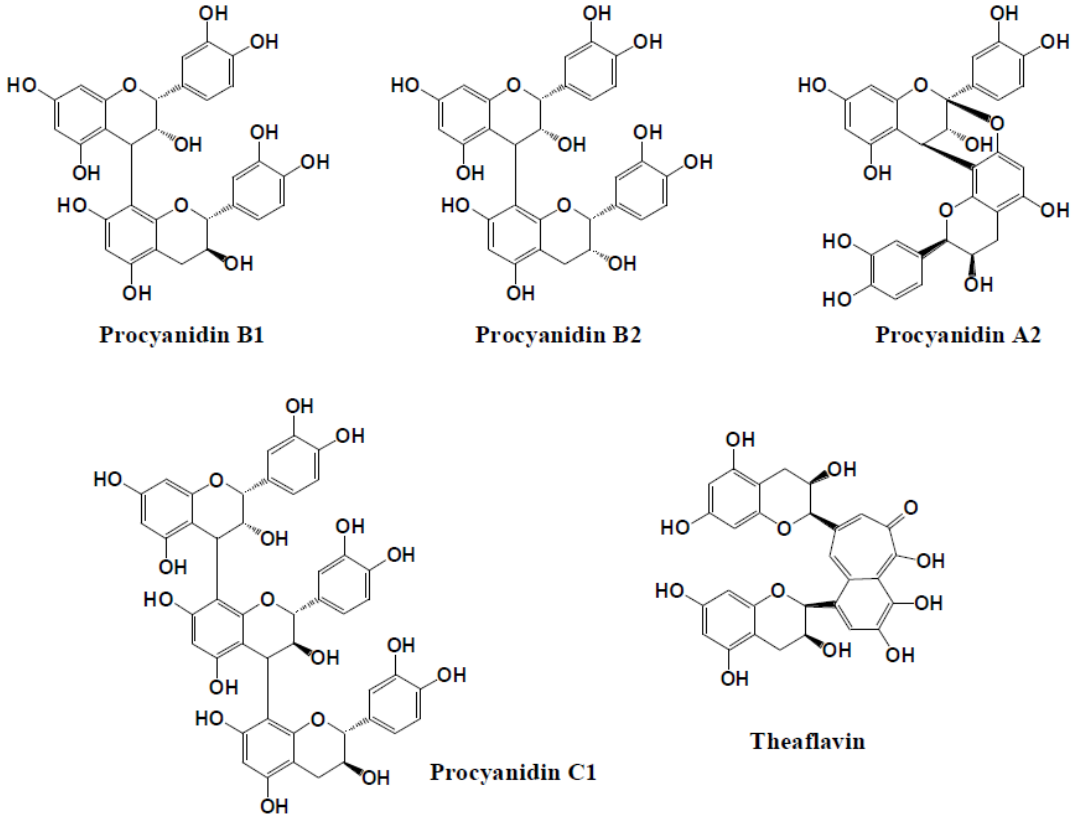
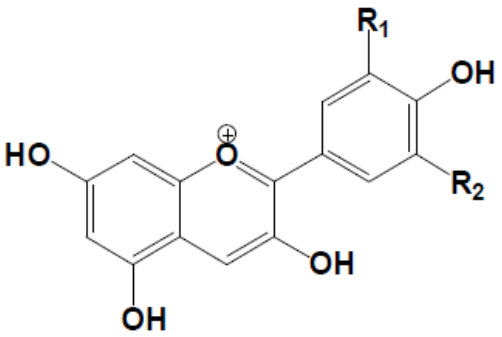


Figure 1.4. Typical procyanidin dimers, trimers, and theaflavin. (Tsao 2010)

Anthocyanindins are water-soluble natural pigments, they can be found in most of plants. Depending on different substitution patterns, there are 6 commonly known anthocyanindins exist: cyanidin, delphinidin, pelargonidin, malvidin, peonidin, and petunidin (Figure 1.5). Anthocyanindins are basic structures of anthocyanins, which consist of anthocyanindins and sugar group on ring B. Anthocyanins are red or pink when environmental pH lower than 7, green or blue when pH higher than 7 (Manach et al. 2004).



Anthocyanidin	R_1	R_2
Cyanidin	-OH	-H
Delphinidin	-OH	-OH
Pelargonidin	-H	-H
Malvidin	$-OCH_3$	$-OCH_3$
Peonidin	$-OCH_3$	-H
Petunidin	-OH	$-OCH_3$

Anthocyanidins

Figure 1.5. Major anthocyanidins. (Tsao 2010)

1.3.2 Polyphenolic compounds in cocoa

Cocoa and cocoa product are the good sources of polyphenols, they contain more than 3 mg/g polyphenols, which is significantly higher than well- known polyphenol-rich source such as black tea, green tea, and red wine, etc. (Pérez-Jiménez et al. 2010). There are three main groups of polyphenols in cocoa beans: catechins, anthocyanidins, and proanthocyanidins (Jalil and Ismail 2008). Catechins in cocoa include (−)-epicatechin, (+)-catechin, (+)-gallocatechin, and (−)-epigallocatechin. The anthocyanidins include cyanidin-3-α-L-arabinoside andcyanidin-3-β-D-galactoside. The procyanidins are composed by dimers, trimers, or oligomers of flavan-3,4-diol (Figure 1.2).

The composition of these polyphenols varies with different cocoa bean species, degree of ripeness, storage, and cocoa processing (Wollgast and Anklam 2000). Fermentation of cocoa is considered as one of the important steps that influence the polyphenolic content of cocoa powder. Fermentation of cocoa bean is usually performed in boxes or baskets, and fermentation lasts from 5 to 7 days (Minifie 2012).

The fermentation mass is covered with banana or plantain leaves to maintain fermentation temperature at about 45°C to 50°C. During fermentation chocolate flavor is produced, pH of cotyledons and pulp rise from 3.5 to 5.0, and color of nibs changes to light or dark brown. Albertini et al. (2015) reported that the total polyphenol content significantly decreased from 60 mg GAE/ g defatted cocoa to 20 mg GAE/ g defatted cocoa in the first 2 days of cocoa bean fermentation. Polyphenols loss may be attributed to diffusion of soluble polyphenols into fermentation sweating, enzymatic oxidation, and nonenzymatic oxidation.

The roasting process causes significant changes of the polyphenolic content. Bordiga et al. (2015) determined total phenolic content, content of anthocyanins and procyanidins in fermented cocoa beans, roasted nibs, cocoa mass, and chocolate from different geographic origins (South America and Africa). The largest polyphenols losses were observed during roasting. There are several possible factors contribute to phenolic compound loss, such as the Maillard reaction, oxidation of phenolic compounds, and formation of complex high molecular weight molecules. Variations in time and temperature combination during roasting result different flavor of final products and stability, chirality, and profile of polyphenolic compounds. Alkalization (Dutching) is another step which can alter or reduce the polyphenolic content of cocoa. Alkalization refers to treat cocoa mass with alkali solution. Usually the duration of the process is about an hour. During alkalization, the cocoa beans, nibs, or powder are treated with alkali solutions, potassium or sodium carbonate is usually

used, and ammonia carbonate is specially used for cocoa powder. Alkalization is usually about 1 h, temperature is 80°C to 100°C, and after alkalization pH of cocoa usually ranges from 6.8 to 7.5, some special cocoa like black cocoa can reach pH 8.5 (Minifie 2012). Alkalization changes cocoa into brown or red color, at the same time flavor of cocoa is enhanced. In last two decades, some studies pointed out the influence of several cocoa processing steps on polyphenolic compound content. Cooper et al. (2007) used ultra-performance liquid chromatography (UPLC) method to separate polyphenolic compounds in chocolate and other cocoa-containing products. They listed catechin, epicatechin, B2 (epicatechin-4β-8-epicatechin, a procyanidin dimer), B5 (epicatechin-4β-6-epicatechin, dimer), C1 (epicatechin-4β-8-epicatechin-4β-8-epicatechin, trimer), and tetramer D (epicatechin-4β-8-epicatechin-4β-8-epicatechin-4β-8-epicatechin) as six of the major polyphenols in chocolate and cocoa products (Table 1.1). The profile and chirality of polyphenols depended on raw material source and manufacturers. The results indicated that some sort of polyphenols was affected by manufacturing conditions and cocoa origin.

Table 1.1. Results of HPLC-MS. (Cooper et al. 2007)

analyte	Determined mass	Theoretical mass	Content (mg/g)
Epicatechin	290.079038	290.079713	0.071-1.942
catechin	290.079038	209.078213	0.043-0.519
dimer B2	578.142427	578.142013	0.041-1.174
dimer B5	578.142427	578.143713	ND-0.236
trimer C	866.205815	866.202813	ND-0.905
tetramer D	1154.269203	1154.270413	ND-0.387

ND: Not detectable

Natsume et al. (2000) reported that the profile of monomers and proanthocaynidin in dark chocolate was similar to cocoa liquor, while the ratio of the monomers to the total amount of polyphenols varied, different cocoa processing and manufacturing processes included alkali treatment, removal of lipids, and milling lead to this variation. Gu et al. (2006) analyzed total antioxidant capacity (AOC), catechins, and procyanidins (PC) in 19 samples of chocolate and cocoa products. In these samples, the natural cocoa powder contained highest AOC and PC, chocolate and cocoa products contained less AOC and PC (Table 1.2). Cocoa processing step such as alkalization can dramatically reduce PC content. Although there were some interesting results from a number of previous studies, it is still far from establishing a well understanding about the changes in the profile of polyphenolic compounds during the processing of cocoa powder manufacturing.

Table 1.2. Catechin and procyanidin contents of cocoa and chocolate croducts. (Gu et al. 2006)

		catechins (mg/g)			procyanidins (mg/g)					
product type	no.	catechin	epicatechin	sum	monomers	2-3 mers	4-6 mers	7-10 mers	polymers	total
milk chocolate	1	0.12	0.18	0.3	0.25	0.49	0.38	0.17	0.88	2.16
	2	0.05	0.18	0.23	0.27	0.57	0.58	0.31	1.11	2.84
	3	0.08	0.24	0.32	0.3	0.61	0.68	0.41	1.14	3.14
dark chocolate	1	0.25	0.52	0.77	0.99	1.84	1.8	1.02	2.87	8.52
	2	0.4	0.64	1.04	1.05	1.68	1.92	1.2	2.86	8.72
	3	0.12	0.75	0.87	0.89	1.78	2.29	1.41	4.47	10.84
	4	0.11	1.06	1.17	1.09	2.73	3.83	2.47	6.64	16.76
	5	0.33	1.25	1.58	1.66	3.74	4.54	2.95	6.67	16.76
baking chips	1	0.35	0.66	1.01	1.66	3.74	4.54	2.95	6.97	19.85
	2	0.5	1.01	1.51	0.95	1.63	1.91	1.21	3.01	8.71
	3	0.26	1.07	1.33	1.51	2.23	2.55	1.52	4.67	12.49
unsweetened chocolate	1	0.52	2.01	2.53	1.24	2.59	3.6	2.47	5.68	15.57
	2	1.17	2	3.17	1.83	3.46	4.17	3.18	6.12	18.76
	3	1.06	1.76	2.82	2.52	4.22	4.16	2.85	6.22	19.97
	Standard reference	0.23	1.24	1.47	2.82	4.78	5.63	3.66	8.3	25.2
natrual powder	1	0.61	2.29	2.9	1.51	4.05	5.53	3.95	10.01	25.05
	2	0.78	1.58	2.36	3.54	7.09	7.36	4.4	9.8	32.19
	3	0.9	2.58	3.48	3.63	7.87	9.06	5.59	15.49	41.64
Dutched powder	1	0.23	0.18	0.41	1.08	1.96	1.47	0.85	1.65	7.02
	2	0.35	0.38	0.73	1.44	2.69	2.39	1.34	2.96	10.82

1.3.3 Health benefits of polyphenolic compounds

Since the 1990's, there have been an increasing number of studies on the chemical properties and health benefits of polyphenolic compounds. Polyphenol compounds are important bioactive components in our diet; they have been recognized as health-beneficial components because of their antioxidant activity and anti-inflammatory potential. Studies have found that catechin may protect against heart disease and stroke (Ding et al. 2006). Schinella et al. (2010) conducted *in vitro* experiments and they found that polyphenols from cocoa extract exhibited both free radical scavenging activity and lipid oxidation inhibiting activity. Mellor et al. (2010) reported that the patients with type 2 diabetes had increased HDL-cholesterol and decreased total cholesterol/ HDL ratio when they were treated with high polyphenol content dark chocolate. Cienfuegos-Jovellanos et al. (2009) conducted a rat study which demonstrated that a flavonoid-enriched cocoa powder (50-600mg/kg) exhibited an antihypertensive effect on spontaneously hypertensive rats, and did not alter blood pressure on control groups. Andújar et al. (2011) demonstrated the anti-inflammatory effects of a cocoa polyphenol-enriched extract (500mg/kg) on an experimental model of Inflammatory Bowel Disease.

1.3.4 Metabolism of polyphenolic compounds in human

The small intestine plays a key role in polyphenol absorption and metabolism. Many dietary polyphenols are absorbed through the small intestine. Then those polyphenols are modified in the small intestine and the liver, which is called

conjugation process. This process includes glucuronidation, sulfation, and methylation (Figure 1.6). Conjugation process can reduce the toxicity of metabolites, and facilitate biliary and urinary elimination of polyphenols (D'Archivio et al. 2010) (Figure 1.7).

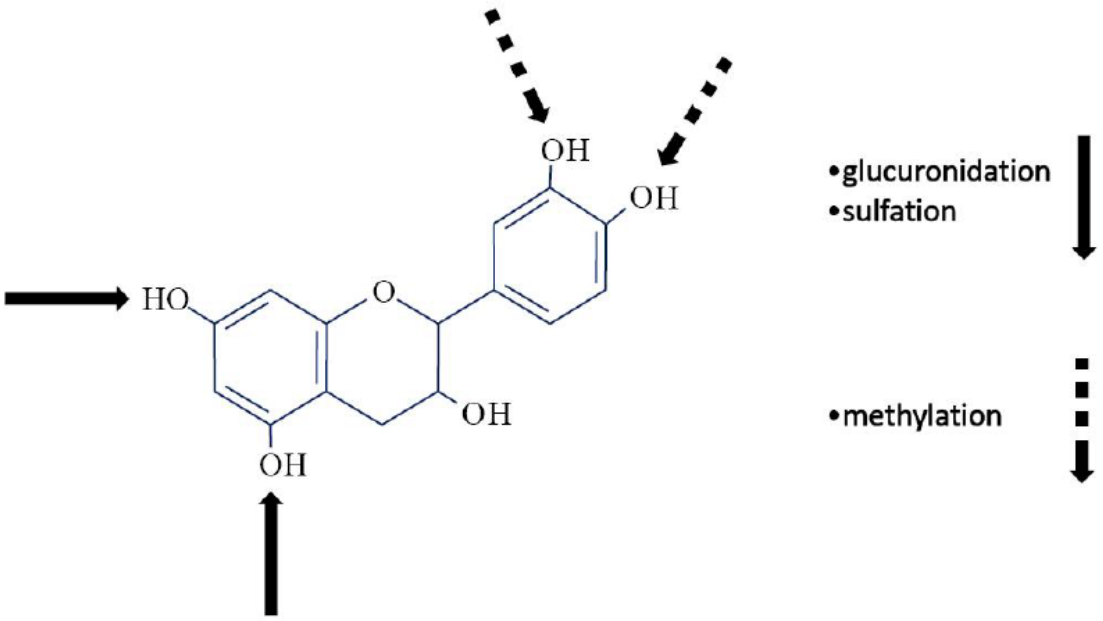


Figure 1.6. The potential sites of the conjugation of the polyphenols. (D'Archivio et al. 2010)

Absorption rate of different polyphenols is varied, and intestinal absorption is affected by polyphenol chemical structure and molecular weight. Small molecules usually show higher absorption rate, while high molecular weight molecules are less or even cannot be absorbed in small intestine.

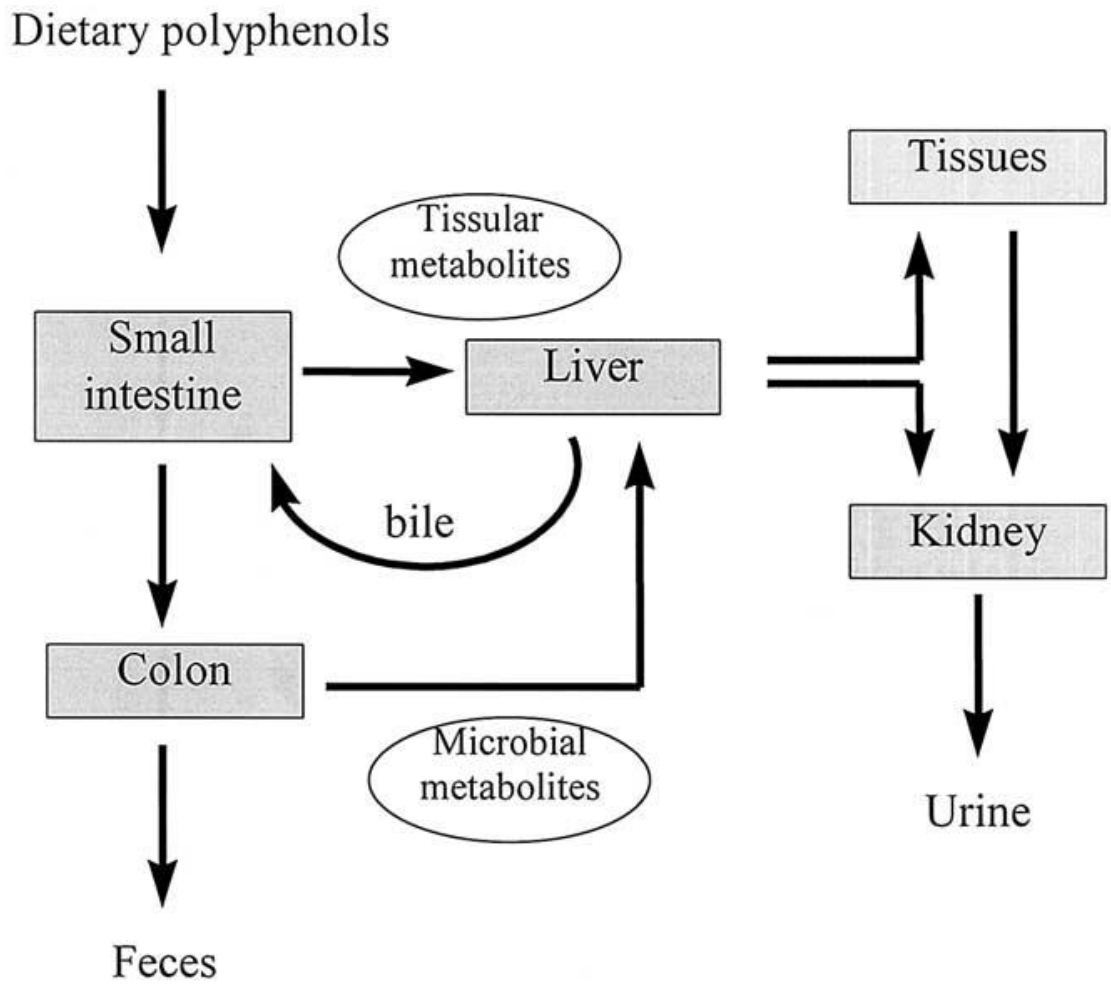


Figure 1.7. Routes for dietary polyphenols and their metabolites in humans. (Scalbert et al. 2002)

By comparing urine recovery rate of commonly known polyphenols, caffeic acid showed highest recovery values tea teaflavins showed lowest urine recovery among 13 polyphenols (Figure 1.8) (Scalbert et al. 2002). Deprez et al. (2001) reported that proanthocyanidin dimer and trimers could be absorbed in human intestine, but polymers could not be absorbed. Holt et al. (2002) also reported that (-)-epicatechin, (+)-catechin, and procyanidin B2 can be detected in human plasma, while polymers were not detected.

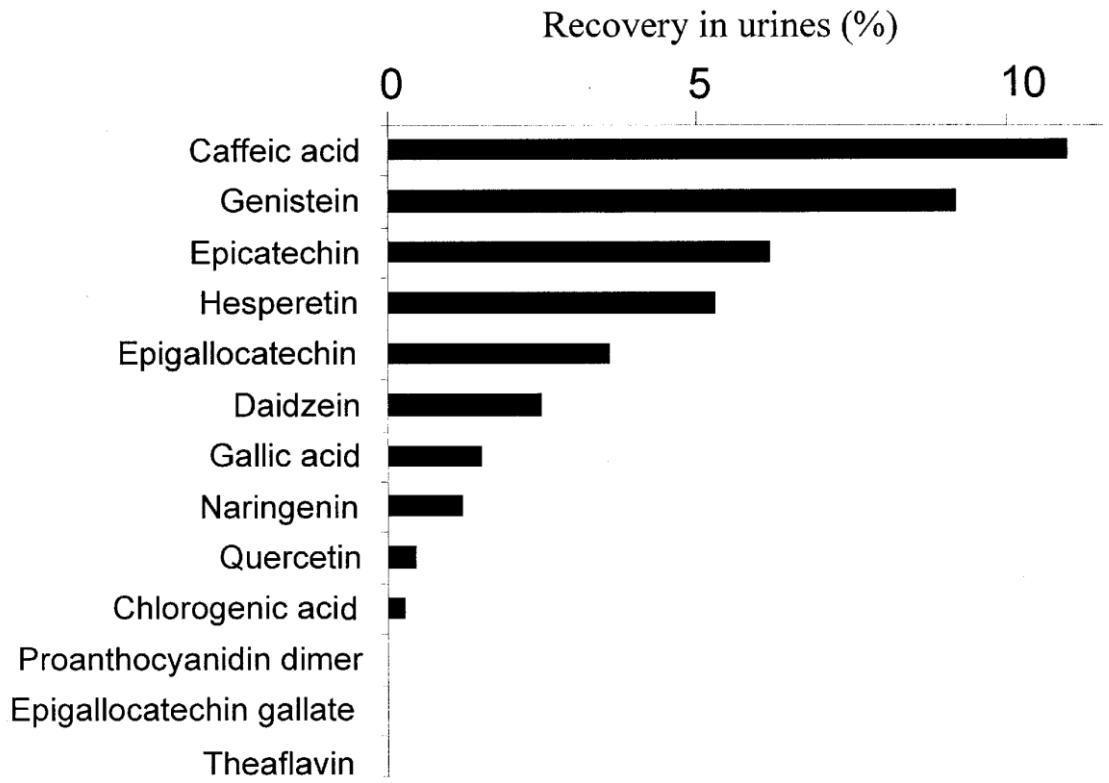


Figure 1.8. Recovery of various dietary polyphenols in urines after dietary intake in humans. (Scalbert et al. 2002)

High molecular weight polyphenols are not efficiently absorbed, as a result they reach the large intestine (Stoupi, Williamson, Viton, et al. 2010a). The gut microbiota can modify phenolic skeletons through enzymatic reactions, which allows the absorption of a range of lower-weight metabolites (Duda-Chodak et al. 2015). Selam et al. (2009) summarized phenolic enzymatic reactions achieved by human intestinal microbiota (Table 1.3). In general, microbial enzymes can hydrolyze glycosides, glucuronides, sulfates, amides, esters, and lactones and operate ring-cleavage, reduction, decarboxylation, and demethylation. After polyphenols are released from food matrix, the initial step of biotransformation of polyphenols by the gut microbiota is deconjugation. During this step the conjugated glycosyl or glucuronosyl are hydrolyzed and removed from ring A or ring B of flavonoids, the products are

phenolic backbone which is called aglycone. Deconjugation is catalyzed by β-glucosidase, β-glucuronidase, α-rhamnosidase, and esterases etc.. The following step is breakdown of phenolic backbone into relatively simple aromatic carboxylic acids. In the first step the ring C is broken down at different positions on the ring C, these breakdown differences result in a large number of phenolic products (Figure1.9). In general, flavonoids (flavonols, flavanones, anthocyanins, flavan-3-ols and procyanidins, and isoflavones) are cleaved into 2 parts, the ring A is converted to hydroxylated aromatic compounds such as phloroglucinol (Figure 1.9(2)); while metabolites from the ring B are phenolic acids, such as 3-(3,4-Dihydroxyphenyl) propionic acid, and these metabolites can be further degraded into small molecular weight metabolites (Figure 1.9 (1)). Different substitution patterns on the C ring and B ring of flavonoids result in formation of various phenolic acids. Depending on fermentation duration, the degree of degradation and the metabolites of polyphenols are highly diverse. Aura et al. (2002) fermented three phenols (flavonols: rutin, isoquercitin, and quercetin glucuronides) with human fecal bacteria. They detected 3,4-dihydroxyphenylacetic acid in first 2 hours of fermentation, and 3-hydroxyphenylacetic acid in 8 hours. These hydroxyphenylacetic acids were not methylated by colon flora *in vitro*, and fermentation pattern were not affected by fermentation scale, inoculation dose, and pH changes (pH 6.0 - 6.9). For flavanones, Rechner et al. (2004) found that 3-(4-hydroxyphenyl)-propionic acid and 3-phenylpropionic acid were the major phenolic products of naringin in an *in vitro*

human gut microorganism fermentation. They also found that degree of degradation was significantly influenced by concentration of the substrate and composition of the human gut microbiota. Czank et al. (2013) performed an *in vivo* study about metabolism of anthocyanidin: cyanidin-3-glucoside (C3G) by using isotope tracer method. Eight male subjects were fed with 500mg ^{13}C-C3G, and blood, breath, urine, and feces samples were collected after 48 hours. Ferulic acid, hippuric acid, phenylpropenoic and phenylacetic acids were detected in serum, urine, and feces as end products. Tzounis et al. (2008) investigated metabolism of 2 most common flavan-3-ols, (-)-epicatechin and (+)-catechin,in human fecal microorganism *in vitro* fermentation system, 5-(3',4'-dihydroxyphenyl)-gamma-valerolactone, 5-phenyl-gamma-valerolactone and phenylpropionic acid were detected as end products. They also found that (+)-catechin can promote the growth of the *Clostridium coccoides/Eubacterium rectale* group, *Bifidobacterium* spp. and *Escherichia coli*, inhibit the growth of the *C. histolyticum* group; while (-)-epicatechin can promote the growth of the *Clostridium coccoides/Eubacterium rectale* group. Appeldoorn et al. (2009) reported that the major metabolites of procyanidin dimers were 2-(3,4-dihydroxyphenyl)acetic acid and 5-(3,4-dihydroxyphenyl)-γ-valerolactone in the *in vitro* fermentation system with human gut microbiota. They proposed a hypothesis that type B procyanidin dimers were converted to small phenolic acids instead of cleavage into flavan-3-ols first. Hur et al. (2002) reported that daidzein was converted into *O*-demethylangolensin (*O*-Dma) when it was fermented with human fecal slurry

for 3 days. Nonflavonoids in this study is mainly focused on phenolic acids, such as gallic acids, protocatechuic acid, and vanillic acid. These hydroxybenzoic acids are decarboxylated and dehydroxylated by microorganisms, then they are converted into benzenetriols, benzenediols, and their derivatives. For example, gallic acid is converted into pyrogallol (Figure 1.9(5)), protocatechuic acid is converted to catechol, vanillic acid is converted to O-methylcatechol (Aura 2005). It is necessary to carefully compare these studies as references, because the experimental conditions of these studies were varied, the parameters like fermentation duration time, pH, and condition of inoculation were different. It has also been observed that different polyphenols may share common metabolites resulting from both *in vitro* and *in vivo* fermentation, however, there were some methylated phenolic compounds that were only found *in vivo*, because these compounds were reconjugated in the small intestine and the liver. For example, reported that epicatechin was reconjugated into epicatechin -glucoside in rats that treated with 10 mg epicatechin (Piskula and Terao 1998).The delivery form of substrates, for example, pure polyphenolic compound *vs.* natural polyphenolic compounds with diet background, should also be considered. Different polyphenols are hydrolyzed, biotransformed, and utilized by different microorganisms in the human colon, which indicates that dietary modulations with polyphenols may play a role in reshaping the gut microbial community and enhancing host microbial interactions to provide beneficial effects. It is possible that cocoa polyphenol-based

functional foods can provide opportunities to modulate the microbial balance in the gut.

