Antimicrobial activity of flavan-3-ols and oligomeric proanthocyanidins from pea seed coats and grape seeds against food and gut bacteria

by

Maria Solis Ares

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

in

Food Science and Technology

Department of Agricultural, Food and Nutritional Science
University of Alberta

Abstract

Proanthocyanidins and their monomeric subunits flavan-*3*-ols belong to the polyphenol group of flavonoids. They have been proposed to exert health promoting effects against cancer, Type 2 diabetes and coronary disease among other conditions. These health promoting effects are believed to be related to the modulation of gastric transit, the inhibition of digestive enzymes, the modulation of the gut microbiota and the direct health promoting effects upon absorption, but the mechanisms remain unknown. This thesis focuses on the properties of modulation of gut microbiota as well as the inhibition of digestive enzymes. The proanthocyanidin extracts from pea seed coats were analyzed using UPCL-ESI-MS prior to their separation using High Speed Counter Current Chromatography (HSCCC), a technique with several advantages over other chromatographic methods. No flavan-*3*-ols or proanthocyanidins were identified in these extracts, hypothesizing that these molecules underwent oxidation and are now highly polymerized. Commercial grape seed extract was selected as the raw material for the separation of proanthocyanidins by their degree of polymerization. It was possible to isolate 3 fractions: Fraction I with trimeric proanthocyanidins, Fraction II with trimeric and dimeric proanthocyanidins and Fraction III with dimeric proanthocyanidins as well as a polymeric proanthocyanidin fraction; flavan-*3*-ols were commercially available. The antimicrobial effect of flavan-*3*-ols increased with the number of hydroxyl groups in the molecule, specifically by OH groups and gallate moieties located on the B ring of the monomer. The antimicrobial effect of oligomeric proanthocyanidins from grape seed extract does not increase with the length of the oligomer. The enzyme inhibition properties of flavan-*3*-ols is increased with the number of hydroxyl groups present in the

monomer, but there was no evidence that this trait increased with the length of the oligomer in the case of grape seed proanthocyanidins. Gut microbiota modulation and enzyme inhibition properties of flavan-*3*-ols and proanthocyanidins cannot entirely explain the health promoting effects exerted by these compounds. Therefore, further research needs to be done in the modulation of the gastric transit by flavan-*3*-ols and oligomeric proanthocyanidins as well as their direct effects upon absorption; the determination of the antimicrobial effect of flavan-*3*-ol metabolites will be useful for a more thoroughly understanding of the modulation of gut microbiota by flavan-*3*-ols and proanthocyanidins.

Preface

This thesis is an original work by Maria Solis Ares. No part of this thesis have been previously published. Separation of proanthocyanidins was carried out in the department of Food Technology from the University of Bonn, Germany with Dr. Fabian Weber and Dr. Andreas Schieber as the lead collaborators. The experimental work in the anaerobic chamber was supported by fellow student, Tinting Ju.

Acknowledgements

First of all I would like to thank Dr. Michael Gänzle for the amazing opportunity to work in his lab, without his guidance and support throughout these two years, this thesis would not have been possible. I feel immensely lucky and honoured to have worked in 2-50 lab with several colleagues which are as passionate for food microbiology as I am. I am especially thankful to Nuanyi Liang, who was my mentor and first friend in the lab, thanks for sharing your knowledge and encouraging me to always look further; to Yalu Yan, for all those amazing coffee breaks that helped me relax and unwind; Kaixing, Janu, Arisha, thanks for all your help and friendship. I also want to thank Tingting Ju, who kindly provided me with the strict anaerobic strains and taught me how to use the anaerobic chamber.

Dr. Jocelyn Ozga, Dr. Jonathan Curtis and Dr. Ben Willing, thank you for your guidance as my committee members. I feel very lucky to have worked with Dr. Andreas Schieber and Dr. Fabian Weber from the University of Bonn, I am very grateful for all their help with the separation of proanthocyanidins. Dr. Lynn McMullen, thank you for all your time, advice and very helpful tips during lab meetings.

I would like to thank my family, specially my mom who taught me to pursue my dreams, to never give up and always encouraged me to follow my path on science. Abuelona, thanks for flying all the way from Mexico to make sure I was doing fine. To my boyfriend Rodri, for being there for me always in the bad and good moments, I am looking forward to what life has for us. Thanks to my friends back in Mexico City: Karen, Miriam, Chris and Jazz, that despite of the distance we are still very close; to my Mexican friends here in Edmonton: Ana and Natalia, thanks for the support and encouragement with my photography hobby. Lastly, I would like to thank CONACYT and the Alberta Pulse Growers and Western Grains for providing the funding to carry out this research project which helped me grow professionally and personally.

Table of contents

List of figures

List of tables

List of abbreviations

Abbreviation	Definition
C	Catechin
c-AMP	Cyclic adenosine monophosphate
CCK	Cholecystokinin
Da	Dalton
EC	Epicatechin
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
ESI	Electrospray ionization
FAB	Fastidious anaerobe broth
GLP-1	Glucagon-like peptide-1
GPCR	G-protein coupled receptors
GO	Glucose oxidase
GSE	Grape seed extract
HPLC	High-performance liquid chromatography
HSCCC	High-speed counter-current chromatography
INT	Iodonitrotetrazolium
IP3	Inositol triphosphate
LB	Luria Bertani (broth)
LC	Liquid chromatography
MALDI-TOF	Matrix assisted laser desorption/ionization-Time of flight
MBC	Minimal bactericidal concentration

mDP	Mean degree of polymerization
MH	Mueller-Hinton (broth)
MIC	Minimal Inhibitory Concentration
MRS	de Man, Rogosa and Sharpe (media)
mMRS	Modified de Man, Rogosa and Sharpe (media)
MS	Mass spectrometry
NADH	Nicotinamide adenine nucleotide
ND	Not determined
NMR	Nuclear magnetic resonance
PA	Proanthocyanidin
POD	Peroxidase
PSC	Pea seed coat
PSCE	Pea seed coat extract
Tas2R	Type 2 taste receptor cells
TRC	Taste receptor cell
UPLC	Ultra-performance liquid chromatography
UV	Ultra violet
v	Volume

1. Introduction

1.1 Dietary polyphenols

Polyphenols are abundant and widely distributed secondary plant metabolites (Harborne, 2013). They are associated in the defense against ultraviolet radiation and microbial attack (Beckman, 2000). More than 8,000 polyphenols have been identified in plants (Pandey and Rizvi, 2009), ranging from very simple molecules such as phenolic acids, to molecules with high degree of polymerization, some polymers with molecular weights up to 30,000 Da (Bravo, 1998). They usually occur in conjugated form with sugars residues (monosaccharides or polysaccharides), glucose being the most common sugar residue; galactose, rhamnose, xylose, arabinose, glucoronic and galacturonic acids are also found (Bravo, 1998). It is common for polyphenols to associate with other compounds such as organic and carboxylic acids, amines and lipids, as well as other phenols (Kondratyuk and Pezzuto, 2004).

Polyphenols are classified into different groups depending on their basic chemical structure: The number of phenolic rings in the molecule and the structural elements that bind the rings together (Harborne, 2013). The main classes are phenolic acids, flavonoids, stilbenes and lignans (Spencer et al., 2008); flavonoids being the widest studied class (Bravo, 1998; Pandey and Rizvi, 2009). Phenolic compounds exhibit a very interesting array of biochemical and physiological effects, acting as antioxidants and radical scavengers as well as metal chelators (Morel et al., 1993). There are several studies on the antioxidant properties of polyphenols, although the results *in vivo* are not conclusive (Middleton, Kandaswami and Theoharides, 2000).

As the antioxidant and radical scavenging effects of polyphenols does not explain the health promoting effects such as glucose homeostasis (Ding et al., 2013) and prevention of cardiovascular disease (Rasmussen et al., 2005) attributed to these compounds, looking more closely on other aspects is encouraged. The health promoting effects exerted by polyphenols are possibly linked to the modulation of the intestinal transit by the bitter taste receptors in the gut, the inhibition of digestive enzymes in the stomach, small and large intestines, the modulation of the gut

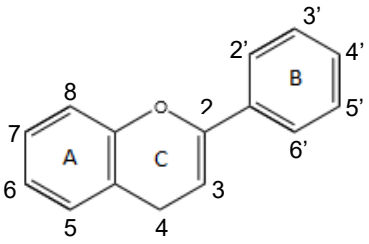
microbiota and the direct health promoting effects upon absorption in digestive tract, mainly stomach, small and large intestines.

This thesis will emphasize on flavan-*3*-ols and oligomeric proanthocyanidins, as they are ubiquitous to almost all plants (Middleton et al., 2000). The experiments described in this thesis aimed to explain how the structure of oligomeric proanthocyanidins and their subunits are related to the antimicrobial effects exerted against an array of food and intestinal bacteria, as well as the digestive enzyme inhibition properties of these molecules.

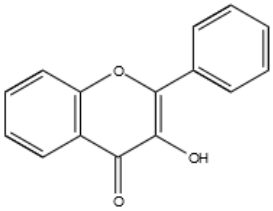
1.2 Flavonoids

Proanthocyanidins are composed of flavon-*3*-ols; these molecules belong to the group of flavonoids. They are also known as condensed tannins as they can denature or 'tan' proteins. Flavonoids are synthesized from phenylalanine and tyrosine (Havsteen, 2002; Pietta, 2000). More than four thousand molecules have been identified (Iwashina, 2000), many of them responsible for the appealing colors of flowers, fruits and leaves (Groot and Rauen, 1998). As plants are not able to move, they use flavonoids as one of several mechanisms to stay fit and survive (Treutter, 2006); they function as plant defense against herbivore and microbial attack (Dixon et al., 2005) and inhibit viral enzymes (Havsteen, 2002).

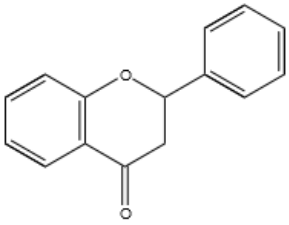
Flavonoids also help the plants to deal with environmental stress, as they were found to help them survive in soils with high concentration of toxic metals such as aluminum (Barcelo and Poschenrieder, 2002), photo protection against ultra-violet radiation has also been reported (Ryan et al., 2002). They have a basic skeleton consisting of two aromatic rings (Ring A and B) and a pyran ring (Ring C). The differences between the flavonoid family compounds lie on the saturation of heteroatomic ring (Ring C), the hydroxylation pattern, the attachment of ring B at carbon-2 or carbon-3 position of the pyran ring, and the molecules conjugated to the aromatic ring (Grotewold, 2005). Based on these differences, flavonoids can be divided in flavonols, flavanones, flavanols, flavones, anthocyanins and isoflavones (Pandey and Rizvi, 2009).



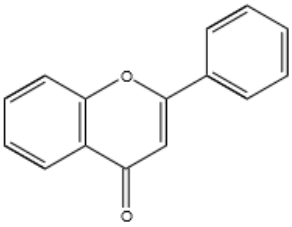
Basic flavonoid structure



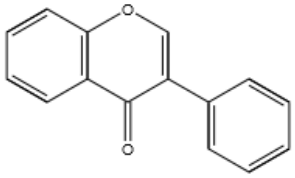
Flavonols



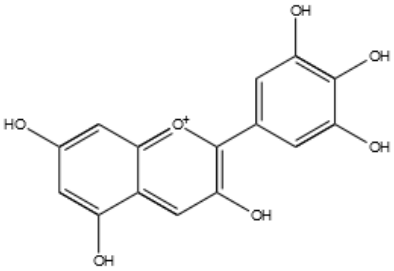
Flavanones



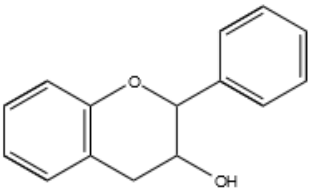
Flavones



Isoflavones



Anthocyanins



Flavan-*3*-ols

Figure 1. Basic flavonoid structure and flavonoid subclasses (modified from Pandey and Rizvi, 2009).

1.3 Proanthocyanidins

Proanthocyanidins are oligomeric and polymeric end products of the flavonoid biosynthetic pathway (Dixon et al., 2005). These compounds are responsible for the aspects of flavor and astringency of beverages such as wine, teas and fruit juices

(Porter, 1989). Another group of tannins, known as hydrolysable tannins, is composed by esters of phenolic acids and a polyol, for example gallic acid in gallotannins plus glucose; but proanthocyanidins are far more common in the human diet (Clifford and Scalbert, 2000).

These polymeric flavonoids are composed by monomer units known as flavan-*3*-ols; being (+)-catechin (2,3-*trans* stereoisomer) and (–)-epicatechin (2,3-*cis* stereoisomer) the most abundant ones in plant materials (Dixon et al., 2005).

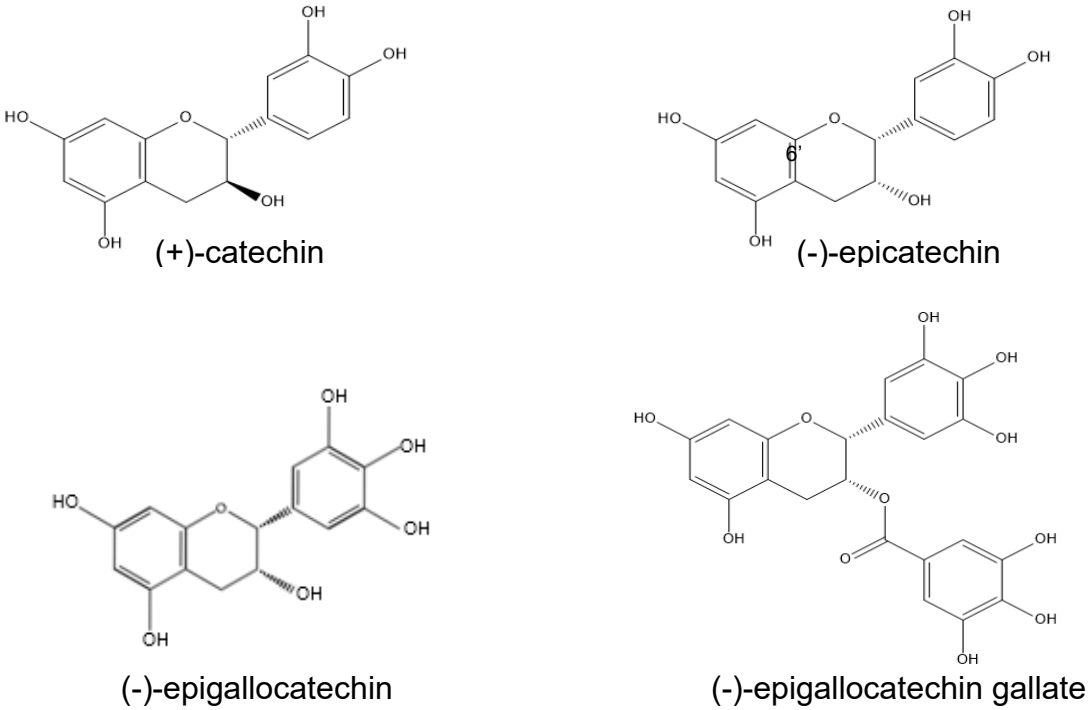


Figure 2. Chemical structure of some common flavan-*3*-ols (modified from Aron and Kennedy, 2007). The structure of an oligomeric proanthocyanidin is shown in Figure 3.

Accordingly, proanthocyanidins vary in terms of their degree of polymerization, the presence or absence of modifications to the 3-hydroxyl group, such as esterification, and the stereochemistry and hydroxylation patterns of the flavan-*3*-ol units (Foo and Porter, 1980).

Flavanol units can be linked to acyl moieties. Gallic acid is the most common substituent forming an ester in the C3 position hydroxyl group (Santos-Buelga and Scalbert, 2000) as in tea (Nonaka et al., 1983) and wine (Prieur et al., 1994). Glycosyl substituents can also link flavanols, the hydroxyl group at C3 position is where the sugar generally attaches (Kashiwada et al., 1986) or less commonly, it attaches to the C5 position (Gujer et al., 1986).

These monomers link successively to each other by C4 of the ‘upper’ unit to C8 position of the ‘starter’ or ‘lower’ unit. Less frequently, C4 from the ‘upper unit’ links to C6 of the ‘lower’ unit, forming B-type proanthocyanidins (Porter, 1989); interflavanoid bonds (C-O) occur, formed by an oxidative coupling and these molecules are known as A-type proanthocyanidins (Köhler et al., 2008). Type B proanthocyanidins are mostly present in fruits, followed by legumes and cereals and also in their derived products (wine, beer, cider, etc.); type A proanthocyanidins are mostly found in cranberry, cinnamon and avocado (Monagas et al., 2010; Wang et al., 2010; Foo et al., 2000).

Proanthocyanidins are susceptible to oxidation, light and moisture and they are not thermostable. From the plant extraction step to their identification, methods must be carefully chosen to avoid degradation. Some measures such as keeping the plant sample frozen or lyophilized are recommended to avoid enzymatic activity as well as filling the container with nitrogen to avoid or reduce oxidation during storage (Yang, 2014).

Recently, proanthocyanidins have been widely studied due to suggested biological activities such as high antioxidant properties (Amarowicz et al., 2000; Dixon et al., 2005) and several interesting health promoting effects (Scalbert et al., 2000). Although proanthocyanidins are believed to be beneficial, they are also linked to anti-nutritive effects, as they bind dietary proteins and fibre as well as minerals, delaying their digestion and absorption (McSweeney et al., 2001). Proanthocyanidins can also interfere with digestion by complexing digestive enzymes and endogenous protein (Butler, 1992). The mechanisms by which proanthocyanidins exert beneficial and adverse health effects are discussed in the next paragraphs.

1.4 Bitter Taste and Modulation of Kinetics of Digestion

Molecular sensing of luminal contents in the intestinal tract is crucial as it is involved in several functions as secretory activity of gastrointestinal glands, blood supply, absorptive activity and motility; it also turns on hormonal/neural pathways that modulate biological responses like caloric intake and pancreatic insulin secretion (Rozengurt, 2006).

The gustatory system has evolved to detect bitter taste as likely harmful; this as a warning against the ingestion of toxic substances like plant alkaloids among other environmental toxins (Scott, 2005).

In the oral cavity, type II taste receptor cells (TRC) is where bitter taste receptors are expressed; these cells located into taste buds on the tongue (Avau and Depoortere, 2016); taste receptors contain several proteins like ion channels, ligand-gated channels, transporters and G protein-coupled receptors (Sternini, 2007). In the case of human genome, it encodes 25 different type 2 receptors that can be characterized in 3 different groups: specialists, promiscuous and intermediate in terms of their selectivity (Meyerhof et al., 2010). These Tas2R are a subgroup of the guanine nucleotide-binding regulatory protein (G-protein) (Lindemann, 1996; Chen et al., 2006), which contain seven transmembrane α-helices (Ji, Grossman and Ji, 1998).

Bitter taste stimuli activate the G-protein coupled receptors (GPCR) which results in the synthesis of secondary messenger molecules, such as cyclic adenosine monophosphate (cAMP) and inositol trisphosphate (IP3), leading to the release of intracellular Ca^{2+} (Herness and Gilbertson, 1999).

Bitter taste receptors have been found in several extra-oral tissues including the gut, heart, thyroid and brain (Avau and Depoortere, 2016). This intriguing finding suggests that bitter taste receptors have additional functions besides identifying poisonous food (Avau et al., 2015; Shaik et al., 2016).

Gut endocrine cells, which resemble the cells in the tongue by having ‘microvilli like’ protrusions (Höfer et al., 1996) have been found to possess taste receptors on their surface. These receptors are responsible for the sensing of nutrients, as well as gut

microbial metabolites (Breer et al., 2012). These cells are known as ‘solitary chemosensory cells’ (Avau and Depoortere, 2016), ‘microvillar cells’ (Kusumakshi et al., 2015), ‘brush cells’ (Krasteva et al., 2012) or ‘neuroendocrine cells’ (Merigo et al., 2005) depending on their properties and their morphology. More research and discussion are needed to unify a name for this kind of cells which can be either different subtypes (based on functional properties or location in the body) or a unique cell type.

Bitter taste receptors are also responsible for regulating the release of gastrointestinal peptides and subsequently modulate ingestive behaviour (Depoortere, 2015). Some of these peptides are glucagon-like peptide-1 (GLP-1), neuropeptide Y, cholecystokinin (CCK), vasoactive intestinal peptide and ghrelin (Dotson, Geraedts and Munger, 2013).

Tas2Rs are believed to be involved in a ‘negative feedback loop’ as the intra-gastric administration of denatonium benzoate, a potent bitter molecule, to healthy patients caused a delay of gastric emptying resulting in an increased satiation feeling, and a decrease in nutrient volume tolerance (Janssen et al., 2011, Avau et al., 2015). The conclusion of these studies was that ingestion of bitter compounds result in decreased food intake (Avau et al., 2015).

Bitter tastants were responsible for changes in the contractility of the intestinal muscle fibres and the delay of gastric emptying in mice gut. In conclusion: bitter taste receptors in the gut slow the rate at what bitter and possibly harmful substances are ingested, providing the body with more time to metabolize these compounds before they go into to the systemic circulation (Glendinning et al., 2008).

As bitter taste receptors modulate the kinetics of digestion, they will influence the availability of other nutrients as well.

1.5 Protein binding and enzyme inhibition by proanthocyanidins

The sensation of astringency generated by proanthocyanidins in the mouth is due to the formation of nonspecific complexes with salivary proteins (Rinaldi et al., 2014; Baxter et al., 1997). Proanthocyanidins bind dietary proteins and digestive enzymes, resulting in the decreased absorption of nutrients (Barrett et al., 2013). This binding property is possible due to the abundant hydroxyl groups in proanthocyanidins (Scalbert et al.,

2000) that bind the amino groups in peptides or other molecules (Siebert, Troukhanova and Lynn, 1996). For hydrolysable tannins as well as condensed tannins, larger polymers with more co-ordinated hydroxyl groups bind proteins more efficiently than smaller molecules (Baxter et al., 1997).

The slower digestion of nutrients due to the inhibition of digestive enzymes is not always detrimental. In the case of starch, if α-amylase and glucoamylase are inhibited, glucose levels in blood after the consumption of a meal will be lower; thus, this inhibition can be used to modulate blood glucose levels in patients with metabolic disorders like diabetes (Barrett et al., 2013; Li et al., 2005; Apostolidis et al., 2003).