Table 1.3. Phenolic enzymatic reactions achieved by the human intestinal microbiota. (Selam et al., 2009)

reaction	compound	enzyme	microbiota containing enzymes refs
Hydrolysis of glucuronides	ellagitannins	β-glucuronidase	*Escherichia coli*
Hydrolysis of glycosides	isoflavones, flavonols, flavanones, anthocyanins, ellagitannins, lignans	β-glucosidase	*Streptococcus faecalis, Eubacterium rectale,Clostridium sphenoides, Clostridium saccharogumia,Clostridium cocleatum, Bacteroides ovatus,Bacteroides fragilis, Bacteroides distasonis*
Hydrolysis of ester	hydroxycinnamates	esterases	*Escherichia coli, Bifidobacterium lactis, Lactobacillus gasseri*
hydrolysis of carbonyl	isoflavones		*Bacteroide ovatus spp., Streptococcus intermedius spp.,Ruminococcus productus, SNU-Julong 732,Enterococcus faecium, Lactobacillus mucosae,Finegoldia magna, and Veillonella spp.*
Reductions	isoflavones, hydroxycinnamates, stilbenes	hydrogenases	
Dehydroxylation	flavonols, flavanones, hydroxycinnamates, ellagitannins, lignans	dehydroxylase	*Clostridium scindens, Eggerthella lenta*

Table cont'd.

reaction	compound	enzyme	microbiota containing enzymes refs
Demethylation	flavonols, flavan-3-ols, anthocyanins, lignans	demethylase	*Eubacterium limosum, Eubacterium callanderi,Streptococcus, Lactobacillus, Clostridium, Butyribacterium,methylotrophicum, Peptostreptococcus productus*
Decarboxylation	benzoic acids,hydroxycinnamates, ellagitannins	decarboxylase	
Isomerization	flavan-3-ols	isomerase	
Fission	isoflavones,flavonols, flavanones,flavan-3-ols,anthocyanins,ellagitannins		*Clostridium spp. HGHA136, Eubacterium ramulus, Clostridium orbiscindens, Eubacterium oxidoreducens, Butyrivibrio spp., Butirivibrio spp.*

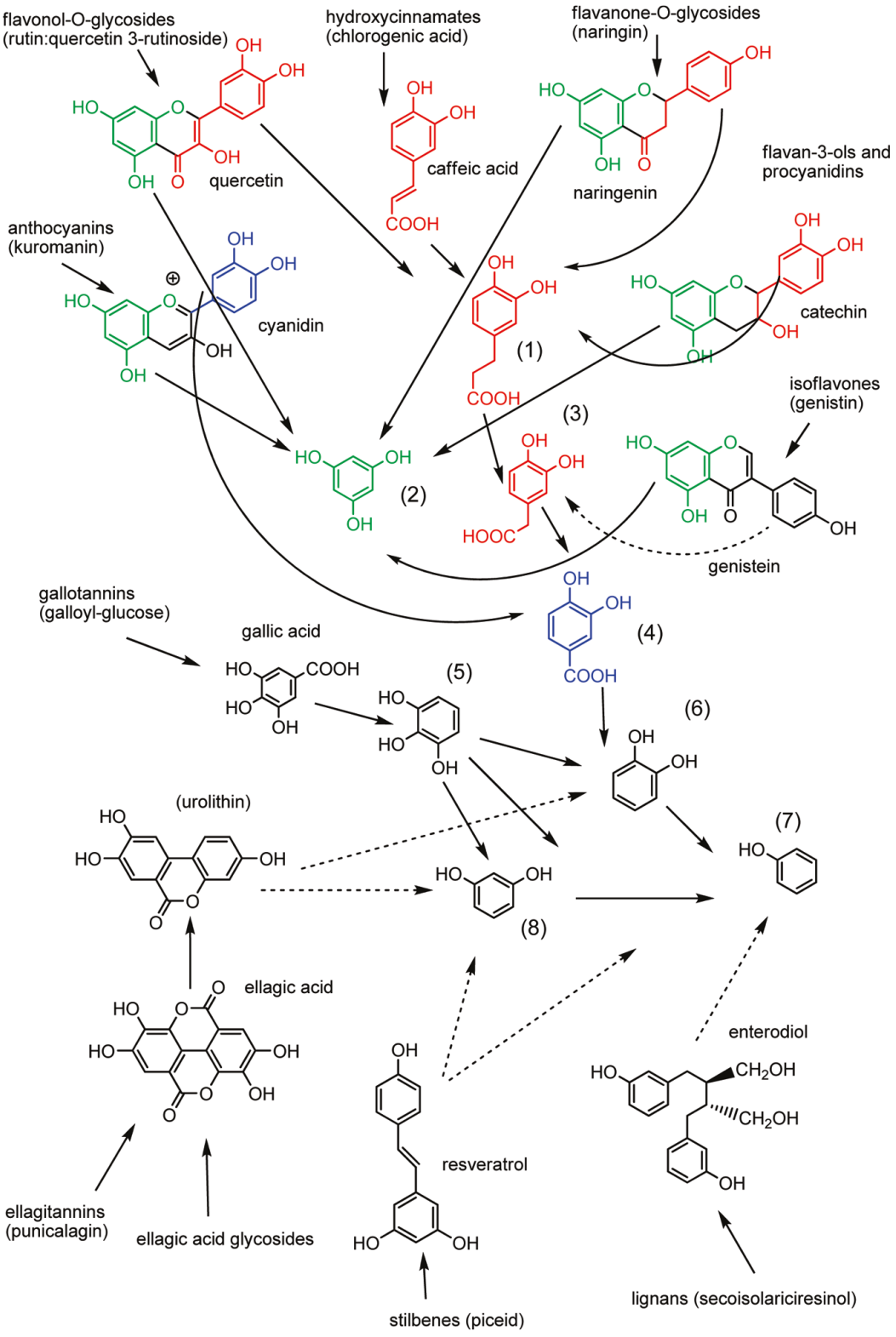


Figure 1.9 Confluence metabolic pathways and common metabolites of dietary phenolic compounds. Solid arrows are demonstrated pathways, dashed arrows are hypothetical pathways. (Selam et al., 2009)

1.4 Short chain fatty acids

1.4.1 Short chain fatty acids in human colon

Short chain fatty acid (SCFA) are organic fatty acids with 1 to 8 carbons, they were mainly produced by microbial fermentation of polysaccharides and oligosaccharides in the colon. Fermentation of protein and peptides also can contribute to SCFAs production. Polysaccharides and oligosaccharides are firstly hydrolyzed to their constituent sugars, then they are further fermented anaerobically. This anaerobic fermentation provides energy for microorganism. The fermentation produces gases (Carbon dioxide, hydrogen, and methane) and organic acids, mainly linear SCFAs: acetate, propionate, and butyrate (Wong et al. 2006) which can be absorbed and used by human. The general reaction of SCFA fermentation can be simplified as: **$59\ C_6H_{12}O_6 + 38\ H_2O \rightarrow 60\ CH_3COOH + 22\ CH_3CH_2COOH + 18\ CH_3CH_2CH_2COOH + 96\ CO_2 + 268\ H^+ +$ heat** (Topping and Clifton 2001).

The typical ratio of acetate, propionate, and butyrate is 3:1:1, but this ratio varies depending on different substrates. The bacterial pathways of anaerobic SCFAs fermentation is shown in Figure 1.10. Sugars are firstly converted to pyruvate. Pyruvate is a key component in the SCFAs fermentation, a part of pyruvate is converted to acetyl-CoA with hydrogen and carbon dioxide. Then acetyl-CoA was hydrolyzed to acetate, and acetate can be also synthesized from CO_2, H_2, and methyl group through Wood-Ljungdahl pathway (Ragsdale and Pierce 2008). Propionate has 3 pathways of synthesis, the first one is from phosphoenlypyruvate (PEP) to pyruvate,

then through acrylate pathway by reducing lactate to propionate. The second one is succinate pathway, which starts from PEP to oxaloacetate, then to fumarate, fumarate is reduced to succinate, finally it is converted to propionate. The third one is propanediol pathway, deoxyhexose sugars are converted to propane-1,2-diol, then propionaldehyde, consequently propionate (Reichardt et al. 2014). Butyrate is formed through acetyl-CoA to butyrl-CoA, and then it is converted by phosphotransbutyrylase and butyrate kinase to butyrate. Another pathway is cross-feeding (growth of one species relies on metabolites of another species) of acetate-producing bacteria and butyrate-producing bacteria, in this pathway butyrate-producing bacterium can be a net user of acetate and acetate is converted to butyrate (Duncan et al. 2004).

SCFAs in large intestine result in several metabolic effects. SCFAs can affect glucose metabolism. den Besten, Lange, et al. (2013b) infused mice with SCFAs directly in the cecum, they reported that SCFAs are mild regulators of glucose homeostasis, propionate is glucogenic in liver, 62% infused propionate are used to produce glucose in mice; while acetate and butyrate are lipogenic in liver, they are converted to palmitate and cholesterol. Layden et al. (2012) reported that plasma acetate level was negatively associated with visceral adipose tissue, and was also negatively associated with plasma insulin levels in the oral glucose tolerance test. SCFAs can affect lipid metabolism. The ratio of propionate to acetate may be an important factor that influence lipid metabolism. As mentioned above, acetate is

lipogenic; while an *in vitro* study showed that propoinate can inhibit synthesis of fatty acids and cholesterol from acetate (Demigné et al. 1995). SCFAs may act as regulators of appetite. Psichas et al. (2015) found that propionate can stimulate the secretion of the gut hormones peptide YY (PYY) and glucagon-like peptide 1 (GLP-1), which both can acutely suppress appetite, in isolated rat colonic crypt cultures.

1.4.2 Short chain fatty acids and human health

The microbiota ecosystem in the human large intestine is influenced by diet, the pH of the contents of the gut lumen may be one of the important indicators of the status of the lumen contents. Different fermentable substrate in diet lead to different fermentation patterns, thus the pH and the composition of fermentation products and ratios may also be different. Duncan et al. (2009) investigated growth of 33 representative human colonic bacteria under different pH environment, they reported that 8 *Bacteroides* species and 19 Gram-positive anaerobes grew poorly at pH 5.5, and these species thrived at pH 6.7; while *Eubacterium rectale,* which is butyrate-producing Gram-positive bacteria, was detected at pH 5.5, but was not detected at pH 6.7. Walker et al. (2005) reported that SCFA can promote large intestine functions, which include modulation of colonic motility, promotion of visceral blood flow, and prevention of the overgrowth of potential pathogens in the lumen. Fu, Shi, and Mo (2004) reported that SCFAs in the human colon may maintain colonocyte differentiation, and may exert a protective effect against carcinogenesis. Acetate is the major component of SCFAs in the colon. One study showed that acetate and

propionate may increase blood cholesterol and triglyceride levels (Wolever et al. 1989). However, another study suggested that substrate-dependent SCFA which produced by fermentation can inhibit cholesterol synthesis (Chen, Anderson, and Jennings 1984). Hosseini et al. (2011) reviewed propionate's effect on health, and stated that propionate has antilipogenic and cholesterol -lowering effects. Propionate also has been shown to exhibit antiproliferative effect towards colon cancer. Butyrate was reported to maintain a normal colonocyte phenotype, which could contribute to lowering the risk of colorectal cancer (McOrist et al. 2011). In general, SCFAs play a key role in colonic health and may play a key role in prevention of certain diseases ((den Besten, van Eunen, et al. 2013a); (Lecumberri, Goya, et al. 2007a)).

1.4.3 Determination of Short chain fatty acids

Measurement of profile of SCFAs can be performed either *in vivo* or *in vitro*. In general, SCFAs are produced and absorbed in the colon and mainly transported to the liver though the portal vein, and partly to the other organs via peripheral vein (Bergman 1990). Therefore, determination of concentration of SCFAs in blood is a direct method for assessing the degree of SCFA formation. However, it is difficult to extract and measure portal vein blood on a large-scale study of humans. Using currently available methodology the concentration of SCFAs in peripheral vein are below the minimum detectable concentration except acetate. Therefore, the application using venous blood for determination of SCFAs is limited. Determination of SCFAs in colostomy patient's colonic content has been used (Clausen, Bonnen, and

Mortensen 1991), but this *in vivo* approach also limited by experimental condition. Fermentation in the large intestine can be reflected by breath hydrogen gas excretion, and this method is a non-invasive test (Oku and Nakamura 2003). Hydrogen gas is produced from fermentation of non-digestible carbohydrates by microorganisms in large intestine. During fermentation, SCFAs, carbon dioxide, and hydrogen is produced simultaneously (Figure 1.10). Hydrogen cannot be metabolized by human cells, it diffuses into blood, subsequently it is breathed out of body through the lungs. Since colonic fermentation is the only source of hydrogen, determination of breath hydrogen can reflect fermentation in the large intestine. However, breath hydrogen gas excretion does not reflect the profile of SCFAs. Consequently, *in vitro* measurement is preferred.

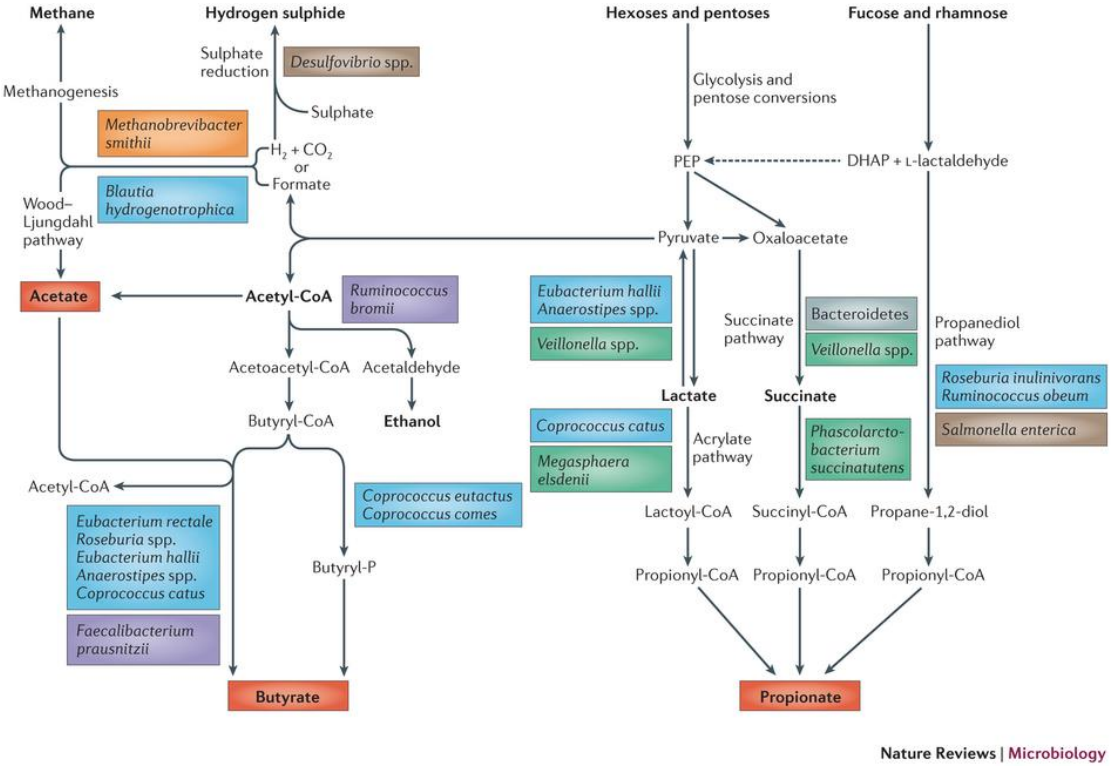


Figure 1.10. Pathways that are responsible for the biosynthesis of the major microbial metabolites that result from carbohydrate fermentation and bacterial cross-feeding. (Louis, Hold, and Flint 2014)

1.4.4 Model system

Both *in vivo* or *in vitro* model system has been used to assess SCFA production in previous studies. In animal studies, both rats (Kotani et al. 2009) and pigs (Liu 2015) were used as models. These studies showed that SCFAs concentration increased after with the inclusion of fermentable carbohydrates. However, the GI tract of rats is vastly different from human's, the microflora and metabolism pattern may not appropriately reflect those of in human gut. Pigs are considered better models than rats, but the pig model system are restricted by availability of resources to conduct studies with large numbers of animals. Therefore, pigs are not utilized as widely as

rats in model. Compared with *in vivo* model system, *in vitro* model system is simpler, faster, cheaper, and less resource-consuming, thus it is frequently employed in human gut microorganism fermentation studies (Gültekin-Özgüven, Berktaş, and Özçelik 2016)

1.5 Objective

The carbohydrates in cocoa that are mainly composed of dietary fibers. The cocoa fibers may be fermented by microorganisms in the human colon to produce beneficial compounds to the host. The variation in the cocoa processing, such as alkalization impacts the polyphenols composition (Wollgast and Anklam 2000), thermal treatment (roasting) affects dietary fiber composition (Valiente et al. 1994), and then consequently affects the fermentation patterns and the composition of the end products. However, the detail of these influences and changes, plus structure of carbohydrates in cocoa and their possible complexes with polyphenols are not well understood. In order to establish a more comprehensive understanding of polyphenolic compounds in cocoa and fermentation products changes, a model system which simulates colonic fermentation in the lower GI tract was developed, five differently treated cocoa samples (Natural cocoa, D-11-S, D-11-B, Lavado, Shell) were subjected to the pooled fecal fermentation, and D-11-B was fermented individual fecal samples, the pH, polyphenol compounds, and short chain fatty acids produced were determined.

CHAPTER 2. INTERACTION OF COCOA SAMPLES WITH MICROBIOTA FROM POOLED FECAL SAMPLE

2.1 Materials and methods

2.1.1 Cocoa sample analysis

Five cocoa samples provided by The Hershey company are labeled as Natural cocoa, D-11-S, D-11-B, Lavado, and Shell. All the samples were from roasted cacao, but underwent different processing. The shell and natural cocoa powder were not subjected to alkalization, the D-11-S were lightly alkalized by potassium carbonate to pH 7.6, while the D-11-B were heavily alkalized by sodium hydroxide and sodium bicarbonate to pH 8.0. To acquire quantified information about the processing treatments of each cocoa sample, the cocoa samples were subjected to Inductively Coupled Plasma Mass Spectrometry (ICP-MS) for plant tissue analysis. Analysis was done by Soil Testing & Plant Analysis Lab at Louisianan State University.

The sample treatment for ICP-MS was followed as below: Cocoa samples (0.5 g) were mixed with 2.2 ml deionized water (DI) water and heated by heat block. When temperature was brought to 125°C, 5 mL concentrated nitric acid (HNO_3) was added, then digested for 2.75 h. Add 3 mL hydrogen peroxide, let mixture cool down to room temperature, and filled to a volume of 20 mL with DI water.

2.1.2 Fecal sample collection

The fecal samples were collected from 7 randomly selected volunteer donors, and are identified as Volunteer Group 1. The donors had no history of gastrointestinal disease, and had not taken antibiotics during the 3 months prior to donation, donors

were asked to keep records of his/her regular diet for 3 to 5 days prior to collection on the last day. When sample collection was completed, the fecal samples were immediately frozen and stored in –80°C in an ultra-low temperature freezer until used.

2.1.3 Cocoa sample predigestion

A protocol simulating the human digestion and absorption process in the stomach and small intestine was established. Cocoa substrates were pretreated in the model *in vitro* digestive system to simulate the products that would reach the colon microbiota for fermentation. The enzymatic digestion consisted of pepsin digestion followed by pancreatin, to remove the digestible, non-fibrous contents of the substrate. Pepsin degrades protein into mostly water-soluble peptides and the pancreatin which is a mixture of amylase, lipase and protease further degrades protein, peptides, starches and lipids. The protease works to hydrolyze proteins into oligopeptides; amylase hydrolyzes starches into oligosaccharides and the disaccharide maltose; and lipase hydrolyzes triglycerides into fatty acids and glycerol. The digestion process was conducted as followed:

Sixty (60) g of freeze dried cocoa sample was added to 200 mL distilled water. Then 2 mol/L hydrochloric acid (HCl) was added to bring the solution to a pH of 2. The mixture was incubated for 10 min in a water bath with shaking to bring the temperature to 37°C. Next, 0.5 g (3500 U/mg) of pepsin was added to the mixture and incubated while shaking for another 3 h. Then the pH of the sample was brought to

7.5 with 2 mol/L sodium hydroxide (NaOH) solution, and incubated for 10 min to bring the temperature back to 37°C before adding 4.0 g of pancreatin (SIGMA-ALDRICH) and 1g of bile salts (mixture of sodium cholate and sodium deoxycholate, from SIGMA-ALDRICH). The solution was mixed thoroughly for 10 min then incubated at 37°C for 2 h. The mixture was microwaved for 3 min to denature the enzymes and then filtered to obtain the solids. Those remaining solids from the mixture were washed with 75% ethanol and filtered again and freeze dried overnight. The dried substrate was later made into a powder and kept frozen in a -80°C freezer until utilized for the fermentation step.

2.1.4 Fecal slurry preparation and Cocoa sample fermentation

The anaerobic buffer solution was prepared according to the following steps and was used throughout the fermentation process. The anaerobic solution was a mixture of two solutions, A and B, at the ratio 9:1 and prepared as follows: Solution A (per liter of distilled water) contained 11.76 g of sodium bicarbonate ($NaHCO_3$), 11.1 ml of Hemin (0.78 mmol/L of water), 1.1ml of menadione (0.36 mmol/L of water) and 1.1 ml of resazurin (3.98 mmol/L of water) as a redox indicator. The solution was autoclaved at 121°C for 15min after preparation. Solution B (per liter of autoclaved distilled water) contained 0.48 mol NaCl, 0.02 mmolK_2HPO_4 (Dipotassium phosphate), and 0.63 mmol L-Cysteine-HCl.

The *in vitro* pooled bacteria inoculums were prepared by blending the feces from 7 donors (20g feces each) with the anaerobic buffer at the 1:4 ratio (g: mL) in a

beaker, then filtered with a filter whirl-pak bag (Nasco). The pooled fecal slurry was incubated at 37°C under anaerobic conditions for 1 h.

One-liter glass bottles used as fermentation vessels were autoclaved at 121°C for 15 min prior to usage. To each vessel was added: 175mL of the anaerobic solution (solution A+B) and 5 g of the fermentation substrate (control: water). Then an aliquot of 25mL of the incubated bacteria fecal solution (slurry) was added to each vessel, the vessel was immediately flushed with an anaerobic gas mixture (10% CO_2, 80% N_2 and 10% H_2) for 10 min. The samples were then incubated at 37°C with stirring using magnetic stirring bars. Samples were collected at 0, 6, 12, and 24 h. The 15 mL samples were placed in falcon tubes and frozen at -80°C until usage. The fermentation in an *in vitro* digestive model system was repeated twice and 3 samples were examined for each separate experiment. The 5 cocoa samples were fermented by the pooled fecal slurry that mentioned above. These batch of fermentation was marked as Pooled Fermentation. (Table 2.1).

Table 2.1. Experimental design of pooled fermentation

	Pooled Fermentation	
	Substrate	Inoculums
Treatment 1	Natural Cocoa	Pooled fecal slurry
Treatment 2	D-11-B	Pooled fecal slurry
Treatment 3	D-11-S	Pooled fecal slurry
Treatment 4	Lavado	Pooled fecal slurry
Treatment 5	Shell	Pooled fecal slurry

2.1.5 Short chain fatty acid determination

For each of the samples, the pH was measured as an indicator of SCFA fermentation using a pH meter (pH meter: Seven2Go pro, pH probe: InLab 413 SG/2m, METTLER TOLEDO). One milliliter (1mL) of the fermentation samples was thoroughly mixed with 4mL of distilled water. A 1mL sample of the diluted extract was acidified with acid solution (metaphosphoric acid (3.72M) plus 2-ethylbutyrate (3.72μM)) and vortexed. The mixture was centrifuged for 10 min at 3000 rpm. The supernatant was collected in auto sampling vials for fatty acid analysis by gas chromatography (GC) measuring short chain fatty acid (acetate, propionate, and butyrate). GC condition: Shimadzu GC2010 equipped with a 15-m EC-1000 column that had an internal diameter of 0.53 mm and a film thickness of 1.2 μm (Alltech Associates, Inc.; Deerfield, IL). The reagent preparation procedure and temperature gradient for volatile short chain fatty acids analysis was adapted from Grigsby et al. (1992) and Bateman et al. (2002), respectively. The polyphenol compounds results are expressed in gallic acid equivalent (GAE) and catechin equivalent (CE). GAE was calculated by the following equation: G= C* V/M. G was the total gallic acid content per gram of certain substrate, C was the concentration of gallic acid of certain sampling in mg/ml, V was sampling volume in ml, M was the weight of substrate in g. CE was calculated by the following equation: Ca= C* V/M. Ca was the total catachin content per gram certain substrate, C was the concentration of catachin of

certain sampling in mg/ml, V was sampling volume in ml, M was the weight of substrate in g.

2.1.6 Phenolic acids determination

Fermented samples were diluted with deionized water at ratio of 1:4, then centrifuged for 15min at 3000rpm, the aliquot of the supernatant was subjected to Waters ACQUITY Class I UPLC system with 50mm phenolic column. The mobile phase is acetonitrile and 5% formic acid. Oven temperature is 30°C. Flow rate is listed in Table 2.2.

Table 2.2. UPLC flow rate

Time (min)	Flow (mL/min)	5%formic acid %	acetonitrile %
Initial	0.1	100	0
2	0.3	100	0
10	0.3	80	20
11	0.3	70	30
12	0.3	70	30
13	0.3	50	50
15	0.3	50	50
16	0.3	100	0
20	0.3	100	0

2.1.7 Data analysis

Data was analyzed by using SAS 9.4. ANOVA with Tukey's adjustment, significance value (alpha) was 0.05. The results were expressed as mean± Standard deviation.

2.2 Results

2.2.1 Mineral elements of the raw cocoa samples.

Inductively Coupled Plasma Mass Spectrometry (ICP-MS) was employed to qualify and quantify the extent of processing that each cocoa sample underwent, especially alkalization processing. Mineral elements data were listed in Table 2.3. Although small amount of sodium (2-3mg/100g) and potassium (approximately 2g/100g) naturally exist in cocoa bean, higher levels were introduced into cocoa powder during manufacture through alkalization. Sodium hydroxide, sodium bicarbonate, potassium hydroxide, and potassium carbonate were commonly used alkali reagents. Thus, content of sodium and potassium in cocoa powder can reflect extent of alkalization, the higher of sodium and potassium content, the more severe of alkalization treatment. Cocoa sample D-11-S contained higher potassium than other 4 cocoa samples ($P<0.05$), no significant difference was observed among content of potassium of Lavado, D-11-B, natural cocoa, and shell. While sodium content of cocoa sample D-11-B was highest among 5 cocoa samples ($P<0.05$).

Table 2.3. Mineral elements results of five cocoa samples.

Element	Lavado	D-11-S	D-11-B	Natural Cocoa	Shell
Aluminum (Al), ppm	50.17±1.07	31.55±2.42	145.42±1.49	60.51±2.78	161.52±22.67
Boron (B), ppm	25.94±0.14	24.82±0.55	21.28±0.25	27.81±0.51	33.45±0.74
Calcium (Ca), %	0.15±0.00	0.14±0.00	0.12±0.00	0.17±0.00	0.19±0.00
Copper (Cu), ppm	35.52±0.37	45.42±1.28	45.89±0.16	45.81±0.68	36.25±0.39
Magnesium (Ma), %	0.59±0.01	0.52±0.01	0.58±0.00	0.59±0.01	0.46±0.01
Manganese (Mn), ppm	25.20±0.28	43.69±1.25	63.34±0.33	43.11±0.45	36.48±0.85
Phosphorus (P), %	0.76±0.01	0.66±0.02	0.74±0.01	0.79±0.01	0.48±0.01
Potassium (K), %	1.64 ± 0.01^{b}	5.24 ± 0.14^{a}	1.79 ± 0.01^{b}	1.75 ± 0.02^{b}	1.76 ± 0.02^{b}
Sodium (Na), ppm	71.44 ± 0.62^{bc}	178.24 ± 8.63^{b}	25155.63 ± 111.21^{a}	37.81 ± 1.97^{c}	65.08 ± 4.09^{bc}
Sulfur (S), %	0.25±0.00	0.26±0.01	0.25±0.00	0.27±0.00	0.17±0.00
Zinc (Zn), ppm	83.78±0.91	78.88±2.15	75.44±0.34	79.89±1.08	57.20±1.70

Results are expressed in Mean ±SD. Significant difference is only compared among cocoa samples within each mineral element. Means that do not share a letter are significantly different. D-11-B and D-11-S are alkalized cocoa samples, natural cocoa, Lavado, and shell are non-alkalized cocoa sample

2.2.2 pH changes in the *in vitro* GI tract model system of Pooled Fermentation.

Initially, the pH of 5 cocoa samples in the in vitro model system ranged from 7.44 to7.63. After 6 hours fermentation in the model system, a significant pH decrease ($P<0.05$) occurred for the Lavado, shell, D-11-S, D-11-B, and Natural cocoa model system, while the pH in control did not have a significantly change ($P>0.05$). After 24 hours fermentation of the five cocoa powder samples, the pH significantly decreased in all of the 5 cocoa sample treatment groups and the control ($P<0.05$), the final pH of those samples was 6.9 to 7.2. From 6 h to the end of fermentation, the pH of D-11-S and D-11-B cocoa samples were higher than pH of Lavado and Natural Cocoa samples ($P<0.05$). Among all the 5 samples, the pH of the Shell sample exhibited highest reduction in pH, from 7.44±0.06 at 0 hour to 6.69±0.14 at the end of fermentation; the D-11-S sample had lowest reduction in pH, from 7.63±0.14 at 0 hour to 7.20±0.03 at the end of fermentation.

Table 2.4. The pH change during in the *in vitro* GI tract model system of cocoa samples fermented with pooled fecal slurry over 24h

Sample	0 Hour	6 Hour	12 Hour	24 Hour
Natural cocoa	7.56 ± 0.11^{Ab}	7.09±0.02	7.02±0.03	6.95 ± 0.05^{Bb}
D-11-S	7.63 ± 0.14^{Ab}	7.34±0.03	7.23±0.02	7.20 ± 0.03^{Bc}
D-11-B	7.61 ± 0.06^{Ab}	7.35±0.04	7.24±0.02	7.11 ± 0.04^{Bc}
Lavado	7.60 ± 0.02^{Ab}	6.96±0.09	7.00±0.04	6.93 ± 0.08^{Bb}
Shell	7.44 ± 0.06^{Abc}	6.92±0.13	6.90±0.09	6.69 ± 0.14^{Ba}
Control	7.85 ± 0.14^{Aa}	7.83±0.08	7.70±0.02	7.59 ± 0.07^{Bd}

pH results are expressed in Mean ±SD. Means that do not share a letter are significantly different, lower-case letters can be only compared within each column, and capital letters can be only compared within each row. D-11-B and D-11-S are alkalized cocoa samples, natural cocoa, Lavado, and shell are non-alkalized cocoa sample. Control was deionized water.