The inhibition of digestive enzymes has been reported since 1960's. β-Glucosidase was partially inhibited by a proanthocyanidin fraction from wattle or Acacia tree (Goldstein and Swain, 1964). In the case of trypsin and α-amylase, they were inhibited by the proanthocyanidin fraction from lentils, cocoa beans and pears (Quesada et al., 1996). Proanthocyanidins from carob (*Ceratonia siliqua*) also strongly inhibited the activity of trypsin, α-amylase and lipase. Proanthocyanidin inhibition was compared to *m*-digallic acid (at the same concentrations) finding that trypsin was inhibited in a greater extent by proanthocyanidins; lipase was more inhibited by the digallic acid, whereas for α-amylase, it was inhibited in the same extent by the two types of tannins (Tamir and Alumot, 1969). The inhibition of trypsin, α-amylase and lipase increases with the degree of polymerization of proanthocyanidins (Horigome et al., 1988).

Proanthocyanidins inhibit bacterial enzymes; one example is the inhibition of urease activity in *H. pylori* (Adeniyi et al., 2009). Urease is used by *H. pylori* to release ammonia and be able to survive in the acid gastric environment (Cires et al., 2016).

Another interesting finding suggests that tea catechins are not able to inhibit the growth of E*scherichia coli* O157:H7 but they affect some virulence factors like biofilm formation (Lee et al., 2009). Tannins bind surface proteins, thus inhibiting adhesion. A-type proanthocyanidin trimers from cranberries (*Vaccinium macrocarpon)* prevented the infection of P-fimbriated *Escherichia coli* in the urinary tract by inhibiting the adherence to the tissue (Foo et al., 2000) again binding surface proteins.

The protein precipitating capacity of proanthocyanidins increases with the increase in the mean degree of polymerization. It also depends on the stereochemical properties, the type of linkage among flavan-*3*-ols and the amount of hydroxyl groups in the molecule (Jonker and Yu, 2017). It has been suggested that the molecular weight of proanthocyanidins is the most important factor for protein precipitation (Ropiak et al., 2017), but with mean degree of polymerization above eight there is no further change in proanthocyanidin-protein interactions (Harbertson et al., 2014).

1.6 Modulation of gut microbiota through microbial metabolism and antimicrobial activity

1.6.1 Gut microbiota

The human gut microbiota is a complex community of microbes that inhabit the human intestine. Gut microbiota has an impact on the health and disease of the host due to its collective metabolic activities and host interactions (Lozupone et al., 2012). Some examples of these interactions are the ability to break down indigestible components of the human diet, such as plant polysaccharides, which are the most abundant biological polymers, and carbohydrate fermentation with short-chain fatty acids as a product (Brune and Friedrich, 2000). It also provides protection against pathogens by competitive exclusion or production of antimicrobial compounds (Sekirov et al., 2010) and plays an important role in the modulation of the immune system (Bäckhed et al., 2005).

The human intestinal microbiota comprises at least 10^{13} microbial cells, the majority of them being strict anaerobes (Bäckhed et al., 2004). Members of the *Firmicutes* and *Bacteroidetes* phyla are the most abundant (Zoetendal et al., 2008; Dueñas et al., 2015), whereas *Proteobacteria, Verrucomicrobia, Actinobacteria, Fusobacteria*, and *Cyanobacteria* are present in minor proportions (Eckburg et al., 2005).

The microbiota changes in number and composition along the intestinal tract; in the stomach and duodenum there are approximately 10^1 to 10^3 cells per gram of intestinal content, jejunum and ileum: 10^4 to 10^7 cells per gram, and 10^{11} to 10^{12} bacteria per gram in the distal colon (Sekirov et al., 2010). Frank et al (2007) compared biopsy

samples from healthy individuals and reported that the small intestine is abundant in bacilli, whereas in the colonic samples they found *Bacteroidetes* and *Firmicutes*.

The composition of the gut microbiota in each person is different and unique (Van Duynhoven et al., 2011), it is acquired at birth and can be influenced by the environmental stress and the changes in diet; it also depends on the genotype and the health status of the host (Turnbaugh et al., 2009; Vrieze et al., 2010). It is considered stable throughout adulthood, but can be modified by changes in diet, general lifestyle or antibiotic intake. These alterations and imbalances in the gut species composition, known as dysbiosis leads to disease (Nicholson et al., 2012); some of these diseases being: obesity, cardiovascular disease, diabetes, inflammatory bowel disease (which includes Crohn's disease and ulcerative colitis), among others (Tremaroli and Bäckhed, 2012; Sekirov et al., 2010).

A recent study analyzed the changes in gut microbiota after inbred, transgenic and outbred mice were fed with high-fat and high-sugar diets finding that the composition of microbes shifted in the same way despite of the different genotypes, concluding that the shaping of the gut microbiota depends mostly on the host diet (Walter, 2015). It was also found that the total dietary composition has a bigger impact in the gut microbiota changes than the total caloric intake (David et al., 2014). Dietary fibre is a significant energy source for members of the gut microbiota and the fermentation of these non-digestible carbohydrates results in the formation of short chain fatty acids (SCFAs) (Tremaroli and Bäckhed, 2012), acetate, propionate and butyrate being the most abundant (den Besten et al., 2013).

A study comparing the faecal microbiota of African children with mostly vegetarian diets vs. European children eating diets high in protein, sugar and starch and low in fibre showed that African children have high numbers of *Bacteroidetes* and a reduction *in Firmicutes* whereas *Prevotella* and *Xylanibacter*, which contain several cellulose and xylan hydrolysis bacterial genes, were particularly abundant. In the case of western children, *Prevotella* and *Xylanibacter* were not present whereas *Firmicutes* and *Proteobacteria* were abundant (De Filippo et al., 2010). In conclusion, diet is able to shift the gut microbiota composition, which leads to very different intestinal microbiomes

in humans. In this previous study, fibre played an important role but the specific components of the diet that have a major impact in shaping the gut microbiota remain to be discovered.

Proanthocyanidins are ubiquitous to almost all the plants (Middleton et al., 2000) where fibre is the major cell wall component (Dhingra et al., 2012) and therefore a significant part of the human diet. Proanthocyanidins influence gut microbiota by serving as substrate for bacterial metabolism, which in turn modified their bioavailability and biological activities, and by exerting direct antimicrobial effects, these properties are discussed in the next paragraphs.

1.6.2 Absorption and metabolism of proanthocyanidins by gut microbiota

The daily intake of PAs varies around 0.1 to 0.5 grams (Santos-Buelga and Scalbert, 2000) depending on how rich the diet is in terms of fruits and vegetables, and some beverages as red wine and tea.

After ingestion, the proanthocyanidins that were not absorbed in the small intestine, reach the colon (Clifford, 2004), where they are metabolized by colonic gut microbiota (Dueñas et al., 2015).

The absorption degree depends on the general structure of the proanthocyanidin, molecular weight and esterification of the molecules (Deprez et al., 2001; Scalbert et al., 2002). Catechins are more easily absorbed than proanthocyanidins and flavan-*3*-ol gallates (Crozier, del Rio and Clifford, 2010). There are several studies on PA rich tea and wine extracts showing that these compounds prevent from some types of cancer and cardiovascular diseases (Santos-Buelga and Scalbert, 2000).

The monomer (+)-catechin and the proanthocyanidin dimer B3 are degraded mainly to phenolic acids by rat caecal microflora (Groenewoud and Hundt, 1984 & 1986). A study using Caco-2 cells, which are a human colon epithelial cancer cell line, as a model suggested that (+)-catechin and PA oligomers can be absorbed by the human gut epithelium (Deprez et al., 2001). Oligomers from dimers to pentamers were detected in rat plasma two hours after the rats were fed with apple proanthocyanidins (Shoji et al., 2006).

The absorption of monomers and dimers had been controversial as a study that used an *in vivo* rat model reported that proanthocyanidin dimers (B1, B2 and B3) were not absorbed nor hydrolyzed (Donovan et al., 2002). Another *in vivo* study showed that proanthocyanidin oligomers were not converted to monomers after rats were fed with grape seed extract (Tsang et al., 2005), monomers are absorbed in the intestine (Kale et al., 2010).

The metabolism and absorption of PAs depend largely on the degree of polymerization. A study which used a rat model suggests that the high DP of the proanthocyanidins resulted in decreased absorption and decreased microbial metabolism; these are as consequence of the tendency of tannins to bind proteins, making them unavailable for microbial attack (Gonthier et al., 2003).

PA polymers that are able to reach the colon were considered relatively indigestible because they were recovered in feces without changes (Scalbert et al., 2000); but Deprez et al (2000) were the first to find evidence on PA polymer bacterial degradation. Briefly, they performed an *in vitro* assay using human fecal microbiota, under anaerobic conditions and a mixture of ^{14}C-labeled PA polymers with a mean degree of polymerization of six; then they measured the degradation PA products by thiolysis. The degradation of the polymers was extensive and the main product was: 3-(*m*-hydroxyphenyl) propanoic acid, followed by hydroxyphenil acetic acid, hydroxyphenyl valeric acid and phenylpropionic acid; these low-molecular-weight aromatic acids can be subsequently absorbed in the gut (Das, 1971).

In the colon, gut microbiota degrade non-digested food components and transform them into microbial metabolites (Kleesen et al., 2000). For degradation of flavonoids by gut microbiota, the first step is the cleavage of the glycosyl moieties known as deglycosylation, which is catalyzed by α-rhamnosidase, β-glucosidase and β-glucuronidase (Aura, 2008). Catechins and proanthocyanidins are rarely glycosylated (Clifford, 2004; Škerget et al., 2005). For glycosylated flavonoids, *Bacteroides distasonis*, *Bacteroides uniformis* and *Bacteroides ovatus* hydrolyze glycosides to aglycones (Bokkenheuser et al., 1987). Other strains hydrolyzing glycosides are

Bacteroides fragilis, Bacteroides distasonis, Clostridium cocleatum and *Clostridium ramnosum* (Clavel et al., 2006).

The metabolism of catechins and proanthocyanidins by the gut microbiota also includes ring-fission and several reactions involving functional groups of the flavonoid aglycones and phenolic acids, such as decarboxylation, dihydroxylation or demethylation (Griffiths, 1982).

All the microbial phenolic metabolites that are absorbed in the intestine go to the liver via the portal vein where they undergo Phase II metabolism: glucuronidation, methylation, sulfation or a mix of these reactions; then, these compounds enter the systemic circulation and are delivered to the organs, or excreted in the urine (Cardona et al., 2013). Figure 3 shows the microbial metabolism proposed for a PA trimer with phenylpropionic acids and benzoic acid as products:

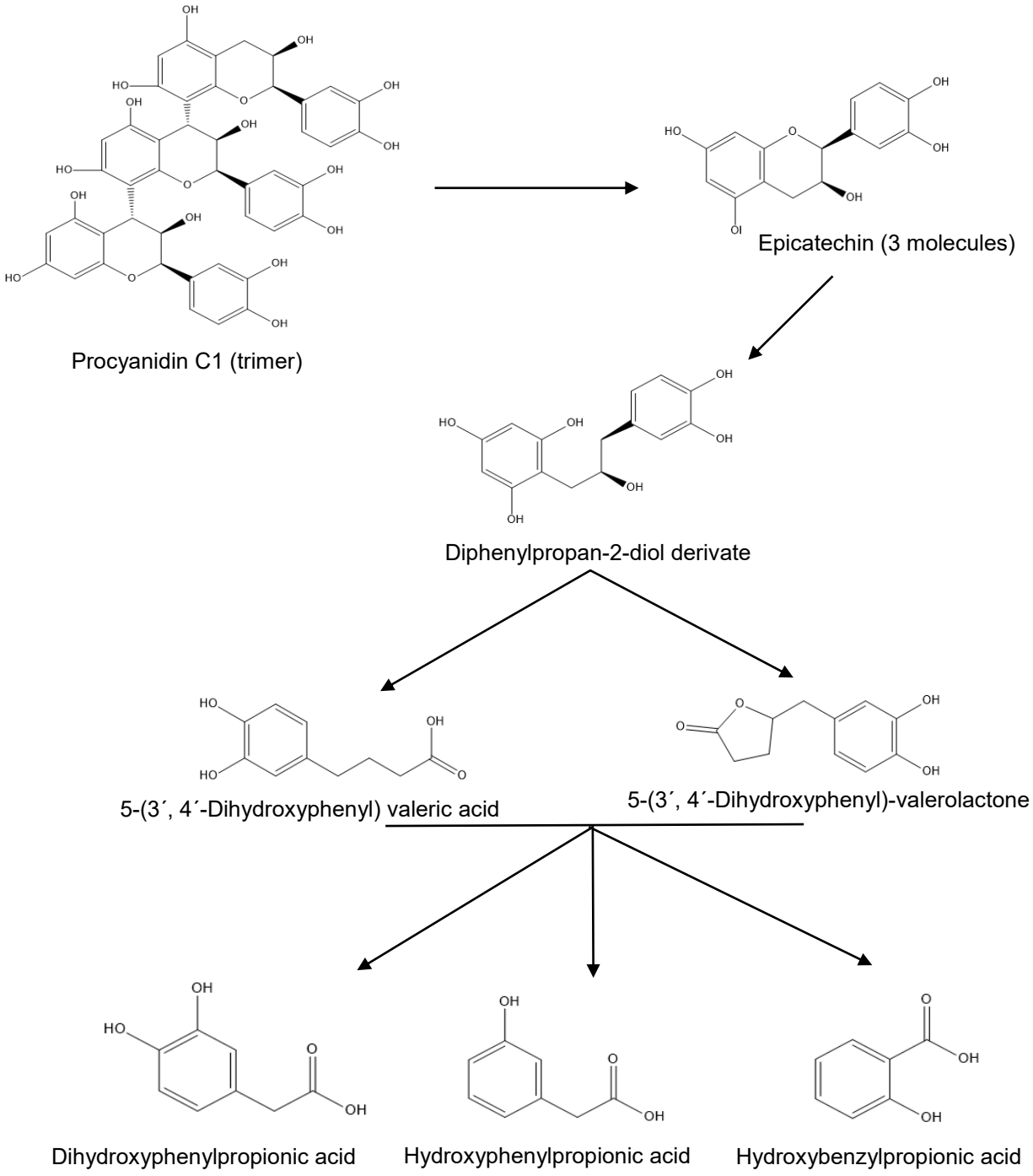


Figure 3. Degradation of proanthocyanidins by colonic microbiota (modified from Marin et al., 2015)

As shown in Figure 3, after the gallate ester has been metabolized, the C-ring of the flavan-*3*-ol is opened forming a diphenylpropan-2-diol derivate, which is further transformed to 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone; the valerolactone ring can be opened forming 5-(3,4-dihydroxyphenyl)valeric acid. These molecules can undergo further transformations giving rise to phenylpropionic and benzoic acids (Marin et al., 2015).

In a group of humans that were given 150 mg of green tea solids, 5-(3,4-dihydroxyphenyl)-γ-valerolactone was found in plasma and urine. This molecule is the product of the ring fission of EGCG (Epigallocatechin gallate), a reaction that is carried by gut microbiota. Before ring fission, EGCG undergoes several reactions such as methylation, glucuronidation and sulfation majorly in the small intestine and liver (Meng et al., 2001).

The *in vitro* degradation of radiolabeled PA oligomers from willow tree shoots by human gut microbiota was analyzed. Briefly, 14C-labeled PA oligomers with a mean DP of 6 were incubated with human gut isolates in anaerobic conditions. The proanthocyanidin concentration was measured by thiolysis, finding a major drop in the PA concentration, meaning they were metabolized. Six phenolic acids were detected using Gas chromatography-Mass spectrometry analysis (Deprez et al., 2000).

Although the colon has a very dense bacterial population, only a few bacterial species that metabolize proanthocyanidins and flavan-*3*-ols have been identified along with their catabolic pathways (Cardona et al., 2013). The human faecal isolates *Eggerthella lenta* and *Flavonifractor plautii (*formerly *Clostridium orbiscindens*) degrade (−)-epicatechin and (+)-catechin into 5-(3,4-dihydroxyphenyl)-γ-valerolactone and 4-hydroxy-5-(3,4-dihydroxyphenyl) valeric acid (Kutschera et al., 2001).

Proanthocyanidins and flavan-*3*-ols, as well as their metabolites let some microorganisms grow and thrive and inhibit other bacteria, including pathogens (Lee et al., 2006; Selma et al., 2009; Hervert-Hernandez and Goñi, 2011; Tzounis et al., 2011). Nevertheless, the mechanisms of the modulation of gut microbiota or the ways in which their bioactive metabolites influence human health are poorly known and yet to be elucidated (Cardona et al., 2013).

1.6.3 Antibacterial Activity of Proanthocyanidins

Antibacterial agents can be 'bacteriostatic', meaning that the agent inhibits bacterial growth (i.e. keeping bacteria in stationary phase), whereas 'bactericidal' antibacterials kill bacteria. The determination of whether an agent is bactericidal or bacteriostatic can be influenced by several factors, such as test duration and growth conditions (Pankey and Sabath, 2004). For the measurement of antimicrobial activity, the most widely used methods are the determination of the Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC).

Proanthocyanidins inhibit bacteria by several mechanisms that depend on the chemical structure and the concentration of the compounds as well as the microbial strain (Almajano et al., 2007; Campos et al., 2003). They can bind to specific proteins in the cell membrane causing the leaking of several compounds/ions such as: potassium ions, glutamic acid, intracellular RNA, among others (Puupponen-Pimiä et al., 2005; Ikigai et al., 1993). The inhibitory effect of tannins can be explained by their iron binding capacity, which mainly affects aerobic microorganisms that need iron to form heme groups (Chung et al., 1998).

The mechanisms proposed for the growth inhibition of bacteria by proanthocyanidins are that the high number of hydroxyl groups in these molecules inhibit extracellular microbial enzymes by binding them; proanthocyanidins interfere directly into the microbial metabolism and that tannins possess metal ions complexation properties (Cos et al., 2004), especially iron and zinc which are essential mineral micronutrients for most microorganisms (Daglia, 2012).

Since 1990's the antimicrobial activity of proanthocyanidins on bacteria, yeasts and fungi has been recognized (Scalbert, 1991). In the case of flavan-3-ols, the monomeric units of the proanthocyanidins, several studies were based on tea extracts (Ahn et al., 1991; Diker et al., 1991; Sakanaka et al., 1992). Tea catechins such as (-)-gallocatechin-3-gallate, (-)-epigallocatechin-3-gallate, (-)-catechin-3-gallate (among others) possess antimicrobial activity at nanomolar concentrations against some foodborne pathogens like *Bacillus cereus* (Friedman et al., 2006).

Epicatechin and Catechin from tea isolates inhibit the growth of *Clostridium perfringens, Clostridium difficile* and *Bacteroides spp.* whereas some commensal anaerobes like *Lactobacillus spp.* and *Bifidobacterium spp.* remained almost unchanged (Lee et al., 2006; Tzounis et al., 2008).

Epigallocatechin gallate, which is the most widely studied flavan-*3*-ol, has demonstrated antibacterial properties against *Staphylococcus epidermidis* (Nishino et al., 1987) methicillin-resistant *Staphylococcus aureus* (MRSA) (Kono et al., 1994), *Streptococcus mutans* (Sakanaka et al., 1989), and *Helicobacter pylori* (Yanagawa et al., 2003). Enhanced antibacterial activity has been observed if a galloyl or gallic molecule is present in the position C3 of the flavan-*3*-ol C ring (Yang, 2014).

In the case of flavan-*3*-ols, the number of hydroxyl groups in the B-ring influences the antimicrobial capability (Scalbert, 1991). The presence of a gallic acid residue on the molecule also increases the antimicrobial activity of flavan-*3*-ols as (epi)gallocatechin and (epi)gallocatechin gallate have shown higher antimicrobial activity than catechin and its isomer: epicatechin (Taguri, Tanaka and Kouno, 2004; Scalbert, 1991). In the case of hydrolysable tannins, the number of gallic acid substituents has an impact on iron binding properties but did not influence the antibacterial activity; a higher number of gallic acid molecules means a higher iron binding capacity (Engels, Schieber and Ganzle, 2011; Engels, Ganzle and Schieber, 2009).

Prodelphinidins, which are oligomers of epigallocatechin and/or epigallocatechin-3-gallate, show higher antimicrobial activity than the monomers (Taguri, Tanaka and Kouno, 2004), supporting that a higher number of hydroxyl groups results in a higher antimicrobial activity.

1.7 Identification and isolation of Proanthocyanidins

Since the elucidation of PA basic structure during the 1960s, more than 200 PA oligomers with DPs as high as five have been identified and fully characterized (Foo and Karchesy, 1991). Usually, plant proanthocyanidins have a much higher degree of polymerization but they are hard to identify because they are present as complex mixtures in food extracts, resulting in poor solubility (Matthews et al., 1997a).

The most common methods for PA analysis are normal phase (Gu et al., 2004) and reversed-phase HPLC (High performance liquid chromatography) with UV detection (Cheynier et al., 1999; Hammerstone et al., 1999; Kennedy and Waterhouse, 2000). The disadvantages with reversed-phase HPLC are that the order of elution does not correspond to the degree of polymerization of the proanthocyanidins and that the separation of polymers with DP larger than four is not possible. The presence of several isomers results in overlapping peaks in the chromatogram. In the case of Normal-phase HPLC, the order of elution does increase with the increase in DP (Lazarus et al., 1999; Waterhouse et al., 1999). Chromatography with Sephadex LH-20 or Toyopearl resin have been frequently used as well (Kennedy and Taylor, 2003). The molecule structures can be identified by applying mass spectrometry (MS) and NMR (Nuclear magnetic resonance) spectroscopy (Foo et al., 1997). NMR analysis makes it possible to know the average DP, stereochemistry and the hydroxylation pattern of the B ring (Czochanska et al., 1980).

Large polymers are characterized by chemical degradation like the phloroglucinolysis reaction (Foo et al., 1997; Kennedy and Jones, 2001) or in the presence of benzyl mercaptan (Matthews et al., 1997b) which are acid-catalyzed condensation reactions (Schofield et al., 2001).

In the case of benzyl mercaptan reaction (also known as thiolysis), proanthocyanidins are heated in the presence of acid and toluene-α-thiol; the terminal unit in the chain is released and the other molecules in the chain are converted to benzyl thioethers (Thompson et al., 1972). With further HPLC analysis, determination of chain length and tannin composition is possible. However, thiolysis can be incomplete if the proanthocyanidins are not highly pure (Matthews et al., 1997b); side reactions also occur (Brown and Shaw, 1974) and some reaction products are very unstable (Hemingway and McGraw, 1983), these resulting in altered yields of the reaction; moreover, benzyl mercaptan is highly toxic and possesses a pungent smell (Schofield et al., 2001).

The phloroglucinolysis reaction has the same principle as the reaction with benzyl mercaptan. Proanthocyanidins are hydrolyzed in acidic conditions and the terminal unit

is released, the extension subunits are converted to electrophilic flavan-*3*-ol intermediates which are cleaved with phloroglucinol leading to the formation of adducts that can be further analyzed by reverse-phase HPLC (Kennedy and Jones, 2001).

These methods give us information about the average degree of polymerization and the terminal and inner units (Santos-Buelga and Scalbert, 2000). Nonetheless, the methods mentioned above involve large volumes of solvent and the irreversible adsorption of the sample to the column packing material resulting in very low yields (Kumar et al., 2009).

1.7.1 HSCCC (High-speed counter-current chromatography)

Recently, HSCCC (High-speed counter-current chromatography) has arisen as a more suitable technique for separation of natural compounds from small ions to macromolecules (Marston and Hostettmann, 1994; Degenhardt et al., 2000a, b) due to the elimination of solid supports, high purity of the fractions, high sample recovery due to no irreversible adsorption, minimal risk of sample denaturation and low sample consumption, resulting in money savings. (Ito, 1981; Ito and Conway, 1986; Marston and Hostettmann, 1994; Degenhardt et al., 2000a, b).

CCC is a term created by Ito (1981) to describe a support-free, liquid-liquid chromatography where the solutes are partitioned into two immiscible phases in a rotating coiled tube. This method has been applied for the separation of green and black tea polyphenols (Degenhardt et al., 2000b; Kumar et al., 2009), apple proanthocyanidins (Shibusawa et al., 2001), grape seed extract proanthocyanidins (Köhler et al., 2008) and cocoa bean proanthocyanidins (Esatbeyoglu, 2015) to name a few.

Proanthocyanidins from apples were isolated by their degree of polymerization using HSCCC with a special type-J multilayer coil centrifuge. Oligomers up to heptamers were isolated plus another fraction with higher polymerized oligomers (5-mers to 13-mers); the fractions were further analyzed with HPLC or MALDI-TOF-MS (Matrix-assisted laser desorption/ionization, time-of-flight mass spectrometry (Shibusawa et al., 2001).

Dimeric to trimeric proanthocyanidins were isolated by HSCCC after solvent precipitation with pentane (used to remove the polymeric compounds). Solid-phase

extraction on polyamide followed to improve the purities of the isolated compounds (Köhler et al., 2008).

Proanthocyanidins from cacao beans were separated using HSCCC in order to study their antioxidant activities. Isolation of catechin, epicatechin, and oligomers up to a pentamer was possible; the fractions were further analyzed with phloroglucinolysis, UPLC (Ultra performance liquid chromatography), mass spectrometry (MS) as well as nuclear magnetic resonance spectroscopy (NMR) (Li et al., 2016)

Separation of proanthocyanidins with HSCCC necessitates optimization of the solvent system (Ito, 1981). The sample must be soluble in the solvent system, the two solvent phases must have similar volumes in order to decrease wastage, partition coefficient K of the analyte should be between $0.5 \leq K \leq 1.0$, and the retention of the stationary phase must be high to improve peak resolution. In the case of polar compounds, 1-butanol-water is used, whereas in non-polar compounds the testing should start with a mixture of hexane–ethyl acetate–methanol–water (1:1:1:1) (Ito, 2005). It is also important to choose suitable support techniques for the identification of the compounds in the isolated fractions.

1.8 Thesis goal and research objectives

Oligomeric proanthocyanidins and flavan-*3*-ols have been proposed to exert several health promoting effects but the mechanisms remain unknown. Their mechanisms may relate to the modulation of gastric transit by bitter taste receptors in the gut, the inhibition of digestive enzymes by binding and/or precipitation, the modulation of the gut microbiota, or to the direct health promoting effects upon absorption. Separation of oligomeric proanthocyanidins from pea seed coats and grape seed extract using High speed counter current chromatography (HSCCC) provides an excellent tool to purify proanthocyanidins by degree of polymerization in sufficient quantities for subsequent evaluation on whether the structure of flavan-*3*-ols and oligomeric proanthocyanidins is related to their antimicrobial activity against common food and gut bacteria and their inhibition properties against digestive enzymes.

It is hypothesized that the enzyme inhibition and antimicrobial activity of flavan-*3*-ols and oligomeric proanthocyanidins is increased by the number of hydroxyl groups as well as the length of the oligomer, and the presence of a galloyl moieties.

In order to test this hypothesis, the objectives were to:

- Separate oligomeric proanthocyanidins from pea seed coats and grape seed extract by their degree of polymerization.
- Determine the inhibitory and bactericidal spectrum of flavan-*3*-ols and oligomeric proanthocyanidins, targeting food and gut bacteria.
- Determine oligomeric proanthocyanidins and flavan-*3*-ol ability to inhibit digestive enzymes.

2. Materials and Methods

2.1 Plant material

Pea Seed coats (from *Pisum sativum* cultivar Solido) were obtained from Mountain Meadows Food Processing Ltd. (Alberta, Canada T0G 1L0, 2009). This specific pea seed cultivar was used as was previously characterized with respect of its proanthocyanidins composition and biological activity. Grape seed extract “exGrape seed OPC40” was purchased from Breko (Bremen, Germany).

2.2 Chemicals

(+)-Catechin hydrate, (−)-epicatechin and (−)-epigallocatechin gallate were purchased from Sigma (Oakville, ON); (−)-Epigallocatechin was purchased from Extrasynthese (Genay,France). Methanol, chloroform and acetone were purchased from Fisher Scientific (Ottawa, ON). *p*- iodonitrotetrazolium, Toyopearl resin (HW-40F) from Sigma.

2.3 Strains and culture conditions

The strains used for this study as well as their origin and growth conditions are shown in Table 1.

L. acidophilus FUA 3066 and *L. reuteri* FUA 3400 were incubated under microaerophilic conditions (GasPak™ EZ anaerobe container system-Becton, Dickinson & Co). The two *C. jejuni* strains were incubated in microaerophilic conditions (CampyPak™-Becton, Dickinson and Company).

Mouse gut isolates were kindly provided by Dr. Ben Willing (University of Alberta). Incubated during 48h in Fastidious Anaerobe broth (LabM, Neogen Co) under strict anaerobic conditions (Anaerobic chamber, Bactron300-Shel Lab).

Stock cultures of each strain were prepared in 50% glycerol and stored at -80°C.

Table 1. Microorganisms and growth conditions used for this thesis.

Organism	Origin or Reference	Growth conditions	Phylum/family
Allobaculum sp.	Mouse gut	FAB, anaerobic, 37°C	Firmicutes/ Erysiopelotrichaceae
Anaerotruncus sp.	Mouse gut	FAB, anaerobic, 37°C	Firmicutes/ Ruminococcaceae
Ruminococcus gnavus	Mouse gut	FAB, anaerobic, 37°C	Firmicutes/ Ruminococcaceae
S. pasteuri FUA 2077	Rat mesenteric lymph nodes carrying colon cancer tumor	LB, aerobic, 37°C	Firmicutes/ Staphylococcaceae
Bacillus subtilis FAD 109	Ropy bread	LB, aerobic, 37°C	Firmicutes/ Bacillaceae
Bifidobacterium adolescentis	Type strain	mMRS, microaerophilic 37°C	Actinobacteria/ Bifidobacteriaceae
L. acidophilus FUA 3066	Probiotic culture	mMRS, microaerophilic 37°C	Firmicutes/ Lactobacillaceae
L. reuteri FUA 3400	Sponge dough, human lineage	mMRS, microaerophilic, 37°C	Firmicutes/ Lactobacillaceae
L. murinus Wm17	Mouse gut	MRS, anaerobic, 37°C	Firmicutes/ Lactobacillaceae
L. reuteri C47	Mouse gut	MRS, anaerobic, 37°C	Firmicutes/ Lactobacillaceae
Enterococcus faecalis ATCC 19433	Type strain	MH, aerobic,37°C	Firmicutes/ Enterococcaceae
Enterococcus faecium ATCC 19434	Type strain	MH,aerobic,37°C	Firmicutes/ Enterococcaceae
Propionibacterium sp.	Mouse skin	FAB, anaerobic, 37°C	Actinobacteria/ Propionibacteriaceae
Escherichia coli AW 1.7	Beef	LB, aerobic, 37°C	Proteobacteria/ Enterobacteriaceae
Campylobacter jejuni FUA 1211	Poultry	MH, microaerophilic, 42°C	Proteobacteria/ Campylobacteraceae
Campylobacter jejuni FUA 1215	Poultry	MH, microaerophilic, 42°C	Proteobacteria/ Campylobacteraceae

2.4 Preparation of Stock solutions for MIC assay

(+)-Catechin hydrate, (−)-epicatechin, (−)-epigallocatechin and (−)-epigallocatechin gallate were dispersed in 80% (v/v) aqueous acetone, whereas the crude and PA-enriched proanthocyanidin extracts, the polymeric grape seed proanthocyanidin fraction and the oligomeric fractions from grape seed were dissolved in 100% methanol. All the stock solutions had a final concentration of 10 g/L.

2.5 Determination of the Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal concentration (MBC).

The Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) assays are used to determine the antimicrobial activity of certain compounds, in this case: flavan-*3*-ols and oligomeric proanthocyanidin extracts from grape seed extract.

MIC and MBC were determined using Critical Dilution Assay in a 96 well microtiter flat bottom plate (Corning INC, NY).

For the MIC determination, wells of a microtiter plate were filled with 100 µL of corresponding sterile medium, 100 µL of the stock solutions were added into the wells (Highest inhibitory concentration). Then, two-fold serial dilution were prepared by pipetting 100 µL of the highest concentration into the next columns, consecutively. For all the assays, a positive and a negative control were used. Acetone and Methanol were evaporated from the plates by placing them into a laminar flow hood for 2 hours. After the solvent removal, 50 µL of diluted overnight bacterial cultures (1 mL culture to 10 mL of medium) were added to all the wells except for the negative control. The plates were incubated depending on the characteristics of each strain used in this project.

To determine bacterial growth, 40 µL of 0.2 mg/mL *p*-iodonitrotetrazolium violet (INT) (Sigma-Aldrich) were added to each well of the plate (Eloff, 1998). The plates were then incubated at 37°C from 30 min to 1 hour to promote the appearance of the red color.

INT is a redox dye used as an indirect indicator of bacterial growth (Haslam et al., 2000). NADH (Nicotinamide adenine nucleotide) which is a natural occurring compound

in living cells, transfers its electrons to the tetrazolium dye forming NAD^+ and INT-formazan, the later compound being responsible for the red color.

For the MBC determination, 10 µL of each well from the MIC plates were transferred to a new plate containing 100 µL of sterile medium. Subsequently, the MBC plates were incubated accordingly. After the incubation, growth was revealed with INT solution using the same procedure as in the MIC plates.

MIC and the MBC were defined as the lowest concentrations where no red color appeared. Three biological replicates were performed.

2.6 Extraction of proanthocyanidins from pea seed coats (cultivar Solido).

Extraction of crude proanthocyanidins from pea seed coats was adapted from the procedure described by Jin et al. (2012), where pea seed coats from the cultivar "Solido" (~10g) were ground to a fine powder using liquid nitrogen, mortar and pestle. The powder was then soaked in 66% (v/v) aqueous acetone (10mL per g of sample) in a 250 mL Erlenmeyer flask. The flask was promptly filled with nitrogen to reduce the oxygen content, and covered with a glass stopper and parafilm; then it was put on the magnetic stirrer (moderate agitation) at 4ºC in the dark for 24 h.

Subsequently, the sample was filtered using a Buchner funnel and Whatman® filter paper #1, under mild vacuum. The residue was rinsed with cold 66% aqueous acetone; the liquid was pooled and the acetone was evaporated using the Rota-vap concentrator (BUCHI rotavapor R-205). The pea seed coat residue was discarded.

The remaining liquid was partitioned 5 times with chloroform (1:3 v/v, chloroform:extract), using a separatory funnel in order to remove lipophilic compounds and flavan-3-ol monomers. The aqueous fraction was freeze-dried and this product was defined as Crude PA Extract.

This proanthocyanidin extraction method was adapted from Jin Lihua et al., (2012).

2.7 Proanthocyanidin-enriched extract from pea seed coats

The crude proanthocyanidin extract was further purified by Size Exclusion Chromatography using a hydroxylated methacrylic polymer: Toyopearl resin HW 40-F

(Sigma-Aldrich), with a pore size of 100-7000 Da molecular weight range as described by Jin et al., 2012. Polypropylene columns were used (1.5 x 12cm, Supelco, ~10ml bed volume). Toyopearl resin HW 40-F was prepared accordingly to the Sigma-Aldrich manual to remove the fines from the slurry and avoid obstruction. After this step, the resin slurry was 50% resin and 50% water, so ~ 10mL of slurry were added to the polypropylene column to have a final resin volume of 5mL. Columns were left 24h for the resin to sit.

The column was preconditioned with 50% (v/v) aqueous methanol + 0.1 % (v/v) trifluoroacetic acid (TFA). The crude extract powder was dissolved in a minimum amount of 50% v/v aqueous methanol + 0.1% v/v TFA (90-100mg extract per column) and then loaded in the column.

The column was washed with 9 bed volumes consisting in 10mL of 50% v/v aqueous methanol + 0.1% v/v TFA and the eluents were monitored each time with a UV-Vis spectrometer (Varioskan flash, Thermo Sci.) at 2 wavelength ranges (260-370 nm and 500-560 nm), washing steps must continue until minimal absorbance is achieved.

The column was then eluted with 4 bed volumes of 66% aqueous acetone + 0.1% TFA. After elution, acetone was removed using the rota-vap concentrator. The remaining sample was freeze-dried and labeled as “PA enriched extract”.

2.8 Inhibition of rat digestion enzymes by proanthocyanidins from pea seed coats and grape seed.

For this experiment, rat intestinal mucosa powder (Sigma Co, St Louis, USA) was used. The reaction mixture contained 1mL water + 1% maltodextrin and 1mL of 50mM sodium maleate buffer (0.2M maleate, 0.2N NaOH, distilled water, pH=6.0) with 1% rat enzyme mixture + 200µL of a 10g/L solution of each flavan-*3*-ol, fractionated proanthocyanidins from grape seed extract, or crude-PA or PA-enriched extracts from pea seed coats. Three glass beads were added to each tube. Controls without proanthocyanidins were also prepared.

The 15mL falcon tubes containing the reaction mixture were incubated at 37°C for 4 hours with agitation at 200 rpm. The reaction was stopped by putting the tubes in a

water bath at 92°C for 10 min. The samples were allowed to cool down and then centrifuged for 3 min. This method was adapted from Tsunehiro et al (1999).

The released glucose was measured with a glucose oxidase assay kit (Megazyme, Bray, Ireland). This method is based on two enzymatic reactions catalysed by glucose oxidase and peroxidase, respectively:

(1) β-D-Glucose + O_2 + H_2O → D-glucono-δ-lactone + H_2O_2

(2) H_2O_2 + p-hydroxybenzoic acid + 4-aminoantipyrine → quinoneimine dye + 4 H_2O

In the first reaction Glucose Oxidase (GO) oxidizes glucose to D-glucono-δ-lactone; for the second reaction hydrogen peroxide, product of (1), reacts with p-hydroxybenzoic acid and 4-aminoantipyrine in the presence of Peroxidase (POD) leading to the formation of quinoneimine dye which is a pink coloured compound that can be measured at 510 nm (Megazyme-International, 2014).

For this reaction 50µL of sample, water or standard and 200µL of GO solution were added to a 96 well microtiter plate. Absorbance was measured after 5 min at 510 nm. Then 50 µL of POD were added to each well and the plate was incubated at 25°C for 20 min, absorbance was measured immediately using a spectrophometer system.

The data in this experiment was statistically analyzed using a one way ANOVA to determine significant differences among the means of each sample as well as a Tukey Test for multiple comparisons.

2.9 Preparative solvent precipitation of the grape seed extract.

The grape seed extract (8g) was dissolved in 160mL of ethanol and thoroughly stirred throughout the experiment. Hexane (450mL) was placed in a separatory funnel and slowly dripped into the solution for the sequential precipitation of proanthocyanidins. After the precipitation ended, the suspension was stirred for 30 min more and then filtered. The aqueous phase containing oligomeric proanthocyanidins and flavan-*3*-ols was freeze-dried. The solid remaining was dissolved in water and freeze-dried to obtain the higher oligomeric and polymeric proanthocyanidins.

2.10 High-speed counter-current chromatography (HSCCC)

A high-speed countercurrent chromatograph model manufactured by Dynamic Extractions (United Kingdom) was used for the separation of proanthocyanidins from grape seed extract in the department of Food Technology and Biotechnology of the University of Bonn. The separation was carried out at room temperature at a revolution speed of 1550 rpm and at a flow rate of 6.0 mL/min. Head to tail elution mode was used so the lower aqueous phase was the mobile phase. All the samples (400-900 mg) were dissolved in 7mL of a 1:1 mixture of upper and lower phase, and injected into the system. The solvent system used was: ethyl acetate/2-propanol/water (40:1:40). Each run took 50 min and the fractions were collected in glass tubes (3 mL each). Subsequently, the fractions were analyzed using an UPLC system.

2.11 Analysis of pea seed coat extract, grape extract and oligomeric proanthocyanidin fractions

An ultra-high-performance liquid chromatograph (UHPLC) system from Nexera (Shimadzu, Kyoto, Japan) was used to carry out chromatographic analyses in the department of Food Technology and Biotechnology of the University of Bonn. This system was equipped with two high-pressure gradient pumps (LC-30AD), a degasser (model DGU-20A5R), an autosampling unit (model SIL-30AD), a column oven (model CTO-20AC) which was used at 40 °C; and a diode-array detector (SPD-M20A).

The column used was an Acquity UPLC HSS-T3 (2.1mm x 150 mm), with a pore size of 100Å and particle size of 1.8 µm (Waters, Eschborn, Germany). It was equipped with a security guard cartridge of the same material.

According to the chromatograms, fractions containing oligomeric proanthocyanidins with the same range of polymerization degrees were put together; the remaining solvents on the samples were evaporated using a rota-vap system (BUCHI Co). Subsequently, samples were freeze-dried and stored at 4°C in the dark.

Proanthocyanidin fractions from grape seed extract, pea seed coat proanthocyanidin extracts and grape seed extract were also analyzed with an Acquity UPLC I-Class system (Milford, MA, USA), coupled with a LTQ-XL ion trap mass spectrometer (Thermo

Scientific, Inc., Braunschweig, Germany) which was used in the negative ionization mode, covering a mass range from m/z 100-2000.

The column used was the same for the two systems; the mobile phase consisted of two solvents: formic acid (B) (1%, v/v) in water (A). The gradient used for both UPLC systems was (min/%B): 0/5; 5/12; 8/12; 20/30; 21/100; 25/100; 26/5; 30/5 of 1%; with a flowrate of 0.4 mL/min and an injection volume of 20 µL.

2.12 Effect of pea seed coat on growth and acidification of *L. acidophilus* and *L. reuteri* in a simulated forestomach

Lactobacillus acidophilus FUA 3066 and *Lactobacillus reuteri* FUA 3400 were cultured overnight in 40mL of modified MRS (De Man, Rogosa and Sharpe) broth at 37 °C, using microaerophilic conditions (GasPak BD, USA). After the incubation, the 50mL falcon tubes containing the cultures were centrifuged at 5000 rpm during 15 min; then, the broth was discarded and exchanged for sterile HPLC water, the centrifugation and washing steps were repeated two more times. The washed cells with 40mL of water were added to a beaker with 40g of wheat flour to prepare the control. For the sample, 32g of wheat flour were mixed with 8g of milled pea seed coat and 40mL of the washed culture. Samples of 1g of the dough were taken at the beginning of the fermentation (0 h), after 4h and 24h. The gram of dough was diluted with 9mL of water and then the pH was measured for the samples as well as the controls. The growth of *Lactobacillus acidophilus* FUA 3066 and *Lactobacillus reuteri* FUA 3400 along the fermentation was also measured; briefly, a volume of the sample dilution was taken and further dilutions were performed. 100 µL of the -5 dilution were added to an mMRS agar plate and the liquid was distributed using 3-6 sterile glass beads. The plates were incubated using microaerophilic conditions, at 37°C for 48h. The number of colonies in the plates was determined and the results were reported in colony forming units per gram of sourdough (CFU/g).

3. Results

3.1 Inhibitory and bactericidal concentrations of flavan-*3*-ols

MIC and MBC assays were performed with four flavan-*3*-ols against an array of food and intestinal bacteria in order to determine the antimicrobial activity of these compounds. The flavan-*3*-ol stock solutions were prepared with a concentration of 10g/L. A two-fold dilution method was used with concentrations ranging from 3.3g/mL to 0.0064 g/L. These results are shown in Table 2 and Table 3, with concentrations indicated in g/L. In the case of the strains selected, *Ruminococcus gnavus, Allobacullum sp*. and *Anaerotruncus sp.* are mouse gut isolates; *E. coli* is a commensal strain, *Campylobacter jejuni* was selected as a common food-borne pathogen originating from animal intestines; *Bacillus subtilis* and *Staphylococcus pasteuri* were used as indicator strains to compare with other studies. Finally, an array of lactic acid bacteria formed by *Lactobacillus sp*., *Enterococcus sp*. and *Bifidobacterium adolescentis*, was selected as the members of this group are found in several food products but are also representatives of gut microbiota.

As Table 2 shows, anaerobic mouse gut bacteria were strongly inhibited by the four flavan-*3*-ols tested, whereas the lactic acid bacteria were very resistant, especially *L. reuteri* FUA 3400 and *L. acidophilus* FUA 3066. *B. subtilis, E. coli, S. pasteuri* and *Propionibacterium sp*. were resistant as well. The two *C. jejuni* strains were sensitive to flavan-*3*-ols especially to epigallocatechin and epigallocatechin gallate. *C. jejuni* FUA 1211 was more sensitive to EC, EGC and EGCG in comparison to *C. jejuni* FUA 1215, showing that the antimicrobial effect of flavan-*3*-ols is strain specific.

Except for *C. jejuni* FUA 1211, there was no difference between the MIC values of catechin and epicatechin, suggesting that the isomeric conformation does not play a role in the antimicrobial action of flavan-*3*-ols.

Table 2. MIC of flavan-3-ols. Values are indicated in g/L.