2.2.3 Procyanidin content of raw cocoa and digested cocoa samples.

As mentioned in section 1.3, cocoa beans contain oligomeric and polymeric procyanidins. In 5 undigested and 5 predigested cocoa samples, caffeine, theobromine, and procyanidins from monomer to decamer polymers were determined

(Table 2.5). Digested Lavado contained highest total procyanidin among five cocoa samples (66.32 mg/g substrate in undigested sample, 118.42 in predigested sample), while procyanidins content of D-11-B and D-11-S were much lower than the other 3 samples. After digestion, total procyanidin content of Shell and Natural cocoa slightly increased; on contrast, total procyanidin content of D-11-B and D-11-S decreased. Dramatic increase was observed in Lavado after digestion. Content of caffeine, theobromine, procyanidin monomer, dimer, and trimer decreased in all cocoa samples after digestion.

Table 2.5. Phenolic acids and procyanidin (monomer - 10mer) content of raw cocoa and digested cocoa samples.

Samples	Caffeine	Theobromine	1mer	2mer	3mer	4mer	5mer	6mer	7mer	8mer	9mer	10mer	total
Shell-raw	0.16%	1.09%	2.04	1.39	1.13	1.49	1.69	2.03	1.55	1.45	2.12	0.54	15.43
Shell-digested	0.08%	0.47%	0.52	0.59	0.75	1.26	1.75	2.43	1.65	2.03	3.64	0.95	15.57
D11S-raw	0.13%	1.79%	0.53	0.38	0.17	0.16	0.11	0.04	0.08	<0.001	<0.001	<0.001	1.47
D11S-digested	0.06%	1.29%	0.07	0.09	0.06	0.06	0.04	0.02	0.04	0.01	<0.001	<0.001	0.39
Natural cocoa-raw	0.17%	1.89%	3.69	2.55	1.72	1.58	1.73	1.77	1.22	0.84	1.02	0.32	16.44
Natural cocoa-digested	0.11%	1.00%	0.91	1.1	1.32	1.96	2.31	2.96	1.75	1.95	3.16	0.55	17.97
D11B-raw	0.12%	2.14%	0.11	0.01	0.02	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.14
D11B-digested	0.03%	0.56%	0.01	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.01
Lavado -raw	0.18%	2.18%	10.75	7.25	5.75	5.27	7.44	8.03	6.47	5.46	8.46	1.44	66.32
Lavado - digested	0.12%	1.15%	3.3	3.99	5.47	9.7	15.29	19.05	12.28	15.78	27.8	5.76	118.42

Caffeine and Theobromine are expressed in wt.%, Procyanidins are expressed in mg/g substrate

2.2.4 Polyphenolic compounds change of cocoa samples during 24h in Individual Fermentation.

There was no detectable phenolic acid found in the control group. The initial GAE of D-11-S and D-11-B were higher than that of Natural cocoa, Lavado, and Shell ($P<0.05$). After 24 hours, GAE significantly increased in the five fermented cocoa samples ($P<0.05$). There were no significant differences between initial GAE of D-11-B and D-11-S, similarly, initial GAE of Natural cocoa and Lavado were not significant different ($P>0.05$). For the final GAE, D-11-B and D-11-S were higher than the rest of the three samples ($P<0.05$). GAE variation in the Shell showed different pattern from the other four samples, it increased significantly in the first 6 hours, while GAE of other samples increased more gradually.

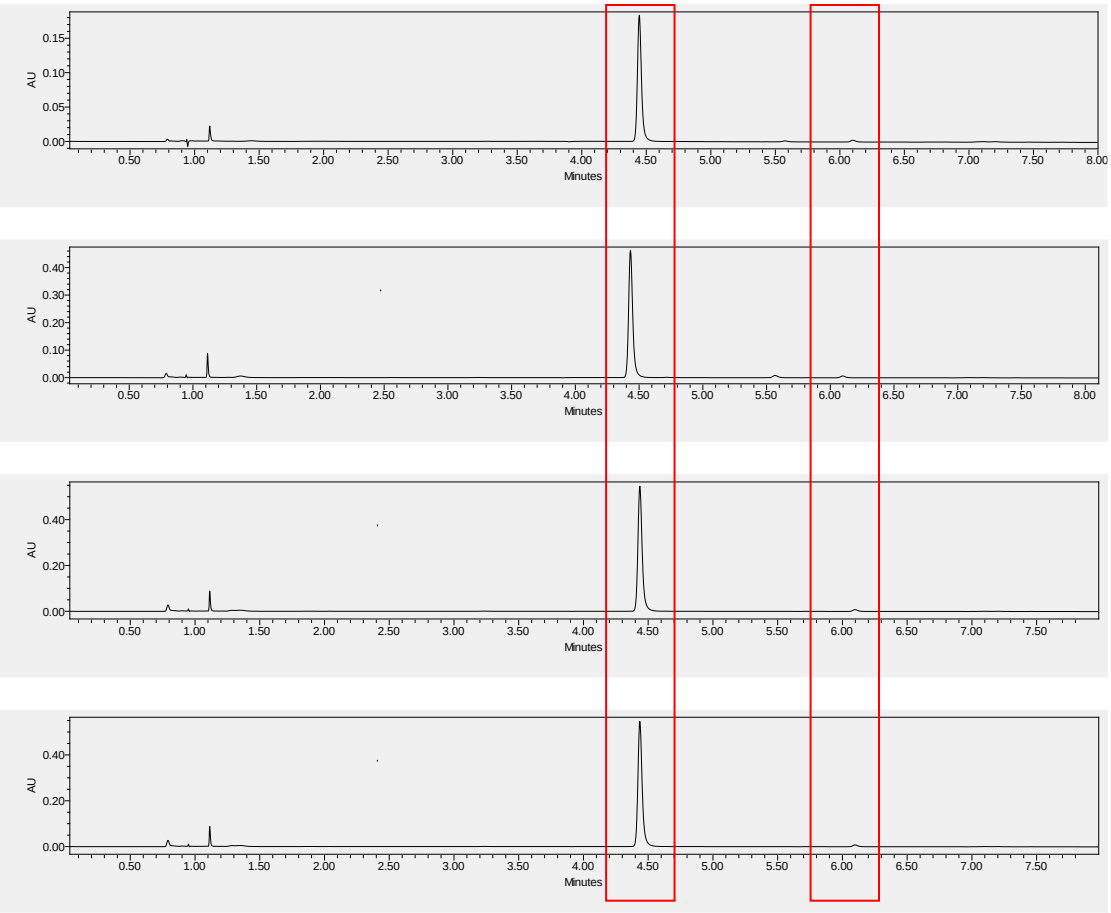


Figure 2.1. Chromatogram changes of cocoa sample D-11-B fermented with pooled fecal slurry. From top to bottom, fermentation time is 0h, 6h, 12h, and 24h. Peak at retention time 4min is Gallic acid, at 6min is catechin.

Table 2.6. Gallic acid equivalent changes of cocoa samples during 24h fermentation of cocoa sample with pooled fecal slurry in the *in vitro* GI tract model system.

	Natural cocoa	D-11-S	D-11-B	Lavado	Shell
0 Hour	0.0783±0.0264^{Aa}	0.2018±0.0000^{Ba}	0.2064±0.0450^{Ba}	0.0376±0.0022^{Aa}	0.0458±0.0213^{Aa}
6 Hour	0.0850±0.0248	0.2225±0.0321	0.2684±0.0178	0.0613±0.0040	0.1673±0.0107
12 Hour	0.1016±0.0324	0.2357±0.0362	0.2700±0.0007	0.0972±0.0113	0.1786±0.0006
24 Hour	0.1228±0.0099^{Ab}	0.3250±0.0883^{Cb}	0.2994±0.0317^{Cb}	0.1013±0.0071^{Ab}	0.1927±0.0079^{C} ^{ABb}

Results are expressed in mean ±SD in mg/g substrate. Means that do not share a letter are significantly different, lower-case letters can be only compared within each column, and capital letters can be only compared within each row.

The lowest initial CE was Lavado; D-11-S, D-11-B, and Natural cocoa were not significantly different from each other. After 24 hours fermentation, CE in all of samples increased ($P<0.05$). CE in the Shell group drastically increased in first 6 hours fermentation, and finial CE was much higher than the other four fermented cocoa samples ($P<0.05$).

Table 2.7. Catechin equivalent changes of cocoa samples during 24h fermentation of cocoa samples with pooled fecal slurry in the *in vitro* GI tract model system

Fermentation time	Natural cocoa	D-11-S	D-11-B	Lavado	Shell
0 Hour	0.3030±0.0870^{Aa}	0.3298±0.0521^{Aa}	0.2474±0.0502^{Aa}	0.1584±0.0266^{Ba}	0.4976±0.2402^{Aa}
6 Hour	0.4493±0.0308	0.4482±0.0172	0.3366±0.0219	0.4200±0.0494	2.2429±0.0162
12 Hour	0.5363±0.0580	0.5391±0.0203	0.3833±0.0503	0.6717±0.1562	2.0050±0.1734
24 Hour	0.6356±0.0585^{Ab}	0.5938±0.0251^{Ab}	0.3978±0.0609^{Ab}	1.0141±0.0345^{Bb}	1.9670±0.0427^{Cb}

Results are expressed in mean ±SD in mg/g substrate. Means that do not share a letter are significantly different, lower-case letters can be only compared within each column, and capital letters can be only compared within each row. D-11-B and D-11-S are alkalized cocoa samples, natural cocoa, Lavado, and shell are non-alkalized cocoa sample.

2.2.5 Short chain fatty acids content changes in the *in vitro* GI tract model system of Pooled Fermentation.

Three SCFAs, propionate, acetate, and butyrate, were detected in cocoa samples after fermentation. Initial propionate concentration of the five cocoa samples were not different from each other, nor initial concentration of acetate and butyrate. Table 2.8 showed the concentration of propionate in Natural cocoa, D-11-S, D-11-B, and control samples, did not significantly change during the 24 h. At the end of cocoa samples fermentation, propionate in Lavado and Shell significantly increased ($P<0.05$). Table 2.9 showed acetate in the control group remained at the original concentration during 24 h fermentation. Conversely, acetate in the other 5 cocoa samples significantly increased ($P<0.05$). The final concentration of acetate in the

Shell sample was highest among the 5 cocoa samples ($P<0.05$). This pattern was also observed in the changes in butyrate. During 24h fermentation of cocoa samples, only butyrate in the shell group exhibited statistically significant increase ($P<0.05$), there were no significant differences among the other 4samples and control group (Table 2.10).

Table 2.8. Propionate changes of fecal sample during 24h fermentation of cocoa samples fermented with pooled fecal slurry in the *in vitro* GI tract model system

	Natural cocoa	D-11-S	D-11-B	Lavado	Shell	Control
0 Hour	0.70 ± 0.07^{Aa}	0.81 ± 0.14^{Aa}	1.29 ± 0.76^{Aa}	0.84 ± 0.20^{Aa}	1.00 ± 0.09^{Aa}	0.89 ± 0.64^{Aa}
6 Hour	1.17 ± 0.11	1.47 ± 0.60	1.36 ± 0.47	1.3 ± 0.33	1.12 ± 0.28	0.89 ± 0.61
12 Hour	0.94 ± 0.20	1.07 ± 0.16	1.16 ± 0.24	1.22 ± 0.23	1.55 ± 0.65	0.79 ± 0.66
24 Hour	1.56 ± 0.27^{Aa}	1.02 ± 0.11^{Aa}	0.67 ± 0.10^{Aa}	3.23 ± 0.3^{Bb}	$6.95\pm0.35^{C}{}_{b}$	$0.32\pm0.14^{A}{}_{c}$

Results are expressed in mean ±SD in mmol/L. Means that do not share a letter are significantly different, lower-case letters can be only compared within each column, and capital letters can be only compared within each row. D-11-B and D-11-S are alkalized cocoa samples, natural cocoa, Lavado, and shell are non-alkalized cocoa sample. Control was deionized water.

Table 2.9. Acetate changes of fecal sample during 24h fermentation of cocoa samples fermented with pooled fecal slurry in the *in vitro* GI tract model system

	Natural cocoa	D-11-S	D-11-B	Lavado	Shell	Control
0 Hour	2.15 ± 0.06^{Aa}	2.81 ± 0.32^{Aa}	3.14 ± 0.66^{Aa}	3.17 ± 0.19^{Aa}	4.16 ± 0.53^{Aa}	2.94 ± 0.67^{Aa}
6 Hour	8.49 ± 0.47	5.79 ± 0.38	4.75 ± 0.05	8.76 ± 0.08	10.56 ± 0.83	2.96 ± 0.31
12 Hour	13.25 ± 0.38	11.02 ± 0.75	11.02 ± 0.10	16.89 ± 0.44	25.16 ± 5.63	2.43 ± 0.76
24 Hour	22.16 ± 0.31^{BCb}	15.94 ± 1.55^{ABb}	12.56 ± 4.16^{Ab}	25.88 ± 1.78^{Cb}	38.10 ± 14.92^{Db}	1.28 ± 0.20^{Aa}

Results are expressed in mean ±SD in mmol/L. Means that do not share a letter are significantly different, lower-case letters can be only compared within each column, and capital letters can be only compared within each row. D-11-B and D-11-S are alkalized cocoa samples, natural cocoa, Lavado, and shell are non-alkalized cocoa sample. Control was deionized water.

Table 2.10. Butyrate changes of fecal sample during 24h fermentation of cocoa samples fermented with pooled fecal slurry in the *in vitro* GI tract model system

	Natural cocoa	D-11-S	D-11-B	Lavado	Shell	Control
0 Hour	0.40±0.01	0.45±0.12	0.59±0.33	0.44±0.05	0.33±0.01	0.58±0.21
6 Hour	0.47±0.04	0.35±0.16	0.42±0.10	0.40±0.06	0.37±0.05	0.53±0.20
12 Hour	0.41±0.08	0.48±0.07	0.43±0.08	0.45±0.05	0.65±0.15	0.30±0.24
24 Hour	0.51±0.09	0.51±0.06	0.67±0.10	0.5±0.03	1.55±0.10 A	0.16±0.12

Results are expressed in mean ±SD in mmol/L. Only value of shell sample at 24h is significantly different from other values. D-11-B and D-11-S are alkalized cocoa samples, natural cocoa, Lavado, and shell are non-alkalized cocoa sample. Control was deionized water.

2.2.6 Microorganism distribution of cocoa samples in Pooled Fermentation

After stringent quality sequence curation, a total of 3799034 sequences were parsed and 3309403 were then clustered. 3308382 sequences identified within the Bacteria and Archaea domains were utilized for final microbial analysis (Figure 2.2). Before fermentation *Firmicutes* was the most abundant phylum, it accounted for more than 70% of total microorganism population in the pooled fecal sample from Volunteer Group 1; next was *Bacteroidetes* phylum, which account for approximate 20% of total microorganism population; *Proteobacteria* and *Actinobacteria* took up only 6% of total microorganisms. After 24 hours fermentation, dramatic increases of *Proteobacteria* were observed in the five fermented cocoa samples, *Proteobacteria* became dominant phylum and it took up 40% - 60% of total microorganism population after fermentation; meanwhile population of *Firmicutes* decreased to lower than 40%. Increases of *Bacteroidetes* were observed in D-11-B, Natural cocoa powder, and Shell after 24 h fermentation, while it decreased in D-11-S, Lavado, and

the control. The highest increase of *Bacteroidetes* was in shell sample, its population account for 40% of total microorganism population, while in the control its population decreased to less than 3%. Decreases of *Actinobacteria* were found in the five fermented cocoa samples, its population went down to 1% in D-11-B and the control.

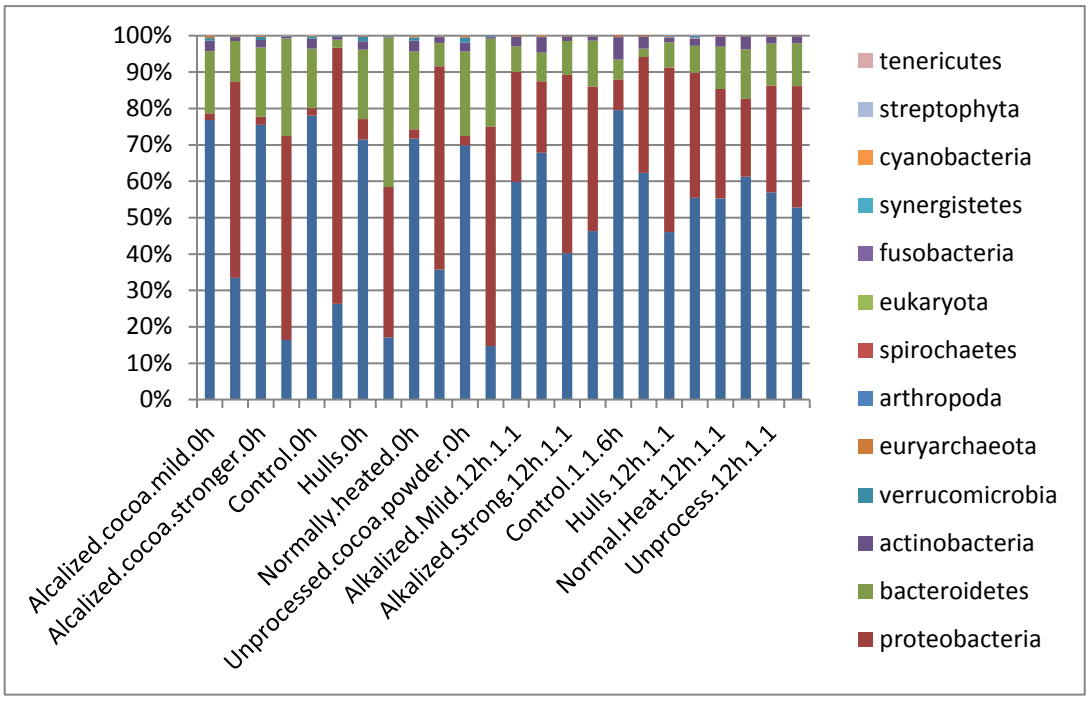


Figure 2.2. Relative abundance of Phyla of fecal microorganism in the *in vitro* GI tract model system of cocoa samples fermented with pooled fecal slurry. In this figure, "alkalized cocoa mild" means cocoa sample D-11-S, "alkalized strong" means D-11-B, " unprocess" means natural cocoa, "normal heat" means Lavado, and "hulls" means shell.

2.2.7 Macronutrients intake of Group 1

Macronutrients data was collected and calculated based on nutrition facts label of food and USDA Food Composition Databases. The detailed daily food intake records of each donor in Volunteer Group 1 was listed in Appendix. In this Macronutrients record table, carbohydrates include monosaccharide, disaccharide, oligosaccharide, and polysaccharide, sugar only refers to monosaccharide and disaccharide. There was no significant difference of average daily intake of calories, protein, and lipid among 7 donors ($P>0.05$). Donor G had higher carbohydrates intake than Donor B and Donor C ($P<0.05$); while Donor A and Donor D had higher dietary fiber intake than Donor C and Donor G ($P<0.05$). Sugar intake of Donor C was much less than Donor D ($P<0.05$).

Table 2.11. Macronutrients average daily intake of 7 donors in Volunteer Group 1

Donor	Calories/Cal	Protein/g	Lipid/g	Carbohydrates/g	Dietary fiber/g	Sugar/g
A	2083.6±1053.3	69.3±15.9	58.6±16.0	203.2±50.9^{ab}	14.1±4.0^{a}	56±29.6^{abc}
B	1103±176.1	24.5±9.2	52.3±10.9	142.3±29.0^{b}	11.4±3.8^{ab}	53.7±31.9^{abc}
C	904.4±359.0	36.2±16.4	43.2±35.2	92.5±20.2^{b}	3.9±1.2^{b}	13.2±8.1^{c}
D	1438.2±504.0	47.1±19.7	44.8±19.6	223.6±73.5^{ab}	15.3±6.0^{a}	105.4±37.9^{a}
E	1549.8±296.0	94.9±64.7	63.6±18.4	167.8±29.3^{b}	9.4±3.9^{ab}	49.0±9.3^{abc}
F	1444.7±382.2	50.7±28.8	43.5±31.3	187.7±88.2^{ab}	9.7±3.3^{ab}	85.6±29.6^{ab}
G	1930.7±468.1	96.1±11.7	28.4±15.8	320.0±85.6^{a}	4.6±1.0^{b}	26.0±7.5^{bc}

Results are expressed in mean ±SD. Significant difference is only compared among 7 donors within each macronutrient. Means that do not share a letter are significantly different. No significant difference of average daily intake of calories, protein, and lipid among 7 donors

2.3 Discussion

This study was carried out to assess the fermentation of cocoa powder with human gut microbiota. It was the first study that investigated the fermentation of various processed cocoa powder with both pooled and individual fecal samples from multiple donors, monitored microorganism activity, short chain fatty acids, and polyphenols at the same time. The results found that cocoa powder can be used by human gut microorganism during fermentation, and produced polyphenols and SCFAs which could possess potential health benefits to the human body.

2.3.1 Chemical properties of cocoa samples.

Total 11 mineral elements were investigated in this study. In general, the most abundant mineral element in each cocoa sample was potassium, followed by magnesium, phosphorus, sulfur, and calcium. The mineral elements profile of cocoa samples was similar to the cocoa bean in study conducted by Afoakwa et al. (2013), and content of elements were roughly at the same level.

Mineral elements content result of cocoa samples showed that D-11-S and D-11-B contained significantly higher amount of sodium and potassium than other samples, this result proved that sample D-11-B and D-11-S received alkalization treatment. This result was also in line with the manufacture information provided by The Hershey Company. D-11-S was treated with potassium carbonate to pH 7.6, so potassium element was introduced, and finally high amount of potassium was left in D-11-S; while D-11-B was treated with sodium hydroxide and sodium bicarbonate to pH 8.0, so high amount of sodium was left in D-11-B.

Result also demonstrated that there was no significant difference of sodium or potassium content among Lavado, natural cocoa, and shell sample. According to manufacture information provided by The Hershey company, natural cocoa and shell

had not received alkalization treatment. Therefore, cocoa sample Lavado may receive no or very slight alkalization treatment. Other possible explanation of relative low content of sodium and potassium in Lavado was that it may receive other special processing to remove these 2 elements. Mineral elements other than sodium and potassium were affected substantially by the elements in the soil, previous study also reported that mineral elements contents of cocoa, including Mn, Fe, Zn, Cu, Cd, and Ba, were affected by cropping site rather than genotype (de Araujo et al. 2017).

Based on result of mineral elements and manufacture information, five cocoa sample can be categorized into two group. D-11-S and D-11-B were alkalized cocoa powder, natural cocoa, Lavado, and shell were non-alkalized sample. In detail, D-11-S was mildly alkalized cocoa powder, and D-11-B was heavily alkalized cocoa powder.

Total procyanidins content of D-11-B and D-11-S were much lower than the other 3 samples (Table 2.4). This suggests that alkalization treatment contributed to reduction of polyphenols content in cocoa during manufacture. Previous studies also reported phenolic compound loss during alkalization. Li et al. (2014) measured total polyphenol content in cocoa powder alkalized by different alkali solution, they found that total polyphenol content of each cocoa powder decreased after alkalization treatment, and cocoa powder treated with potassium carbonate solution contained lower amount of total polyphenol than cocoa powder treated with sodium hydroxide solution. Miller et al. (2008) measured total polyphenol content of commercial cocoa powder which were lightly alkalized (pH 62.0-7.20), medium alkalized (pH 7.21-7.60), and heavily alkalized (pH 7.61 and higher). They found that the more severe alkalization it received, the lower polyphenol content in the product. The total polyphenol content of cocoa powder even showed a linear decrease as pH of cocoa

powder increased. Result of these 2 studies also roughly corresponded to relationship between alkalization of cocoa sample and polyphenol content. The polyphenol content of cocoa powder will be further discussed in 2.3.3. It should be noted that alkalization was not the only factor that affected polyphenol content during cocoa powder manufacture. Previous studies also reported that cocoa origin and roasting can also have affected polyphenol content (Miller et al. 2008); (Ramli et al. 2006)). Alkalization is one of the factors contributed to polyphenol loss.

2.3.2 pH, SCFAs and gut microbiota changes during 24h in Pooled Fermentation.

The pH is an important parameter in the fermentation because it can reflect the growth of microflora. The pH decrease during the fermentation of the cocoa samples was mainly due to the production of short chain fatty acid (Maekivuokko et al. 2007).The substrates for short chain fatty acid production including dietary fibers and other carbohydrates (Walker et al. 2005). Previous work in our lab (unpublished data) identified acetate, propionate, and butyrate are produced during cocoa fermentation. The levels of the short chain fatty acid concentrations vary with the cocoa samples and microflora during fermentation.

In cocoa powder samples fermented with pooled fecal slurry experiments, the pH continuously decreases during cocoa samples fermentation were observed in all of 5 in vitro model systems. The SCFAs results showed that when fermentation duration time increased, more SCFAs were produced. The SCFAs analysis results were in accord with pH results. Furthermore, the lowest pH was found in the shell sample fermentation in vitro model system, and the shell sample was used to produce the highest amount of SCFAs were detected in this model system (Table 2.7 to Table 2.9). It was also noted that although both alkali treated and non- alkali treated cocoa

samples exhibited pH decrease during cocoa samples fermentation with fecal slurry, after 24 h fermentation the final pH of non- alkalized cocoa samples were lower than pH of alkalized cocoa samples (P<005). The mildly alkalized cocoa sample D-11-S (treated with potassium carbonate) had lowest reduction in pH at the end of fermentation. This result indicated that alkalization treatment changed fermentability of dietary fiber in cocoa powder.

Acetate was the main contributor to the pH decrease of the cocoa samples, the acetate content was about 8- 20 times higher than propionate and butyrate content (Table 2.7 to Table 2.9). This result indicated that the bacteria species which produce acetate were more active than propionate-producing bacteria and butyrate-producing bacteria in the cocoa samples fermentation. This result was consistent with previous studies, which reported that acetate is the dominant SCFA in the column of human and rats ((Ríos-Covián et al. 2016); (Vogt and Wolever 2003);(Campbell, Fahey, and Wolf 1997); (Treem et al. 1996)). As mentioned above, pH change was the one of the primary indicators of microbial activity, in this study the pH decrease confirmed the existence of the human gut microbial activities in the human gut fermentation model system. Increases of the SCFAs in model system further confirmed the microbial activity. Combination of both the pH and the SCFAs results indicated that human gut microorganism from human fecal slurry metabolized carbohydrates in cocoa powder samples. The SCFAs were the metabolites of the microorganism activity, and the increased SCFAs content in model system subsequently induced pH decreases.

The butyrate increase was only observed at the latter period (12 h- 24 h) in the Shell sample fermentation, during this time period the pH further decreased and finally reached a value lower than 7. The butyrate content change in other 4 groups showed that butyrate content in model systems were much lower than content of

acetate. Walker et al. (2005) showed that in anaerobic fermentation, the butyrate production preferred lower pH environment, sample in the pH 5.5 produced higher amount of butyrate than sample in pH 6. In this study the pH in model systems were higher than 6, butyrate-producing rate may be relatively low under neutral environment, this may be one of the reasons that content of butyrate was much lower than content of acetate. On the other hand, some of the butyrate-producing bacteria, such as F. Prausnitzii, need an acetate rich environment to grow and consume acetate to produce butyrate (Duncan et al. 2002), it is reasonable that butyrate content increase was only detected in the model system with highest acetate production.

The pH in the control model system decreased after 24 hours incubation (Table 2.3). Theoretically, since no substrate was added into in vitro fermentation model system, human gut microorganism was not able to grow without carbohydrates, therefore SCFAs would not be produced, thus the pH in model system would not decrease. However, although the pooled fecal sample was filtered, it was still possible that a tiny amount of carbohydrates existed in fecal slurry, microorganism could ferment these carbohydrates then produce SCFAs. This may be the reason of pH decrease in control. The pH reduction extent in the control model system was less than other 5 cocoa powder samples fermentation model system. This meant the SCFAs content in cocoa samples fermentation model system was higher than in the control. This result indicated that during the cocoa powder samples fermentation, even if a part of SCFAs was fermented from carbohydrates originally existed in fecal slurry, the other part of SCFAs was from cocoa powder samples fermentation.

It was noted that increases of Bacteroidetes phylum were observed in D-11-B, Natural cocoa powder, and Shell after 24 h fermentation, while it decreased in D-11-S, Lavado, and the control. Meanwhile the highest increase of Bacteroidetes was in

shell sample, its population account for 40% of total microorganism population, while in the control its population decreased to less than 3%. These results indicated that proliferation of human gut microbes was affected by different cocoa samples. The effects of polyphenol -rich food on growth of human gut microorganisms is complicated. For example, an in vitro fermentation of grape seed extract study conducted by Cueva et al. (2013) showed profile of microbiota change during fermentation. They found that grape seed extract promoted growth of Lactobacillus/Enterococcus but decrease in the Clostridium histolyticum group during fermentation. However, the results shown in grape seed experiment was contrary to a study which employed an in vitro fermentation of a red wine extract by human gut microbiota (Sánchez-Patán et al. 2012). Microbial activity was observed during fermentation, but the profile of main bacterial groups did not significantly change. Although the studies focused on fiber-microbiota relationship is increasing in numbers, understanding of effect of fiber on microbiota profile changing is still poor and conclusion of those studies are controversial.

In summary, during 24h fermentation of cocoa samples with human fecal slurry, pH decrease was observed in both alkalized and non-alkalized cocoa samples. Reduction of pH was caused by production of short chain fatty acids by human gut microbiota. At the end of fermentation, pH of non-alkalized cocoa samples were lower than pH of alkalized cocoa samples. Alkalization reduced fermentability of dietary fiber in cocoa powder. This may be because some of the dietary fiber was converted to simple sugars and lost during alkalization.