Organism	CATECHIN	EPICATECHIN	EGC	EGCG
Allobaculum sp.	0.2±0.0	0.2±0.0	0.025±0.0	0.012±0.0
Anaerotruncus sp.	0.17±0.06	0.17±0.06	0.02±0.007	0.02±0.007
Ruminococcus gnavus	0.05±0.0	0.066±0.028	<0.0064	<0.0064
Staphylococcus pasteuri	>3.3	>3.3	0.6±0.23	0.4±0.0
Bacillus subtilis	3.3±0.0	3.3±0.0	2.77±0.92	0.67±0.23
Bifidobacterium adolescentis FUA 2018	3.3±0.0	3.3±0.0	X	0.8±0.0
L. acidophilus FUA 3066	>3.3	>3.3	>3.3	3.3±0.0
L. reuteri FUA 3400	>3.3	>3.3	>3.3	3.3±0.0
L. reuteri C47	3.3±0.0	3.3±0.0	X	0.8±0.0
L. murinus Wm17	3.3±0.0	3.3±0.0	X	0.8±0.0
Enterococcus faecalis	3.3±0.0	3.3±0.0	1.7±0.0	0.33±0.12
Enterococcus faecium	3.3±0.0	3.3±0.0	0.8±0.0	ND
Propionibacterium sp.	>3.3	3.3±0.0	3.3±0.0	0.4±0.0
E. coli AW 1.7	>3.3	3.3±0.0	1.1±0.52	1.1±0.52
C. jejuni FUA 1211	1.97±0.46	0.33±0.12	0.025±0.0	0.021±0.007
C. jejuni FUA 1215	1.55±0.2	1.7±0.0	1.1±0.52	0.071±0.031

ND: not determined due to excessive turbidity
X: not performed
Three replicates were performed for each strain.

Table 3. MBC of flavan-*3*-ols. Values are indicated in g/L.

Organism	CATECHIN	EPICATECHIN	EGC	EGCG
Allobaculum sp.	0.2±0.0	0.2±0.0	0.025±0.0	0.012±0.0
Anaerotruncus sp.	0.17±0.06	0.17±0.06	0.02±0.007	0.02±0.007
Ruminococcus gnavus	0.05±0.0	0.066±0.028	<0.0064	<0.0064
Staphylococcus pasteuri	>3.3	>3.3	2.23±0.83	0.8±0.0
Bacillus subtilis	>3.3	>3.3	>3.3	3.3±0.0
Bifidobacterium adolescentis FUA 2018	>3.3	>3.3	X	>3.3
L. acidophilus FUA 3066	>3.3	>3.3	>3.3	>3.3
L. reuteri FUA 3400	>3.3	>3.3	>3.3	>3.3
L. reuteri C47	>3.3	>3.3	X	>3.3
L. murinus Wm17	>3.3	>3.3	X	>3.3
Enterococcus faecalis	>3.3	>3.3	3.3±0.0	2.77±0.92
Enterococcus faecium	>3.3	>3.3	2.5±0.8	1.97±0.46
Propionibacterium sp.	>3.3	3.3±0.0	3.3±0.0	0.4±0.0
E. coli AW 1.7	>3.3	>3.3	3.03±0.46	3.03±0.46
C. jejuni FUA 1211	1.97±0.46	0.53±0.12	0.038±0.013	0.021±.007
C. jejuni FUA 1215	2.08±1.08	2.77±0.92	2.6±1.18	0.27±0.15

ND: not determined due to excessive turbidity
X: not performed
Three biological repeats were performed for each strain.

The comparison of the four flavan-*3*-ols makes possible to determine whether a higher number of hydroxyl groups in the molecule increases the antimicrobial activity. EGCG is the flavan-*3*-ol with more hydroxyl groups, it has 3-OH in the B ring and 3-OH from the gallic acid ester; followed by EGC, which has 3 hydroxyl groups in the B ring. In this study EGCG had substantially higher inhibition than EGC for *Allobacullum sp*., B*. subtilis, E. faecalis* and *Propionibacterium sp.* The inhibition of EGCG compared to EGC was always equal or higher.

For the case of EC and EGC, with 2 and 3 hydroxyl groups in the B ring respectively, EGC had higher inhibition for all the strains except for *Lactobacillus reuteri* FUA 3400, *Lactobacillus acidophilus* FUA 3066 and *Bifidobacterium adolescentis* FUA 2018. The inhibition of mice gut bacteria was substantially higher for EGC compared to EC; the MIC values were 10 fold lower for EGC.

The Minimal Bactericidal concentration (MBC) was performed as well to determine if the antimicrobial effect of flavan-*3*-ols is bactericidal or just bacteriostatic. Results of MBC of flavan-*3*-ols are presented in Table 3, with the concentrations indicated in g/L.

As Table 3 shows, the antibacterial activity of flavan-*3*-ols is bactericidal for *C. jejuni* FUA 1211 and the mouse gut microbiota strains except for *L. reuteri* C47 and *L. murinus* Wm17. For all the other strains, the effect of EGC and EGCG was bacteriostatic and they were resistant to C and EC.

3.2 Separation of proanthocyanidins from pea seed coats and grape seed extract.

Ultra-high performance liquid chromatography combined with electrospray ionization tandem mass spectrometry was the method used to identify and characterize the compounds present in the crude and enriched pea seed coat extracts and grape seed extracts, prior to the separation of proanthocyanidins using High-speed counter-current chromatography. The base peak chromatograms are shown below in Figures 4, 5 and 6, where the upper number refers to the retention time, whereas the lower number indicates the mass to charge ratio. Previous publications identified oligomeric proanthocyanidins as major compounds in pea seed coat extracts (Ferraro et al.,2014; Yang, 2014; Jin et al., 2012) therefore; base peak chromatograms shown in this thesis were used to verify the presence of these compounds. The lack of these compounds in the base peak chromatograms does not mean that they are absent; however, oligomeric proanthocyanidins are no longer major components in pea seed coat extract, likely due to oxidative polymerization during storage since 2009.

Figures 4 and 5 show the separation of crude and enriched extracts respectively. None of the peaks in the chromatograms corresponded to an oligomeric proanthocyanidin with degree of polymerization of five or less, or a flavan-*3*-ol molecule. It was

hypothesized that since the pea seed coats are from the 2009 harvest, flavan-3-ols and oligomeric proanthocyanidins might have undergone auto-oxidation, resulting in highly polymerized proanthocyanidins, which cannot be detected as their molecular weight is higher than 2000 g/mol.

The crude pea seed coat extract underwent a purification step with Toyopearl HW-40F resin in order to remove impurities that are very common in plant materials, such as organic acids, sugars, flavonols, among others; Figure 5 shows less peaks than Figure 4, suggesting that the purification step did get rid of some contaminants; however, procyanidins could not be detected and none of the peaks was identified.

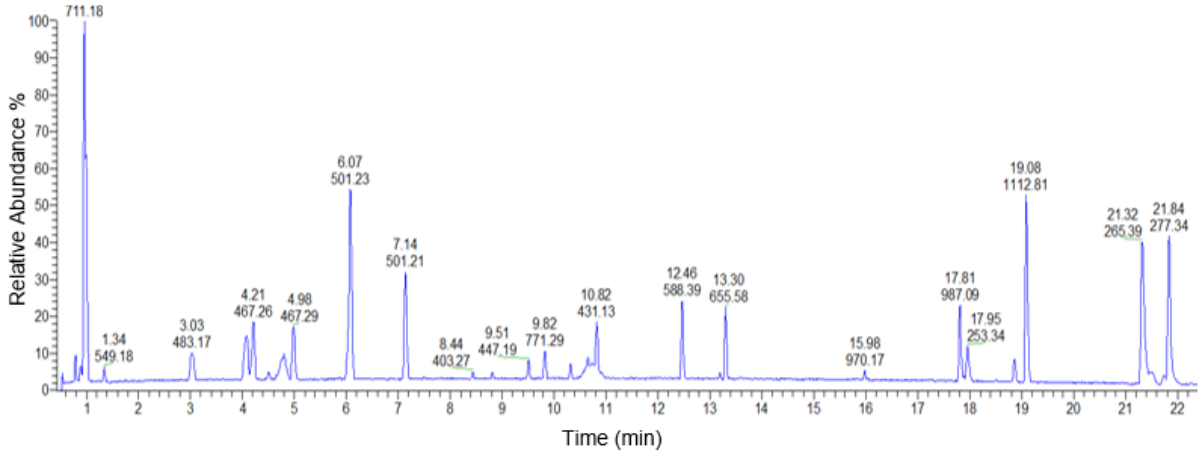


Figure 4. Base peak chromatogram of the crude pea seed coat extract using UHPLC-ESI-MS analysis (Top number: retention time, bottom number: mass to charge ratio).

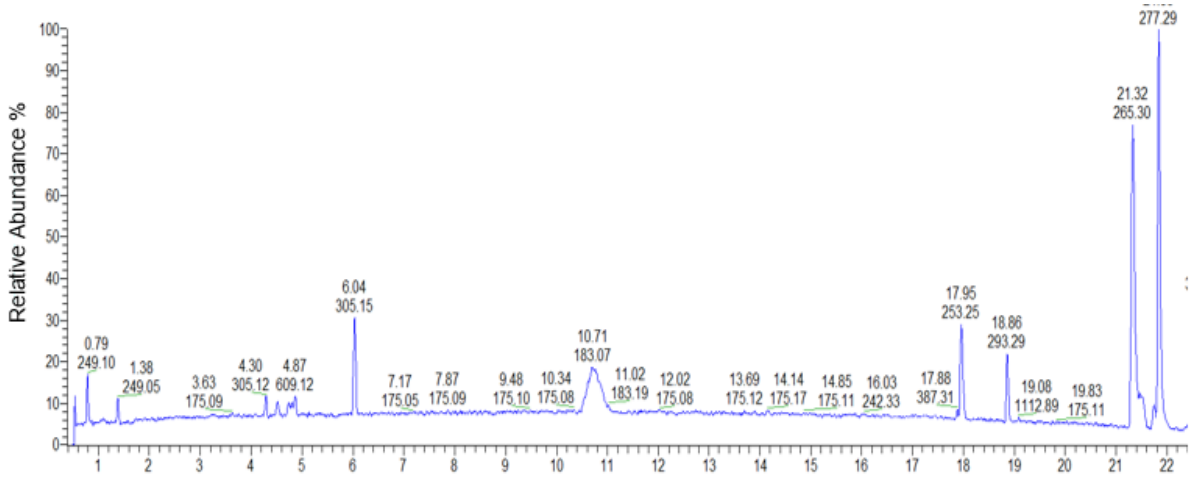


Figure 5. Base peak chromatogram of the enriched pea seed coat extract using UHPLC-ESI-MS analysis (Top number: retention time, bottom number: mass to charge ratio).

As large quantities of proanthocyanidin oligomers with different degree of polymerization were needed to determine the antibacterial and enzyme inhibitory activity of proanthocyanidin oligomers, it was decided to purify proanthocyanidins from the commercial grape seed extract “exGrape seed OPC40” from (Breko, Germany). According to the company, this extract contains >90% polyphenols, where >40% are oligomeric proanthocyanidins. Figure 6 shows the base peak chromatogram for the commercial grape seed extract. Several compounds were identified as oligomeric proanthocyanidins as shown in Table 4 and Figure 6.

Table 4. Tentatively identified compounds from UHPLC-ESI-MS chromatograms. Figures 6 to 9.

Peak	$[M - H]^-$	Compound
A	865	Proanthocyanidin trimer
B	729	Proanthocyanidin dimer gallate
C	577	Proanthocyanidin dimer
D	441	epicatechin monogallate
E	305	epigallocatechin
F	289	epicatechin

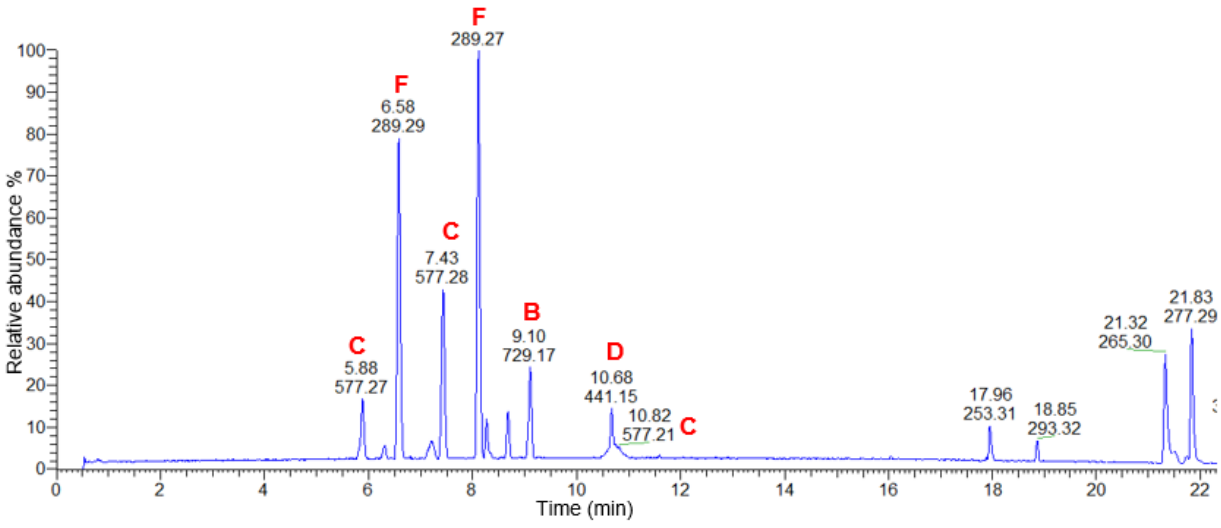


Figure 6. Base peak chromatogram of the grape seed extract using UHPLC-ESI-MS analysis. (Top number: retention time, bottom number: mass to charge ratio).

In Figure 6, the most abundant peaks at *m/z* 289 correspond to the monomer (-)-catechin or its isomer (+)-epicatechin, *m/z* 577 corresponds to a (epi)catechin dimer,

m/z 441 corresponds to a (epi)catechin plus a galloyl ester (Rockenbach, 2012; Friederich, Eberhardt and Galensa, 2000).

Peaks at *m/z* 265 and 277 are shown in the chromatograms of the three extracts analyzed (crude and enriched pea seed coat and grape seed extracts), suggesting that they are contaminants in the column. Grape seed extract may possess oligomers with higher degree of polymerization but the mass spectrophotometer used is just able to detect *m/z* up to 2000. There are several peaks that remain unidentified but it is very difficult to identify and characterize all the compounds in a complex plant extract.

The commercial grape seed extract was labeled as having >40% proanthocyanidins and the base peak chromatogram confirms the presence of flavan-*3*-ols and oligomeric proanthocyanidins. This extract was therefore suitable to separate oligomeric proanthocyanidins by their degree of polymerization using High-speed counter-current chromatography.

3.2.1 Separation of grape seed extract oligomeric proanthocyanidins using HSCCC

High-speed counter-current chromatography was the method chosen to separate the grape seed oligomeric proanthocyanidins by their degree of polymerization. This chromatographic method was chosen because it possesses many advantages over traditional column chromatography techniques. HSCCC provides high yields, up to several grams in one hour; it also provides fractions with high purity, elimination of solid supports and the use of large solvent volumes (Ito, 1981; Ito and Conway, 1986). The solvent system selected was ethyl acetate/2-propanol/water (40:1:40) as suggested by Kohler et al (2012); it was possible to isolate three fractions with different degrees of polymerization, known as ‘Fraction I, II and III’. The base peak of the fractions are shown below in Figures 7, 8 and 9.

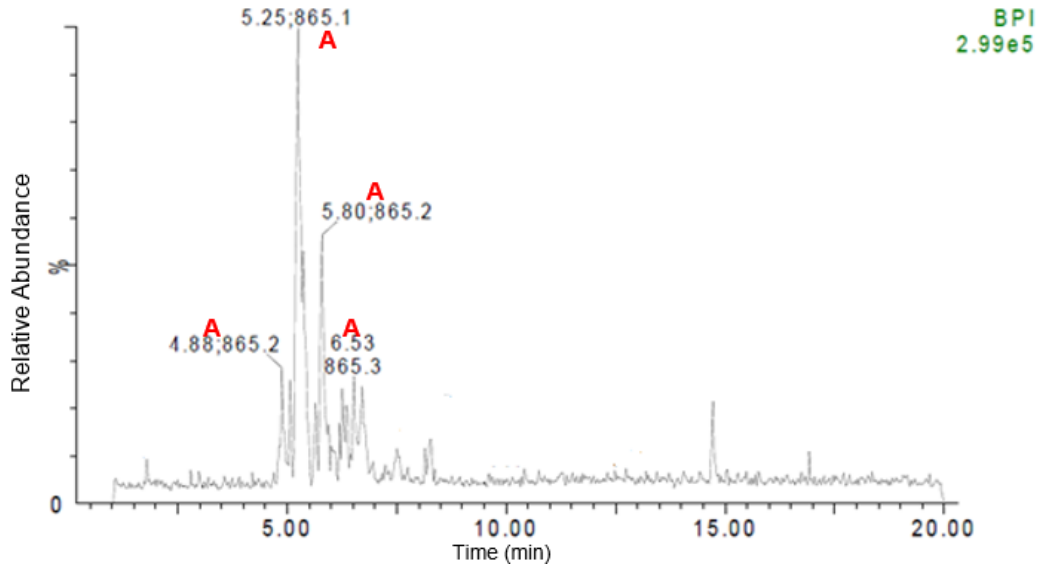


Figure 7. Base peak chromatogram of Fraction I using UHPLC-ESI-MS analysis in negative ion mode (number to the left: retention time, number to right: mass to charge ratio).

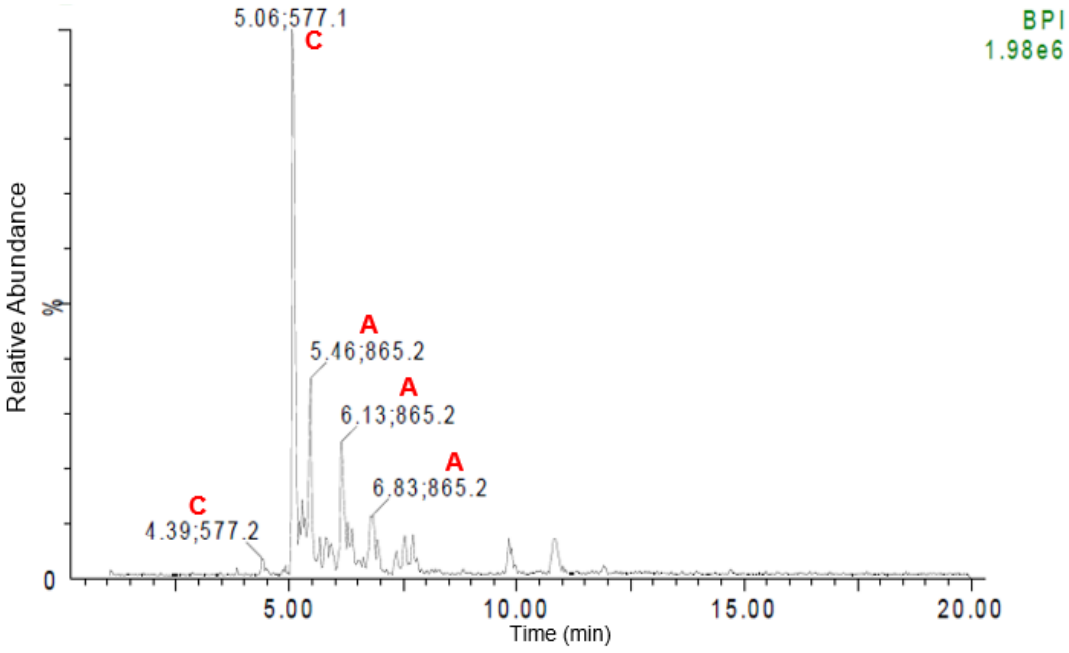


Figure 8. Base peak chromatogram of Fraction II using UHPLC-ESI-MS analysis in negative ion mode (number to the left: retention time, number to right: mass to charge ratio).

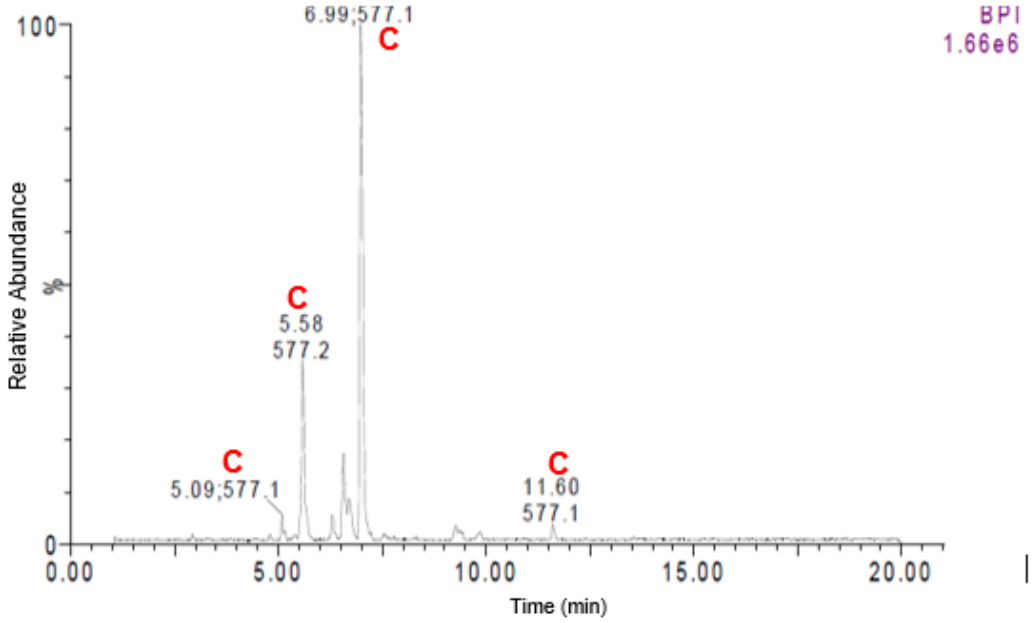


Figure 9. Base peak chromatogram of Fraction III using UHPLC-ESI-MS analysis in negative ion mode (number to the left: retention time, number to right: mass to charge ratio).

The base peak chromatogram of 'fraction I' shows 4 major peaks identified as 'A', with an *m/z* 865 corresponding to proanthocyanidin trimers. The fact that several peaks have the same *m/z* suggests that the monomers forming the trimers have different patterns of isomerization. In the case of 'fraction II', the major peak 'C' with m/z 577 corresponds to a proanthocyanidin dimer; three more peaks with m/z 865 are shown, corresponding to proantocyanidin trimers with different isomerization patterns. For 'fraction III', four peaks with m/z 577 corresponding to a proanthocyanidin dimers with different isomerization patterns are shown. With the use of HSCCC it was possible to isolate three oligomeric fractions with different degree of polymerization in enough quantities to test their antibacterial properties as well as their capability to inhibit digestive enzymes.

3.3 Inhibitory and bactericidal concentrations of pea seed coats and grape seed proanthocyanidins fractions.

MIC and MBC assays were performed with the crude and enriched pea seed coat extracts as well as the fractions obtained from the commercial grape seed extract to

assess their antimicrobial activity against the same array of food and gut bacteria tested for flavan-*3*-ols. The stock solutions were prepared in a concentration of 10g/L dissolved in 100% methanol; the solvent was evaporated for two hours to avoid interference of the solvent in the growth of the selected strains. A two-fold dilution method was used in a 96-well microtiter plate, with concentrations ranging from 3.3g/L to 0.0064 g/L. This results are shown in Table 5, 6 and 7, three biological repeats were performed for each strain.

Table 5. MIC and MBC of the crude and enriched pea seed coat extracts. Values are indicated in g/L.

	Crude pea seed coat extract		**PA-enriched pea seed coat extract**	
Organism	MIC	MBC	MIC	MBC
Allobaculum sp.	0.05±0.0	0.05±0.0	0.025±0.0	0.04±0.01
Anaerotruncus sp.	0.04±0.01	0.04±0.01	0.025±0.0	0.025±0.0
Ruminococcus gnavus	0.016±0.007	0.016±0.007	0.033±0.014	0.05±0.0
Staphylococcus pasteuri	0.47±0.12	0.95±0.26	0.4±0.0	1.7±0.0
Bacillus subtilis	2.77±0.92	>3.3	2.23±0.92	>3.3
L. acidophilus FUA 3066	>3.3	>3.3	>3.3	>3.3
L. reuteri FUA 3400	>3.3	>3.3	>3.3	>3.3
Enterococcus faecalis	0.8±0.0	3.03±0.65	0.67±0.23	2.23±0.92
Enterococcus faecium	0.33±0.10	2.77±0.83	0.27±0.12	2.23±0.92
Propionibacterium sp.	1.7±0.0	1.7±0.0	1.7±0.0	>3.3
E. coli AW 1.7	3.3±0.0	>3.3	1.4±0.52	3.3±0.0
C. jejuni FUA 1211	0.015±0.005	0.2±0.0	0.083±0.029	0.083±0.029
C. jejuni FUA 1215	0.1±0.0	0.2±0.0	0.05±0.0	0.05±0.0

Table 6. MIC of the polymeric fraction and the oligomeric proanthocyanidins fractions from grape seed extract. Values are indicated in g/L.

Organism	Polymeric Fraction	Fraction 1 Trimers	Fraction 2 Dimers/Trimers	Fraction 3 Dimers
Allobaculum sp.	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0
Anaerotruncus sp.	0.04±0.01	0.04±0.01	0.04±0.01	0.04±0.01
R. gnavus	0.05±0.0	0.05±0.0	0.05±0.0	0.05±0.0
S. pasteuri	0.53±0.24	0.53±0.24	0.66±0.24	0.8±0.0
Bacillus subtilis	1.7±0.0	1.7±0.0	1.7±0.0	2.23±0.92
L. acidophilus FUA 3066	>3.3	>3.3	>3.3	>3.3
L. reuteri FUA 3400	>3.3	>3.3	>3.3	>3.3
E. faecalis	1.1±0.52	1.4±0.52	2.23±0.92	3.3±0.0
E. faecium	0.4±0.0	0.4±0.0	0.8±0.0	0.8±0.0
Propionibacterium sp.	1.7±0.0	2.77±0.92	2.77±0.92	2.77±0.92
E. coli AW 1.7	1.7±0.0	1.7±0.0	1.7±0.0	1.7±0.0
C. jejuni FUA 1211	0.27±0.12	0.33±0.12	0.33±0.1	0.26±0.1
C. jejuni FUA 1215	0.1±0.0	0.17±0.058	0.2±0.0	0.33±0.12

Table 7. MBC of the polymeric fraction and the oligomeric proanthocyanidins fractions from grape seed extract. Values are indicated in g/L.

Organism	Polymeric Fraction	Fraction 1 Trimers	Fraction 2 Dimers/Trimers	Fraction 3 Dimers
Allobaculum sp.	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0
Anaerotruncus sp.	0.04±0.01	0.04±0.01	0.04±0.01	0.04±0.01
R. gnavus	0.05±0.0	0.05±0.0	0.05±0.0	0.05±0.0
S. pasteuri	1.7±0.0	3.3±0.0	3.3±0.0	3.3±0.0
Bacillus subtilis	>3.3	>3.3	>3.3	>3.3
L. acidophilus FUA 3066	>3.3	>3.3	>3.3	>3.3
L. reuteri FUA 3400	>3.3	>3.3	>3.3	>3.3
E. faecalis	2.23±0.92	>3.3	>3.3	>3.3
E. faecium	3.3±0.0	3.3±0.0	3.3±0.0	>3.3
Propionibacterium sp.	>3.3	>3.3	>3.3	>3.3
E. coli AW 1.7	>3.3	>3.3	>3.3	>3.3
C. jejuni FUA 1211	0.27±0.12	0.33±0.1	0.33±0.1	0.26±0.12
C. jejuni FUA 1215	0.2±0.0	0.4±0.0	0.4±0.0	0.53±0.23

According to Table 5, the inhibitory effect of crude and PA-enriched pea seed coat extract was not different for *Ruminococcus gnavus, S. pasteuri, B. subtilis, E. faecalis, E. faecium* and *Propionibacterium sp*. The PA-enriched extract showed higher inhibition for *Allobaculum sp., Anaerotruncus sp., E. coli* and *C. jejuni FUA 1215*, whereas the crude extract showed higher inhibition for *C. jejuni* FUA 1211. The two Lactobacillus strains tested were resistant to both extracts. The composition of the extracts remains unknown but it is hypothesized that both of them contain highly polymerized proanthocyanidins.

Grape seed extract fractions inhibited mouse intestinal bacteria and the inhibitory effects of the four isolated proanthocyanidin fractions tested towards these strains was not different. *L. reuteri* and *L. acidophilus* were resistant to all the compounds tested. There was no substantial difference between the inhibitory effect of the four fractions tested towards *S. pasteuri, B. subtilis, E. coli* and *C. jejuni* FUA 1211. In the case of both *Enterococcus* strains tested, the polymeric fraction and Fraction I showed higher inhibitory effect than Fraction II and III. For Propionibacterium sp. and C. jejuni FUA 1215, the inhibitory effect of the polymeric fraction was higher compared to the other three fractions tested.

The antibacterial effect of the grape seed extract proanthocyanidin fractions was not affected by the degree of polymerization of the oligomer. Nevertheless, the inhibitory effect of monomers is substantially higher than the oligomeric proanthocyanidins, especially for EGCG; these results are shown in Table 2.

The minimal bactericidal concentration (MBC) assay was performed in order to determine if the antibacterial effect of crude and enriched extract as well as the proanthocyanidin fractions from grape seed extract were bactericidal or just bacteriostatic. Data is presented in Table 7, with the concentrations indicated in g/L.

As Table 7 shows, the antibacterial effect against members of the mouse gut bacteria was bactericidal, as well as for *C. jejuni* FUA 1211. The antibacterial effect was bacteriostatic for *B. subtilis, E. coli, E. faecalis* and *S. pasteuri*. *L. reuteri* FUA 3400 was resistant to all of the compounds tested.

3.4 Inhibition of rat digestive enzymes by flavan-*3*-ols, crude and PA-enriched pea seed coat extracts and grape seed proanthocyanidins.

In order to determine the inhibition of digestive enzymes by flavan-*3*-ols as well as crude and PA-enriched pea seed coat extracts and grape seed proanthocyanidin fractions, maltodextrin was digested *in vitro* by rat digestive enzymes at 37°C in the presence or absence of flavan-*3*-ols or proanthocyanidins. After four hours of incubation, the reaction was stopped and the released glucose was measured with a

glucose oxidase assay kit. Data of released glucose is shown in Figure 10. The values are indicated in g/L.

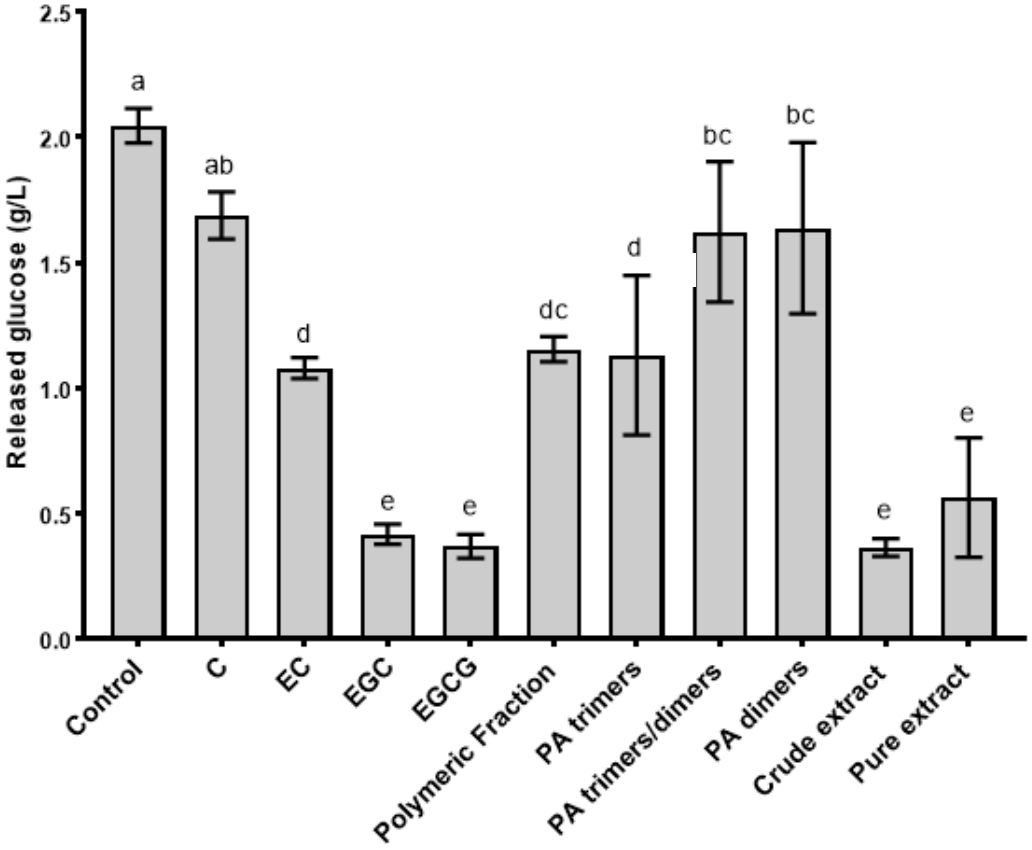


Figure 10. Released glucose after incubation of maltodextrin with rat digestive enzymes in the presence of flavan-3-ols and proanthocyanidins from pea seed coat and grape seed extracts. Values shown in g/L. (Abbreviations: C: catechin, EC: epicatechin, EGC: epigallocatechin, EGCG: epigallocatechin gallate, PA: proanthocyanidin, GSE: grape seed extract). Three biological replicates were performed for each compound. Statistical analysis was done by one way ANOVA and Tukey post hoc analysis. If bars do not share the same superscript, values differ significantly (P<0.05).

All of the compounds tested inhibited rat digestive enzymes in different extents except for Catechin (C) (Fig. 10). EGCG, EGC and crude pea seed coat extract showed the

highest inhibitory effect, followed by the enriched extract, EC, Fraction I and the polymeric PA grape seed extract.

Epicatechin showed higher inhibition of digestive enzymes compared to catechin, both of these molecules possess 2 hydroxyl groups in their B rings and just differ in their stereochemical conformation. This suggests that this attribute plays a role in the digestive enzyme inhibition by flavan-*3*-ols. Difference was found between EC and EGC and between EC and EGCG. The inhibitory effect of EGC, which has 3 hydroxyl groups was not different from EGCG, which has 6 hydroxyl groups. However, maltodextrin digestion was already fully inhibited by EGC so that an additional inhibitory effect of EGCG could not be measured. Further experiments should use a lower concentration of EGC and EGCG to determine whether their inhibitory activity is different.

It was hypothesized that the higher the number of hydroxyl groups in the molecule, the protein binding and precipitating properties of flavan-*3*-ols will be higher as well, as more OH groups are able to bind a larger number of digestive enzymes inactivating them; these findings suggest that the inhibition of enzymes by flavan-*3*-ols increases with the number of hydroxyl groups in the molecule.

Determination of the inhibitory activity of grape seed proanthocyanidin fractions revealed that inhibition by the polymeric proanthocyanidin fraction and Fractions II and III was not different; nevertheless, there was difference in the inhibitory effect of Fraction I and all the other grape seed fractions.

The findings in this experiment suggest that the degree of hydroxylation of flavan-*3*-ols changes inhibition of digestive enzymes, but the degree of polymerization of oligomeric grape seed extract proanthocyanidins does not play a role in their inhibitory properties. Pea seed coat crude extract showed inhibition of digestive enzymes although their composition is unknown.

3.4 Effect of pea seed coat on growth and acidification of *L. acidophilus* and *L. reuteri* in a simulated forestomach

The lactic acid bacteria strains used in this study were highly resistant to flavan-*3*-ols and proanthocyanidins from pea seed coats and grape seed extracts, especially *L.*

reuteri FUA 3400 and *L. acidophilus* FUA 3066; so further testing on antimicrobial properties of pea seed coat were performed with these two strains, using a more complex matrix.

L. reuteri FUA 3400 and *L. acidophilus* FUA 3066 were grown in a matrix of wheat flour and water + 10% pea seed coat extract at 37°C to simulate the conditions of the forestomach; a control without pea seed coat was used for comparison. One gram of sample was taken at 0h, 4h and 24h, and diluted with 9mL of sterile distilled water and pH was measured, plate counts were also performed. Figure 11 and Figure 12 show the pH values as well as the growth of the strains along the fermentation in colony forming units per gram of sourdough (CFU/g).

As figure 11 shows, the pH along the fermentation for both controls of *L. reuteri* and *L. acidophilus* were significantly lower than the pH values of the fermentation with 10% crude pea seed coat extract, suggesting that the polymeric proanthocyanidins in the crude pea seed coat extract affect Lactobacillus growth, as *L. reuteri* and *L. acidophilus* are acid producers, a lower pH indicates that the strains grew well.

Figure 12 shows that the crude pea seed coat extract significantly inhibits the growth of *L. acidophilus* whereas the growth of *L. reuteri* is just affected after 4 hours of the fermentation.

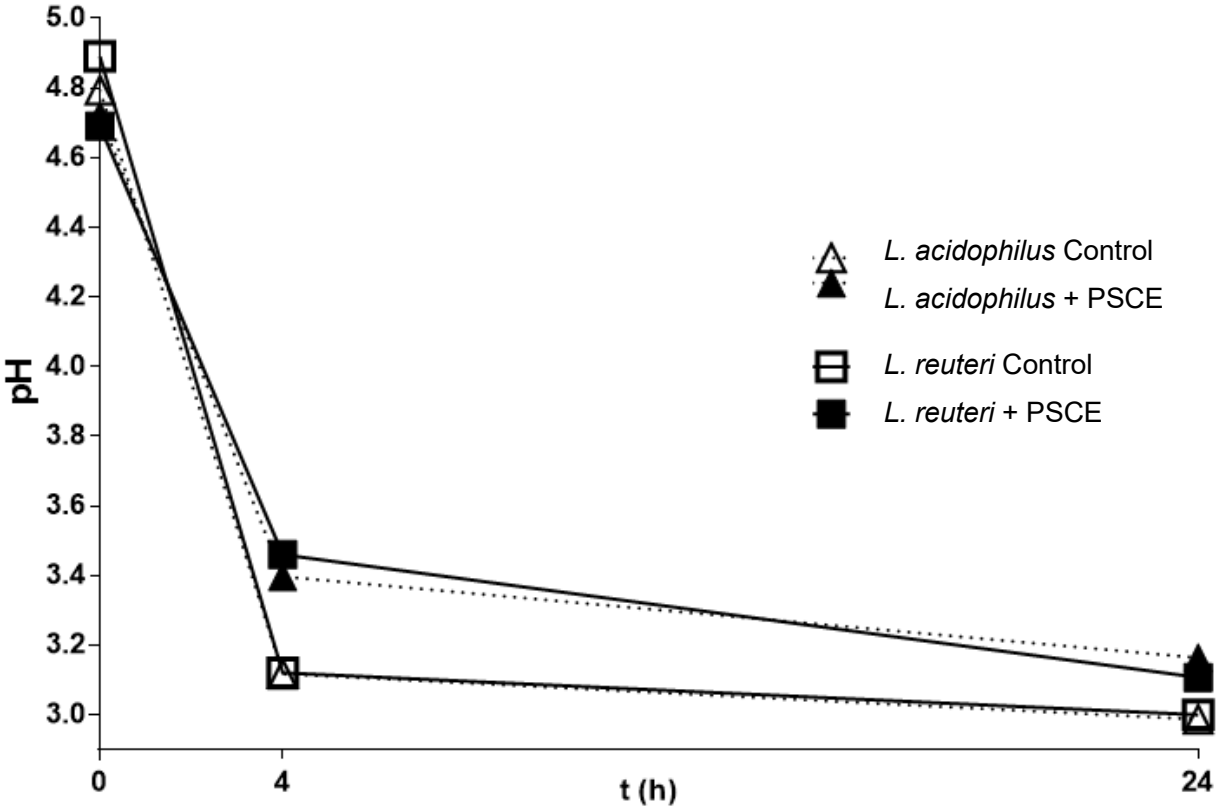


Figure 11. Acidification of sourdough with *L. reuteri* or *L. acidophilus* with or without pea seed coat extract (PSCE). Error bars are smaller than symbol size. Three biological replicates performed.

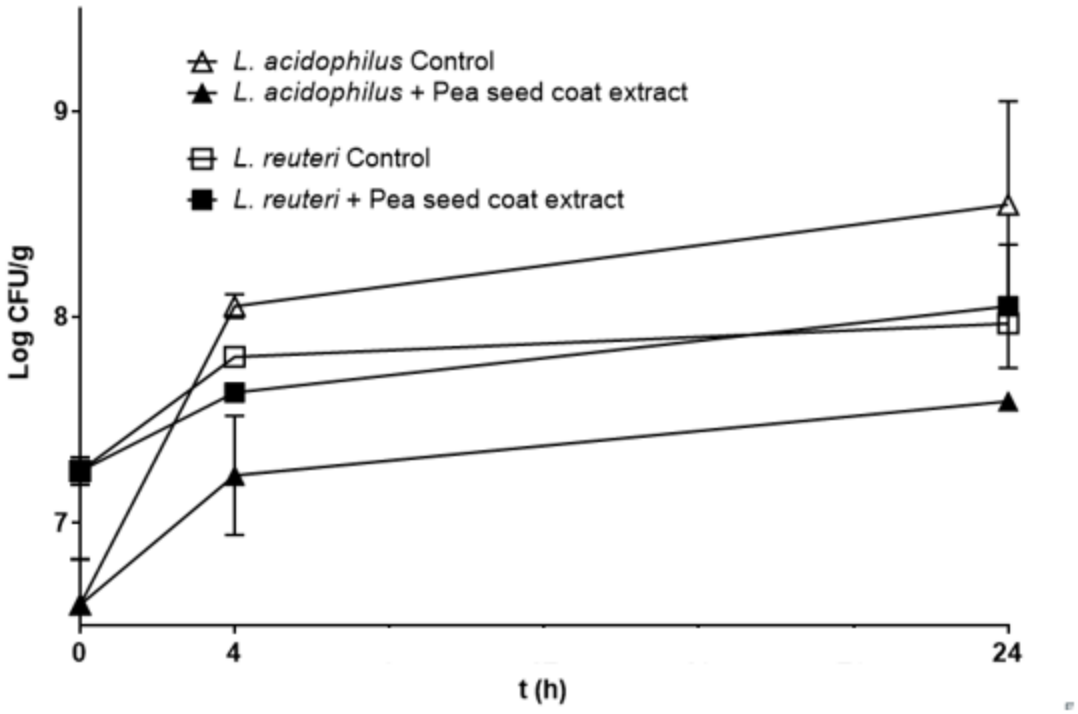


Figure 12. Cell counts of *L. reuteri* or *L. acidophilus* during growth in wheat sourdough with or without pea seed coat extract. Data show means ± standard deviation of three biological replicates.

3. Discussion

This study evaluated the antimicrobial effect of flavan-*3*-ols and oligomeric proanthocyanidins from pea seed coats and grape seed extract towards a broad array of bacteria: common food-borne pathogens, lactic acid bacteria, members of the mouse gut microbiota among others. The digestive enzyme inhibition by flavan-*3*-ols and oligomeric proanthocyanidins from pea seed coats and grape seed extracts were determined as well, with the aim of explaining the health promoting effects exerted by these flavonoid compounds.

4.1 HSCCC (High-speed counter-current chromatography)

This chromatographic method was chosen to separate oligomeric proanthocyanidins by their degree of polymerization. It possesses many advantages over traditional column chromatography techniques such as: high yields due to no irreversible adsorption of the

sample to the column material (up to several grams in few hours); fractions with high purity, elimination of solid support, lower solvent volumes and good reproducibility (Ito, 1981; Ito and Conway, 1986; Marston and Hostettmann, 1994). Counter-current chromatography utilizes a mixture of immiscible solvents referred to as “two-phase solvent system”; one phase acts as stationary phase whereas the other one works as the mobile phase (Ito, 2005). The solvent system used for HSCCC runs was: ethyl acetate/2-propanol/water (40:1:40) (Köhler, Wray and Winterhalter, 2008), a suitable system for the separation for oligomeric proanthocyanidins from plant extracts.

Putman and Butler (1985) were the first to report separation of a proanthocyanidin extract from sorghum grain into 9 fractions with different degrees of polymerization. This technique was also applied to separate flavan-*3*-ols and proanthocyanidins from green and black tea, where the separation of epigallocatechin gallate and epicatechin gallate was achieved. In the case of proanthocyanidins, the extract was purified using a polyamide column before the actual separation using HSCCC; separation of dimeric and trimeric proanthocyanidins was achieved (Degenhardt et al., 2000b). Shibusawa et al (2001) accomplished the separation of apple proanthocyanidins by their degree of polymerization, from dimers and trimers up to highly polymerized oligomers with thirteen units. In more recent years, dimeric, trimeric, tetrameric and pentameric proanthoycianidins were isolated and separated from unroasted cocoa beans (Esatbeyoglu, Wray and Winterhalter, 2015) using countercurrent chromatography. Köhler, Wray and Winterhalter (2008) achieved the separation of several dimers, a trimer and a tetramer from grape seed extract; in this experiment, the proanthocyanidin extract was purified using a polyamide column and highly polymerized compounds were removed by solvent precipitation to avoid interference in the separation of oligomeric proanthocyanidins.