2.3.3 Procyanidin content in raw cocoa samples and Polyphenolic compounds change during 24h in Pooled Fermentation.

2.3.3.1 Procyanidin content in raw cocoa samples

Procyanidin distribution in each cocoa sample were very different from each other (Table 2.4), this result illustrated that different processing resulted in greatly modified polyphenol content. As mentioned in 2.3.1, total procyanidins content of alkalized cocoa samples (D-11-B and D-11-S) were much lower than non- alkalized cocoa samples (natural cocoa, Lavado, and shell) (Table 2.4). This suggested that alkalization treatment contributed to polyphenols compound loss in cocoa during manufacture. This is in line with previous studies. Roasting, alkali treatment, milling, and removal of lipids can lead to loss of polyphenol content ((Bordiga et al. 2015) (Cooper et al. 2007); (Natsume et al. 2000)). A study also reported that cocoa processing, such as roasting and Dutching, can significantly reduce the polyphenolic compound content (Mazor Jolić et al. 2011). Pre-digestion reduced content of caffeine, theobromine, and procyanidin monomer, dimer, trimer. This loss may be attributed to washing steps in pre-digestion, these compounds are relatively small and low molecular weight molecules, and their solubility in water and ethanol are higher than procyanidins with higher degree of polymerization (DP), thus they may be washed off during pre-digestion. This is similar to absorption of polyphenols in humans. In human body, these low molecular weight compounds are released from food matrix, and they are absorbed in the small intestine ((Holt et al. 2002); (Scalbert et al. 2002);(Deprez et al. 2001)). Procyanidin polymers cannot be absorbed in the small intestine and passed into the large intestine (Stoupi, Williamson, Viton, et al. 2010a).

2.3.3.2 Polyphenolic compounds change during 24h in Pooled Fermentation.

The main polyphenol compounds of cocoa are catechin, epicatechin, and catechin polymers (Ortega et al. 2008). The polyphenolic content varied greatly due to different cocoa species and treatments during processing (Lecumberri, Mateos, et al. 2007b). Polyphenolic compounds (polymer) accumulated more in shells and skins of the cocoa bean (Afoakwa et al. 2013), so it so it accounts for the shell sample containing higher initial and final CE content. Shell sample interacted with microbiota and had highest polyphenolic compound content at the end of fermentation, it may be used as a low-cost phenolic-rich health food ingredient. The other 4 cocoa samples also had different final content of GAE and CE. At the end of fermentation, GAE content of alkalized cocoa samples (D-11-S and D-11-B) were higher than non-alkalized cocoa samples ($P<0.05$), while high or low CE content of each cocoa samples did not necessarily correspond to light or heavy alkalization treatment that each cocoa sample received. This result implied that alkalization may affect availability of polyphenol, however, influence of alkalization varied from one kind of polyphenol to another.

In this study concentrations of polyphenolic compounds in the cocoa samples increased during fermentation. This implied that colonic bacteria may convert bounded polyphenols into measurable free polyphenols in cocoa. The polyphenol polymers that are larger than dimers are not absorbed, so they can reach the lower GI tract and interact with microbiome, then these polymers may be broken down to dimer, monomer, or simple phenolic compounds. The results of other studies showed that only about10% procyanidin oligomer underwent scission of the interflavan bond and release catechins ((Stoupi, Williamson, Drynan, et al. 2010b); (Appeldoorn et al. 2009). Hein et al. (2008) used a pig intestinal microbiota model system to investigate

the microbial degradation ability on flavonol glycosides, they found that flavonols were hydrolyzed by the pig intestinal microbiota to simple phenolic compound such as phloroglucinol. The source of increased polyphenols in this study suggest phenolic compounds are being released from biopolymers by action of the human gut microorganisms, phenolics are also produced from degradation of procyanidin oligomers and polymers. The details of this chemistry are not clear and need further investigation.

In summary, alkalization reduced polyphenol content of cocoa powder during manufacture. Cocoa powder can be fermented by human gut microorganism, and polyphenol content of cocoa powder increased during fermentation regardless alkalization treatment of cocoa powder.

CHAPTER 3. INTERACTION OF COCOA SAMPLE WITH MICROBIOTA FROM INDIVIDUAL FECAL SAMPLES

3.1 Materials and methods

3.1.1 Fecal sample collection

In Pooled Fermentation, Lavado showed relatively high polyphenol and SCFAs content after 24 h fermentation. Based on the results, cocoa sample Lavado was selected and subsequently fermented with fecal slurries from 6 donors. These 6 donors were called Volunteer Group 2. The requirements of donors and fecal sample collection protocol were the same as Pooled Fermentation.

3.1.2 Cocoa sample predigestion

The cocoa powder Lavado used in this Individual Fermentation was from the same raw cocoa sample that used in the Pooled Fermentation. The digestion condition of the protocol was strictly followed, all the enzymes and reagents were purchased from the same supplier.

3.1.3 Fecal slurry preparation and Cocoa sample fermentation

The *in vitro* bacteria inoculums were prepared using 20 g of the feces from a donor that was mixed with 80 mL of the anaerobic buffer then filtered with a filter whirlpak bag. The anaerobic buffer was prepared by following the same protocol mentioned in 2.1.4. The solution was incubated at 37°C under anaerobic conditions for 1 h.

Cocoa sample Lavado were fermented with 6 different fecal slurries (Table 3.1). The inoculation and fermentation steps were described in 2.1.4. Deionized water was used as substrate in the control group. The fermentation in an *in vitro* digestive model system was repeated twice and 3 samples were examined for each separate experiment. Samples were collected at times 0, 6, 12, and 24, stored in 15 mL falcon

tubes and frozen at -80°C until usage. This batch of fermentation was marked as Individual Fermentation, the pooled fecal slurry was mixture of fecal sample from all 6 donors (Table 3.1).

Table 3.1. Experimental design of Individual Fermentation

	Individual Fermentation	
	Substrate	Inoculums
Treatment 1	Lavado	Donor 1
Treatment 2	Lavado	Donor 2
Treatment 3	Lavado	Donor 3
Treatment 4	Lavado	Donor 4
Treatment 5	Lavado	Donor 5
Treatment 6	Lavado	Donor 6
Treatment 7	Lavado	Pooled fecal slurry

3.1.4 Determination of pH, short chain fatty acid, and phenolic acids.

The samples collected from each individual fermentation of cocoa sample Lavado were subjected to determination of pH, short chain fatty acids, and phenolic acids of cocoa sample Lavado. The instruments and reagents employed in the Individual Fermentation were the same as the items which used in Pooled Fermentation.

3.1.5 Data analysis

Data was analyzed by using SAS 9.4. ANOVA with Tukey's adjustment, significance value (alpha) was 0.05. The results were expressed as mean± Standard deviation.

3.2 Results

3.2.1 pH changes in the *in vitro* GI tract model system of Individual Fermentation.

In the Pooled Fermentation study, Lavado showed relatively high polyphenol and SCFAs content after 24h fermentation. Therefore, the cocoa sample Lavado was selected and fermented with fecal slurry from 6 individual donors. Similar as the result of Pooled Fermentation experiments (Table 3.2), variation of the initial pH,

final pH, and pH changing patterns were observed in six individual donor fecal samples and pooled fecal sample fermentation, except the control, which had no pH change during 24 hours fermentation. The initial pH of model system ranged from 7.62±0.04 to 7.12±0.01, the final pH of model system ranged from 7.54±0.02 to 6.79±0.19. The pH of the control (Table 3.2) exhibited no significant change, as well as Donor 6. The pH decreased in Donor 1 to the Donor 5, and the Pooled fecal group ($P<0.05$). The pH of the Donor 4 and the Pooled showed significant reduction after the first 6 hours of fermentation($P<0.05$); while significant pH decrease of Donor 1 and Donor 2 occurred after 12 hours.

Table 3.2. The pH change during in the *in vitro* GI tract model system of cocoa sample Lavado fermented with individual fecal slurries over 24h.

	Fermentation Time			
Inoculums	0 Hour	6 Hour	12 Hour	24 Hour
Donor 1	7.62 ± 0.04^{Ac}	7.56±0.01	7.09±0.04	7.01 ± 0.04^{Bb}
Donor 2	7.35 ± 0.02^{Ab}	7.29±0.04	7.06±0.02	6.92 ± 0.02^{Bb}
Donor 3	7.27 ± 0.02^{Ab}	7.18±0.01	7.16±0.03	7.06 ± 0.04^{Bb}
Donor 4	7.36 ± 0.03^{Ab}	7.01±0.20	7.00±0.14	6.79 ± 0.19^{Ba}
Donor 5	7.12 ± 0.01^{Aa}	7.11±0.02	7.01±0.03	6.86 ± 0.10^{Ba}
Donor 6	7.59 ± 0.04^{Ac}	7.52±0.03	7.55±0.02	7.54 ± 0.02^{Ac}
Pooled	7.60 ± 0.08^{Ac}	7.10±0.12	7.07±0.08	7.02 ± 0.06^{Bb}
Control	7.45 ± 0.01^{Ab}	7.45±0.01	7.45±0.02	7.43 ± 0.03^{Bc}

Result was expressed as mean ±SD. Means that do not share a letter are significantly different, lower-case letters can be only compared within each column, and capital letters can be only compared within each row. Pooled fecal slurry was prepared from mixture of fecal sample from all 6 donors. Control was deionized water.

3.2.2 Polyphenols content changes of cocoa sample Lavado during 24h in Individual Fermentation.

The gallic acid changes were measured in cocoa powder sample Lavado fermented with 6 individual and 1 pooled fecal slurries. No gallic acid was detected in the control. Results showed that except the Donor 4 and the Donor 6, GAE content significantly increased in other 5 treatment groups ($P<0.05$). The initial GAE of each treatment were close to 0.03 mg/g substrate. Over 24h fermentation, each treatment group exhibited distinctive GAE content increase pattern. At the end of fermentation, GAE content in the Pooled reached 0.2185±0.0128mg/g substrate, which was the highest among 7 treatments. GAE content in other treatments were much lower than the Pooled fecal group ($P<0.05$), they value ranged from 0.0615±0.0125 mg/g to 0.1030±0.0097 mg/g substrate.

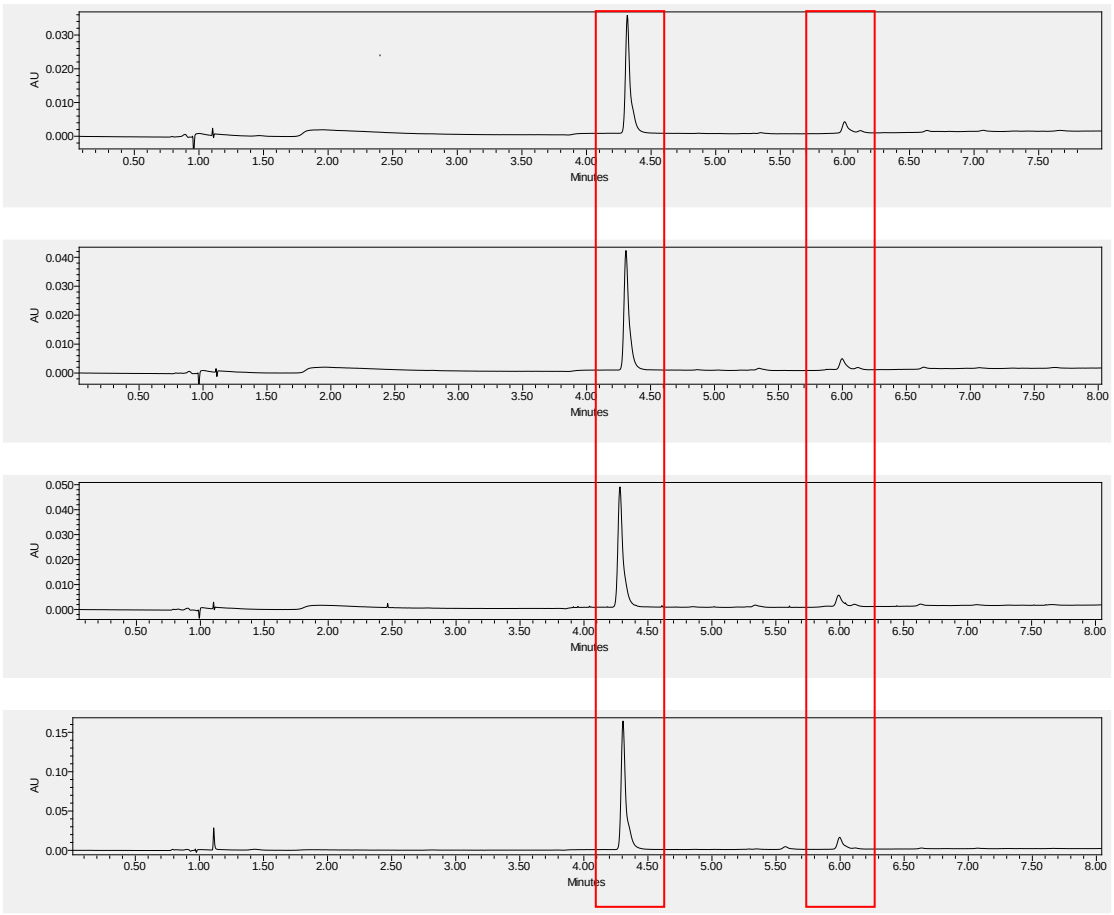


Figure 3.1. Chromatogram changes of cocoa samples Lavado fermented with individual fecal sample Donor 3. From top to bottom, fermentation time is 0 h, 6 h, 12 h, and 24 h. Peak at retention time 4min is Gallic acid, at 6 min is catechin.

Table 3.3. Gallic acid equivalent changes of cocoa sample Lavado during 24 h fermentation of cocoa sample Lavado fermented with individual fecal slurries in the *in vitro* GI tract model system.

	Fermentation time			
Inoculums	0 Hour	6 Hour	12 Hour	24 Hour
Donor1	0.0172±0.0067^{Aa}	0.0252±0.0034	0.0565±0.0099	0.0898±0.0179^{Bb}
Donor2	0.02±0.0028^{Aa}	0.0236±0.003	0.0312±0.0031	0.0651±0.0125^{Bb}
Donor3	0.0243±0.0055^{Aa}	0.0346±0.001	0.0509±0.0097	0.103±0.0097^{Bb}
Donor4	0.03±0.0066^{Aa}	0.0305±0.0137	0.0334±0.0047	0.0258±0.0032^{Aa}
Donor5	0.0267±0.0021^{Aa}	0.0234±0.0073	0.0476±0.002	0.0901±0.0193^{Bb}
Donor6	0.0108±0.0036^{Aa}	0.0101±0.0018	0.0180±0.0087	0.0194±0.0078^{Aa}
Pooled	0.0171±0.0067 ^{Aa}	0.0709±0.0008	0.0748±0.013	0.2185±0.0128^{Bc}

Results are expressed in mean ±SD in mg/g substrate. Means that do not share a letter are significantly different, lower-case letters can be only compared within each column, and capital letters can be only compared within each row. Pooled fecal slurry was prepared from mixture of fecal sample from all 6 donors.

Table 3.4. Catechins change over 24 h cocoa samples fermented with individual fecal slurry in the *in vitro* GI tract model system.

	Fermentation time			
Treatment	0 Hour	6 Hour	12 Hour	24 Hour
Donor1	0.1357 ± 0.0297^{Aa}	0.2766±0.0312	0.5755±0.0032	0.9849 ± 0.0821^{Bc}
Donor2	0.1139 ± 0.0515^{Aa}	0.2552±0.0251	0.2507±0.0447	0.4733 ± 0.0169^{Aab}
Donor3	0.1718 ± 0.0693^{Aa}	0.4329±0.0479	0.5166±0.1043	1.1925 ± 0.133^{Bc}
Donor4	0.1407 ± 0.021^{Aa}	0.2462±0.0532	0.4054±0.0286	0.6152 ± 0.1309^{Bb}
Donor5	0.1436 ± 0.0457^{Aa}	0.2788±0.0889	0.342±0.0017	0.7724 ± 0.1572^{Bb}
Donor6	0.142 ± 0.0601^{Aa}	0.0867±0.032	0.114±0.0654	0.1489 ± 0.0262^{Aa}
Pooled	0.151 ± 0.0673^{Aa}	0.4947±0.007	0.8055±0.0258	1.6182 ± 0.1217^{Bd}

Result was expressed as mean ±SD in mg/g substrate. Means that do not share a letter are significantly different, lower-case letters can be only compared within each column, and capital letters can be only compared within each row. Pooled fecal slurry was prepared from mixture of fecal sample from all 6 donors.

Catechin was detected in all of 7 treatment groups, there was no catechin found in the control group. The initial CE content in all of 7 treatment groups were close to 0.1 mg/g substrate. During 24 h fermentation, CE content increase was observed in the Donor 1, 3, 4, 5, and the Pooled fecal group ($P<0.05$). The final CE content in the Pooled was 1.6182±0.1217 mg/g substrate, which was the highest value among all of 7 treatment groups. CE content in the other treatment groups ranged from 0.4733±0.0169 to 1.1925±0.1330 mg/g substrate.

3.2.3 Short chain fatty acids content changes in the *in vitro* GI tract model system of Individual Fermentation

Butyrate content in all of 7 treatments and the control were measured. Butyrate content increased significantly after 24 hours fermentation of cocoa sample Lavado

with pooled fecal slurry ($P<0.05$). No significant change of butyrate content was observed in the Donor 1 to 6 and the control. The concentration of butyrate in all of 7 treatments and the control were no more than 2 mmol/L, the highest concentration of butyrate was found in the Pooled at the end of fermentation (1.77±0.01 mmol/L), which was higher than butyrate concentration of Lavado in the Pooled Fermentation.

Table 3.5. Butyrate changes of individual fecal samples in individual during 24h fermentation of cocoa sample Lavado with individual fecal slurries in the *in vitro* GI tract model system.

	Fermentation Time			
Inoculums	0 Hour	6 Hour	12 Hour	24 Hour
Donor 1	1.28 ± 0.54^{Ad}	0.84±0.15	1.14±0.20	0.86 ± 0.03^{Ab}
Donor 2	1.24 ± 0.28^{Ad}	0.78±0.08	1.03±0.25	0.69 ± 0.22^{Ab}
Donor 3	0.85 ± 0.07^{Ac}	0.58±0.05	0.54±0.15	0.43 ± 0.09^{Aab}
Donor 4	0.56 ± 0.30^{Ab}	0.21±0.03	0.41±0.15	0.30 ± 0.04^{Aa}
Donor 5	0.51 ± 0.20^{Ab}	0.24±0.05	0.39±0.19	0.18 ± 0.02^{Aa}
Donor 6	0.16 ± 0.03^{Aa}	0.32±0.03	0.51±0.14	0.46 ± 0.25^{Aab}
Pooled	0.39 ± 0.17^{Ab}	0.16±0.01	0.46±0.23	1.77 ± 0.01^{Ac}
Control	0.22 ± 0.00^{Aa}	0.22±0.03	0.59±0.32	0.44 ± 0.22^{Aab}

Result was expressed as mean ±SD in mmol/L. Means that do not share a letter are significantly different, lower-case letters can be only compared within each column, and capital letters can be only compared within each row. Pooled fecal slurry was prepared from mixture of fecal sample from all 6 donors. Control was deionized water.

Table 3.6. Acetate changes of individual fecal samples in individual during 24h fermentation of cocoa sample Lavado with individual fecal slurries in the *in vitro* GI tract model system.

	Fermentation Time			
Inoculums	0 Hour	6 Hour	12 Hour	24 Hour
Donor 1	4.56 ± 1.40^{Ab}	4.39±0.35	13.42±1.91	21.24 ± 3.53^{Bd}
Donor 2	5.19 ± 1.33^{Ab}	6.4±0.34	18±1.73	18.99 ± 3.52^{Bcd}
Donor 3	4.11 ± 1.06^{Ab}	5.63±0.28	11.67±0.7	21.3 ± 1.27^{Bd}
Donor 4	1.88 ± 0.80^{Aab}	2.63±0.27	4.05±0.18	9.14 ± 1.63^{Bb}
Donor 5	1.97 ± 0.79^{Aab}	2.73±0.28	5.1±0.12	8.01 ± 0.09^{Bb}
Donor 6	1.57 ± 0.64^{Aab}	5.18±0.84	4.77±1.31	9.3 ± 1.91^{Bb}
Pooled	1.62 ± 0.67^{Aab}	5.87±0.09	8.54±1.06	17.67 ± 0.93^{Bc}
Control	$0.42\pm0.0900.09^{Aa}$	0.88±0.06	0.96±0.23	2.23 ± 0.81^{Aa}

Result was expressed as mean ±SD in mmol/L. Means that do not share a letter are significantly different, lower-case letters can be only compared within each column, and capital letters can be only compared within each row. Pooled fecal slurry was prepared from mixture of fecal sample from all 6 donors. Control was deionized water.

After 24h fermentation of cocoa sample Lavado, acetate concentration increased for all of 6 individual fecal slurries. However, the amount of acetate produced during fermentation varied. The acetate concentration of Donor 1, 2, 3, and the Pooled groups at the end of fermentation were around 20 mmol/L, higher than the rest of donors ($P<0.05$). Acetate concentration of Donor 4, 5, 6 at the end of fermentation were around 10 mmol/L. The acetate concentration in the control showed limited increase and at the end of fermentation the acetate concentration (2.23±0.81 mmol/L) was much lower than the rest of treatment groups ($P<0.05$).

Table 3.7. Propionate changes of individual fecal samples in individual during 24h fermentation of cocoa sample Lavado with individual fecal slurries in the *in vitro* GI tract model system.

	Fermentation Time			
Treatment	0 Hour	6 Hour	12 Hour	24 Hour
Donor 1	2.21 ± 0.68^{Abc}	1.52 ± 0.37	2.57 ± 0.94	1.62 ± 0.21^{Aa}
Donor 2	2.82 ± 0.72^{Ac}	1.52 ± 0.25	2.34 ± 0.89	8.11 ± 0.36^{Bc}
Donor 3	1.04 ± 0.27^{Ab}	1.89 ± 0.69	2.77 ± 0.95	2.61 ± 0.19^{Aa}
Donor 4	1.28 ± 0.80^{Ab}	0.43 ± 0.07	1.13 ± 0.46	1.15 ± 0.7^{Aa}
Donor 5	0.52 ± 0.13^{Aa}	0.57 ± 0.07	0.90 ± 0.52	1.20 ± 0.45^{Aa}
Donor 6	0.34 ± 0.02^{Aa}	0.87 ± 0.51	1.35 ± 0.43	1.14 ± 0.62^{Aa}
Pooled	0.47 ± 0.01^{Aa}	1.08 ± 0.57	1.99 ± 0.58	4.30 ± 0.49^{Bb}
Control	0.37 ± 0.02^{Aa}	0.40 ± 0.09	0.89 ± 0.17	0.79 ± 0.26^{Aa}

Result was expressed as mean ±SD in mmol/L. Means that do not share a letter are significantly different, lower-case letters can be only compared within each column, and capital letters can be only compared within each row. Pooled fecal slurry was prepared from mixture of fecal sample from all 6 donors. Control was deionized water.

Propionate content was measured at 6 h intervals up to 24 h fermentation of the cocoa sample Lavado with individual fecal slurry. At the end of fermentation, only Donor 2 and the Pooled exhibited significant propionate increase ($P<0.05$), while the other 6 treatments showed no significant change for 24 h fermentation. The propionate concentration in the Donor 2 reached 8.11±0.36 mmol/L, which was the highest among all the 7 treatments.

Table 3.8. Macronutrients average daily intake of 6 donors in Volunteer Group 2.

Donor	Calories/kcal	Protein/g	Lipid/g	Carbohydrates/g	Dietary fiber/g	Sugar/g
1	821.5±504.5^{b}	27.6±17.4^{c}	34.5±20.6	91.0±51.7^{bc}	7.9±0.9^{b}	17.9±5.5^{b}
2	1622.0±0.0^{ab}	75.4±0.0^{bc}	34.3±0.0	259.4±0.0^{a}	18.4±0.0^{ab}	30.7±0.0^{b}
3	2052.7±383.2^{a}	41.5±9.1^{bc}	89.0±39.5	274.0±28.7^{a}	21.9±7.3^{a}	59.2±43.1^{ab}
4	1739.7±413.2^{ab}	86.5±20.7^{b}	67.0±15.2	197.3±68.7^{ab}	8.7±4.7^{b}	43.4±2.9^{ab}
5	1664.5±735.8^{ab}	56.0±37.4^{bc}	67.5±30.6	207.0±81.2^{ab}	13.5±8.7^{ab}	102.7±37.6^{a}
6	1102.0±0.0^{ab}	142.5±0.0^{a}	29.7±0.0	57.0±0.0^{c}	18.0±0.0^{ab}	16.9±0.0^{b}

Results are expressed in mean ±SD. Significant difference is only compared among 6 donors within each macronutrient. Means that do not share a letter are significantly different.

Table 3.9. Ratio of macronutrients to dry matter (DM) in diet

Donor	Dry Matter/g	Protein : DM	Lipid : DM	Carbohydrates : DM	Fiber : DM
1	153.1±83.7^{b}	0.17±0.05^{bc}	0.22±0.09	0.61±0.13^{b}	0.06±0.02^{ab}
2	369.2±0.0^{a}	0.20±0.00^{bc}	0.09±0.00	0.70±0.00^{a}	0.05±0.00^{ab}
3	404.6±46.9^{a}	0.10±0.01^{c}	0.22±0.08	0.68±0.08^{a}	0.06±0.03^{ab}
4	350.7±94.7^{ab}	0.25±0.02^{b}	0.20±0.05	0.55±0.05^{a}	0.03±0.01^{b}
5	330.5±143.7^{ab}	0.16±0.08^{bc}	0.21±0.03	0.64±0.05^{a}	0.04±0.01^{ab}
6	229.2±0.0^{ab}	0.62±0.00^{a}	0.13±0.00	0.25±0.00^{a}	0.08±0.00^{a}

Results are expressed in mean ±SD. Significant difference is only compared among 6 donors within each column. Means that do not share a letter are significantly different.

The detailed daily food intake records of each donor in Volunteer Group 2 was listed in Appendix. According to the record of donors, Donor 2 and Donor 6 kept their diet the same every day when they participated in this experiment, thus there was no fluctuation of macronutrients of these 2 donors. Majority of average Macronutrients daily intake data showed significant difference among 6 donors, except average lipid

daily intake. Protein to dry matter ratio of Donor 6 was higher than other 5 donors ($P<0.05$), while carbohydrates to dry matter ratio of Donor 6 was lower than other donors ($P<0.05$). No significant difference of lipid to dry matter ratio was observed among all 6 donors ($P>0.05$).

3.3 Discussions

3.3.1 pH and SCFAs changes during 24h in Individual Fermentation.

The cocoa sample Lavado was fermented by 6 individual donor fecal slurries and 1 pooled fecal slurry. Six out of 7 treatments exhibited significant pH decrease compared to the control at the end of fermentation, and the SCFAs concentration increased in all of 7 treatments (Donor 1 to 6, and the control) for 24 h fermentation. The pH change and the SCFAs results implied that human gut microorganism from various donors metabolized carbohydrates in cocoa sample Lavado.

The results of Individual Fermentation showed similar tendency, such as decrease of pH, increase of polyphenolic compounds, and production of SCFAs, in most of treatments. However, each treatment showed unique variation patterns of pH, polyphenolic content, and concentration of SCFAs. Firstly, the pH and SCFAs results of the cocoa sample Lavado fermented with pooled fecal slurry in Individual Fermentation showed similar inclination of change as the that of results of the same cocoa sample fermented with pooled fecal sample slurry in Pooled Fermentation. This similarity reflected that the experimental operations in this study, included substrate preparation, digestion, fermentation, sampling, and measurements, were stable and consistent. Secondly, the pH decreases and the SCFAs increase was observed in the most of treatment, this suggested that despite there was huge variation of the composition of human gut microbiota among different fecal sample from various

donors (Ursell et al. 2012), the SCFAs-producing microorganisms that are known to existed in human gut (De Filippo et al. 2010), these microorganisms can utilize the fibers in cocoa samples and produce the SCFAs. Thirdly, the initial pH and pH change pattern of each of treatment exhibited some differences, and the SCFAs content change of each treatment were varied, these variations may reflect the composition of the human gut microbiota.

In this study, the pH decreases were observed both in Pooled fermentation and Individual fermentation, and the results suggested that fermentation of cocoa powder which made through different manufacture process may alter human large intestine to a neutral or acidic environment. The gut pH environmental change was induced by the metabolism of human gut microbiota in various individuals (Conlon and Bird 2014). SCFAs can modify pH environment in human gut, human gut pH environment can affect the composition of the human gut microbiota in return, and finally change the SCFAs production (den Besten, van Eunen, et al. 2013a). A low pH environment in human gut can prevent growth of certain pathogenic bacteria, like Clostridium difficile (Gupta et al. 2016), and promote bacteria species with health benefits, such as Lactobacillus spp. and Bifidobacterium spp. (Holzapfel et al. 1998). In this study, the pH changes and SCFAs production indicated that carbohydrates in cocoa powder can be utilized by human gut microorganisms as prebiotics.

Again, butyrate increase was only observed in the Pooled fecal slurry, which also showed large pH reduction and significant acetate increase. Previous study reported that some butyrate-producing bacteria preferred lower pH environment and acetate was needed for their proliferation (Duncan et al. 2004), thus the Pooled fecal slurry may provide an environment for growth of this bacteria. However, although the Donor 1 also showed significant pH decrease and acetate increase, it did not show

significant butyrate content increase during fermentation, butyrate was not produced in Donor 1 probably due to insufficient proliferation of butyrate-producing bacteria, or absence of those bacteria in Donor 1. The difference between fecal sample Donor 1 and Pooled revealed diversity of human gut microorganism between different individual. Microbiota in fecal sample Donor 1 may be less diverse. Individual variations were also found in previous SCFA study. McOrist et al. (2011) measured fecal SCFAs of 46 healthy adult volunteers before they received resistant starch fortified diet, they reported that fecal butyrate levels varied widely among individual, butyrate concentrations ranged 3.5 to 32.6 mmol/kg body weight, butyrate excretions ranged from 0.3 to 18.2mmol/48 h.