Pea seed coats (*Pisum sativum L*. cultivar Solido) were chosen as the raw material for this thesis as they are a rich source of flavan-*3*-ols, oligomeric and polymeric proanthocyanidins (Ferraro et al., 2014). Cultivar Solido in particular, possesses a peculiar brown color given by abundant proanthocyanidin oxidation products caused by polyphenol oxidase *in planta* (Marles, Vandenberg, & Bett, 2008). This seed coats were found to have 4.51 g of proanthocyanidins per 100 g of seed coats and a mean degree

of polymerization of five (Ferraro et al., 2014). Epigallocatechin and gallocatechin were the most abundant sub-units for this cultivar; epicatechin was present in small amounts; no gallic acid substituents are present in pea seed coat flavan-*3*-ols (Ferraro et al., 2014).

Even though pea seed coats were reported to have a mean degree of polymerization of five (Ferraro et a., 2014), no oligomeric proanthocyanidins or flavan-*3*-ols were detected after the analysis of enriched and crude pea seed coat extracts using ultra performance liquid chromatography combined with electrospray ionization tandem mass spectrometry (UPLC-ESI-MS).

The pea seed coats used for the study belong to the 2009 harvest so it is possible that the flavan-*3*-ols as well as the proanthocyanidins may have undergone non-enzymatic oxidation leading to the formation of quinoidal molecules (Pourcel et al., 2007), these molecules are highly reactive and can oxidize other compounds, forming secondary quinones, resulting in the development of heterogenous polymers (Mochizuki et al., 2002; Cheynier et al., 1994; Hathway and Seakins, 1957). Previous studies reported the presence of oligomeric proanthocyanidins with mean degree of polymerization of five as well as flavan-3-ols in pea seed coat extract (Ferraro et al., 2014; Yang, 2014; Jin et al., 2012).

The mass range for the UHPLC-ESI-MS equipment is limited to a *m/z* of up to 2000, so larger polymeric proanthocyanidins are out of the detection range. Proanthocyanidins in pea seed coats have been previously characterized using the phloroglucinolysis method, finding that the extension units as well as the terminal units were mainly composed of epigallocatechin and gallocatechin (Ferraro et al., 2014). Pea seed coats also contain organic acids, anthocyanidins and flavonols in their composition (Jin, 2011).

To obtain a suitable raw material for the extraction and separation of oligomeric proanthocyanidins, a commercial grape seed extract (Breko, Germany) with a proanthocyanidin content of more than 40% was employed. Three fractions were isolated: PA trimers fraction, PA trimer and dimer fraction and dimer fraction.

Grape seeds are known to have high concentrations of condensed tannins (Saito et al., 1998), but in contrast to the pea seed coat proanthocyanidins, grape seed extract proanthocyanidins contain approximately 20%-30% of esterified gallate groups (Cheynier et al., 1999; Prieur et al., 1994); their mean degrees of polymerization range between 2 to 16 units in the polymer and their dominant stereochemistry is *cis* conformation (Prieur et al., 1994).

Hydrolysable tannins composed mainly of gallic and ellagic acids (Seikel and Hillis, 1970), possess higher protein binding properties per unit mass than proanthocyanidins (Haslam, 1974). This might suggest that a higher concentration of gallic acid substituents in a proanthocyanidin fraction will result in higher inhibition of digestive enzymes. However, crude and PA-enriched pea seed coat extracts which don't contain gallates exhibited a higher enzyme inhibition compared to the polymeric fraction rich in gallic acid substituents.

With the HSCCC chromatographic method, it was possible to isolate approximately 90 mg of Fraction I, 135 mg of Fraction II and 80 mg of Fraction III after the separation of 3 grams of grape seed extract after solvent precipitation with hexane to eliminate highly polymerized proanthocyanidins. These quantities were enough to perform MIC in triplicates with fifteen strains, as well as to determine the inhibition of rat digestive enzymes; this would not have been possible if other chromatographic methods had been applied.

All the compounds obtained from the pea seed coat extract and grape seed extract as well as the commercial flavan-*3*-ols were tested to assess their digestive enzyme properties as well as the antimicrobial effects against an array of common food and gut bacteria.

4.2 Antibacterial properties of flavan-*3*-ols and oligomeric proanthocyanidins.

The antibacterial activities of flavan-*3*-ols and oligomeric proanthocyanidins from grape seed extract toward an array of lactic acid bacteria, food-borne pathogens, indicator strains and anaerobic mouse gut microbiota members were demonstrated in this study. All compounds exerted selective bacterial inhibition in some extent depending on their

chemical structure as well as the strain tested. It was hypothesized that flavan-*3*-ols with higher number of hydroxyl groups in the B ring of the molecule or esterified with gallic acid, would have higher bacterial inhibition.

The MIC values for *Staphylococcus pasteuri* agree with a previous data reported by Yang (2014) as the MICs were the same for EGC and EGCG, despite EGCG having 3 more active hydroxyl groups than EGC. The difference between studies lies in the MIC concentration, as Yang (2014) found lower MIC values. This might be because in this study, *p*-iodonitrotetrazolium violet dye (INT) was used to determine the MIC and MBC values. INT is a redox dye used as an indirect indicator of bacterial growth (Haslam et al., 2000); this reaction is based on the electron transference between NADH (Nicotinamide adenine nucleotide), which is a natural occurring compound in living cells and the tetrazolium dye forming NAD+ and a red INT-formazan. Yang (2014) used bromocresol green to asses MIC and MBC concentration, method based on the measurement of the acidification of the bacterial broth on the plate wells. As the two methods measure two different characteristics of bacteria, it is possible that MIC results are somewhat different.

In the case of *Bacillus subtilis*, the MIC determined for EGCG corresponds to the results published by Engels et al (2011). Engels et al (2011) also reported that *C. jejuni* FUA 1220 had a MIC value for EGCG with a concentration 10 times higher than the ones determined in this study for *C. jejuni* FUA 1211 and *C. jejuni* FUA 1215. These findings suggest that the antimicrobial effect of flavan-*3*-ols is strain specific as *C. jejuni FUA* 1211 was also more sensitive to EC and EGC in comparison to *C. jejuni* FUA 1215.

EGCG, which is the most widely studied flavan-*3*-ol, is the molecule tested with the most hydroxyl groups, 3 –OH in the B ring and 3-OH from the esterified gallic acid, followed by EGC, which has 3 hydroxyl groups in the B ring. In this study EGCG had the same or higher inhibition than EGC; the same pattern was observed for EC and EGC. EGC had the same or a higher antimicrobial activity than EC for all the stains tested while lactic acid bacteria were resistant to both. It was reported that a higher number of hydroxyl substitutions in the B ring of the flavan-*3*-ol (Scalbert, 1991; Yang, 2014) as well as the presence of esterified gallic acid enhanced the antimicrobial activity of these

monomers. (Epi)gallocatechin and (epi)gallocatechin gallate have shown higher antimicrobial activity than catechin and its isomer: epicatechin (Taguri, Tanaka and Kouno, 2004; Yam et al., 1997; Scalbert, 1991). This study confirms these reports with bacterial strains representing rodent intestinal microbiota.

With the exception of *C. jejuni* FUA 1211, there was no difference between the MIC values of C and EC, suggesting that the isomeric conformation does not play a role in the antimicrobial action of flavan-*3*-ols. This result agrees with Yang (2014), although generally flavan-*3*-ols with *cis* configuration have higher antimicrobial activities (Yam et al., 1997; Nishino et al., 1987). Nevertheless it was found that catechin has higher growth inhibition compared to epicatechin when using a batch-culture fermentation (Tzounis et al., 2008).

A comparison of the antimicrobial activity of flavan-*3*-ols against *Staphylococcus aureus* found that the number of hydroxyl groups in the molecule as well as the presence of gallic acid esterifications changes the inhibitory effect of these molecules, as epigallocatechin gallate was the most inhibitory compound followed by epicatechin gallate; epicatechin was the least inhibitory compound with just two hydroxyl groups in the B-ring (Akiyama et al., 2001). Epigallocatechin gallate had substantially higher antimicrobial effect against *E. coli* and *S. aureus* compared to epicatechin (Ikigai et al., 1993), supporting the hypothesis that higher number of hydroxyl groups present in the molecule results in higher antibacterial effect.

In the case of the pea seed coat crude and enriched extracts, no difference was found between the antimicrobial activities of these extracts; this agrees with the hypothesis that both extracts are composed of highly polymerized proanthocyanidins; It was reported that proanthocyanidins from pea seed coats cultivar ‘Solido’ were mostly composed by epigallocatechin and gallocatechin, with a mean degree of polymerization of five (Ferraro et al., 2014). Other components in the pea seed coat are proteins (8%) and approximately 38% of starch from which 7% is resistant; the main pea seed coat fibre components are glucose and xylose (Yang, 2014).

To determine the influence of the degree of polymerization on antibacterial activity, the inhibitory and bactericidal effect of a fraction mixed with (epi) catechins, a dimeric

proanthocyanidin (B2) as well as catechin alone were tested (Shan et al., 2007). Catechin was not able to inhibit any of the five pathogenic strains tested; the inhibition effect of the mixed fraction and proanthocyanidin B2 was very similar and these two compounds were able to inhibit all the strains tested with *E. coli* among them (Shan et al., 2007). This thesis confirms that *E. coli* was resistant to catechin, but was inhibited in some extent by the oligomeric proanthocyanidins from grape seed extract.

The inhibition effect of tetrameric, trimeric and dimeric proanthocyanidins as well as a monomeric fractions was tested finding that all the fractions inhibited *S. aureus* to some extent and being the monomeric fraction the one that showed the highest antimicrobial activity (Kumar et al., 2014). This finding also conforms to this study as all the strains tested were more sensitive to the flavan-*3*-ols than to the oligomeric proanthocyanidins from grape seed extract, except for *L. reuteri* and *L. acidophilus* which were resistant to all the compounds tested.

The lactic acid bacteria, especially *L. reuteri* FUA 3400 and *L. acidophilus* FUA 3066 were resistant to all the compounds tested in this study; this was seen before with tea flavan-*3*-ols, where the growth of *Bifidobacterium breve, Bifidobacterium adolescentis* as well as *Lactobacillus casei* Shirota and *Lactobacillus rhamnosus* isolated from a human fecal sample, remained almost unchanged after the supply of catechin and epicatechin. These strains were also resistant to 3-phenylpropionic acid, 4-OH phenylacetic acid and gallic acid (Lee et al., 2006).

The substantial inhibition of some anaerobic mouse gut bacteria by flavan-3-ols in this study agrees with several publications in which these compounds inhibit gut microbiota selectively depending on the chemical structure of the compound and the bacterial species. For example, it was observed that several members of the *Clostridium* and *Bacteroides* genera were inhibited by flavan-*3*-ols from tea, whereas *Bifidobacterium spp*. and *Lactobacillus spp.* remained unchanged (Lee et al., 2006). In the case of oligomeric proanthocyanidins from grape seed extract, mice gut isolates were also substantially inhibited, but the antibacterial effect of these fractions is lower compared to the flavan-*3*-ols, especially for EGCG. The antimicrobial effect against members of

mouse gut microbiota was bactericidal for both flavan-*3*-ols and oligomeric proanthocyanidins from grape seed extract.

Previously, the inhibitory and bactericidal activities of hydrolysable tannins from mango kernels with different degrees of galloylation were determined towards an array of food-borne bacteria and fungi, finding no significant difference in the MIC values between penta-, hexa-, hepta-, octa-, nona-, and deca-*O*-galloylglucose. (Engels et al., 2011). This finding relates to the present study as there was no difference between the antimicrobial action of EGC and EGCG although EGCG has 3 more active hydroxyl groups in its gallate ester than EGC. Moreover, oligomeric proanthocyanidins were did not have a higher activity when compared to monomers.

Lactic acid bacteria and *E. coli* were resistant to mango seed kernel extract containing gallotannins with different degrees of polymerization. Two *C. jejuni* strains were tested as well and they were sensitive to the mango kernel extract but resistant to catechins from green tea, the latter being contrary to what was found in the present study (Kabuki et al., 2000). Engels et al (2011) reported *C. jejuni* as the most inhibited strain by hepta-*O*-galloylglucose and found that this strain was very sensitive toward EGCG.

Catechin was found to promote the growth of human fecal strains of *Lactobacillus spp*. and *Bifidobacterium spp*. using a batch-culture fermentation model (Tzounis et al., 2008). Finding that agrees with this study as *Bifidobacterium adolescentis, L. reuteri and L. acidophilus* were resistant to all the compounds tested.

Catechin and epicatechin do not inhibit *E. coli* but the flavan-*3*-ol gut microbiota metabolites 3-(4-OH phenyl) propionic acid, 3-Phenylpropionic acid and 4-OH phenylacetic acid do. Several pathogenic strains were also inhibited: *Listeria monocytogenes, Pseudomonas aeruginosa, Salmonella enterica* serovar Typhymurium, among others (Lee et al., 2006). Thereby, flavan-*3*-ols and their metabolites are suggested to play a role in the intestinal health maintenance. These two flavan-3-ols, catechin and epicatechin do not inhibit the growth of the *Clostridium coccoides - Eubacterium rectale* group (Tzounis et al., 2008). These microorganisms are relevant as they are short chain fatty acid producers, molecules that have been associated to health promoting effects (Gibson 1999).

4.3 Enzyme inhibition properties of flavan-*3*-ols and oligomeric proanthocyanidins

Proanthocyanidins bind dietary proteins and digestive enzymes including pancreatic and brush-border enzymes, due to the abundant hydroxyl groups in the molecules (Scalbert et al., 2000) that bind the amino groups in peptides or other molecules (Siebert, Troukhanova and Lynn, 1996). For hydrolysable tannins as well as condensed tannins, larger polymers with more co-ordinated hydroxyl groups bind proteins more efficiently than smaller molecules (Horigome et al., 1988; Baxter et al., 1997; Jonker and Yu, 2017), although no difference in protein binding properties were found in proanthocyanidins with mean degree of polymerization above eight (Harbertson et al., 2014). Based on this, it was hypothesized that the higher the number of hydroxyl groups in flavan-*3*-ols as well as the oligomer length in proanthocyanidins the enzyme inhibition ability will be higher as well, as more OH groups are able to bind a larger number of digestive enzymes inactivating them. The enzyme binding properties also depend on the stereochemistry and the type of linkage among monomers (Jonker and Yu, 2017).

All of the compounds tested inhibited rat digestive enzymes in different extents except for catechin which had no difference from the control. EGCG, EGC and crude pea seed coat extract showed the highest inhibitory effect, followed by the PA-enriched extract, EC, Fraction I and the polymeric PA grape seed extract. Epicatechin was able to inhibit digestive enzymes in a greater way compared to catechin, both of these molecules possess 2 hydroxyl groups in their B rings and just differ in their stereochemistry conformation, suggesting that this attribute plays a role in the enzyme binding properties of flavan-*3*-ols against digestive enzymes, differing from the antimicrobial activity were there was no difference between catechin and epicatechin.

Difference was found between EC and EGC as well as between EC and EGCG. EGC counts with 3 hydroxyl groups which are able to bind enzymes whereas EGCG has 6, nevertheless there was no difference between these two flavan-*3*-ols. This may be because the enzyme action was too exhaustive and both compounds reached the maximum glucose release. For further experiments and digestive enzymes inhibition determination, the concentration of phenolic compounds should be lowered to observe if

there is an actual difference between these two flavan-*3*-ols. In the case of the grape seed proanthocyanidin fractions, there was no difference between the polymeric proanthocyanidin fraction and Fractions II and III. These results suggest that the degree of hydroxylation of flavan-*3*-ols changes inhibition of digestive enzymes. This results agree with previous publication where the number of hydroxyl groups matches the enzyme inhibition properties of flavan-*3*-ols (Jonker and Yu, 2017).

The degree of polymerization of oligomeric grape seed extract proanthocyanidins does not play a role in their inhibitory properties, contrary to previous findings that suggest that protein binding properties are dependent on the degree of polymerization of the proanthocyanidins (Horigome et al., 1988; Baxter et al., 1997; Harbertson et al., 2014).

The enzyme inhibition properties of flavan-*3*-ols and proanthocyanidins extend beyond digestive enzymes and they can act against pathogens. One example is the inhibition of urease activity in *Helicobacter pylori* (Adeniyi et al., 2009), the enzyme that helps *H. pylori* to release ammonia and survive in the acid gastric environment (Cires et al., 2016). Another interesting example is the ability of tea catechins to inhibit biofilm formation in E*scherichia coli* O157:H7 (Lee et al., 2009) since they bind surface proteins.

4.4 Lactobacillus resistance to compounds tested.

The *Lactobacillus* strains tested in this study were resistant to all the compounds tested: flavan-*3*-ols and proanthocyanidins from pea seed coat and grape seed extract. These results are in accordance to previous publications where the growth of *Lactobacillus sp*. and *Bifidobacterium sp*., which were not affected by the presence of catechin or epicatechin in batch fermentations (Lee et al., 2006; Tzounis et al., 2008).

In the MIC assays, *Lactobacillus reuteri* FUA 3400 and *Lactobacillus acidophilus* FUA 3066 were resistant, but in the experiment with wheat flour, the pH along the fermentation for both controls of *L. reuteri* and *L. acidophilus* were significantly lower than the pH values of the fermentation with 10% crude pea seed coat extract. This finding suggests that the crude pea seed coat extract affects the growth of *L. reuteri* and *L. acidophilus* as these strains are acid producers and a lower pH indicates higher

growth. *Pisum sativum L*. cultivar Solido seed coats were reported to contain 4.51mg of proanthocyanidins per 100mg of seed coats (Ferrero et al., 2014), so even at a low concentration, as in the case of the sourdough experiment, proanthocyanidins from the crude extract can influence microbial ecology. These results are in accordance with a previous publication where a fermentation with sorghum was carried out and *Lactobacillus casei* and *Lactobacillus reuteri* were inhibited (Lin and Gänzle, 2014); sorghum contains high concentration of phenolic compounds with proanthocyanidins among them, especially in the bran (Awika, McDonough and Rooney, 2005). Lactobacillus does not need iron to grow in anaerobic conditions (Archibald, 1983), in contrast with other bacterial species; this trait can explain the resistance to flavan-3-ols and oligomeric proanthocyanidins shown by this genus. Also, the composition of the cell wall in terms of peptidoglycan varies in composition among bacterial species; flavan-3-ols and oligomeric proanthocyanidins have proved to bind cell wall components, thus the inhibition of bacterial species will vary (Engels et al., 2011).

4.5 Bioavailability and antinutritional effect of flavan-*3*-ols and proanthocyanidins

Oligomeric proanthocyanidins and flavan-*3*-ols have been proposed to exert several health promoting effects. Their mechanisms may relate to the modulation of gastric transit, the inhibition of digestive enzymes, the modulation of the gut microbiota, or to the direct health promoting effects upon absorption. In this study, the enzyme inhibtion as well as antimicrobial properties of flavan-*3*-ols and oligomeric proanthocyanidins were tested. The inhibition of digestive enzymes is closely linked to the biological activity of flavan-*3*-ols and proanthocyanidins. One example of this is the inhibition of α-amylase and glucoamylase that results in lowered glucose levels in blood after meal consumption: thus, this inhibition could be applied to modulate blood glucose levels in patients with metabolic disorders like diabetes (Barrett et al., 2013; Li et al., 2005; Apostolidis et al., 2003).

At the bacterial scale, α-amylase binding by flavan-*3*-ols and proanthocyanidins in the forestomach is not beneficial for the *Lactobacillus* strains as they need maltose released from starch to thrive (Stolz et al., 1993). Amylase inhibition in the small intestine,

however, benefits colonic bacteria since starch is the substrate for some short chain fatty acid producers (Leitch et al., 2007; Tremaroli and Bäckhed, 2012).

The antinutritional effects or the health benefits exerted by flavan-3-ols and proanthocyanidins depend on the general health and nutritional status of the host. If the diet of the host is low in protein and caloric intake, proanthocyanidin consumption will be counterproductive due to the binding of dietary protein and digestive enzymes which will result in delayed digestion of dietary components and their absorption (McSweeney et al., 2001; Butler, 1992). Nevertheless, if the diet of the host is low in dietary fibre the digestive enzyme inhibition of proanthocyanidins is beneficial, as starch will reach the colon where it can be metabolized by microbiota (De Filippo et al., 2010; Tremaroli and Bäckhed, 2012).

4. Conclusions

The objective of this study was to separate oligomeric proanthocyanidins from pea seed coats, unfortunately proanthocyanidins were not found in enough qualities for an extraction with HSCCC by their degree of polymerization. Commercial grape seed extract was chosen as it possesses >40% of proanthocyanidins in its composition. Using HSCCC, three fractions with different degrees of polymerization were isolated and together with flavan-3-ols, it was possible to determine the antibacterial effect of these compounds as well as their ability to inhibit digestive enzymes.

It was found that the antimicrobial effect of flavan-3-ols increases with the number of hydroxyl groups in the molecule, specifically by those located on the B ring of the monomer and the ones from gallate moieties attached to them. In the case of oligomeric proanthocyanidins from grape seed extract, the antimicrobial effect is not affected by the length of the oligomer.

Regarding the enzyme binding effect of flavan-3-ols, it increases with the number of hydroxyl groups present in the monomer, but there was no evidence that the enzyme binding properties increased with the length of the oligomer in the case of grape seed proanthocyanidins.

The health promoting effects exerted by gut microbiota depend on several factors and cannot be entirely explained by gut microbiota modulation or the enzyme binding properties of flavan-*3*-ols and proanthocyanidins alone. Further research needs to be done in the modulation of the gastric transit by flavan-*3*-ols and oligomeric proanthocyanidins as well as their direct effects upon absorption. It would be interesting to determine the antimicrobial effect of flavan-*3*-ol metabolites as well, for a more thorough understanding of the modulation of gut microbiota by these compounds.

References

Adeniyi, B. A., Onwubuche, B. C., Anyiam, F. M., Ekundayo, O., & Mahady, G. B. (2009). Anti-*Helicobacter pylori* activities of *Eucalyptus grandis*: Effects on susceptibility, urease activity and cell surface hydrophobicity. *Pharmaceutical Biology, 47(1), 13-17*.

Ahn, Y. J., Kawamura, T., Kim, M., Yamamoto, T., & Mitsuoka, T. (1991). Tea polyphenols: selective growth inhibitors of Clostridium spp. *Agricultural and Biological Chemistry*, *55*(5), 1425-1426.

Akiyama, H., Fujii, K., Yamasaki, O., Oono, T., & Iwatsuki, K. (2001). Antibacterial action of several tannins against *Staphylococcus aureus*. *Journal of antimicrobial chemotherapy, 48(4), 487-491.*

Almajano, M. P., Carbo, R., Jiménez, J. A. L., & Gordon, M. H. (2008). Antioxidant and antimicrobial activities of tea infusions. *Food chemistry*, *108*(1), 55-63.