In summary, result of pH and SCFA change in Individual Fermentation further proved that carbohydrates in cocoa powder can be utilized by human gut microorganisms as prebiotics, and microorganism produced SCFA that lead to pH decrease. Pattern and extent of pH and SCFA change was largely affected by human gut microbiota.

3.3.2 Polyphenolic compounds change during 24h in Individual Fermentation.

Polyphenols increase was observed in Donor 1, 2, 3, 5 and Pooled during the 24h fermentation. There was no significant difference among the initial GAE content in each treatment group, neither initial CE content did. This result showed stability of experimental operation. Despite the possible variations of the composition of human gut microbiota of each donor (Lozupone et al. 2012), and the variation of polyphenol increase pattern, polyphenol increase indicated that the polyphenol metabolism reaction existed in human large intestinal fermentation of Donor 1, 2, 3, and 5 used in the study. It was in line with previous studies which reported that polyphenols from various sources was metabolized by microorganism. Gross et al. (1996) reported that

quercetin derivatives was found in human urine; Meng et al. (2002) detected (-)-Epigallocatechin gallate from green tea in human urine. Aura et al. (2008) reported that metabolites from catechin was detected human gut microbiota in vitro model system. However, well understanding of microorganism that played roles in the polyphenol metabolism is still not established yet. Further microorganism identification by quantitative PCR is needed to confirm whether the bacteria that hydrolyze polyphenol polymers into monomers was ubiquitous in human gut microbiota of various individuals.

The change of gallic acid and catechins in each fermentation showed distinctive pattern. This reflected variation of the composition of human gut microbiota. The GAE and CE content in the Donor 6 did not significantly change during 24 h fermentation, and we also noticed that the pH of Donor 6 did not significantly change, and the final acetate content was lower than others. It implied that the composition of human gut microbiota of Donor 6 differs with that of other donors, or it was possible that activity of certain kind of bacteria was suppressed under current experimental environment. The GAE and CE content within each treatment did not correspondingly change, the GAE content in the Donor 4 group did not change, but the CE content significantly increased after 24h fermentation. This may also due to variations among individual human microbiota.

Diversity of diet and ratio of macronutrients was a possible explanation of the difference of polyphenol content change among donors. Protein to dry matter ratio of Donor 1 to Donor 5 were lower than 0.3:1, while this ratio of donor 6 was 0.62:1 (Table 3.9). Carbohydrate to dry matter ratio of donor 1 to donor 5 were above 0.5:1, while the ratio of donor 6 was only 0.25:1. In contrast to normal diet of donor 1 to donor 5, the diet of donor 6 was a low-carbohydrate high-protein diet. Beside of

different macronutrients ratio, the diet of donor 6 was less diverse than diet of other 5 donors, it mainly comprised of fine protein powder and supplements, and this diet was strictly kept on a daily basis. Previous studies reported that ratio, rather than amount, of macronutrients can impact composition of human gut microbiota. Kim, Kim, and Park (2016) employed animal study and used mouse as model system, they fed mice either a normal protein/carbohydrate diet (ND) or a high protein/low-carbohydrate diet (HPLCD) for 2 weeks. Microbiota in mice fed with HPLCD was less diverse than mice fed with ND, and composition of microbiota in HPLCD mice was also altered. HPLCD finally led to a deleterious luminal environment and may be healthy harmful to people who consume this kind of diet. In this study, a low-carbohydrate high-protein diet may bring relative low diverse microbiota to donor 6, and fermentation of cocoa sample Lavado with low diverse microbiota induced insignificant change of pH, polyphenol, and SCFAs.

This study suggested that the polyphenol metabolism was related to SCFAs production, and this relationship was possibly due to metabolism of certain microorganism species. However, results of previous studies have been controversial. Parkar, Trower, and Stevenson (2013) reported that polyphenols, including rutin, quercetin, chlorogenic acid, and caffeic acid, can stimulate proliferation of certain bacteria, like bifidobacteria, and can also stimulate bacteria metabolism, made bacteria produce more SCFAs. Duda-Chodak (2012) reported that rutin and catechin had no impact on bacteria metabolism, and some metabolites from those polyphenols can even inhibit growth of bacteria. It was noticeable that both two studies were in vitro experiment, Parkar et al. employed fecal slurry in model system, while Duda-Chodak used culture that was mixed of 6 species bacteria in their experiment. This may partially explain the difference between their results. Both of them used pure

polyphenolic compound but not natural dietary fiber, thus their result may be different from fermentation of food mass in human gut. In general, diversity of human gut microbiota was an important factor that influenced many aspects of human gut fermentation.

In summary, content of polyphenolic compounds increased during cocoa sample Lavado fermented with various fecal slurries. Diversity and composition of human gut microbiota affected fermentation pattern and composition of final polyphenolic compounds.

CHAPTER 4. CONCLUSION AND FUTURE STUDIES

In this study, five different treated cocoa samples were subjected to ICP/MS analysis, a human gastrointestinal digestion and fermentation model system was established, five cocoa samples were subjected to digestion, then fermented with pooled fecal slurry in the *in vitro* model system for 24h. The mineral elements, pH, short chain fatty acids, procyanidin distribution, and polyphenolic compounds were measured. Based on mineral elements results, D-11-S and D-11-B were alkalized cocoa powder; while natural cocoa, Lavado, and shell were non-alkalized cocoa sample. Over 24h fermentation, the pH in all of 5 cocoa samples significantly decreased, and SCFAs increased at the same time. The polyphenolic compounds also increased during cocoa fermentation. The pH and SCFAs results indicated that cocoa samples can be fermented by human gut microbiota. Procyanidin distribution result indicated that alkalization reduced polyphenol content of cocoa powder during manufacture. The polyphenolic compounds results showed that polyphenol content of cocoa powder increased during fermentation regardless alkalization treatment of cocoa powder.

The cocoa sample Lavado was fermented with 6 individual fecal slurries in the *in vitro* GI tract model system. The pH decrease, polyphenol increase, and SCFAs increase were observed in majority of individual fecal fermentation groups, the results indicated that cocoa sample Lavado can be metabolized by human gut microbiota with various composition, and then SCFAs were produced, which lead to pH decrease. Catechins and gallic acid were also produced. The change patterns of pH, SCFAs, and polyphenols in each treatment group were distinctive, the results reflected diversity of human gut microorganism among individual donors. Diversity and

composition of human gut microbiota affected cocoa sample fermentation pattern and composition of final polyphenolic compounds.

Well understanding of microorganism that played roles in the polyphenol metabolism is still not fully established, and the microorganism species that involved in polyphenol metabolism and SCFAs production are not clear, the form of polyphenols exist in carbohydrates complexes is unknown. Future exploration could be conducted as fellow. Microbiota composition varied not only upon fermentation duration but also at site of large intestine, it is worth to set up a real time monitored human gut microbiota *in vitro* system which can continuously perform sampling and analyze data about concentration of various substrates and products during fermentation. Identification of human gut microorganism on large scale and comparison human gut microbiota composition before and after fermentation with cocoa samples by big data technology will help building up a solid and comprehensive understanding on interaction between cocoa powder and human microbiota, and also find out key genus and species of microorganisms which play role in polyphenol metabolism. An *in vitro* fermentation of cocoa with pure culture of identified species can figure out mechanism of polyphenolic compound metabolism in human gastrointestinal tract. The form of polyphenols exist in carbohydrates complexes could be investigated by combination of MALDI and NMR.

REFERENCES

Adolphe, Jennifer L, Susan J Whiting, Bernhard HJ Juurlink, Lilian U Thorpe, and Jane Alcorn. 2010. 'Health effects with consumption of the flax lignan secoisolariciresinol diglucoside', British Journal of Nutrition, 103: 929-38.

Afoakwa, Emmanuel Ohene, Jennifer Quao, Jemmy Takrama, Agnes Simpson Budu, and Firibu Kwesi Saalia. 2013. 'Chemical composition and physical quality characteristics of Ghanaian cocoa beans as affected by pulp pre-conditioning and fermentation', Journal of food science and technology, 50: 1097-105.

Albertini, Barbara, Aurélie Schoubben, Davide Guarnaccia, Filippo Pinelli, Mirco Della Vecchia, Maurizio Ricci, Gian Carlo Di Renzo, and Paolo Blasi. 2015. 'Effect of fermentation and drying on cocoa polyphenols', Journal of agricultural and food chemistry, 63: 9948-53.

Andújar, Isabel, Ma Carmen Recio, Rosa Ma Giner, Elena Cienfuegos-Jovellanos, Sara Laghi, Begoña Muguerza, and José Luis Ríos. 2011. 'Inhibition of ulcerative colitis in mice after oral administration of a polyphenol-enriched cocoa extract is mediated by the inhibition of STAT1 and STAT3 phosphorylation in colon cells', Journal of agricultural and food chemistry, 59: 6474-83.

Appeldoorn, Maaike M, Jean-Paul Vincken, Anna-Marja Aura, Peter CH Hollman, and Harry Gruppen. 2009. 'Procyanidin dimers are metabolized by human microbiota with 2-(3, 4-dihydroxyphenyl) acetic acid and 5-(3, 4-dihydroxyphenyl)-γ-valerolactone as the major metabolites', Journal of agricultural and food chemistry, 57: 1084-92.

Aura, A-M, KA O'leary, G Williamson, M Ojala, M Bailey, R Puupponen-Pimiä, AM Nuutila, K-M Oksman-Caldentey, and K Poutanen. 2002. 'Quercetin derivatives are deconjugated and converted to hydroxyphenylacetic acids but not methylated by human fecal flora in vitro', Journal of agricultural and food chemistry, 50: 1725-30.

Aura, Anna-Marja. 2005. In vitro digestion models for dietary phenolic compounds (VTT Technical Research Centre of Finland).

Aura, Anna-Marja, Ismo Mattila, Tuulikki Seppänen-Laakso, Jarkko Miettinen, Kirsi-Marja Oksman-Caldentey, and Matej Orešič. 2008. 'Microbial metabolism of catechin stereoisomers by human faecal microbiota: comparison of targeted analysis and a non-targeted metabolomics method', Phytochemistry Letters, 1: 18-22.

Bateman, RH, R Carruthers, JB Hoyes, C Jones, JI Langridge, A Millar, and JPC Vissers. 2002. 'A novel precursor ion discovery method on a hybrid quadrupole orthogonal acceleration time-of-flight (Q-TOF) mass spectrometer for studying protein phosphorylation', Journal of the American Society for Mass Spectrometry, 13: 792-803.

Bergman, EN. 1990. 'Energy contributions of volatile fatty acids from the gastrointestinal tract in various species', Physiological reviews, 70: 567-90.

Bhattacharya, Amita, Priyanka Sood, and Vitaly Citovsky. 2010. 'The roles of plant phenolics in defence and communication during Agrobacterium and Rhizobium infection', Molecular plant pathology, 11: 705-19.

Biedermann, Luc, and Gerhard Rogler. 2015. 'The intestinal microbiota: its role in health and disease', European journal of pediatrics, 174: 151-67.

Bordiga, Matteo, Monica Locatelli, Fabiano Travaglia, Jean Daniel Coïsson, Giuseppe Mazza, and Marco Arlorio. 2015. 'Evaluation of the effect of processing on cocoa polyphenols: antiradical activity, anthocyanins and procyanidins profiling from raw beans to chocolate', International journal of food science & technology, 50: 840-48.

Buijsse, Brian, Edith JM Feskens, Frans J Kok, and Daan Kromhout. 2006. 'Cocoa intake, blood pressure, and cardiovascular mortality: the Zutphen Elderly Study', Archives of internal medicine, 166: 411-17.

Burns, Jennifer, Takao Yokota, Hiroshi Ashihara, Michael EJ Lean, and Alan Crozier. 2002. 'Plant foods and herbal sources of resveratrol', Journal of agricultural and food chemistry, 50: 3337-40.

Campbell, Joy M, George C Fahey, and Bryan W Wolf. 1997. 'Selected indigestible oligosaccharides affect large bowel mass, cecal and fecal short-chain fatty acids, pH and microflora in rats', The Journal of nutrition, 127: 130-36.

Chen, Wen-Ju Lin, James W Anderson, and Darrell Jennings. 1984. 'Propionate may mediate the hypocholesterolemic effects of certain soluble plant fibers in cholesterol-fed rats', Proceedings of the society for experimental biology and medicine, 175: 215-18.

Cienfuegos-Jovellanos, Elena, María del Mar Quiñones, Begoña Muguerza, Leila Moulay, Marta Miguel, and Amaya Aleixandre. 2009. 'Antihypertensive effect of a polyphenol-rich cocoa powder industrially processed to preserve the original flavonoids of the cocoa beans', Journal of agricultural and food chemistry, 57: 6156-62.

Clausen, MR, H Bonnen, and PB Mortensen. 1991. 'Colonic fermentation of dietary fibre to short chain fatty acids in patients with adenomatous polyps and colonic cancer', Gut, 32: 923-28.

Clemente, Jose C, Luke K Ursell, Laura Wegener Parfrey, and Rob Knight. 2012. 'The impact of the gut microbiota on human health: an integrative view', Cell, 148: 1258-70.

Conlon, Michael A, and Anthony R Bird. 2014. 'The impact of diet and lifestyle on gut microbiota and human health', Nutrients, 7: 17-44.

Cooper, Karen A, Esther Campos-Giménez, Diego Jiménez Alvarez, Kornél Nagy, Jennifer L Donovan, and Gary Williamson. 2007. 'Rapid reversed phase ultra-performance liquid chromatography analysis of the major cocoa polyphenols and inter-relationships of their concentrations in chocolate', Journal of agricultural and food chemistry, 55: 2841-47.

Crozier, Alan, Hiroshi Ashihara, and Francisco TomÃ. 2011. Teas, cocoa and coffee: plant secondary metabolites and health (John Wiley & Sons).

Cueva, Carolina, Fernando Sánchez-Patán, María Monagas, Gemma E Walton, Glenn R Gibson, Pedro J Martín-Álvarez, Begoña Bartolomé, and M Victoria Moreno-Arribas. 2013. 'In vitro fermentation of grape seed flavan-3-ol fractions by human faecal microbiota: changes in microbial groups and phenolic metabolites', FEMS microbiology ecology, 83: 792-805.

Czank, Charles, Aedín Cassidy, Qingzhi Zhang, Douglas J Morrison, Tom Preston, Paul A Kroon, Nigel P Botting, and Colin D Kay. 2013. 'Human metabolism and elimination of the anthocyanin, cyanidin-3-glucoside: a 13C-tracer study', The American journal of clinical nutrition, 97: 995-1003.

D'Archivio, Massimo, Carmelina Filesi, Rosaria Varì, Beatrice Scazzocchio, and Roberta Masella. 2010. 'Bioavailability of the polyphenols: status and controversies', International journal of molecular sciences, 11: 1321-42.

de Araujo, QR, VC Baligar, GAH de A Loureiro, JO de Souza Júnior, and NB Comerford. 2017. 'Impact of soils and cropping systems on mineral composition of dry cacao beans', Journal of soil science and plant nutrition, 17: 410-28.

De Filippo, Carlotta, Duccio Cavalieri, Monica Di Paola, Matteo Ramazzotti, Jean Baptiste Poullet, Sebastien Massart, Silvia Collini, Giuseppe Pieraccini, and Paolo Lionetti. 2010. 'Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa', Proceedings of the National Academy of Sciences, 107: 14691-96.

Demigné, Christian, Christine Morand, Marie-Anne Levrat, Catherine Besson, Corinne Moundras, and Christian Rémésy. 1995. 'Effect of propionate on fatty acid and cholesterol synthesis and on acetate metabolism in isolated rat hepatocytes', British Journal of Nutrition, 74: 209-19.

den Besten, Gijs, Katja Lange, Rick Havinga, Theo H van Dijk, Albert Gerding, Karen van Eunen, Michael Müller, Albert K Groen, Guido J Hooiveld, and Barbara M Bakker. 2013. 'Gut-derived short-chain fatty acids are vividly assimilated into host carbohydrates and lipids', American Journal of Physiology-Gastrointestinal and Liver Physiology, 305: G900-G10.

den Besten, Gijs, Karen van Eunen, Albert K Groen, Koen Venema, Dirk-Jan Reijngoud, and Barbara M Bakker. 2013. 'The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism', Journal of lipid research, 54: 2325-40.

Deprez, Stephanie, Isabelle Mila, Jean-François Huneau, Daniel Tome, and Augustin Scalbert. 2001. 'Transport of proanthocyanidin dimer, trimer, and polymer across monolayers of human intestinal epithelial Caco-2 cells', Antioxidants and redox signaling, 3: 957-67.

Dilis, Vardis, Effie Vasilopoulou, and Antonia Trichopoulou. 2007. 'The flavone, flavonol and flavan-3-ol content of the Greek traditional diet', Food chemistry, 105: 812-21.

Ding, Eric L, Susan M Hutfless, Xin Ding, and Saket Girotra. 2006. 'Chocolate and prevention of cardiovascular disease: a systematic review', Nutrition & metabolism, 3: 2.

Djoussé, Luc, Paul N Hopkins, Kari E North, James S Pankow, Donna K Arnett, and R Curtis Ellison. 2011. 'Chocolate consumption is inversely associated with prevalent coronary heart disease: The National Heart, Lung, and Blood Institute Family Heart Study', Clinical nutrition, 30: 182-87.

Duda-Chodak, A. 2012. 'The inhibitory effect of polyphenols on human gut microbiota', J. Physiol. Pharmacol, 63: 497-503.

Duda-Chodak, Aleksandra, Tomasz Tarko, Paweł Satora, and Paweł Sroka. 2015. 'Interaction of dietary compounds, especially polyphenols, with the intestinal microbiota: a review', European journal of nutrition, 54: 325-41.

Duncan, Sylvia H, Adela Barcenilla, Colin S Stewart, Susan E Pryde, and Harry J Flint. 2002. 'Acetate utilization and butyryl coenzyme A (CoA): acetate-CoA transferase in butyrate-producing bacteria from the human large intestine', Applied and environmental microbiology, 68: 5186-90.

Duncan, Sylvia H, Grietje Holtrop, Gerald E Lobley, A Graham Calder, Colin S Stewart, and Harry J Flint. 2004. 'Contribution of acetate to butyrate formation by human faecal bacteria', British Journal of Nutrition, 91: 915-23.

Duncan, Sylvia H, Petra Louis, John M Thomson, and Harry J Flint. 2009. 'The role of pH in determining the species composition of the human colonic microbiota', Environmental microbiology, 11: 2112-22.

Eckburg, Paul B, Elisabeth M Bik, Charles N Bernstein, Elizabeth Purdom, Les Dethlefsen, Michael Sargent, Steven R Gill, Karen E Nelson, and David A Relman. 2005. 'Diversity of the human intestinal microbial flora', science, 308: 1635-38.

El Kaoutari, Abdessamad, Fabrice Armougom, Jeffrey I Gordon, Didier Raoult, and Bernard Henrissat. 2013. 'The abundance and variety of carbohydrate-active enzymes in the human gut microbiota', Nature reviews Microbiology, 11: 497-504.

Elleuch, Mohamed, Dorothea Bedigian, Olivier Roiseux, Souhail Besbes, Christophe Blecker, and Hamadi Attia. 2011. 'Dietary fibre and fibre-rich by-products of

food processing: Characterisation, technological functionality and commercial applications: A review', Food chemistry, 124: 411-21.

Etxeberria, Usune, Alfredo Fernández-Quintela, Fermín I Milagro, Leixuri Aguirre, J Alfredo Martínez, and María P Portillo. 2013. 'Impact of polyphenols and polyphenol-rich dietary sources on gut microbiota composition', Journal of agricultural and food chemistry, 61: 9517-33.

FDA. 2014. 'CFR-code of federal regulations title 21', Current good manufacturing practice for finished pharmaceuticals Part, 211.

Flint, Harry J. 2012. 'The impact of nutrition on the human microbiome', Nutrition reviews, 70.

Fu, Hong, Ying Qiang Shi, and Shan Jin Mo. 2004. 'Effect of short‐chain fatty acids on the proliferation and differentiation of the human colonic adenocarcinoma cell line Caco‐2', Journal of Digestive Diseases, 5: 115-17.

Garti, Nissim, and Neil R Widlak. 2015. Cocoa butter and related compounds (Elsevier).

Gibson, Glenn R. 2009. Food Science and Technology Bulletin: Functional Foods (IFIS Publishing).

Graf, Daniela, Raffaella Di Cagno, Frida Fåk, Harry J Flint, Margareta Nyman, Maria Saarela, and Bernhard Watzl. 2015. 'Contribution of diet to the composition of the human gut microbiota', Microbial ecology in health and disease, 26: 26164.

Grassi, Davide, Giovambattista Desideri, Stefano Necozione, Cristina Lippi, Raffaele Casale, Giuliana Properzi, Jeffrey B Blumberg, and Claudio Ferri. 2008. 'Blood pressure is reduced and insulin sensitivity increased in glucose-intolerant, hypertensive subjects after 15 days of consuming high-polyphenol dark chocolate', The Journal of nutrition, 138: 1671-76.

Grigsby, KN, MS Kerley, JA Paterson, and JC Weigel. 1992. 'Site and extent of nutrient digestion by steers fed a low-quality bromegrass hay diet with incremental levels of soybean hull substitution', Journal of animal science, 70: 1941-49.

Gross, Myron, Michelle Pfeiffer, Margaret Martini, Deborah Campbell, Joanne Slavin, and John Potter. 1996. 'The quantitation of metabolites of quercetin flavonols in human urine', Cancer Epidemiology and Prevention Biomarkers, 5: 711-20.

Gu, Liwei, Suzanne E House, Xianli Wu, Boxin Ou, and Ronald L Prior. 2006. 'Procyanidin and catechin contents and antioxidant capacity of cocoa and chocolate products', Journal of agricultural and food chemistry, 54: 4057-61.

Gültekin-Özgüven, Mine, Ijlal Berktaş, and Beraat Özçelik. 2016. 'Change in stability of procyanidins, antioxidant capacity and in-vitro bioaccessibility during processing of cocoa powder from cocoa beans', LWT-Food Science and Technology, 72: 559-65.

Gupta, Purba, Stanley Yakubov, Kevin Tin, Diego Zea, Olga Garankina, Monica Ghitan, Edward K Chapnick, Peter Homel, Yu Shia Lin, and Michael M Koegel. 2016. 'Does Alkaline Colonic pH Predispose to Clostridium difficile Infection?', Southern medical journal, 109: 91-96.

Hammerstone, John F, Sheryl A Lazarus, Alyson E Mitchell, Robert Rucker, and Harold H Schmitz. 1999. 'Identification of procyanidins in cocoa (Theobroma cacao) and chocolate using high-performance liquid chromatography/mass spectrometry', Journal of agricultural and food chemistry, 47: 490-96.

Haslam, Edwin, and Y Cai. 1994. 'Plant polyphenols (vegetable tannins): gallic acid metabolism', Natural product reports, 11: 41-66.

Heim, Kelly E, Anthony R Tagliaferro, and Dennis J Bobilya. 2002. 'Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships', The Journal of nutritional biochemistry, 13: 572-84.

Hein, Eva-Maria, Katrin Rose, Gordon van't Slot, Alexander W Friedrich, and Hans-Ulrich Humpf. 2008. 'Deconjugation and degradation of flavonol glycosides by pig cecal microbiota characterized by fluorescence in situ hybridization (FISH)', Journal of agricultural and food chemistry, 56: 2281-90.

Holt, Roberta R, Sheryl A Lazarus, M Cameron Sullards, Qin Yan Zhu, Derek D Schramm, John F Hammerstone, Cesar G Fraga, Harold H Schmitz, and Carl L Keen. 2002. 'Procyanidin dimer B2 [epicatechin-(4β-8)-epicatechin] in human plasma after the consumption of a flavanol-rich cocoa', The American journal of clinical nutrition, 76: 798-804.

Holzapfel, Wilhelm H, Petra Haberer, Johannes Snel, Ulrich Schillinger, and Jos HJ Huis in't Veld. 1998. 'Overview of gut flora and probiotics', International journal of food microbiology, 41: 85-101.

Hosseini, Elham, Charlotte Grootaert, Willy Verstraete, and Tom Van de Wiele. 2011. 'Propionate as a health-promoting microbial metabolite in the human gut', Nutrition reviews, 69: 245-58.

Hur, Hor-Gil, Richard D Beger, Thomas M Heinze, Jackson O Lay, James P Freeman, Joel Dore, and Fatemeh Rafii. 2002. 'Isolation of an anaerobic intestinal bacterium capable of cleaving the C-ring of the isoflavonoid daidzein', Archives of Microbiology, 178: 8-12.

ICCO. 2016. "Quarterly Bulletin of Cocoa Statistics, Vol. XLII, No. 3, Cocoa year 2015/16." In.: International Cocoa Organization London, UK.

Ignat, Ioana, Irina Volf, and Valentin I Popa. 2011. 'A critical review of methods for characterisation of polyphenolic compounds in fruits and vegetables', Food chemistry, 126: 1821-35.

Jalil, Abbe Maleyki Mhd, and Amin Ismail. 2008. 'Polyphenols in cocoa and cocoa products: is there a link between antioxidant properties and health?', Molecules, 13: 2190-219.

Kim, Eunjung, Dan-Bi Kim, and Jae-Yong Park. 2016. 'Changes of mouse gut microbiota diversity and composition by modulating dietary protein and carbohydrate contents: a pilot study', Preventive nutrition and food science, 21: 57.

Kim, Ok-Sun, Yong-Joon Cho, Kihyun Lee, Seok-Hwan Yoon, Mincheol Kim, Hyunsoo Na, Sang-Cheol Park, Yoon Seong Jeon, Jae-Hak Lee, and Hana Yi. 2012. 'Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species', International journal of systematic and evolutionary microbiology, 62: 716-21.

Knight, Ian. 1999. "Chocolate and cocoa: health and nutrition." In.: Blackwell Science.

Kotani, Akira, Yuji Miyaguchi, Mototaka Kohama, Takafumi Ohtsuka, Taisei Shiratori, and Fumiyo Kusu. 2009. 'Determination of short-chain fatty acids in rat and human feces by high-performance liquid chromatography with electrochemical detection', Analytical Sciences, 25: 1007-11.

Kumar, Shashank, and Abhay K Pandey. 2013. 'Chemistry and biological activities of flavonoids: an overview', The Scientific World Journal, 2013.

Laparra, José Moisés, and Yolanda Sanz. 2010. 'Interactions of gut microbiota with functional food components and nutraceuticals', Pharmacological Research, 61: 219-25.

Lattimer, James M, and Mark D Haub. 2010. 'Effects of dietary fiber and its components on metabolic health', Nutrients, 2: 1266-89.

Layden, Brian T, Sudha K Yalamanchi, Thomas MS Wolever, Andrea Dunaif, and William L Lowe Jr. 2012. 'Negative association of acetate with visceral adipose tissue and insulin levels', Diabetes, metabolic syndrome and obesity: targets and therapy, 5: 49.

Lecumberri, Elena, Luis Goya, Raquel Mateos, Mario Alía, Sonia Ramos, María Izquierdo-Pulido, and Laura Bravo. 2007. 'A diet rich in dietary fiber from cocoa improves lipid profile and reduces malondialdehyde in hypercholesterolemic rats', Nutrition, 23: 332-41.

Lecumberri, Elena, Raquel Mateos, María Izquierdo-Pulido, Pilar Rupérez, Luis Goya, and Laura Bravo. 2007. 'Dietary fibre composition, antioxidant capacity and physico-chemical properties of a fibre-rich product from cocoa (Theobroma cacao L.)', Food chemistry, 104: 948-54.

Ley, Ruth E, Peter J Turnbaugh, Samuel Klein, and Jeffrey I Gordon. 2006. 'Microbial ecology: human gut microbes associated with obesity', Nature, 444: 1022-23.

Li, Yue, Song Zhu, Yun Feng, Feifei Xu, Jianguo Ma, and Fang Zhong. 2014. 'Influence of alkalization treatment on the color quality and the total phenolic and anthocyanin contents in cocoa powder', Food Science and Biotechnology, 23: 59-63.

Liu, Yulan. 2015. 'Fatty acids, inflammation and intestinal health in pigs', Journal of animal science and biotechnology, 6: 41.

Louis, Petra, and Harry J Flint. 2009. 'Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine', FEMS microbiology letters, 294: 1-8.

Louis, Petra, Georgina L Hold, and Harry J Flint. 2014. 'The gut microbiota, bacterial metabolites and colorectal cancer', Nature reviews Microbiology, 12: 661-72.

Lozupone, Catherine A, Jesse I Stombaugh, Jeffrey I Gordon, Janet K Jansson, and Rob Knight. 2012. 'Diversity, stability and resilience of the human gut microbiota', Nature, 489: 220-30.

Maekivuokko, Harri, Hannele Kettunen, Markku Saarinen, Tatsuya Kamiwaki, Yasuhiro Yokoyama, Julian Stowell, and Nina Rautonen. 2007. 'The effect of cocoa and polydextrose on bacterial fermentation in gastrointestinal tract simulations', Bioscience, biotechnology, and biochemistry, 71: 1834-43.

Manach, Claudine, Augustin Scalbert, Christine Morand, Christian Rémésy, and Liliana Jiménez. 2004. 'Polyphenols: food sources and bioavailability', The American journal of clinical nutrition, 79: 727-47.

Mardis, Elaine R. 2011. 'A decade/'s perspective on DNA sequencing technology', Nature, 470: 198-203.

Mazor Jolić, Slavica, Ivana Radojčić Redovniković, Ksenija Marković, Đurđica Ivanec Šipušić, and Karmela Delonga. 2011. 'Changes of phenolic compounds and antioxidant capacity in cocoa beans processing', International journal of food science & technology, 46: 1793-800.

McOrist, Alexandra L, Rosalind B Miller, Anthony R Bird, Jennifer B Keogh, Manny Noakes, David L Topping, and Michael A Conlon. 2011. 'Fecal butyrate levels vary widely among individuals but are usually increased by a diet high in resistant starch', The Journal of nutrition, 141: 883-89.

Mellor, DD, T Sathyapalan, ES Kilpatrick, S Beckett, and SL Atkin. 2010. 'High‐cocoa polyphenol‐rich chocolate improves HDL cholesterol in Type 2 diabetes patients', Diabetic Medicine, 27: 1318-21.

Meng, Xiaofeng, Shengmin Sang, Nanqun Zhu, Hong Lu, Shuqun Sheng, Mao-Jung Lee, Chi-Tang Ho, and Chung S Yang. 2002. 'Identification and characterization of methylated and ring-fission metabolites of tea catechins formed in humans, mice, and rats', Chemical research in toxicology, 15: 1042-50.

Miller, Kenneth B, William Jeffery Hurst, Mark J Payne, David A Stuart, Joan Apgar, Daniel S Sweigart, and Boxin Ou. 2008. 'Impact of alkalization on the antioxidant and flavanol content of commercial cocoa powders', Journal of agricultural and food chemistry, 56: 8527-33.

Minifie, Bernard. 2012. Chocolate, cocoa and confectionery: science and technology (Springer Science & Business Media).

Natsume, Midori, Naomi Osakabe, Megumi Yamagishi, Toshio Takizawa, Tetsuo Nakamura, Haruka Miyatake, Tsutomu Hatano, and Takashi Yoshida. 2000. 'Analyses of polyphenols in cacao liquor, cocoa, and chocolate by normal-phase and reversed-phase HPLC', Bioscience, biotechnology, and biochemistry, 64: 2581-87.

Oku, T, and S Nakamura. 2003. 'Comparison of digestibility and breath hydrogen gas excretion of fructo-oligosaccharide, galactosyl-sucrose, and isomalto-oligosaccharide in healthy human subjects', European journal of clinical nutrition, 57: 1150-56.

Ortega, Nàdia, Maria-Paz Romero, Alba Macià, Jordi Reguant, Neus Anglès, José-Ramón Morelló, and Maria-Jose Motilva. 2008. 'Obtention and characterization of phenolic extracts from different cocoa sources', Journal of agricultural and food chemistry, 56: 9621-27.

Parkar, Shanthi G, Tania M Trower, and David E Stevenson. 2013. 'Fecal microbial metabolism of polyphenols and its effects on human gut microbiota', Anaerobe, 23: 12-19.

Pérez-Jiménez, J, Vos Neveu, F Vos, and A Scalbert. 2010. 'Identification of the 100 richest dietary sources of polyphenols: an application of the Phenol-Explorer database', European journal of clinical nutrition, 64: S112-S20.

Piskula, Mariusz K, and Junji Terao. 1998. 'Accumulation of (−)-epicatechin metabolites in rat plasma after oral administration and distribution of conjugation enzymes in rat tissues', The Journal of nutrition, 128: 1172-78.

Psichas, A, ML Sleeth, KG Murphy, L Brooks, GA Bewick, AC Hanyaloglu, MA Ghatei, SR Bloom, and G Frost. 2015. 'The short chain fatty acid propionate stimulates GLP-1 and PYY secretion via free fatty acid receptor 2 in rodents', International journal of obesity, 39: 424-29.

Ragsdale, Stephen W, and Elizabeth Pierce. 2008. 'Acetogenesis and the Wood–Ljungdahl pathway of CO 2 fixation', Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics, 1784: 1873-98.

Ramiro, Emma, Àngels Franch, Cristina Castellote, Francisco Pérez-Cano, Joan Permanyer, Maria Izquierdo-Pulido, and Margarida Castell. 2005. 'Flavonoids from Theobroma cacao down-regulate inflammatory mediators', Journal of agricultural and food chemistry, 53: 8506-11.

Ramli, Nazaruddin, Osman Hassan, Mamot Said, Wahid Samsudin, and Nor Aini Idris. 2006. 'Influence of roasting conditions on volatile flavor of roasted Malaysian cocoa beans', Journal of Food Processing and Preservation, 30: 280-98.

Rechner, Andreas R, Martin A Smith, Gunter Kuhnle, Glenn R Gibson, Edward S Debnam, S Kaila S Srai, Kevin P Moore, and Catherine A Rice-Evans. 2004. 'Colonic metabolism of dietary polyphenols: influence of structure on microbial fermentation products', Free Radical Biology and Medicine, 36: 212-25.

Reichardt, Nicole, Sylvia H Duncan, Pauline Young, Alvaro Belenguer, Carol McWilliam Leitch, Karen P Scott, Harry J Flint, and Petra Louis. 2014. 'Phylogenetic distribution of three pathways for propionate production within the human gut microbiota', The ISME journal, 8: 1323-35.

Ríos-Covián, David, Patricia Ruas-Madiedo, Abelardo Margolles, Miguel Gueimonde, Clara G de los Reyes-Gavilán, and Nuria Salazar. 2016. 'Intestinal short chain fatty acids and their link with diet and human health', Frontiers in microbiology, 7.

Sánchez-Patán, Fernando, Carolina Cueva, Maria Monagas, Gemma E Walton, Glenn R Gibson M, Jesús E Quintanilla-López, Rosa Lebrón-Aguilar, PJ Martín-Álvarez, M Victoria Moreno-Arribas, and Begoña Bartolomé. 2012. 'In vitro fermentation of a red wine extract by human gut microbiota: changes in microbial groups and formation of phenolic metabolites', Journal of agricultural and food chemistry, 60: 2136-47.

Sarriá, Beatriz, Sara Martínez-López, Aránzazu Fernández-Espinosa, Miren Gómez-Juaristi, Luis Goya, Raquel Mateos, and Laura Bravo. 2012. 'Effects of regularly consuming dietary fibre rich soluble cocoa products on bowel habits in healthy subjects: a free-living, two-stage, randomized, crossover, single-blind intervention', Nutrition & metabolism, 9: 33.

Scalbert, Augustin, Christine Morand, Claudine Manach, and Christian Rémésy. 2002. 'Absorption and metabolism of polyphenols in the gut and impact on health', Biomedicine & Pharmacotherapy, 56: 276-82.

Scalbert, Augustin, and Gary Williamson. 2000. 'Dietary intake and bioavailability of polyphenols', The Journal of nutrition, 130: 2073S-85S.

Schinella, Guillermo, Susana Mosca, Elena Cienfuegos-Jovellanos, María Ángeles Pasamar, Begoña Muguerza, Daniel Ramón, and José Luis Ríos. 2010. 'Antioxidant properties of polyphenol-rich cocoa products industrially processed', Food Research International, 43: 1614-23.

Schwan, Rosane F, and Alan E Wheals. 2004. 'The microbiology of cocoa fermentation and its role in chocolate quality', Critical reviews in food science and nutrition, 44: 205-21.

Sies, Helmut, Tankred Schewe, Christian Heiss, and Malte Kelm. 2005. 'Cocoa polyphenols and inflammatory mediators', The American journal of clinical nutrition, 81: 304S-12S.

Stoupi, Stavroula, Gary Williamson, J Warren Drynan, Denis Barron, and Michael N Clifford. 2010. 'A comparison of the in vitro biotransformation of (−) - epicatechin and procyanidin B2 by human faecal microbiota', Molecular nutrition & food research, 54: 747-59.

Stoupi, Stavroula, Gary Williamson, Florian Viton, Denis Barron, Laurence J King, Jonathan E Brown, and Michael N Clifford. 2010. 'In vivo bioavailability, absorption, excretion, and pharmacokinetics of [14C] procyanidin B2 in male rats', Drug Metabolism and Disposition, 38: 287-91.

Topping, David L, and Peter M Clifton. 2001. 'Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides', Physiological reviews, 81: 1031-64.

Treem, William R, Naheedt Ahsan, Geraldine Kastoff, and Jeffrey S Hyams. 1996. 'Fecal short-chain fatty acids in patients with diarrhea-predominant irritable bowel syndrome: in vitro studies of carbohydrate fermentation', Journal of pediatric gastroenterology and nutrition, 23: 280-86.

Tsao, Rong. 2010. 'Chemistry and biochemistry of dietary polyphenols', Nutrients, 2: 1231-46.

Tzounis, Xenofon, Jelena Vulevic, Gunter GC Kuhnle, Trevor George, Jadwiga Leonczak, Glenn R Gibson, Catherine Kwik-Uribe, and Jeremy PE Spencer. 2008. 'Flavanol monomer-induced changes to the human faecal microflora', British Journal of Nutrition, 99: 782-92.

Ursell, Luke K, Jessica L Metcalf, Laura Wegener Parfrey, and Rob Knight. 2012. 'Defining the human microbiome', Nutrition reviews, 70: S38-S44.

USDA. 2013. "International Agricultural Trade Report: Chocolate Candy." In, edited by Foreign Agricultural Service.

Valiente, C, RM Esteban, E Mollá, and FJ LÓPEZ - ANDRÉU. 1994. 'Roasting effects on dietary fiber composition of cocoa beans', Journal of Food science, 59: 123-24.

Venter, MJ, N Schouten, R Hink, NJM Kuipers, and AB De Haan. 2007. 'Expression of cocoa butter from cocoa nibs', Separation and purification technology, 55: 256-64.

Vogt, Janet A, and Thomas MS Wolever. 2003. 'Fecal acetate is inversely related to acetate absorption from the human rectum and distal colon', The Journal of nutrition, 133: 3145-48.

Walker, Alan W, Sylvia H Duncan, E Carol McWilliam Leitch, Matthew W Child, and Harry J Flint. 2005. 'pH and peptide supply can radically alter bacterial populations and short-chain fatty acid ratios within microbial communities from the human colon', Applied and environmental microbiology, 71: 3692-700.

Walker, Alan W, Jennifer Ince, Sylvia H Duncan, Lucy M Webster, Grietje Holtrop, Xiaolei Ze, David Brown, Mark D Stares, Paul Scott, and Aurore Bergerat. 2011. 'Dominant and diet-responsive groups of bacteria within the human colonic microbiota', The ISME journal, 5: 220-30.

Wang, Zhiling, Zhong Zhao, Xiaofei Cheng, Suqi Liu, Qin Wei, and Ian M Scott. 2016. 'Conifer flavonoid compounds inhibit detoxification enzymes and synergize insecticides', Pesticide biochemistry and physiology, 127: 1-7.

Wolever, Thomas, Furio Brighenti, Dawna Royall, Alexandra L Jenkins, and David JA Jenkins. 1989. 'Effect of rectal infusion of short chain fatty acids in human subjects', American Journal of Gastroenterology, 84.

Wollgast, Jan, and Elke Anklam. 2000. 'Review on polyphenols in Theobroma cacao: changes in composition during the manufacture of chocolate and methodology for identification and quantification', Food Research International, 33: 423-47.

Wong, Julia MW, Russell De Souza, Cyril WC Kendall, Azadeh Emam, and David JA Jenkins. 2006. 'Colonic health: fermentation and short chain fatty acids', Journal of clinical gastroenterology, 40: 235-43.

Wood, George Alan Roskruge, and RA Lass. 2008. Cocoa (John Wiley & Sons).

Wu, Gary D, Jun Chen, Christian Hoffmann, Kyle Bittinger, Ying-Yu Chen, Sue A Keilbaugh, Meenakshi Bewtra, Dan Knights, William A Walters, and Rob Knight. 2011. 'Linking long-term dietary patterns with gut microbial enterotypes', science, 334: 105-08.

Xiao, Chao Wu. 2008. 'Health effects of soy protein and isoflavones in humans', The Journal of nutrition, 138: 1244S-49S.

Yoshida, Atsuko, Keisuke Sonoda, Yoichi Nogata, Takashi Nagamine, Maki Sato, Tomoyuki Oki, Shunjiro Hashimoto, and Hideaki Ohta. 2010. 'Determination of free and bound phenolic acids, and evaluation of antioxidant activities and total polyphenolic contents in selected pearled barley', Food science and technology research, 16: 215-24.

Ze, Xiaolei, Sylvia H Duncan, Petra Louis, and Harry J Flint. 2012. 'Ruminococcus bromii is a keystone species for the degradation of resistant starch in the human colon', The ISME journal, 6: 1535-43.

Zhang, Ying, Ying Li, Can Cao, Jie Cao, Wei Chen, Yu Zhang, Cheng Wang, Jia Wang, Xin Zhang, and Xiujuan Zhao. 2010. 'Dietary flavonol and flavone intakes and their major food sources in Chinese adults', Nutrition and cancer, 62: 1120-27.

APPENDIX: SUPPLEMENTAL DATA AND FIGURES

Detailed daily food intake records of Volunteer Group 1

Donor A:

Day	Diet	Amount/g	Calories/cal	Protein/g	Lipid/g	Carbohydrates/g	Dietary fiber/g	Sugar/g
1	Half banana	59	53	0.64	0.19	13.48	1.5	7.22
	corn flake with fruits	30	110	2	0	26	1	2
	shredded chicken sandwiches	177	241	16.99	16	6	1	2.99
	trail mix	57	280	10	20	22	4	14
	frozen yogurt	144	229	5.76	8.06	34.85	0	34.56
	cookies	20	94	1.03	3.51	14.45	0.4	6.53
	mixed fruit cheese	128	250	7	13	25	2.9	2
	cinnamon tea	2	5	0	0	1	0	0
2	scrambles egg	72	153	9.96	11.65	1.5	0	1.18
	corn tortillas	51.6	160	4	2	32	2	0
	coffee	473	43	0.47	0.85	8	0	0
	Spaghetti	257	131	3.62	3.8	20.71	4.6	14.13
	coke	614	240	0	0	65	0	65
	rum	350	808	0	0	0	0	0
	pizza	400	1120	46.92	50.24	119.72	6.8	12.84
	beer	712	306	3.28	0	25.28	0	0
	rum	350	808	0	0	0	0	0
3	chocolate donut	60	250	2.7	11.94	34.44	1.3	19.15
	tea	2	0	0	0	0	0	0
	corn flake with fruits	30	110	2	0	26	1	2
	Grilled pork loin	326	441	26.99	13.99	41.99	4.9	10.01
	grilled beef/pork sausage	56	203	9.77	17.04	1.86	0	0.48

	BBQ pork ribs	184	460	20.99	20	46.99	2.9	16.01
	salad	184	180	6.99	13.01	6.99	2	3
	cookies	10	47	0.52	1.75	7.22	0.2	3.26
	beer	1780	765	8.19	0	63.19	0	0
4	cookies	10	47	0.52	1.75	7.22	0.2	3.26
	corn flake with fruits	30	110	2	0	26	1	2
	cookies	20	94	1.03	3.51	14.45	0.4	6.53
	rice	120	420	14	4	80	10	0
	lean meat	192.5	481	49.92	29.66	0	0	0
	cookies	50	234	2.58	8.78	36.12	0.9	16.32
	coffee	473	43	0.47	0.85	8	0	0
	orange juice	249	134	0.5	0	33.39	0.5	23.31
5	cookies	10	47	0.52	1.75	7.22	0.2	3.26
	corn flake with fruits	30	110	2	0	26	1	2
	rice	180	630	21.01	5.99	120.1	14.9	0
	lean meat	192.5	481	49.92	29.66	0	0	0
	greek yogurt	150	100	12	0	13.99	5	7

Donor B:

Day	Diet	Amount/g	Calories/cal	Protein/g	Lipid/g	Carbohydrates/g	Dietary fiber/g	Sugar/g
1	walnuts	28.35	185	4.32	18.49	3.89	1.9	0.74
	fries mc	117	378	3.99	18.1	49.82	4.6	0.25
	coke	614.4	240	0	0	65	0	65
	avocado	139	227	2.67	20.96	11.75	9.2	0.41
	frozen yogurt	72	114	2.88	4.03	17.42	0	17.28
2	banana	118	105	1.29	0.39	26.95	3.1	14.43
	Sausage burrito	109	302	12.1	17.04	25.04	1.3	2.8
	coffee	473	43	0.47	0.85	8	0	0

	hashbrown	31	130	2	5	21	1	2
	chicken taquitos whole food	128	330	15	17	30	4	1
3	banana	118	105	1.29	0.39	26.95	3.1	14.43
	pancake	231	524	14.78	22.41	65.37	0	0
	margarine	28.2	205	0.09	22.65	0.22	0	0
	chicken enchiladas	225	283	12.49	9.44	37.99	5.9	5.1
	coke	355	138	0	0	37.56	0	37.56

Donor C:

Day	Diet	Amount/g	Calories/cal	Protein/g	Lipid/g	Carbohydrates/g	Dietary fiber/g	Sugar/g
1	Groud beef	225	702	36.16	60.28	0	0	0
	French bread with mayonaise & cheese	125	420	14	22	41	2	1
	chicken tomato lettuce salad	269	401	13.99	22	29.99	1.1	1.99
2	Chex corn cereal	31	115	1.98	0.74	26.35	1.5	3.44
	French bread with mayonaise & cheese	125	420	14	22	41	2	1
	milk	240	120	7.99	4.99	12	0	10.99
	roasted chicken	84	160	4.99	2.99	28.93	2	2.99
3	Fried chicken	85	230	17	15	8	1	0
	biscuit	25	130	1	7	16	0.6	7
	rice	68	244	7.09	0.93	51.89	0.8	1.04
	chex corn cereal	31	115	1.98	0.74	26.35	1.5	3.44
	milk	240	120	7.99	4.99	12	0	10.99
4	Bean	240	113	4.32	7.68	6.72	0.2	0
	cheeseburger	155	437	24.02	24.94	29.12	1.2	6.56
	French fries	153	203	3.35	5.19	35.97	2.9	0.31
5	Bean	240	113	4.32	7.68	6.72	0.2	0

	rice	68	244	7.09	0.93	51.89	0.8	1.04
	Chex corn cereal	31	115	1.98	0.74	26.35	1.5	3.44
	milk	240	120	7.99	4.99	12	0	10.99

Donor D:

Day	Diet	Amount/g	Calories/cal	Protein/g	Lipid/g	Carbohydrates/g	Dietary fiber/g	Sugar/g
1	rice krispybar	28	128	1.96	5.71	18.09	1	0
	2% milk	240	120	7.99	4.99	12	0	10.99
	blackberries	72	31	1	0.35	6.92	3.8	3.51
	coke	355	138	0	0	37.56	0	37.56
	personal pepperon pizza	174	458	19.51	23.99	39.74	2.3	3.76
	ham swiss sandwich	227	449	30.01	13.01	65.99	2	14.01
	wheat bread	57	146	0.01	1.08	25.99	4.3	2.51
	green snickers	47	220	4	10	29	1	24
	fun size snickers	15	73	1.03	3.66	9.43	0.3	6.98
	reeses heart	100	25	2	0	5	2	0
2	2% milk	240	120	7.99	4.99	12	0	10.99
	lucky charm cereal	18	68	1.38	0.9	14.56	0.9	6.5
	pulled porn sandwich	68	150	7.5	4.5	20	1	4.5
	sweet potato baked fries	12	47.5	0.43	3	6.86	0.4	1.29
	hot dog	56	151	6.93	2.45	25.16	3.4	3.32
	doritos nacho cheese	28	150	2	8	16	1	1
	Pepsi cola	355	149	0	0	41	0	41
	reeses heart	100	25	2	0	5	2	0
3	rice krispybar	28	128	1.96	5.71	18.09	1	0
	green grapes	160	520	4	0	124	8	116
	keebler peanut butter crackers	39	191	4.13	9.28	23.48	1.2	4.52
	ham swiss sandwich	227	449	30.01	13.01	65.99	2	14.01
	blackberries	36	15	0.5	0.18	3.46	1.9	1.76

	waffle fries	84	150	2	7	20	2	1
	fun size snickers	15	73	1.03	3.66	9.43	0.3	6.98
	popcorn	33	140	2	9	16	4	0

Donor E:

Day	Diet	Amount/g	Calories/cal	Protein/g	Lipid/g	Carbohydrates/g	Dietary fiber/g	Sugar/g
1	breakfast cereal	38	2.17	4.41	1.03	27.25	2.8	9.84
	coffee	473	43	0.47	0.85	8	0	0
	shrimp alfredo	297	490	22.01	16.99	62.01	3	9
	tea	2	0	0	0	0	0	0
	Roast beef	609	1114	162.3	51.52	0	0	0
	orange juice	249	134	0.5	0	33.39	0.5	23.31
2	egg	100	140	12	9	2	0	0
	buttermilk biscuit	64	151	4.1	1.79	30.12	1	2.85
	orange juice	249	134	0.5	0	33.39	0.5	23.31
	Chicken and sausage jambalaya	340	360	14.99	12	46.99	3.1	2.01
	berry tea	2	5	0	0	1	0	1
	chicken and shrimp alfredo	213	100	8.01	9.01	18	0	1
	Whole milk	266	237	8.7	8.43	31.57	1	30.83
3	egg and sausage fast food	162	505	18.03	33.65	34.1	0.3	1.54
	turkey and mozzarella cheese sandwich	269	541	34	29	51	5.1	6.99
	Chicken nuggets	88	238	13.79	11.35	20.12	1.9	0.43
	cheese pizza	130	303	15.7	9.76	38.05	4.9	8.79
	orange juice	249	134	0.5	0	33.39	0.5	23.31
4	egg and sausage fast food	162	505	18.03	33.65	34.1	0.3	1.54
	buttermilk biscuit	64	151	4.1	1.79	30.12	1	2.85
	orange juice	249	134	0.5	0	33.39	0.5	23.31
	cheese pizza	130	303	15.7	9.76	38.05	4.9	8.79

	peanut butter and jelly sandwich	57	220	7	12	22	1	8
	spaghetti and meat sauce	283	255	14.29	2.86	43.13	5.1	7.36

Donor F:

Day	Diet	Amount/g	Calories/cal	Protein/g	Lipid/g	Carbohydrates/g	Dietary fiber/g	Sugar/g
1	Coffee	120	5	0	0	1.5	0	0
	Cheez-its	56.7	281	4.87	13.83	35.06	1.2	0.73
	Trader joes south west salad	255	408	12.75	30.6	22.95	5.1	7.65
	Peanut butter monster trail mix	35	180	4	11	18	1	13
	Gooey butter cake	28.3	146	0.91	8.22	16.43	0	15.52
	tilapia fish filets	232	223	46.59	3.94	0	0	0
	Spaghetti	85	43	1.2	1.26	6.85	1.5	4.67
	Peach soda	118	68	0	0	16.99	0	16.32
2	Coffee	180	7	0	0	2.25	0	0
	Milk chocolate Jell-o pudding cup	56.7	223	0	0	50.63	2	36.45
	Applejack cereal w/ 2% milk	354	160	5	5	24.7	3	12.24
	chili's chipotle fresh mex bowl	170	185	10.51	3.5	28	2	2.5
	Dr. Pepper	40	100	2	0	26	0	17
	Jack Daniels whiskey	88.2	145	0.09	0.02	13.98	0	13.94
	Corona beers	710	296	0	0	13	0	0
3	Ginger Ale	122	41	0	0	10.69	0	10.86
	Crackers	56.7	246	8.08	7.57	36.49	2.2	1.33
	Coffee	180	7	0	0	2.25	0	0
	chicken nugget	211.2	570	33.1	27.24	48.28	4.6	1.03
	fries	153	203	3.35	5.19	35.97	2.9	0.31
	lemonade	627	220	0	0	58	0	55
	Sour fruit slice	50	175	0	0	42.5	0	35
	Chicken Salad meal	56.5	115	11.5	5.5	5.5	1.5	1

	Crackers	56.7	246	8.08	7.57	36.49	2.2	1.33
	Ginger Ale	122	41	0	0	10.69	0	10.86

Donor G:

Day	Diet	Amount/g	Calories/cal	Protein/g	Lipid/g	Carbohydrates/g	Dietary fiber/g	Sugar/g
1	bread	58	154	5.13	1.93	28.66	1.6	3.29
	milk	240	120	7.99	4.99	12	0	10.99
	rice	200	680	12	0	156	0	0
	chicken breast	112	110	23	2.5	0	0	0
	green bell pepper	125	60	2	0.5	7	2	4
	celery	50	8	0.34	0.09	1.49	0.8	0.67
	egg	50	70	6	4.5	1	0	0
	sausage	85	272	10.2	24.42	2.06	0	0
	rice	200	680	12	0	156	0	0
	chicken breast	112	110	23	2.5	0	0	0
	milk	240	120	7.99	4.99	12	0	10.99
2	bread	58	154	5.13	1.93	28.66	1.6	3.29
	milk	240	120	7.99	4.99	12	0	10.99
	rice	200	680	12	0	156	0	0
	chicken breast	112	110	23	2.5	0	0	0
	celery	50	8	0.34	0.09	1.49	0.8	0.67
	carrots	50	20	0.47	0.12	4.79	1.4	2.37
	egg	50	70	6	4.5	1	0	0
	rice	200	680	12	0	156	0	0
	chicken breast	112	110	23	2.5	0	0	0
	Coffee	180	7	0	0	2.25	0	0
3	bread	58	154	5.13	1.93	28.66	1.6	3.29
	milk	240	120	7.99	4.99	12	0	10.99

	rice	200	680	12	0	156	0	0
	chicken breast	112	110	23	2.5	0	0	0
	green bell pepper	125	60	2	0.5	7	2	4
	celery	50	8	0.34	0.09	1.49	0.8	0.67
	egg	50	70	6	4.5	1	0	0
	chicken breast	112	110	23	2.5	0	0	0
	milk	240	120	7.99	4.99	12	0	10.99
	broccoli	50	17	1.41	0.18	3.32	1.3	0.85

Detailed daily food intake records of Volunteer Group 2

Donor 1

Day	Diet	Amount/g	Calories/cal	Protein/g	Lipid/g	Carbohydrates/g	Dietary fiber/g	Sugar/g
1	walnuts	20	131	3.05	13.04	2.74	1.3	0.52
	chicken nuggets	113	305	17.71	14.58	25.83	2.5	0.55
	fries	56	175	1.92	8.25	23.21	2.1	0.17
	diet coke	239.5	0	0	0	0	0	0
	Strawberry salad	170	280	12	14.01	25.99	3.1	21
2	sushi crab roll	226	330	6.86	9.14	50.28	2.3	3.44
	Eggplant	170	60	1.41	0.39	14.84	4.2	5.44
	spaghetti sauce	28	11	0.45	0	2.48	0.2	1.58
3	rice crispies treat	23	90	1	2	17	0	7
	miso soup	226	35	3	1	4	0	0
	green salad	170	362	14.72	22.76	30.79	8	14.72
4	egg	113	174	11.94	13.18	0.72	0	0.35
	Bacon	56	234	7.07	22.23	0.72	0	0.56
	Biscuit	51	205	3.06	12.09	20.88	1.9	1.76
	seafood pasta	56	59	2.96	2.72	7.65	0.5	0.74

	wine	242	201	0.87	0	9.21	0	9.21
	pasta	6	634	22.3	2.58	127.69	5.5	4.57

Donor 2

Day	Diet	Amount/g	Calories/cal	Protein/g	Lipid/g	Carbohydrates/g	Dietary fiber/g	Sugar/g
1	Noosa yogurt	113	150	6	6.99	18	0.5	16
	rice	185	370	6.81	0.55	81.68	2.8	0
	large bell pepper	76	16	0.71	0.14	3.8	1.4	1.97
	jumbo egg	63	90	7.91	5.99	0.45	0	0.23
	frozen green peas	67	52	3.5	0.27	9.13	3	3.35
	broccoli	150	51	4.23	0.55	9.96	3.9	2.55
	chicken breast	112	110	23	2.5	0	0	0
	ginger root	5	4	0.09	0.04	0.89	0.1	0.09
	garlic	9	13	0.57	0.04	2.98	0.2	0.09
	oliver oil	15	120	0	14	0	0	0
	thin spaghetti	181	646	22.62	3.24	132.51	6.5	6.46
2	Noosa yogurt	113	150	6	6.99	18	0.5	16
	rice	185	370	6.81	0.55	81.68	2.8	0
	large bell pepper	76	16	0.71	0.14	3.8	1.4	1.97
	jumbo egg	63	90	7.91	5.99	0.45	0	0.23
	frozen green peas	67	52	3.5	0.27	9.13	3	3.35
	broccoli	150	51	4.23	0.55	9.96	3.9	2.55
	chicken breast	112	110	23	2.5	0	0	0
	ginger root	5	4	0.09	0.04	0.89	0.1	0.09
	garlic	9	13	0.57	0.04	2.98	0.2	0.09
	oliver oil	15	120	0	14	0	0	0

	thin spaghetti	181	646	22.62	3.24	132.51	6.5	6.46
3	Noosa yogurt	113	150	6	6.99	18	0.5	16
	rice	185	370	6.81	0.55	81.68	2.8	0
	large bell pepper	76	16	0.71	0.14	3.8	1.4	1.97
	jumbo egg	63	90	7.91	5.99	0.45	0	0.23
	frozen green peas	67	52	3.5	0.27	9.13	3	3.35
	broccoli	150	51	4.23	0.55	9.96	3.9	2.55
	chicken breast	112	110	23	2.5	0	0	0
	ginger root	5	4	0.09	0.04	0.89	0.1	0.09
	garlic	9	13	0.57	0.04	2.98	0.2	0.09
	oliver oil	15	120	0	14	0	0	0
	thin spaghetti	181	646	22.62	3.24	132.51	6.5	6.46