Amarowicz, R., Naczk, M., Zadernowski, R., & Shahidi, F. (2000). Antioxidant activity of condensed tannins of beach pea, canola hulls, evening primrose, and faba bean. *Journal of Food Lipids*, 7(3), 195-205.

Apostolidis, E., Kwon, Y. I., & Shetty, K. (2006). Potential of cranberry-based herbal synergies for diabetes and hypertension management. *Asia Pacific journal of clinical nutrition*, *15*(3), 433.

Archibald, F. (1983). Lactobacillus plantarum, an organism not requiring iron. FEMS microbiology letters, 19(1), 29-32.

Aron, P. M., & Kennedy, J. A. (2008). Flavan-3-ols: Nature, occurrence and biological activity. *Molecular nutrition & food research, 52(1), 79-104*.

Aura, A. M. (2008). Microbial metabolism of dietary phenolic compounds in the colon. *Phytochemistry Reviews*, *7*(3), 407-429.

Avau, B., & Depoortere, I. (2015). The bitter truth about bitter taste receptors: Beyond sensing bitter in the oral cavity. *Acta Physiologica*

Avau, B., Rotondo, A., Thijs, T., Andrews, C. N., Janssen, P., Tack, J., & Depoortere, I. (2015). Targeting extra-oral bitter taste receptors modulates gastrointestinal motility with effects on satiation. *Scientific reports*, *5*, 15985.

Awika, J. M., McDonough, C. M., & Rooney, L. W. (2005). Decorticating sorghum to concentrate healthy phytochemicals. *Journal of Agricultural and Food Chemistry, 53(16)*, 6230-6234.

Bäckhed, F., Ding, H., Wang, T., Hooper, L. V., Koh, G. Y., Nagy, A., ... & Gordon, J. I. (2004). The gut microbiota as an environmental factor that regulates fat storage. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(44), 15718-15723.

Bäckhed, F., Ley, R. E., Sonnenburg, J. L., Peterson, D. A., & Gordon, J. I. (2005). Host-bacterial mutualism in the human intestine. *Science*, *307*(5717), 1915-1920.

Barcelo, J., & Poschenrieder, C. (2002). Fast root growth responses, root exudates, and internal detoxification as clues to the mechanisms of aluminium toxicity and resistance: a review. *Environmental and Experimental Botany*, *48*(1), 75-92.

Barrett, A., Ndou, T., Hughey, C. A., Straut, C., Howell, A., Dai, Z., & Kaletunc, G. (2013). Inhibition of α-amylase and glucoamylase by tannins extracted from cocoa, pomegranates, cranberries, and grapes. *Journal of agricultural and food chemistry*, *61*(7), 1477-1486.

Baxter, N. J., Lilley, T. H., Haslam, E., & Williamson, M. P. (1997). Multiple interactions between polyphenols and a salivary proline-rich protein repeat result in complexation and precipitation. *Biochemistry, 36(18),* 5566-5577.

Beckman, C. H. (2000). Phenolic-storing cells: keys to programmed cell death and periderm formation in wilt disease resistance and in general defence responses in plants? *Physiological and Molecular Plant Pathology*, *57*(3), 101-110.

Bokkenheuser, V. D., Shackleton, C. H., & Winter, J. (1987). Hydrolysis of dietary flavonoid glycosides by strains of intestinal Bacteroides from humans. *Biochemical Journal*, *248*(3), 953-956.

Bravo, L. (1998). Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. *Nutrition reviews*, *56*(11), 317-333.

Breer, H., Eberle, J., Frick, C., Haid, D., & Widmayer, P. (2012). Gastrointestinal chemosensation: chemosensory cells in the alimentary tract. *Histochemistry and cell biology*, *138*(1), 13-24.

Brown, B. R., & Shaw, M. R. (1974). Reactions of flavanoids and condensed tannins with sulphur nucleophiles. J*ournal of the Chemical Society, Perkin Transactions* 1, 2036-2049.

Brune, A., & Friedrich, M. (2000). Microecology of the termite gut: structure and function on a microscale. *Current opinion in microbiology*, *3*(3), 263-269.

Butler, L. G. (1992). Antinutritional effects of condensed and hydrolysable tannins. In Plant polyphenols (pp. 693-698). Springer US.

Campos, F. M., Couto, J. A., & Hogg, T. A. (2003). Influence of phenolic acids on growth and inactivation of *Oenococcus oeni* and *Lactobacillus hilgardii*. *Journal of Applied Microbiology*, *94*(2), 167-174.

Cardona, F., Andrés-Lacueva, C., Tulipani, S., Tinahones, F. J., & Queipo-Ortuño, M. I. (2013). Benefits of polyphenols on gut microbiota and implications in human health. *The Journal of nutritional biochemistry*, *24*(8), 1415-1422.

Chen, M. C., Wu, S. V., Reeve, J. R., & Rozengurt, E. (2006). Bitter stimuli induce Ca 2+ signaling and CCK release in enteroendocrine STC-1 cells: role of L-type voltage-sensitive Ca 2+ channels. *American Journal of Physiology-Cell Physiology, 291(4),* C726-C739.

Cheynier, V., Souquet, J. M., Kontek, A., & Moutounet, M. (1994). Anthocyanin degradation in oxidising grape musts. *Journal of the Science of Food and Agriculture, 66(3),* 283-288.

Cheynier, V., Souquet, J. M., Le Roux, E., Guyot, S., & Rigaud, J. (1999). Size separation of condensed tannins by normal-phase high-performance liquid chromatography. *Methods in enzymology*, *299*, 178-184.

Chung, K. T., Lu, Z., & Chou, M. W. (1998). Mechanism of inhibition of tannic acid and related compounds on the growth of intestinal bacteria. *Food and Chemical Toxicology*, *36*(12), 1053-1060.

Cires, M. J., Wong, X., Carrasco-Pozo, C., & Gotteland, M. (2016). The gastrointestinal tract as a key target organ for the health-promoting effects of dietary proanthocyanidins. *Frontiers in Nutrition*, *3*.

Clavel, T., Henderson, G., Engst, W., Doré, J., & Blaut, M. (2006). Phylogeny of human intestinal bacteria that activate the dietary lignan secoisolariciresinol diglucoside. *FEMS microbiology ecology*, *55*(3), 471-478

Clifford, M. N. (1999). Chlorogenic acids and other cinnamates–nature, occurrence and dietary burden. *Journal of the Science of Food and Agriculture*, *79*(3), 362-372.

Clifford, M. N. (2004). Diet-derived phenols in plasma and tissues and their implications for health. *Planta medica*, *70*(12), 1103-1114.

Clifford, M. N., & Scalbert, A. (2000). Ellagitannins–nature, occurrence and dietary burden. *Journal of the Science of Food and Agriculture*, *80*(7), 1118-1125.

Cos, P., Bruyne, T. D., Hermans, N., Apers, S., Berghe, D. V., & Vlietinck, A. J. (2004). Proanthocyanidins in health care: current and new trends. *Current medicinal chemistry*, *11*(10), 1345-1359.

Crozier, A., Del Rio, D., & Clifford, M. N. (2010). Bioavailability of dietary flavonoids and phenolic compounds. *Molecular aspects of medicine*, *31*(6), 446-467.

Czochanska, Z., Foo, L. Y., Newman, R. H., & Porter, L. J. (1980). Polymeric proanthocyanidins. Stereochemistry, structural units, and molecular weight. *Journal of the Chemical Society, Perkin Transactions 1*, 2278-2286.

Daglia, M. (2012). Polyphenols as antimicrobial agents. *Current opinion in biotechnology*, *23*(2), 174-181.

Das, N. P. (1971). Studies on flavonoid metabolism: absorption and metabolism of (+)-catechin in man. *Biochemical pharmacology*, *20*(12), 3435-3445.

David, L.A., Maurice, C.F., Carmody, R.N., Gootenberg, D.B., Button, J.E., Wolfe, B.E., Ling, A.V., Devlin, A.S., Varma, Y., Fischbach, M.A., et al. (2014). Diet rapidly and reproducibly alters the human gut microbiome. *Nature* 505, 559–563.

De Filippo, C., Cavalieri, D., Di Paola, M., Ramazzotti, M., Poullet, J. B., Massart, S., & Lionetti, P. (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(33),* 14691-14696.

Degenhardt, A., Engelhardt, U. H., Lakenbrink, C., & Winterhalter, P. (2000b). Preparative separation of polyphenols from tea by high-speed countercurrent chromatography. *Journal of agricultural and food chemistry*, *48*(8), 3425-3430.

Degenhardt, A., Knapp, H., & Winterhalter, P. (2000a). Separation and purification of anthocyanins by high-speed countercurrent chromatography and screening for antioxidant activity. *Journal of Agricultural and Food Chemistry*, *48*(2), 338-343.

den Besten, G., van Eunen, K., Groen, A. K., Venema, K., Reijngoud, D. J., & Bakker, B. M. (2013). The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *Journal of lipid research, 54(9),* 2325-2340.

Depoortere, I. (2013). Taste receptors of the gut: emerging roles in health and disease. *Gut*, gutjnl-2013.

Déprez, S., Brezillon, C., Rabot, S., Philippe, C., Mila, I., Lapierre, C., & Scalbert, A. (2000). Polymeric proanthocyanidins are catabolized by human colonic microflora into low-molecular-weight phenolic acids. *The Journal of nutrition*, *130*(11), 2733-2738.

Deprez, S., Mila, I., Huneau, J. F., Tome, D., & Scalbert, A. (2001). Transport of proanthocyanidin dimer, trimer, and polymer across monolayers of human intestinal epithelial Caco-2 cells. *Antioxidants and redox signaling*, *3*(6), 957-967.

Dhingra, D., Michael, M., Rajput, H., & Patil, R. T. (2012). Dietary fibre in foods: a review. *Journal of food science and technology, 49(3)*, 255-266.

Diker, K. S., Akan, M., Hascelik, G., & Yurdakök, M. (1991). The bactericidal activity of tea against *Campylobacter jejuni* and *Campylobacter coli. Letters in Applied Microbiology*, *12*(2), 34-35.

Ding, Y., Dai, X., Jiang, Y., Zhang, Z., Bao, L., Li, Y., ... & Gu, J. (2013). Grape seed proanthocyanidin extracts alleviate oxidative stress and ER stress in skeletal muscle of low-dose streptozotocin-and high-carbohydrate/high-fat diet-induced diabetic rats. Molecular nutrition & food research, 57(2), 365-369.

Dixon, R. A., Xie, D. Y., & Sharma, S. B. (2005). Proanthocyanidins–a final frontier in flavonoid research? *New phytologist*, *165*(1), 9-28.

Donovan, J. L., Lee, A., Manach, C., Rios, L., Morand, C., Scalbert, A., & Rémésy, C. (2002). Procyanidins are not bioavailable in rats fed a single meal containing a grapeseed extract or the procyanidin dimer B 3. *British Journal of Nutrition*, *87*(04), 299-306.

Dotson, C. D., Geraedts, M. C., & Munger, S. D. (2013, March). Peptide regulators of peripheral taste function. *In Seminars in cell & developmental biology* (Vol. 24, No. 3, pp. 232-239). Academic Press.

Du, Q., Jerz, G., & Winterhalter, P. (2004). Isolation of two anthocyanin sambubiosides from bilberry (*Vaccinium myrtillus*) by high-speed counter-current chromatography. *Journal of Chromatography A, 1045(1)*, 59-63.

Dueñas, M., Muñoz-González, I., Cueva, C., Jiménez-Girón, A., Sánchez-Patán, F., Santos-Buelga, C.,... & Bartolomé, B. (2015). A survey of modulation of gut microbiota by dietary polyphenols. *BioMed research international*, *2015*.

Eckburg, P. B., Bik, E. M., Bernstein, C. N., Purdom, E., Dethlefsen, L., Sargent, M., ... & Relman, D. A. (2005). Diversity of the human intestinal microbial flora. *Science*, *308*(5728), 1635-1638.

Eloff, J. N. "A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria." *Planta medica* 64.08 (1998): 711-713.

Engels, C., Gänzle, M. G., & Schieber, A. (2009). Fractionation of gallotannins from mango (*Mangifera indica L.*) kernels by high-speed counter-current chromatography and determination of their antibacterial activity. *Journal of agricultural and food chemistry, 58(2),* 775-780.

Engels, C., Schieber, A., & Gänzle, M. G. (2011). Studies on the inhibitory spectrum and mode of antimicrobial action of gallotannins from mango kernels (*Mangifera indica L.*). *Applied and environmental microbiology.*

Esatbeyoglu, T., Wray, V., & Winterhalter, P. (2015). Isolation of dimeric, trimeric, tetrameric and pentameric procyanidins from unroasted cocoa beans (*Theobroma cacao L.*) using countercurrent chromatography. *Food chemistry*, *179*, 278-289.

Ferraro, K., Jin, A. L., Nguyen, T. D., Reinecke, D. M., Ozga, J. A., & Ro, D. K. (2014). Characterization of proanthocyanidin metabolism in pea (*Pisum sativum*) seeds. *BMC plant biology*, *14*(1), 238.

Foo, L. Y., & Karchesy, J. J. (1991). Procyanidin tetramers and pentamers from Douglas fir bark. *Phytochemistry*, *30*(2), 667-670.

Foo, L. Y., & Porter, L. J. (1980). The phytochemistry of proanthocyanidin polymers. *Phytochemistry*, *19*(8), 1747-1754.

Foo, L. Y., Lu, Y., Howell, A. B., & Vorsa, N. (2000). A-Type proanthocyanidin trimers from cranberry that inhibit adherence of uropathogenic P-Fimbriated *Escherichia coli*. *Journal of Natural Products*, *63*(9), 1225-1228.

Foo, L. Y., Lu, Y., McNabb, W. C., Waghorn, G., & Ulyatt, M. J. (1997). Proanthocyanidins from *Lotus pedunculatus*. *Phytochemistry*, *45*(8), 1689-1696.

Frank, D. N., Amand, A. L. S., Feldman, R. A., Boedeker, E. C., Harpaz, N., & Pace, N. R. (2007). Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proceedings of the National Academy of Sciences*, *104*(34), 13780-13785.

Friedman, M., Henika, P. R., Levin, C. E., Mandrell, R. E., & Kozukue, N. (2006). Antimicrobial activities of tea catechins and theaflavins and tea extracts against *Bacillus cereus*. *Journal of Food Protection®*, *69*(2), 354-361.

Friedrich, W., Eberhardt, A., & Galensa, R. (2000). Investigation of proanthocyanidins by HPLC with electrospray ionization mass spectrometry. *European Food Research and Technology, 211(1)*, 56-64.

Gibson, G. R., & Fuller, R. (2000). Aspects of in vitro and in vivo research approaches directed toward identifying probiotics and prebiotics for human use. *The Journal of nutrition, 130(2),* 391S-395S.

Gibson, G. R., & Wang, X. (1994). Regulatory effects of bifidobacteria on the growth of other colonic bacteria. *Journal of Applied Microbiology, 77(4),* 412-420.

Gibson, G. R. (1999). Dietary modulation of the human gut microflora using the prebiotics oligofructose and inulin. *The Journal of nutrition, 129(7),* 1438S-1441s.

Glendinning, J. I., Yiin, Y. M., Ackroff, K., & Sclafani, A. (2008). Intragastric infusion of denatonium conditions flavor aversions and delays gastric emptying in rodents. *Physiology & behavior, 93(4),* 757-765.

Goldstein, J. L., & Swain, T. (1965). The inhibition of enzymes by tannins. *Phytochemistry*, *4*(1), 185-192.

Gonthier, M. P., Donovan, J. L., Texier, O., Felgines, C., Remesy, C., & Scalbert, A. (2003). Metabolism of dietary procyanidins in rats. *Free Radical Biology and Medicine*, *35*(8), 837-844.

Griffiths, L. A. (1982). Mammalian metabolism of flavonoids. In *The flavonoids* (pp. 681-718). Springer US.

Groenewoud, G., & Hundt, H. K. L. (1984). The microbial metabolism of (+)-catechin to two novel diarylpropan-2-ol metabolites in vitro. *Xenobiotica*, *14*(9), 711-717.

Groenewoud, G., & Hundt, H. K. L. (1986). The microbial metabolism of condensed (+)-catechins by rat-caecal microflora. *Xenobiotica*, *16*(2), 99-107.

Groot, H. D., & Rauen, U. (1998). Tissue injury by reactive oxygen species and the protective effects of flavonoids. *Fundamental & clinical pharmacology*, *12*(3), 249-255.

Grotewold E (2005). The science of flavonoids. Springer.

Gu, L., Kelm, M. A., Hammerstone, J. F., Beecher, G., Holden, J., Haytowitz, D., & Prior, R. L. (2004). Concentrations of proanthocyanidins in common foods and estimations of normal consumption. *The Journal of nutrition*, *134*(3), 613-617.

Gu, L., Kelm, M. A., Hammerstone, J. F., Beecher, G., Holden, J., Haytowitz, D., & Prior, R. L. (2003). Screening of foods containing proanthocyanidins and their structural characterization using LC-MS/MS and thiolytic degradation. *Journal of Agricultural and Food Chemistry, 51(25*), 7513-7521.

Gu, L., Kelm, M. A., Hammerstone, J. F., Zhang, Z., Beecher, G., Holden, J., ... & Prior, R. L. (2003). Liquid chromatographic/electrospray ionization mass spectrometric studies of proanthocyanidins in foods. *Journal of Mass Spectrometry, 38(12),* 1272-1280.

Gujer, R., Magnolato, D., & Self, R. (1986). Glucosylated flavonoids and other phenolic compounds from sorghum. *Phytochemistry*, *25*(6), 1431-1436.

Hammerstone, J. F., Lazarus, S. A., Mitchell, A. E., Rucker, R., & Schmitz, H. H. (1999). Identification of procyanidins in cocoa (*Theobroma cacao*) and chocolate using high-performance liquid chromatography/mass spectrometry. *Journal of Agricultural and Food Chemistry*, *47*(2), 490-496.

Hamosh, M., & Burns, W. A. (1977). Lipolytic activity of human lingual glands (Ebner). *Laboratory investigation; a journal of technical methods and pathology*, *37*(6), 603-608.

Harbertson, J. F., Kilmister, R. L., Kelm, M. A., & Downey, M. O. (2014). Impact of condensed tannin size as individual and mixed polymers on bovine serum albumin precipitation. *Food chemistry, 160,* 16-21.

Harborne, J. B. (2013). *The flavonoids: advances in research since 1980*. Springer.

Haslam, E. (1974). Polyphenol–protein interactions. *Biochemical Journal*, *139*(1), 285.

Haslam, G., Wyatt, D., & Kitos, P. A. (2000). Estimating the number of viable animal cells in multi-well cultures based on their lactate dehydrogenase activities. *Cytotechnology*, *32*(1), 63-75.

Hathway, D. E., & Seakins, J. W. T. (1957). Enzymic oxidation of catechin to a polymer structurally related to some phlobatannins. *Biochemical Journal, 67(2)*, 239.

Havsteen, B. H. (2002). The biochemistry and medical significance of the flavonoids. *Pharmacology & therapeutics*, *96*(2), 67-202.

Hemingway, R. W., & McGraw, G. W. (1983). Kinetics of acid-catalyzed cleavage of procyanidins. *Journal of Wood Chemistry and Technology*, *3*(4), 421-435.

Herness, M. S., & Gilbertson, T. A. (1999). Cellular mechanisms of taste transduction. *Annual review of physiology, 61(1),* 873-900.

Hervert-Hernández, D., & Goñi, I. (2011). Dietary polyphenols and human gut microbiota: a review. *Food Reviews International*, *27*(2), 154-169.

Höfer, D., Püschel, B., & Drenckhahn, D. (1996). Taste receptor-like cells in the rat gut identified by expression of alpha-gustducin. *Proceedings of the National Academy of Sciences, 93(13),* 6631-6634.

Horigome, T., Kumar, R., & Okamoto, K. (1988). Effects of condensed tannins prepared from leaves of fodder plants on digestive enzymes in vitro and in the intestine of rats. *British journal of nutrition*, *60*(02), 275-285.

Ikigai, H., Nakae, T., Hara, Y., & Shimamura, T. (1993). Bactericidal catechins damage the lipid bilayer. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, *1147*(1), 132-136.

Ito, Y. (1981). Countercurrent chromatography. *Journal of biochemical and biophysical methods*, *5*(2), 105-129.

Ito, Y. (2005). Golden rules and pitfalls in selecting optimum conditions for high-speed counter-current chromatography. *Journal of Chromatography A*, *1065*(2), 145-168.

Ito, Y., & Conway, W. D. (1986). High-speed countercurrent chromatography.

Iwashina, Tsukasa. "The structure and distribution of the flavonoids in plants." *Journal of Plant Research* 113.3 (2000): 287-299.

Janssen, S., Laermans, J., Verhulst, P. J., Thijs, T., Tack, J., & Depoortere, I. (2011). Bitter taste receptors and α-gustducin regulate the secretion of ghrelin with functional effects on food intake and gastric emptying. *Proceedings of the National Academy of Sciences, 108(5)*, 2094-2099.

Ji, T. H., Grossmann, M., & Ji, I. (1998). G protein-coupled receptors I. Diversity of receptor-ligand interactions. *Journal of Biological Chemistry, 273(28),* 17299-17302.

Jin Lihua (2011), Flavonoids in Saskatoon Fruits, Blueberry Fruits, and Legume Seeds. Department of Agricultural, Food and Nutritional Science, University of Alberta. PhD Thesis.

Jin, A. L., Ozga, J. A., Lopes-Lutz, D., Schieber, A., & Reinecke, D. M. (2012). Characterization of proanthocyanidins in pea (*Pisum sativum L.*), lentil (*Lens culinaris L.*), and faba bean (*Vicia faba L.*) seeds. *Food Research International*, *46*(2), 528-535.

Johnson LR (1997) Gastrointestinal Physiology. St Louis, MO: Mosby-Year Book, Inc.

Jonker, A., & Yu, P. (2017). The Occurrence, Biosynthesis, and Molecular Structure of Proanthocyanidins and Their Effects on Legume Forage Protein Precipitation, Digestion and Absorption in the Ruminant Digestive Tract. *International journal of molecular sciences, 18(5),* 1105.

Kale, A., Gawande, S., Kotwal, S., Netke, S., Roomi, W., Ivanov, V., ... & Rath, M. (2010). Studies on the effects of oral administration of nutrient mixture, quercetin and red onions on the bioavailability of epigallocatechin gallate from green tea extract. Phytotherapy Research, 24(S1).

Kashiwada, Y., Nonaka, G. I., & Nishioka, I. (1986). Tannins and Related Compounds. XLV. Rhubarb. (5): Isolation and Characterization of Flavan-*3*-ol and Procyanidin Glucosides. *Chemical and pharmaceutical bulletin*, *34*(8), 3208-3222.

Kennedy, J. A., & Jones, G. P. (2001). Analysis of proanthocyanidin cleavage products following acid-catalysis in the presence of excess phloroglucinol. *Journal of Agricultural and Food Chemistry*, *49*(4), 1740-1746.

Kennedy, J. A., & Taylor, A. W. (2003). Analysis of proanthocyanidins by high-performance gel permeation chromatography. *Journal of Chromatography A*, *995*(1), 99-107.

Kennedy, J. A., & Waterhouse, A. L. (2000). Analysis of pigmented high-molecular-mass grape phenolics using ion-pair, normal-phase high-performance liquid chromatography. *Journal of Chromatography A*, *866*(1), 25-34.

Kleessen, Eugenia Bezirtzoglou, Jaana Mättö, B. (2000). Culture-based knowledge on biodiversity, development and stability of human gastrointestinal microflora. *Microbial ecology in health and disease*, *12*(2), 53-63.

Köhler, N., Wray, V., & Winterhalter, P. (2008). Preparative isolation of procyanidins from grape seed extracts by high-speed counter-current chromatography. *Journal of Chromatography A*, *1177*(1), 114-125.

Kondratyuk, T. P., & Pezzuto, J. M. (2004). Natural product polyphenols of relevance to human health. *Pharmaceutical biology*, *42*(sup1), 46-63.

Kono, K., Tatara, I., Takeda, S., Arakawa, K., & Hara, Y. (1994). Antibacterial activity of epigallocatechin gallate against methicillin-resistant *Staphylococcus aureus*. *Kansenshogaku Zasshi. The Journal of the Japanese Association for Infectious Diseases*, *68*(12), 1518-1522.

Krasteva, G., Canning, B. J., Papadakis, T., & Kummer, W. (2012). Cholinergic brush cells in the trachea mediate respiratory responses to quorum sensing molecules. *Life sciences, 91(21),* 992-996.

Kumar, N. S., Wijekoon, W. M. B., Kumar, V., Punyasiri, P. N., & Abeysinghe, I. S. B. (2009). Separation of proanthocyanidins isolated from tea leaves using high-speed counter-current chromatography. *Journal of Chromatography A*, *1216*(19), 4295-4302.

Kumar, N. S., Bandara, B. R., Hettihewa, S. K., & Panagoda, G. J. (2014). Oligomeric proanthocyanidin fractions from fresh tea leaves and their antibacterial activity against *Staphylococcus aureus*. *Journal of the National Science Foundation of Sri Lanka*, 42(3).

Kusumakshi, S., Voigt, A., Hübner, S., Hermans-Borgmeyer, I., Ortalli, A., Pyrski, M., & Montmayeur, J. P. (2015). A binary genetic approach to characterize TRPM5 cells in mice. *Chemical senses*, *40*(6), 413-425.

Kutschera, M., Engst, W., Blaut, M., & Braune, A. (2011). Isolation of catechin-converting human intestinal bacteria. *Journal of applied microbiology*, *111*(1), 165-175.

Lazarus, S. A., Adamson, G. E., Hammerstone, J. F., & Schmitz, H. H. (1999). High-performance liquid chromatography/mass spectrometry analysis of proanthocyanidins in foods and beverages. *Journal of Agricultural and Food Chemistry*, *47*(9), 3693-3701.

Lee, H. C., Jenner, A. M., Low, C. S., & Lee, Y. K. (2006). Effect of tea phenolics and their aromatic fecal bacterial metabolites on intestinal microbiota. *Research in microbiology*, *157*(9), 876-884.

Lee, K. M., Kim, W. S., Lim, J., Nam, S., Youn, M., Nam, S. W., & Park, S. (2009). Antipathogenic properties of green tea polyphenol epigallocatechin gallate at concentrations below the MIC against enterohemorrhagic *Escherichia coli* O157: H7. *Journal of food protection*, *72*(2), 325-331.

Leitch, E. C. M. W., Walker, A. W., Duncan, S. H., Holtrop, G., & Flint, H. J. (2007). Selective colonization of insoluble substrates by human faecal bacteria. Environmental microbiology, 9(3), 667-679.

Li, L., Zhang, S., Cui, Y., Li, Y., Luo, L., Zhou, P., & Sun, B. (2016). Preparative separation of cacao bean procyanidins by high-speed counter-current chromatography. *Journal of Chromatography B*, *1036*, 10-19.

Li, Y., Wen, S., Kota, B. P., Peng, G., Li, G. Q., Yamahara, J., & Roufogalis, B. D. (2005). *Punica granatum* flower extract, a potent α-glucosidase inhibitor, improves postprandial hyperglycemia in Zucker diabetic fatty rats. *Journal of Ethnopharmacology*, *99*(2), 239-244.

Lin, X. B., & Gänzle, M. G. (2014). Quantitative high-resolution melting PCR analysis for monitoring of fermentation microbiota in sourdough. *International journal of food microbiology, 186,* 42-48.

Lindemann, B. (1996). Chemoreception: tasting the sweet and the bitter. *Current Biology*, *6*(10), 1234-1237.

Lozupone, C. A., Stombaugh, J. I., Gordon, J. I., Jansson, J. K., & Knight, R. (2012). Diversity, stability and resilience of the human gut microbiota. *Nature*, *489*(7415), 220-230.

Manach, C., Williamson, G., Morand, C., Scalbert, A., & Rémésy, C. (2005). Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *The American journal of clinical nutrition*, *81*(1), 230S-242S.

Marin, L., Miguélez, E. M., Villar, C. J., & Lombó, F. (2015). Bioavailability of dietary polyphenols and gut microbiota metabolism: antimicrobial properties. BioMed research international, 2015.

Marles, A. Vandenberg, K.E. Bett Polyphenol oxidase activity and differential accumulation of polyphenolics in seed coats of pinto bean (*Phaseolus vulgaris* L.) characterize postharvest color changes. *Journal of Agricultural and Food Chemistry, 56 (16)* (2008), pp. 7049–7056

Marston, A., & Hostettmann, K. (1994). Counter-current chromatography as a preparative tool—applications and perspectives. *Journal of Chromatography A*, *658*(2), 315-341.

Martin, L. T., & Dupré, D. J. (2016). Bitter Taste Receptors.

Matthews, S., Mila, I., Scalbert, A., & Donnelly, D. M. (1997a). Extractable and non-extractable proanthocyanidins in barks. *Phytochemistry, 45(2),* 405-410.

Matthews, S., Mila, I., Scalbert, A., Pollet, B., Lapierre, C., Hervé du Penhoat, C. L., & Donnelly, D. M. (1997b). Method for estimation of proanthocyanidins based on their acid depolymerization in the presence of nucleophiles. *Journal of Agricultural and Food Chemistry*, *45*(4), 1195-1201.

McSweeney, C. S., Palmer, B., McNeill, D. M., & Krause, D. O. (2001). Microbial interactions with tannins: nutritional consequences for ruminants. *Animal Feed Science and Technology, 91(1),* 83-93.

Meng, X., Sang, S., Zhu, N., Lu, H., Sheng, S., Lee, M. J., ... & Yang, C. S. (2002). Identification and characterization of methylated and ring-fission metabolites of tea catechins formed in humans, mice, and rats. *Chemical research in toxicology*, *15*(8), 1042-1050.

Merigo, F., Benati, D., Tizzano, M., Osculati, F., & Sbarbati, A. (2005). a-Gustducin immunoreactivity in the airways. *Cell and tissue research, 319(2),* 211-219.

Meyerhof, W., Batram, C., Kuhn, C., Brockhoff, A., Chudoba, E., Bufe, B., & Behrens, M. (2010). The molecular receptive ranges of human TAS2R bitter taste receptors. *Chemical senses*, *35*(2), 157-170.

Middleton, E., Kandaswami, C., & Theoharides, T. C. (2000). The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacological reviews*, *52*(4), 673-751.

Mochizuki, M., Yamazaki, S. I., Kano, K., & Ikeda, T. (2002). Kinetic analysis and mechanistic aspects of autoxidation of catechins. *Biochimica et Biophysica Acta (BBA)-General Subjects, 1569(1),* 35-44.

Molan, A. L. (2013). Antioxidant and prebiotic activities of selenium-containing green tea. *Nutrition*, *29*(2), 476.

Monagas, M., Urpi-Sarda, M., Sánchez-Patán, F., Llorach, R., Garrido, I., Gómez-Cordovés, C., & Bartolomé, B. (2010). Insights into the metabolism and microbial biotransformation of dietary flavan-*3*-ols and the bioactivity of their metabolites. *Food & function*, *1*(3), 233-253.

Morel, I., Lescoat, G., Cogrel, P., Sergent, O., Pasdeloup, N., Brissot, P., ... & Cillard, J. (1993). Antioxidant and iron-chelating activities of the flavonoids catechin, quercetin and diosmetin on iron-loaded rat hepatocyte cultures. *Biochemical pharmacology*, *45*(1), 13-19.

Nicholson, J. K., Holmes, E., Kinross, J., Burcelin, R., Gibson, G., Jia, W., & Pettersson, S. (2012). Host-gut microbiota metabolic interactions. *Science*, *336*(6086), 1262-1267.

Nishino, C., Enoki, N., Tawata, S., KOBAYASHI, K., & FUKUSHIMA, M. (1987). Antibacterial activity of flavonoids against *Staphylococcus epidermidis*, a skin bacterium. *Agricultural and biological chemistry*, *51*(1), 139-143.

Nonaka, G., Kawahara, O., & Nishioka, I. (1983). Tannins and related compounds. XV. A new class of dimeric flavan-*3*-ol gallates, theasinensins A and B, and proanthocyanidin gallates from green tea leaf.(1). *Chemical and Pharmaceutical Bulletin*, *31*(11), 3906-3914.

Pandey, K. B., & Rizvi, S. I. (2009). Plant polyphenols as dietary antioxidants in human health and disease. *Oxidative medicine and cellular longevity*, *2*(5), 270-278.

Pankey, G. A., & Sabath, L. D. (2004). Clinical relevance of bacteriostatic versus bactericidal mechanisms of action in the treatment of Gram-positive bacterial infections. *Clinical infectious diseases*, *38*(6), 864-870.

Pietta, P. G. (2000). Flavonoids as antioxidants. *Journal of natural products*, *63*(7), 1035-1042.

Porter, L. J. (1989). Tannins. *Methods in plant biochemistry*, *1*, 389-419.

Pourcel, L., Routaboul, J. M., Cheynier, V., Lepiniec, L., & Debeaujon, I. (2007). Flavonoid oxidation in plants: from biochemical properties to physiological functions. *Trends in plant science, 12(1),* 29-36.

Prieur, C., Rigaud, J., Cheynier, V., & Moutounet, M. (1994). Oligomeric and polymeric procyanidins from grape seeds. *Phytochemistry*, *36*(3), 781-784.

Puupponen-Pimiä, R., Nohynek, L., Hartmann-Schmidlin, S., Kähkönen, M., Heinonen, M., Määttä-Riihinen, K., & Oksman-Caldentey, K. M. (2005). Berry phenolics selectively inhibit the growth of intestinal pathogens. *Journal of applied microbiology*, *98*(4), 991-1000.

Quesada, C., Bartolomé, B., Nieto, O., Gómez-Cordovés, C., Hernández, T., & Estrella, I. (1996). Phenolic inhibitors of α-amylase and trypsin enzymes by extracts from pears, lentils, and cocoa. *Journal of Food Protection*, *59*(2), 185-192.

Rasmussen, S. E., Frederiksen, H., Struntze Krogholm, K., & Poulsen, L. (2005). Dietary proanthocyanidins: occurrence, dietary intake, bioavailability, and protection against cardiovascular disease. Molecular nutrition & food research, 49(2), 159-174.

Rinaldi, A., Jourdes, M., Teissedre, P. L., & Moio, L. (2014). A preliminary characterization of Aglianico (*Vitis vinifera L. cv*.) grape proanthocyanidins and evaluation of their reactivity towards salivary proteins. *Food chemistry*, *164*, 142-149.

Rockenbach, I. I., Jungfer, E., Ritter, C., Santiago-Schübel, B., Thiele, B., Fett, R., & Galensa, R. (2012). Characterization of flavan-*3*-ols in seeds of grape pomace by CE, HPLC-DAD-MS n and LC-ESI-FTICR-MS. *Food research international*, *48*(2), 848-855.

Ropiak, H. M., Lachmann, P., Ramsay, A., Green, R. J., & Mueller-Harvey, I. (2017). Identification of structural features of condensed tannins that affect protein aggregation. *PloS one, 12(1),* e0170768.

Rozengurt, E. (2006). Taste receptors in the gastrointestinal tract. I. Bitter taste receptors and α-gustducin in the mammalian gut. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, *291*(2), G171-G177.

Ryan, K. G., Swinny, E. E., Markham, K. R., & Winefield, C. (2002). Flavonoid gene expression and UV photoprotection in transgenic and mutant Petunia leaves. *Phytochemistry*, *59*(1), 23-32.

Saito, M., Hosoyama, H., Ariga, T., Kataoka, S., & Yamaji, N. (1998). Antiulcer activity of grape seed extract and procyanidins. *Journal of Agricultural and Food Chemistry, 46(4),* 1460-1464.

Sakanaka, S., Kim, M., Taniguchi, M., & Yamamoto, T. (1989). Antibacterial substances in Japanese green tea extract against *Streptococcus mutans*, a cariogenic bacterium. *Agricultural and Biological Chemistry*, *53*(9), 2307-2311.

Sakanaka, S., Shimura, N., Aizawa, M., Mujo, K. I. M., & Yamamoto, T. (1992). Preventive effect of green tea polyphenols against dental caries in conventional rats. *Bioscience, biotechnology, and biochemistry*, *56*(4), 592-594.

Santos-Buelga, C., & Scalbert, A. (2000). Proanthocyanidins and tannin-like compounds–nature, occurrence, dietary intake and effects on nutrition and health. *Journal of the Science of Food and Agriculture*, *80*(7), 1094-1117.

Scalbert, A. (1991). Antimicrobial properties of tannins. *Phytochemistry*, *30*(12), 3875-3883.

Scalbert, A., Mila, I., Albrecht, A. M., & Rabot, S. (2000). Proanthocyanidins and human health: systemic effects and local effects in the gut. *Biofactors*, *13*(1-4), 115-120.

Scalbert, A., Morand, C., Manach, C., & Rémésy, C. (2002). Absorption and metabolism of polyphenols in the gut and impact on health. *Biomedicine & Pharmacotherapy*, *56*(6), 276-282.

Schofield, P., Mbugua, D. M., & Pell, A. N. (2001). Analysis of condensed tannins: a review. *Animal Feed Science and Technology*, *91*(1), 21-40.

Scott, K. (2005). Taste recognition: food for thought. *Neuron, 48(3),* 455-464.

Seikel, M. K., & Hillis, W. E. (1970). Hydrolysable tannins of Eucalyptus delegatensis wood. *Phytochemistry, 9(5),* 1115-1128.

Sekirov, I., Russell, S. L., Antunes, L. C. M., & Finlay, B. B. (2010). Gut microbiota in health and disease. *Physiological reviews*, *90*(3), 859-904.

Selma, M. V., Espín, J. C., & Tomás-Barberán, F. A. (2009). Interaction between phenolics and gut microbiota: role in human health. *Journal of agricultural and food chemistry*, *57*(15), 6485-6501.

Shaik, F. A., Singh, N., Arakawa, M., Duan, K., Bhullar, R. P., & Chelikani, P. (2016). Bitter taste receptors: extraoral roles in pathophysiology. *The international journal of biochemistry & cell biology*, *77*, 197-204.

Shan, B., Cai, Y. Z., Brooks, J. D., & Corke, H. (2007). Antibacterial properties and major bioactive components of cinnamon stick (*Cinnamomum burmannii*): activity against foodborne pathogenic bacteria. *Journal of Agricultural and Food Chemistry, 55(14),* 5484-5490.

Shibusawa, Y., Yanagida, A., Isozaki, M., Shindo, H., & Ito, Y. (2001). Separation of apple procyanidins into different degrees of polymerization by high-speed counter-current chromatography. *Journal of Chromatography A*, *915*(1), 253-257.

Shoji, T., Masumoto, S., Moriichi, N., Akiyama, H., Kanda, T., Ohtake, Y., & Goda, Y. (2006). Apple procyanidin oligomers absorption in rats after oral administration: analysis of procyanidins in plasma using the porter method and high-performance liquid chromatography/tandem mass spectrometry. *Journal of Agricultural and Food Chemistry*, *54*(3), 884-892.

Siebert, K. J., Troukhanova, N. V., & Lynn, P. Y. (1996). Nature of polyphenol− protein interactions. *Journal of Agricultural and Food Chemistry, 44(1),* 80-85.

Škerget, M., Kotnik, P., Hadolin, M., Hraš, A. R., Simonič, M., & Knez, Ž. (2005). Phenols, proanthocyanidins, flavones and flavonols in some plant materials and their antioxidant activities. *Food chemistry*, *89*(2), 191-198.

Spencer, J. P., El Mohsen, M. M. A., Minihane, A. M., & Mathers, J. C. (2008). Biomarkers of the intake of dietary polyphenols: strengths, limitations and application in nutrition research. *British Journal of Nutrition*, *99*(01), 12-22.

Sternini, C. (2007). Taste receptors in the gastrointestinal tract. IV. Functional implications of bitter taste receptors in gastrointestinal chemosensing. *American Journal of Physiology-Gastrointestinal and Liver Physiology, 292(2)*, G457-G461.

Stolz, P., Böcker, G., Vogel, R. F., & Hammes, W. P. (1993). Utilisation of maltose and glucose by lactobacilli isolated from sourdough. *FEMS Microbiology Letters, 109(2-3),* 237-242.

Taguri, T., Tanaka, T., & Kouno, I. (2004). Antimicrobial activity of 10 different plant polyphenols against bacteria causing food-borne disease. *Biological and Pharmaceutical Bulletin, 27(12),* 1965-1969.

Tamir, M., & Alumot, E. (1969). Inhibition of digestive enzymes by condensed tannins from green and ripe carobs. *Journal of the Science of Food and Agriculture*, *20*(4), 199-202.

Tremaroli, V., & Bäckhed, F. (2012). Functional interactions between the gut microbiota and host metabolism. *Nature*, *489*(7415), 242-249.

Treutter, D. (2006). Significance of flavonoids in plant resistance: a review. *Environmental Chemistry Letters*, *4*(3), 147-157.

Tsang, C., Auger, C., Mullen, W., Bornet, A., Rouanet, J. M., Crozier, A., & Teissedre, P. L. (2005). The absorption, metabolism and excretion of flavan-*3*-ols and procyanidins following the ingestion of a grape seed extract by rats. *British Journal of Nutrition*, *94*(02), 170-181.

Tsunehiro, Jun, et al. "Digestibility of the hydrogenated derivative of an isomaltooligosaccharide mixture by rats." *Bioscience, biotechnology, and biochemistry* 63.9 (1999): 1515-1521.

Turnbaugh, P. J., Hamady, M., Yatsunenko, T., Cantarel, B. L., Duncan, A., Ley, R. E., & Egholm, M. (2009). A core gut microbiome in obese and lean twins. *Nature*, *457*(7228), 480-484.

Tzounis, X., Rodriguez-Mateos, A., Vulevic, J., Gibson, G. R., Kwik-Uribe, C., & Spencer, J. P. (2011). Prebiotic evaluation of cocoa-derived flavanols in healthy humans by using a randomized, controlled, double-blind, crossover intervention study. *The American journal of clinical nutrition*, *93*(1), 62-72.

Tzounis, X., Vulevic, J., Kuhnle, G. G., George, T., Leonczak, J., Gibson, G. R., & Spencer, J. P. (2008). Flavanol monomer-induced changes to the human faecal microflora. British Journal of *Nutrition, 99(4),* 782-792.

Van Duynhoven, J., Vaughan, E. E., Jacobs, D. M., Kemperman, R. A., Van Velzen, E. J., Gross, G.,... & Westerhuis, J. A. (2011). Metabolic fate of polyphenols in the human superorganism. *Proceedings of the national academy of sciences*, *108*(Supplement 1), 4531-4538.

Vanhaelen, M., & Vanhaelen-Fastre, R. (1989). TLC-densitometric determination of 2, 3-cis-procyanidin monomer and oligomers from hawthorn (*Crataegus laevigata* and *C. monogyna*). *Journal of pharmaceutical and biomedical analysis, 7(12),* 1871-1875.

Vrieze, A., Holleman, F., Zoetendal, E. G., De Vos, W. M., Hoekstra, J. B. L., & Nieuwdorp, M. (2010). The environment within: how gut microbiota may influence metabolism and body composition. *Diabetologia*, *53*(4), 606-613.

Walter, J. (2015). Murine gut microbiota—Diet trumps genes. *Cell host & microbe*, *17*(1), 3-5.

Wang, W., Bostic, T. R., & Gu, L. (2010). Antioxidant capacities, procyanidins and pigments in avocados of different strains and cultivars. *Food Chemistry*, *122*(4), 1193-1198.

Waterhouse, A. L., Price, S. F., & McCord, J. D. (1999). [11] Reversed-phase high-performance liquid chromatography methods for analysis of wine polyphenols. *Methods in enzymology*, *299*, 113-121.

Yam, T. S, Shah, S. & Hamilton-Miller, J. M. T. (1997). Microbiological activity of whole and fractionated crude extracts of tea (*Camellia sinensis*), and of tea components. *FEMS Microbiology Letters 152*, 169–74.

Yanagawa, Y., Yamamoto, Y., Hara, Y., & Shimamura, T. (2003). A combination effect of epigallocatechin gallate, a major compound of green tea catechins, with antibiotics on *Helicobacter pylori* growth in vitro. *Current microbiology*, *47*(3), 0244-0249.

Yanagida, A., Kanda, T., Shoji, T., Ohnishi-Kameyama, M., & Nagata, T. (1999). Fractionation of apple procyanidins by size-exclusion chromatography. *Journal of Chromatography A, 855(1),* 181-190.

Yanagida, A., Kanda, T., Takahashi, T., Kamimura, A., Hamazono, T., & Honda, S. (2000). Fractionation of apple procyanidins according to their degree of polymerization by normal-phase high-performance liquid chromatography. *Journal of Chromatography A*, *890*(2), 251-259.

Yang, H. (2014) Characterization of nutrient profiles from legume seeds. Department of Agricultural, Food and Nutritional Science, University of Alberta.

Zoetendal, E. G., Rajilić-Stojanović, M., & De Vos, W. M. (2008). High-throughput diversity and functionality analysis of the gastrointestinal tract microbiota. *Gut*, *57*(11), 1605-1615.