Donor 3

Day	Diet	Amount/g	Calories/cal	Protein/g	Lipid/g	Carbohydrates/g	Dietary fiber/g	Sugar/g
1	Toasted hamburger bun	50	142	0.01	1.89	25.5	0.9	3.93
	peanut butter	16	95	3.5	7.5	4	1	1.5
	fig preserves	34	100	0	0	26	0	24
	large coffee	600	24	0	0	7.5	0	0
	coffee creamer	15	35	0	1.5	6	0	6
	Nachos	454	1589	19.61	97.61	158.49	14.5	9.85
	mixed nuts	28.35	172	5.53	15.17	6.36	1.8	1.42
	burger patty	112	180	21	9	0	0	0
	steak sauce	15	10	0	0	2	0	1
	Toasted hamburger bun	50	142	0.01	1.89	25.5	0.9	3.93

2	butter	14.1	103	0.04	11.33	0.11	0	0
	fruit jelly	28	30	0	0	6	0	2
	orange juice	480	221	3.98	0	54	0	48
	crunchy granola bar	63	285	7.5	10.5	42	3	16.5
	large coffee	600	24	0	0	7.5	0	0
	coffee creamer	15	35	0	1.5	6	0	6
	pretzels and hummus	99	280	7	14	52	3	8
	Nutella Berry Crepes	240	390	13	15	52	4	20
	tea	480	0	0	0	0	0	0
	crackers	38.1	165	5.43	5.09	24.52	1.4	0.9
	red beans	157	223	6.22	5.43	37.27	4.1	0.24
	Toasted hamburger bun	50	142	0.01	1.89	25.5	0.9	3.93
3	butter	14.1	103	0.04	11.33	0.11	0	0
	strawberry jam	20	50	0	0	13	0	10
	peanut butter	16	95	3.5	7.5	4	1	1.5
	large coffee	600	24	0	0	7.5	0	0
	coffee creamer	15	35	0	1.5	6	0	6
	baked potato	278	1193	19.85	29.77	198.58	19.7	0
	butter	14.1	103	0.04	11.33	0.11	0	0
	mushrooms	59	30	0.75	1.61	3.25	0.2	0
	sour cream	12	22	0.84	1.69	0.84	0	0.04
	broccoli	91	31	2.57	0.34	6.04	2.4	1.55
	artichoke	128	60	4.19	0.19	13.45	6.9	1.27
	black olives	16	25	0	2.5	1	0	0

Donor 4

Day	Diet	Amount/g	Calories/cal	Protein/g	Lipid/g	Carbohydrates/g	Dietary fiber/g	Sugar/g
1	milk	367.5	206	14.52	7.28	20.18	0	20.18
	chocolate cereal	28	111	1.96	3.11	21.31	2.8	10
	special pastry cresp bar	30	150	2	6	19	1	6
	Bean Burrito	185	387	13.6	11.19	57.78	7.8	3.2
	chicken breast tenders	226.7	572	37.11	29.26	39.86	0	0
	macaroni and cheese	227	440	14.73	26.67	35.43	2.5	6.42
2	milk	367.5	206	14.52	7.28	20.18	0	20.18
	chocolate cereal	28	111	1.96	3.11	21.31	2.8	10
	special pastry cresp bar	30	150	2	6	19	1	6
	pepper omie pizza	135	279	14	10	32.99	2	4
	Jimmy John's The J.J. Gargantuan	249	532	34.7	27.16	37.05	0	0
3	milk	367.5	206	14.52	7.28	20.18	0	20.18
	chocolate cereal	28	111	1.96	3.11	21.31	2.8	10
	special pastry cresp bar	30	150	2	6	19	1	6
	Jimmy John's The J.J. Gargantuan	249	532	34.7	27.16	37.05	0	0
	pork chops	227	347	42.72	18.7	2.68	0	0
	rice	200	680	12	0	156	0	0
	granny smith	85	49	0.37	1.6	11.57	2.4	8.15

Donor 5

Day	Diet	Amount/g	Calories/cal	Protein/g	Lipid/g	Carbohydrates/g	Dietary fiber/g	Sugar/g
1	Sour patch kids candy	80	300	0	0	74	0	52
	Strawberry nutrigrain bar	37	120	1.74	3.55	24.83	2.6	11.58
	coffee	224	7	0.2	0	1.68	0	0
	cream	105	201	3.11	20.06	2.96	0	3.85
	sugar	1.5	6	0	0	1.47	0	1.46

	cheese	30	90	3	7	4	0	3
	egg white	99	51	10.79	0.17	0.72	0	0.7
	spniach	10	2	0.29	0.04	0.36	0.2	0.04
	ham	81	77	4.2	2.83	9.04	3.7	0
	onion	40	16	0.44	0.04	3.74	0.7	1.7
	Bacon	350	10.6	33.34	1.08	0	0	0.84
	Plain Cheesecake	80	280	6	15	31	0.3	18
	Champagne	226	200	0.16	0	6.4	0	1.84
	orange juice	93.3	44	0.63	0.14	10.27	0.3	8.17
2	Champagne	113	100	0.08	0	3.2	0	0.92
	Jack Daniels whiskey	29.4	48	0.03	0.01	4.66	0	4.65
	ginger ale	90	34	0	0	8.62	0	8.62
	Strawberry nutrigrain bar	37	120	1.74	3.55	24.83	2.6	11.58
	coffee	224	7	0.2	0	1.68	0	0
	cream	105	201	3.11	20.06	2.96	0	3.85
	sugar	1.5	6	0	0	1.47	0	1.46
	pancakes	100	265	0	0.1	69.6	0	16
	egg	50	72	6.28	4.75	0.36	0	0.18
	syrup	56	146	0.02	0.03	37.54	0	33.86
	Strawberry	110.5	39	0.48	0.12	10.09	2.3	5.04
	blueberry	74	42	0.55	0.24	10.72	1.8	7.37
	Strawberry nutrigrain bar	37	120	1.74	3.55	24.83	2.6	11.58
	Kung pao Chicken	604	779	58.95	42.16	41.49	9.1	18.3
	Vegetable crackers	62	268	6.2	10.34	41.34	2	4.14
	Peanuts	36.5	207	9.42	17.97	5.89	3.1	1.72
	Ginger ale	122	41	0	0	10.69	0	10.86
3	Lemonade	185.25	83	0.13	0	20.6	1.1	19.27
	Fried chicken sandwich	152	175	5.85	7.45	21.26	2.1	2.66
	Fries	88	133	2.19	3.39	23.51	1.9	0.2

	Fruit snacks	44	143	1.1	0	34.1	0	19.8
	White chocolate pecan candy		330	3	31	15	1	11
	Cranberry orange biscuits (Belvita)	50	230	3	8	36	3	12

Donor 6

Day	Diet	Amount/g	Calories/cal	Protein/g	Lipid/g	Carbohydrates/g	Dietary fiber/g	Sugar/g
1	Protein drink mix	60	220	30	6	10	0	2
	Formula 1 Herbalife	53	210	24	4.5	17	9	6
	Raspberry Tea herbalife	1.7	5	0	0	1	0	0
	eggwhites	132	69	14.39	0.22	0.96	0	0.94
	Tuna in water	85	109	20.08	2.52	0	0	0
	Mayonnaise reduced fat	15	54	0.06	6	0	0	0
	Protein drink mix	60	220	30	6	10	0	2
	Formula 1 Herbalife	53	210	24	4.5	17	9	6
	Raspberry Tea herbalife	1.7	5	0	0	1	0	0
2	Protein drink mix	60	220	30	6	10	0	2
	Formula 1 Herbalife	53	210	24	4.5	17	9	6
	Raspberry Tea herbalife	1.7	5	0	0	1	0	0
	eggwhites	132	69	14.39	0.22	0.96	0	0.94
	Tuna in water	85	109	20.08	2.52	0	0	0
	Mayonnaise reduced fat	15	54	0.06	6	0	0	0
	Protein drink mix	60	220	30	6	10	0	2
	Formula 1 Herbalife	53	210	24	4.5	17	9	6
	Raspberry Tea herbalife	1.7	5	0	0	1	0	0
3	Protein drink mix	60	220	30	6	10	0	2
	Formula 1 Herbalife	53	210	24	4.5	17	9	6
	Raspberry Tea herbalife	1.7	5	0	0	1	0	0
	eggwhites	132	69	14.39	0.22	0.96	0	0.94

Tuna in water	85	109	20.08	2.52	0	0	0
Mayonnaise reduced fat	15	54	0.06	6	0	0	0
Protein drink mix	60	220	30	6	10	0	2
Formula 1 Herbalife	53	210	24	4.5	17	9	6
Raspberry Tea herbalife	1.7	5	0	0	1	0	0

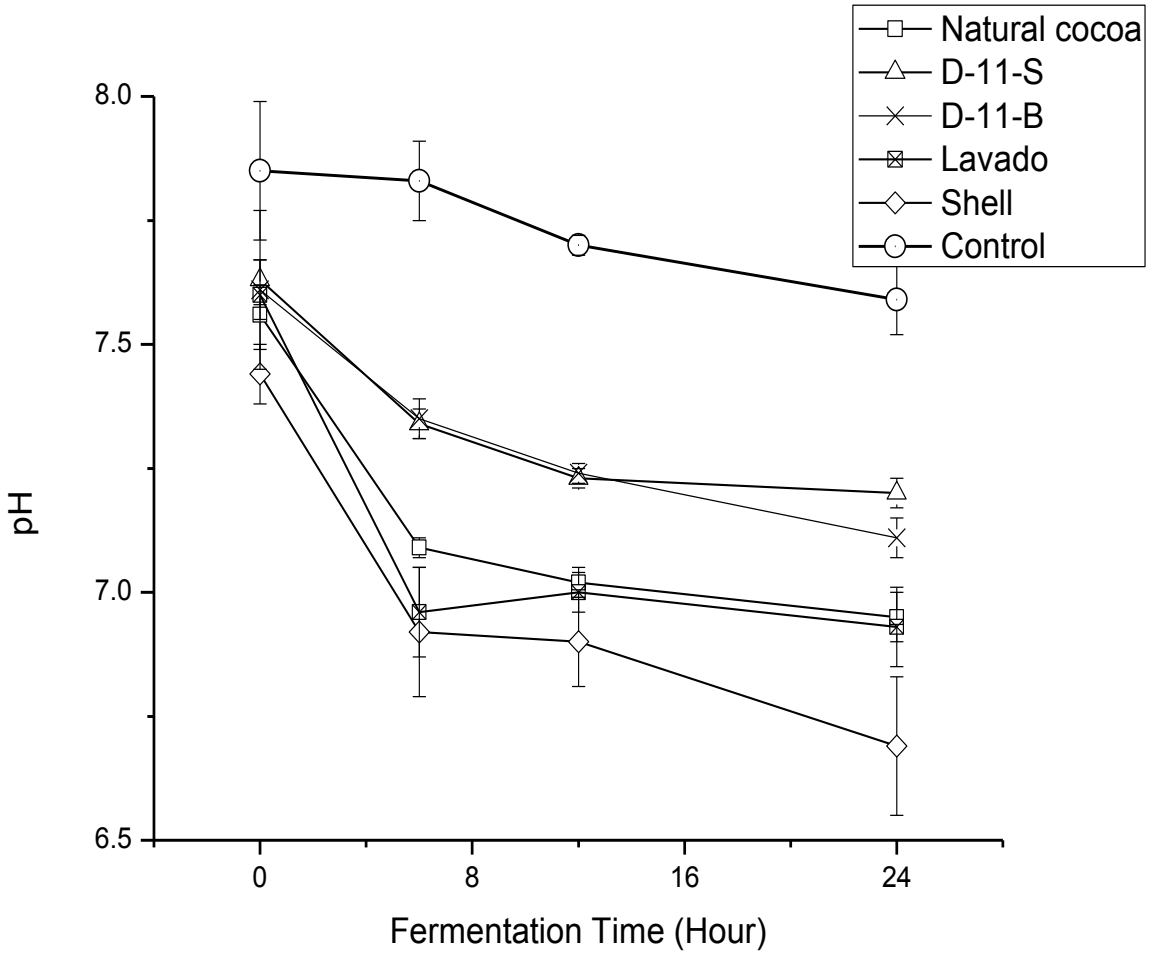


Figure A.1. The pH change of cocoa samples when fermented with pooled fecal slurry in an *in vitro* digestive model system.

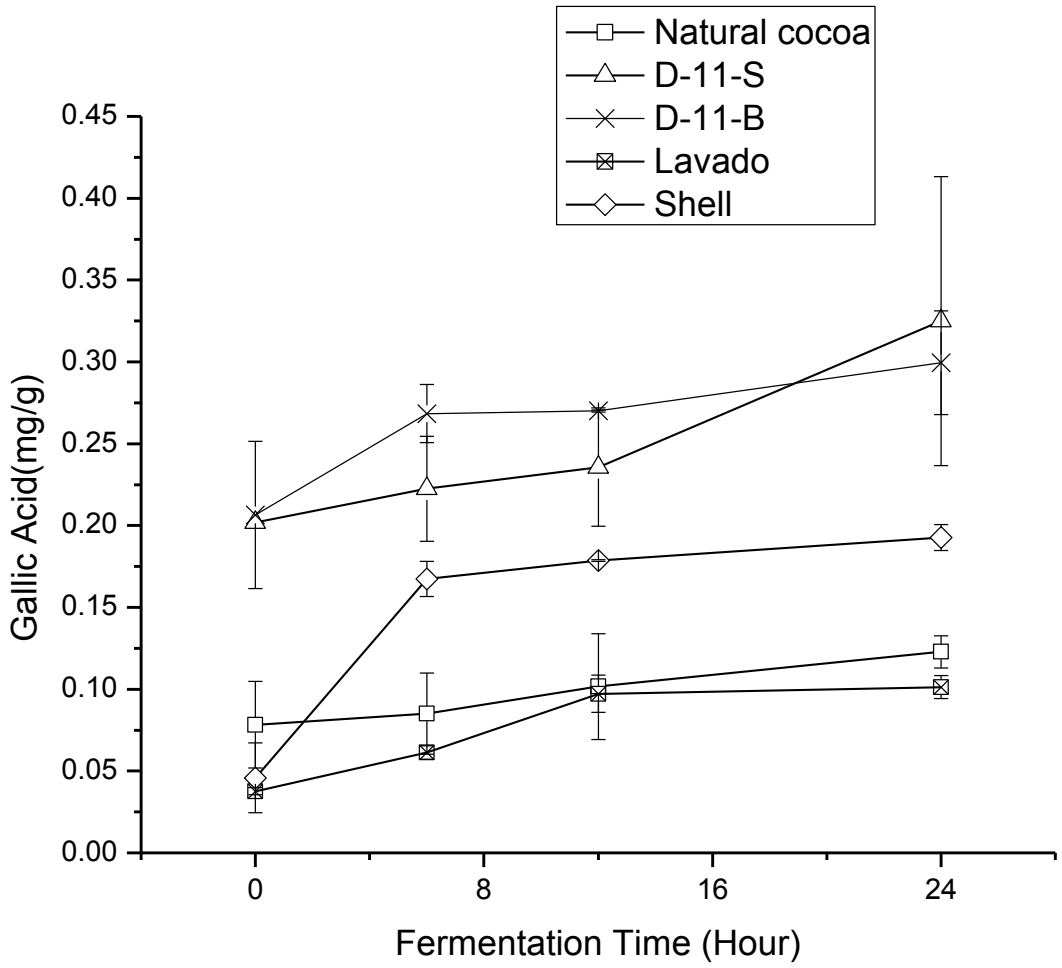


Figure A.2. Gallic acid changes over 24 hour cocoa samples fermentation in the *in vitro* GI tract model system.

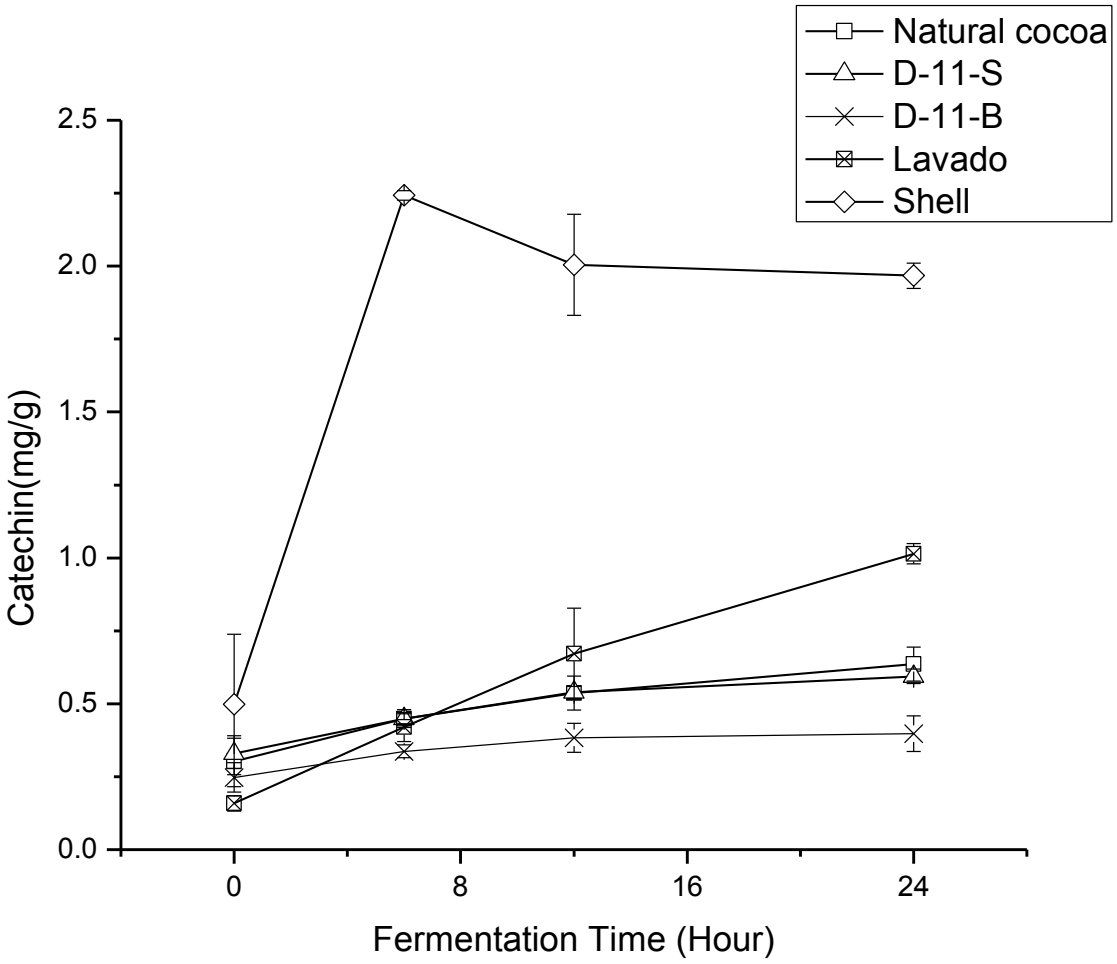


Figure A.3. Catechin changes over 24 hour cocoa samples fermentation in the *in vitro* GI tract model system.

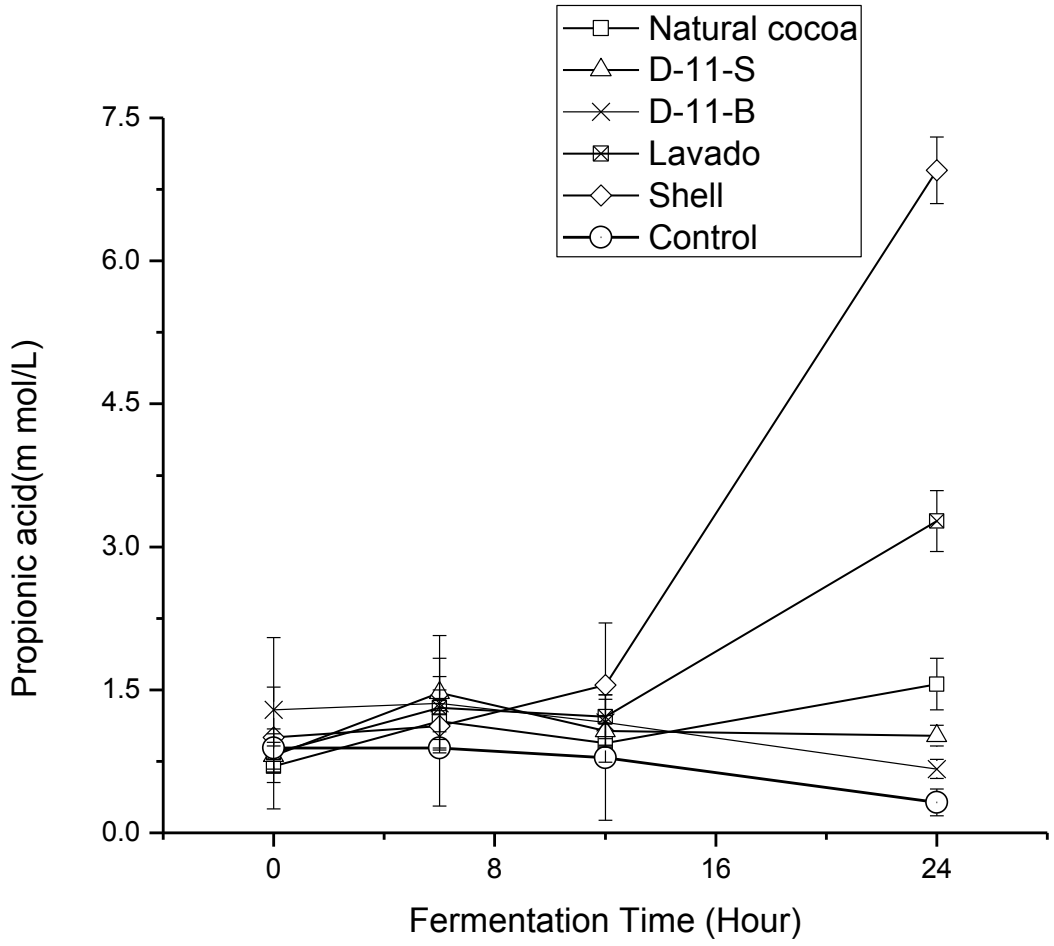


Figure A.4. Propionate changes over 24 hour cocoa samples fermentation in the *invitro* GI tract model system.

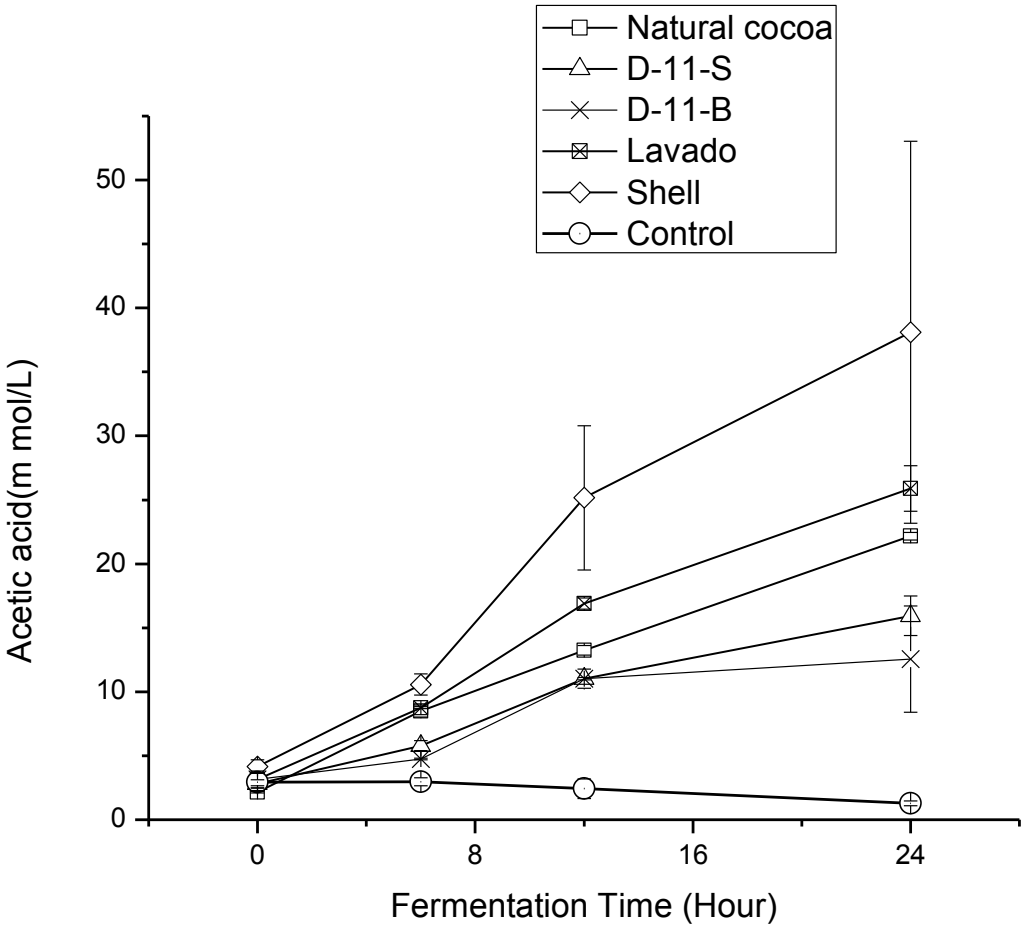


Figure A.5. Acetate changes over 24 hour cocoa samples fermentation in the *in vitro* GI tract model system.

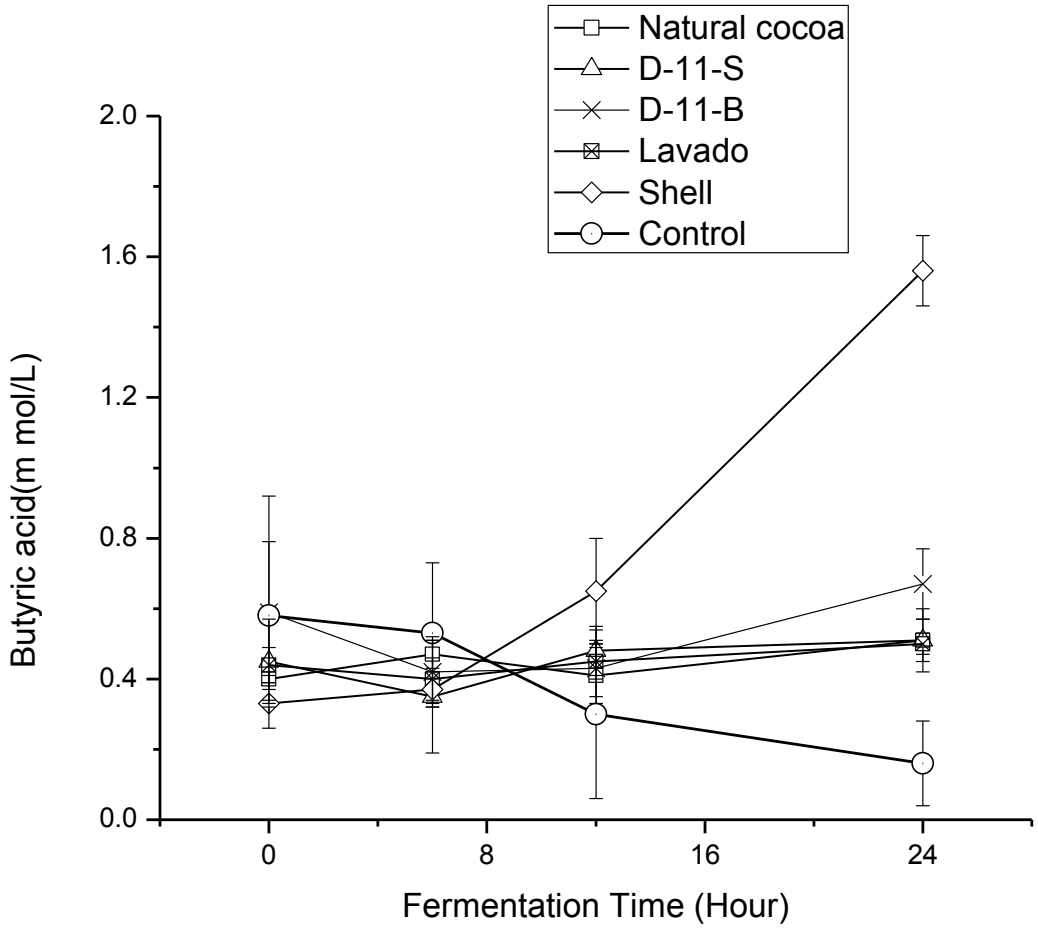
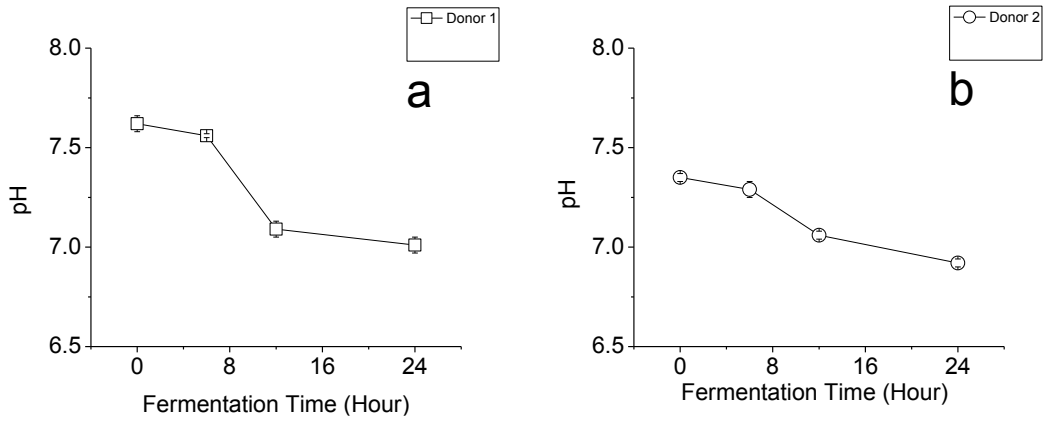


Figure A.6. Butyrate changes of fecal slurry over 24 hour fermentation of different cocoa samples in the *in vitro* GI tract model system.



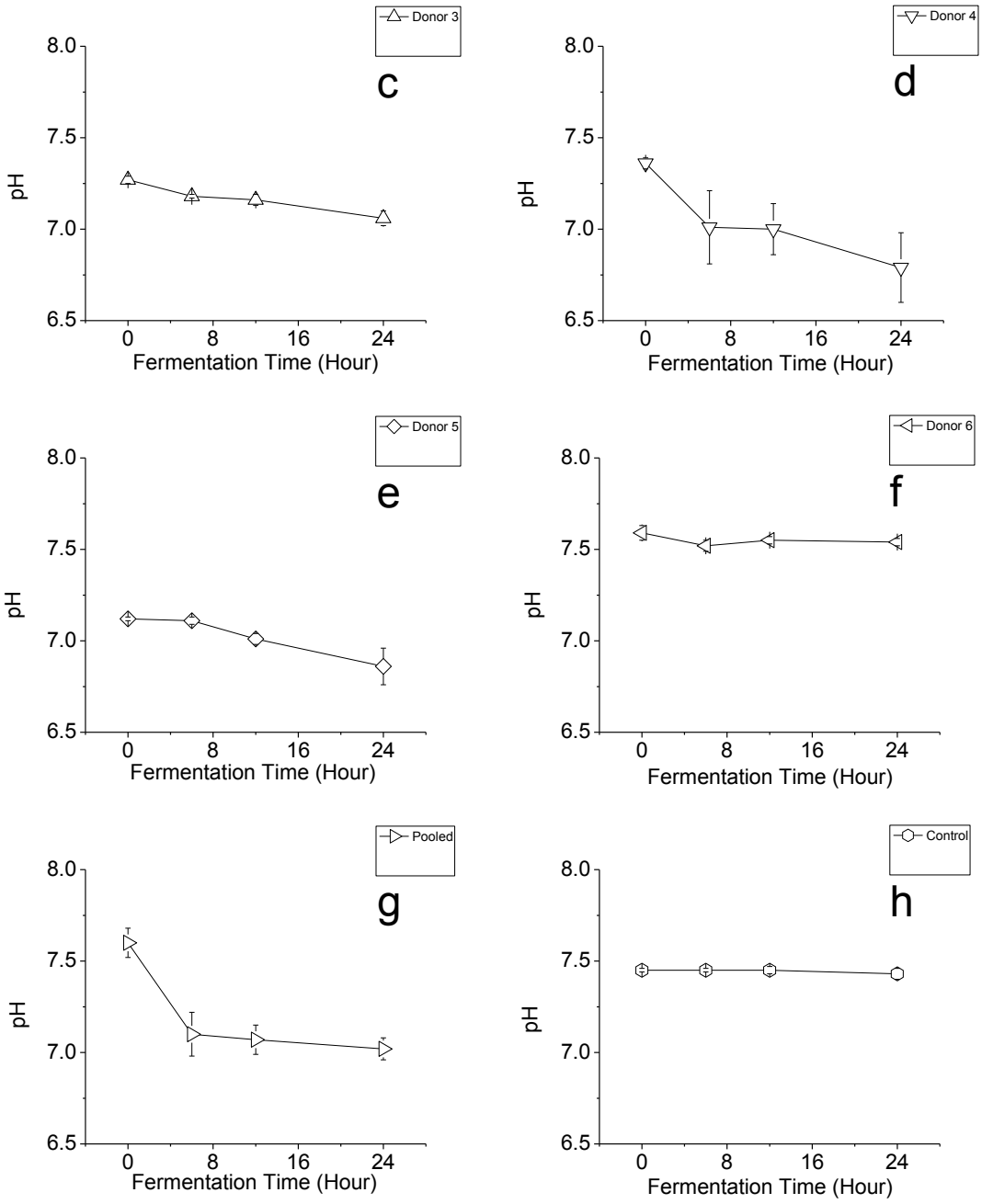
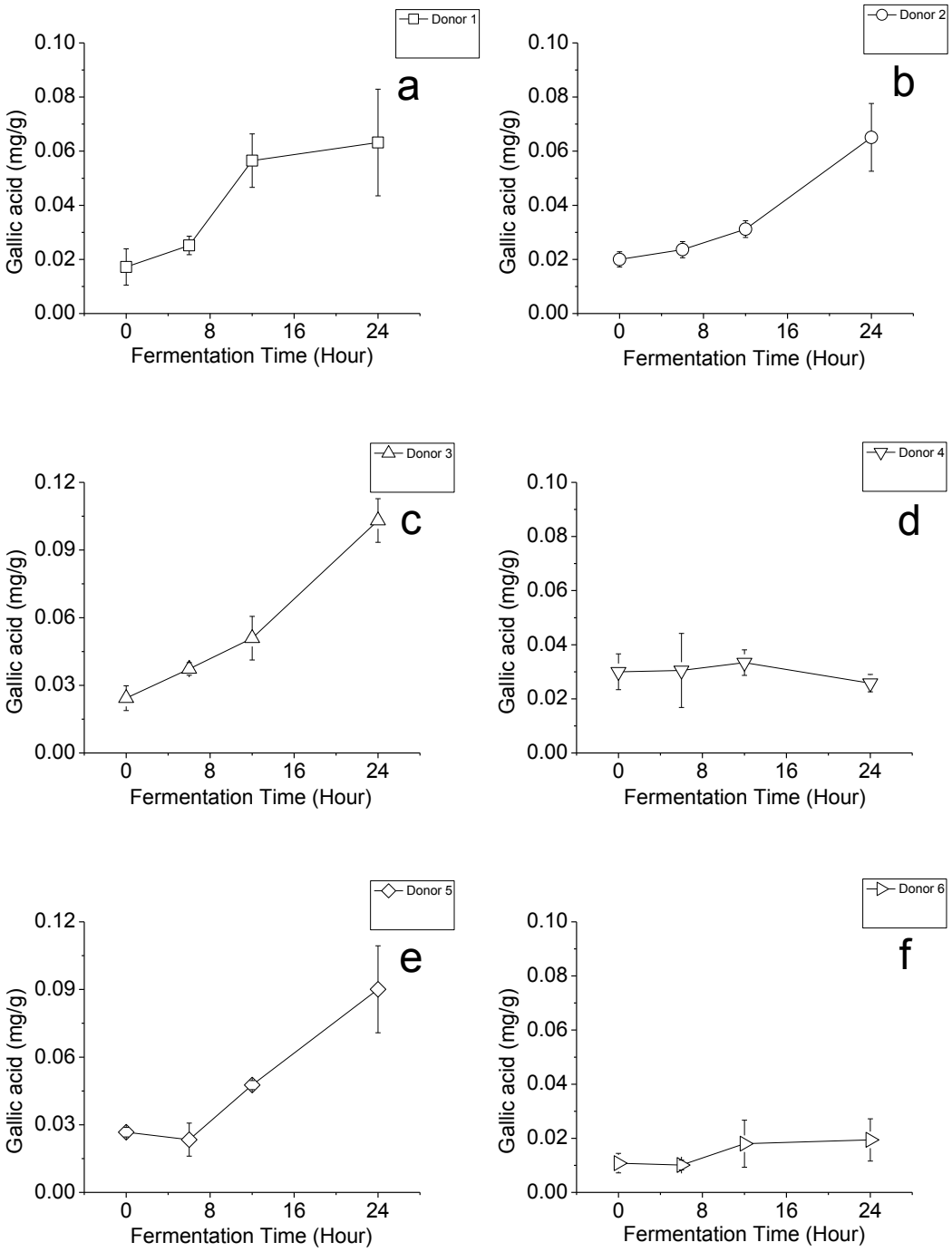


Figure A.7. The pH change of cocoa sample Lavado fermented with individual fecal slurry in an *in vitro* digestive model system


Donor 1
a
Gallic acid (mg/g)
0.00
0.02
0.04
0.06
0.08
0.10
0
8
16
24
Fermentation Time (Hour)
Donor 2
b
Gallic acid (mg/g)
0.00
0.02
0.04
0.06
0.08
0.10
0
8
16
24
Fermentation Time (Hour)
Donor 3
c
Gallic acid (mg/g)
0.00
0.03
0.06
0.09
0.12
0
8
16
24
Fermentation Time (Hour)
Donor 4
d
Gallic acid (mg/g)
0.00
0.02
0.04
0.06
0.08
0.10
0
8
16
24
Fermentation Time (Hour)
Donor 5
e
Gallic acid (mg/g)
0.00
0.03
0.06
0.09
0.12
0
8
16
24
Fermentation Time (Hour)
Donor 6
f
Gallic acid (mg/g)
0.00
0.02
0.04
0.06
0.08
0.10
0
8
16
24
Fermentation Time (Hour)

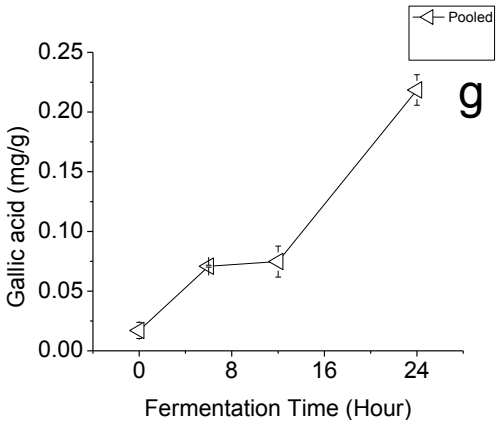
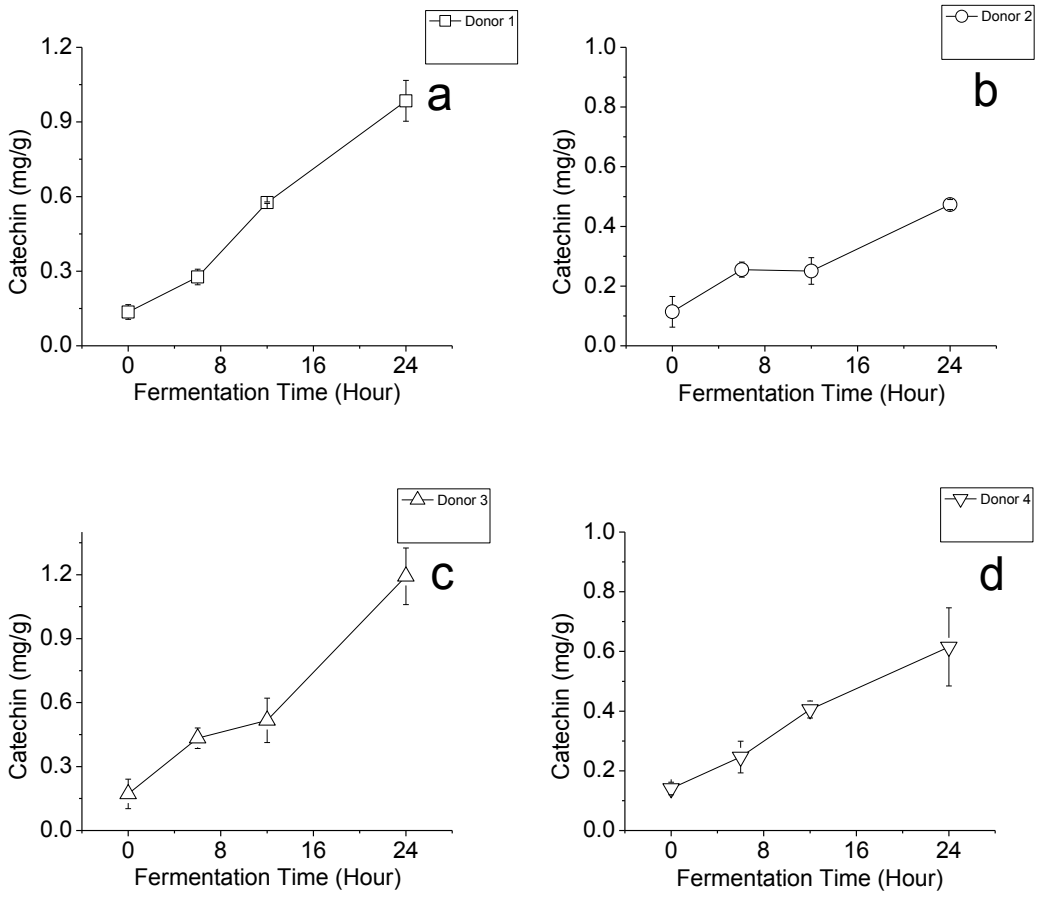


Figure A.8. Gallic acid changes over 24 hour cocoa samples fermented with individual fecal slurry in the *in vitro* GI tract model system.



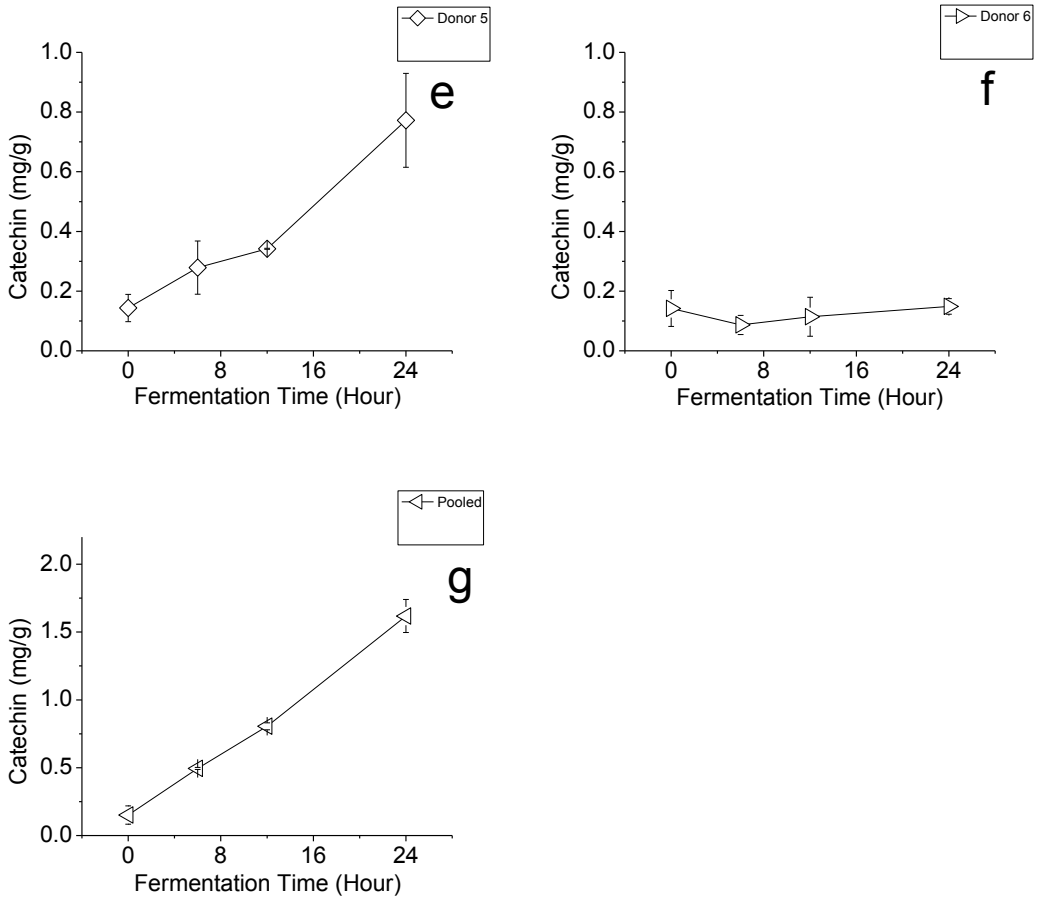
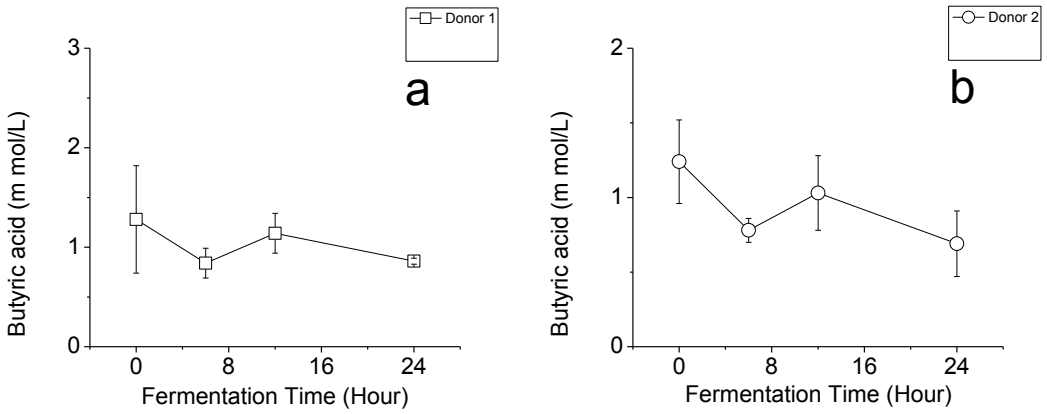


Figure A.9. Catechins change over 24 hour cocoa samples fermented with individual fecal slurry in the *in vitro* GI tract model system.



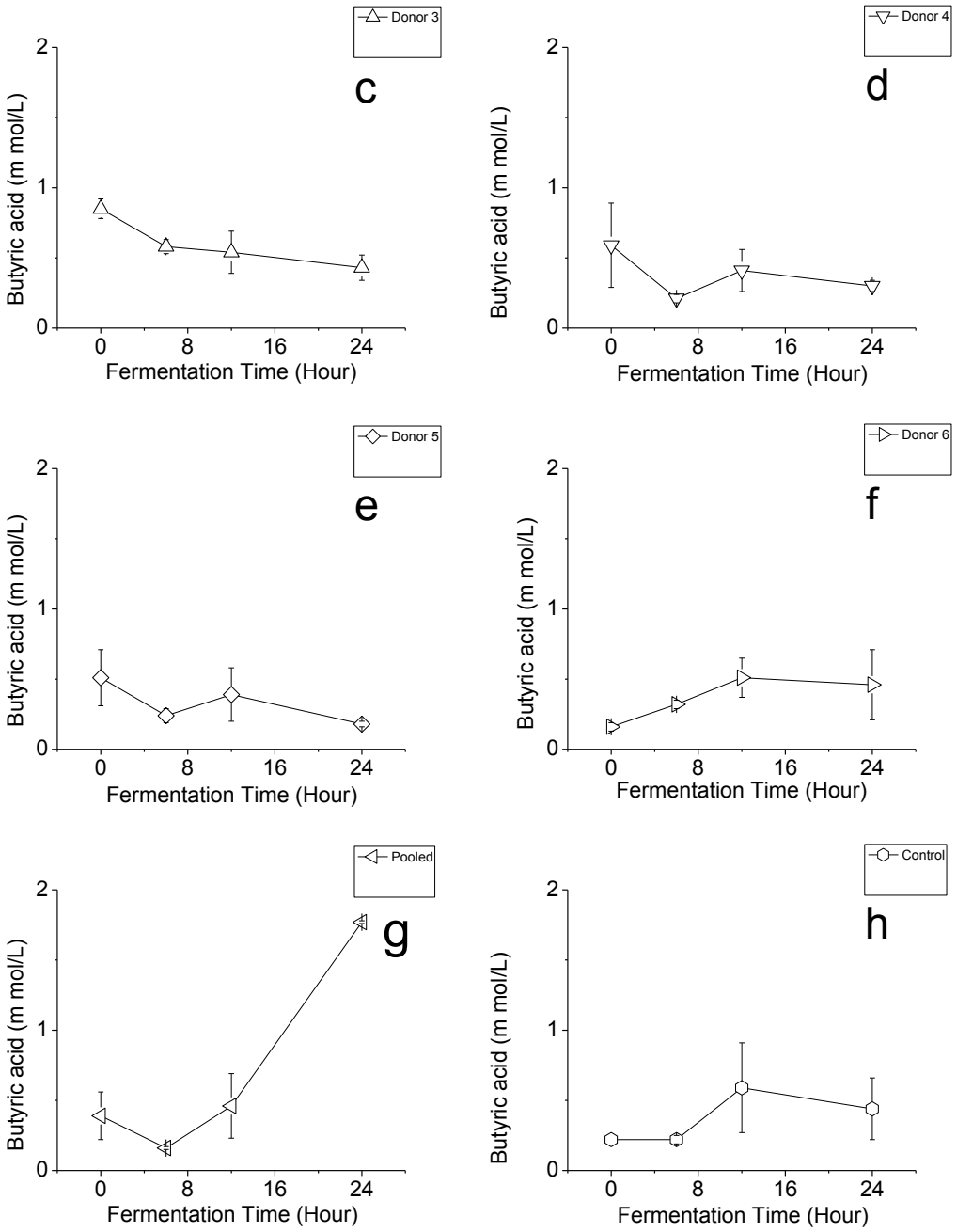


Figure A.10. Butyrate changes in individual fecal slurry over 24 hour of fermentation of cocoa sample Lavado in the *in vitro* GI tract model system.

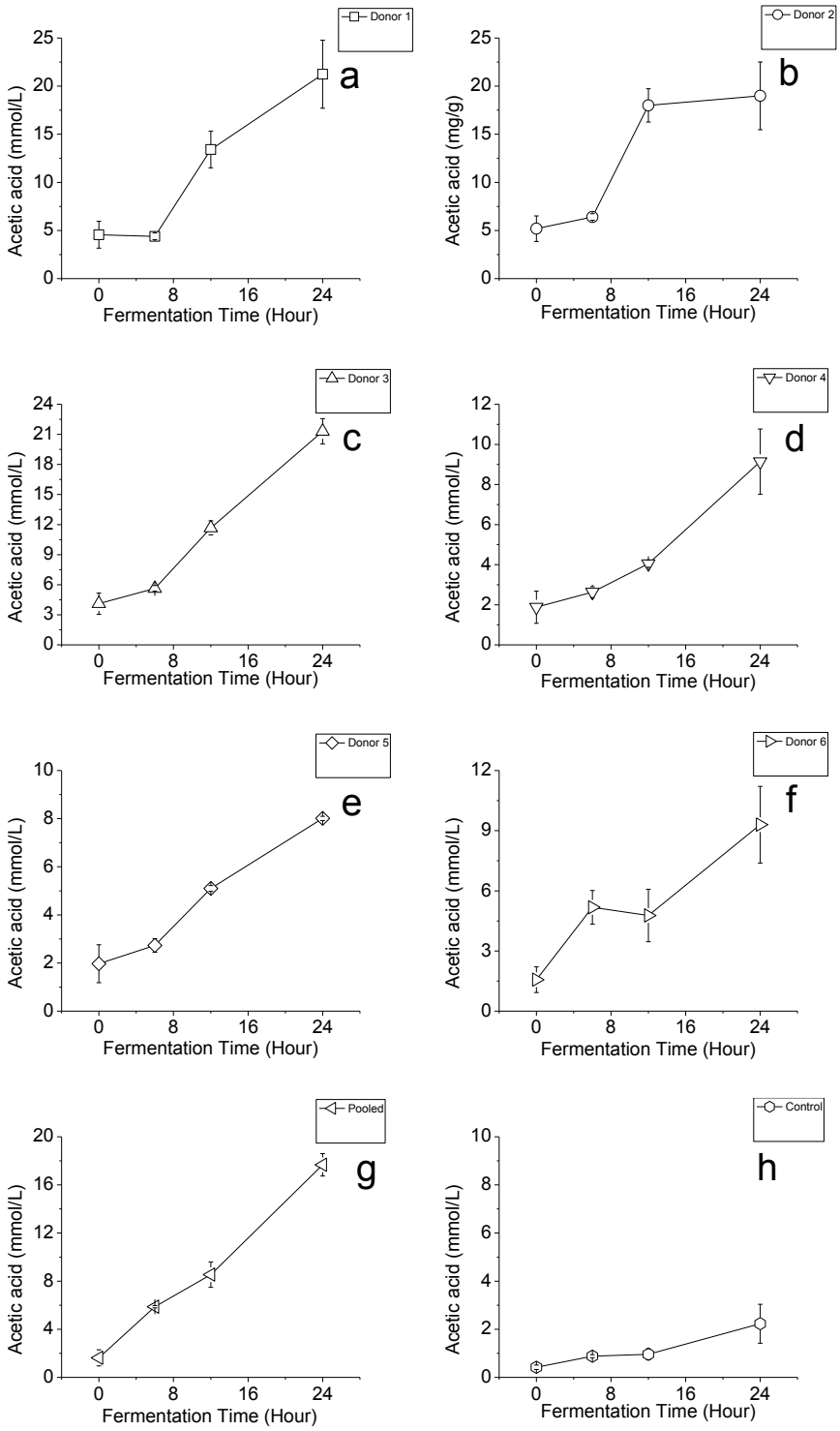


Figure A.11. Acetate changes in individual fecal slurry over 24 hour of fermentation of cocoa sample Lavado in the *in vitro* GI tract model system.

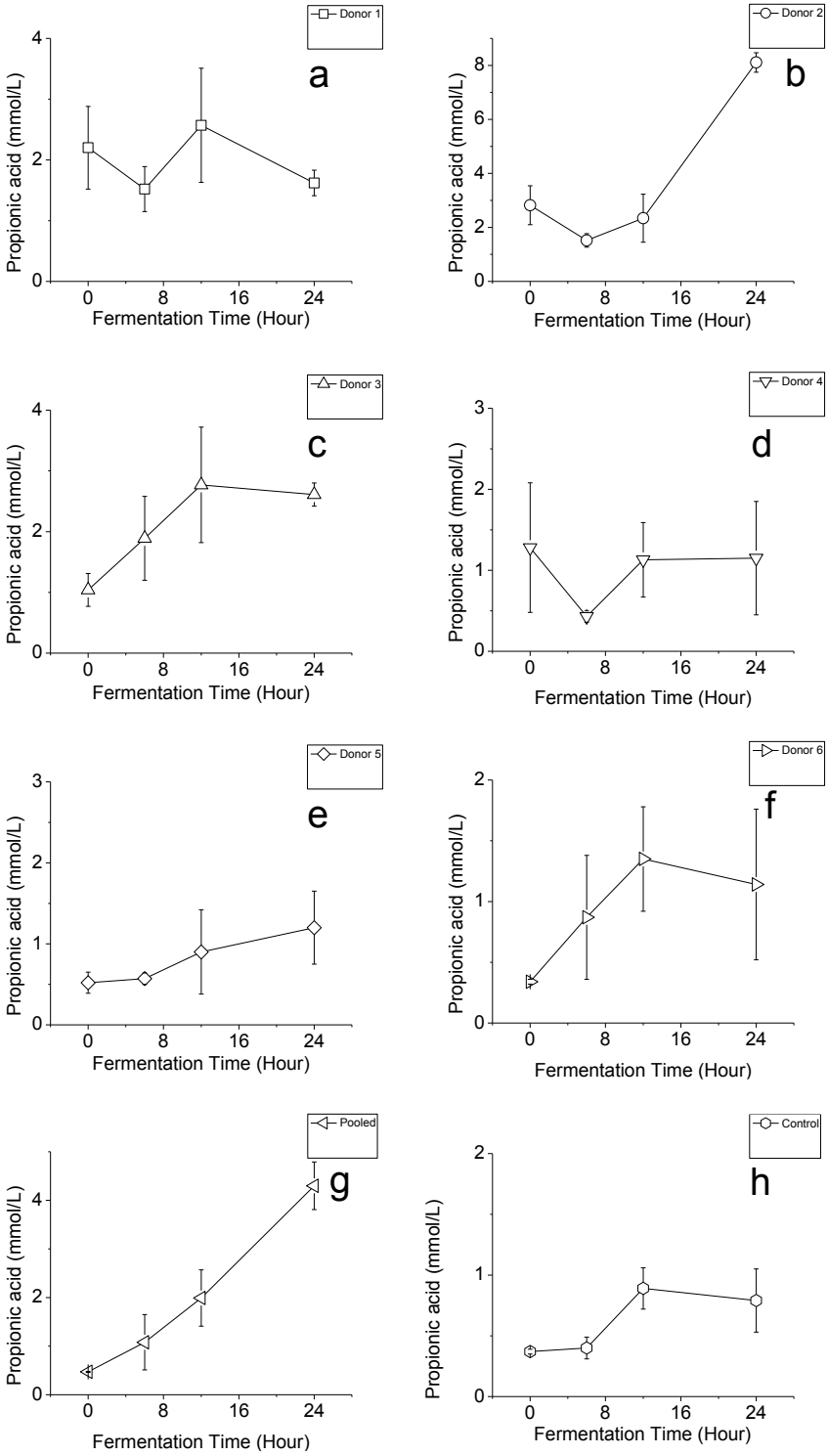


Figure A.12. Propionate changes in individual fecal slurry over 24 hour of fermentation of cocoa sample Lavado in the *in vitro* GI tract model system.

VITA

Zuyin Li was born in Hunan, China on February. He received his Bachelor of Engineering degree in Food Science and Technology in 2009 from Jiangnan University, China. He received his Master of Science degree in Food Science in 2012 from Jiangnan University, China. Zuyin Li started his PhD program of Food Science at Louisiana State University in June 2013. He plans to graduate in May 2018.