Universitat de Lleida

# Colonic metabolism of phenolic compounds: from in vitro to in vivo approaches

Juana Mosele

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JUANA I. MOSELE

# COLONIC METABOLISM OF PHENOLIC COMPOUNDS: FROM *IN VITRO* TO *IN VIVO* APPROACHES

Doctoral Thesis

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Antioxidants Research Group

Department of Food Technology

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Dissertation presented by Juana Inés Mosele to obtain the PhD degree from the University of Lleida. This Doctoral Thesis has been supervised by María José Motilva Casado and Alba Macià Puig. The present work has been carried out in the Antioxidant Research Goup in the Department of Food Technology and is included in "Ciencia i Tecnologia Agraria i Alimentaria" doctoral program.

The present Doctoral Thesis is framed on the context of two different projects:

The MEFOP project: *Metabolic Fate of Olive Oil Phenolic Compounds in Humans: Study of metabolic pathways and tissue distribution*.

The INCOMES project: *Guide for the substantiation of health claims in foods: immune, cognitive functions and metabolic syndrome* (Collaborative project between Food Companies & Research Centers).

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*De todos los instrumentos del hombre, el más asombroso es, sin duda, el libro. Los demás son extensiones de su cuerpo. El microscopio, el telescopio, son extensiones de su vista; el teléfono es extensión de la voz; luego tenemos el arado y la espada, extensiones del brazo. Pero el libro es otra cosa: el libro es una extensión de la memoria y la imaginación.*

Jorge Luis Borges

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Publication II: **Mosele, J.I.**, Martín-Peláez, S., Macià, A., Farràs, M., Valls, R.M., Catalán, U., Motilva, M.J. Study of the catabolism of thyme phenols combining in vitro fermentation and human intervention. *Journal of Agricultural and Food Chemistry*, 2014, 62, 10954-10961.

Publication III: Martín-Peláez, S., **Mosele, J.I.**, Pizarro, N., Farràs, M., de la Torre, R., Subirana, I., Pérez-Cano, F.J., Castañer, O., Solà, R., Fernández-Castillejo, S., Heredia, S., Farré, M., Motilva, M.J., Fitó, M. Effect of virgin olive oil and thyme phenolic compounds on blood lipid profile: implications of human gut microbiota. *European Journal of Nutrition*, 2015; 13. Article in Press. DOI: 10.1007/s00394-015-1063-2.

Publication IV: **Mosele, J.I.**, Macià, A., Romero, M.P., Motilva, M.J., Rubió, L. Application of in vitro gastrointestinal digestion and colonic fermentation models to pomegranate products (juice, pulp and peel extract) to study the stability and catabolism of phenolic compounds. *Journal of Functional Foods*, 2015 14, 529-540.

Publication V: **Mosele, J.I.**, Gosalbes, M.J., Macià, A., Rubió, L., Vázquez-Castellanos, J.F., Jiménez Hernández, N., Moya, A., Latorre, A., Motilva, M.J. Effect of daily intake of pomegranate juice on fecal microbiota and feces metabolites from healthy volunteers. *Molecular Nutrition and Food Research*, 2015, 59, 1942-1953.

Publication VI**: Mosele, J.I.**, Macià, A., Romero, M.P., Motilva, M.J. Stability and metabolism of *Arbutus unedo* bioactive compounds (phenolics and antioxidants) under *in vitro* digestion and colonic fermentation. *Food Chemistry*, 2016, 201, 120-130.

Publication VII: **Mosele, J.I.**, Macià, A., Motilva, M.J. Understanding of human metabolic pathways of different sub-classes of phenols from *Arbutus unedo* fruit after an acute intake. *Food and Function*. 2016., DOI: 10.1039/C6FO00181E.

Publication VIII: **Mosele, J.I.**, Macià, A., Motilva, M.J. Metabolic and microbial modulation of the large intestine ecosystem by non-absorbed diet phenolic compounds: A review. *Molecules*, 2015, 20, 17429-17468.

## ABSTRACT

Phenolic compounds are a group phytochemicals widely but not uniformly distributed in the plant kingdom. These compounds have generated great expectations in the scientific community as natural therapeutics due to their antioxidant, anti-inflammatory and anticarcinogenic properties. In an attempt to associate the intake of phenolic compounds with reduced risk of chronic diseases, much effort has been invested in the phenolic characterization of plant-based products.

Phenolic compounds are usually taken orally through food or supplements intake. The gastrointestinal tract, therefore, is the main route of entrance to the body. Initial attention has been focused on studying the bioavailability of the native compounds present in food. Nevertheless, their apparent low bioavailability has awoken the possibility of contemplating other related metabolites rather than the original forms. The obstinacy to really cover the general metabolism of phenolic compounds in the human body provided their colonic fate with a central role. This is partly explained because phenolic compounds are modestly absorbed and, therefore, a considerable amount of unmetabolized products reaches the colon.

The different works that make up the body of this Doctoral Thesis are focused on the study of the colonic metabolism of the more representative phenolic compounds present in three plant-based products. The products concerned were enriched olive oil in its own phenolic compounds and in combination with thyme phenolic compounds, pomegranate and *Arbutus unedo* (strawerry tree) fruit.

*In-vitro* fermentation and human interventions were conducted for the purpose of exploring the fate of phenolic compounds in the gut. Different metabolic pathways have been proposed based on *in-vitro* fermentations and corroborated through the analysis of human faeces after dietary treatment. Because of the potential benefits that phenolic compounds can cause to the local ecosystem, it is vital to monitor the behaviour of these phytochemicals in the large intestine. For this reason, the impact of phenolic compounds in the activity and composition of gut microbiota was also evaluated.

## RESUMEN

Los compuestos fenólicos son una clase de fitoquímicos que se encuentran amplia pero no uniformemente distribuidos en el reino vegetal. Dadas sus propiedades antioxidantes, anti-inflamatorias y anticancerígenas, dichos compuestos han despertado gran interés en la comunidad científica como posibles sustancias terapéuticas de origen natural. Con el fin de encontrar asociaciones positivas entre la ingesta de compuestos fenólicos y mejora de marcadores de salud, muchos esfuerzos se han invertido para definir el perfil fenólico de muchos productos.

Los compuestos fenólicos normalmente se consumen de forma oral a través de alimentos o suplementos, por lo tanto, el tracto gastrointestinal constituye la principal vía de entrada al organismo. En un principio, muchos trabajos de investigación se centraron en estudiar la biodisponibilidad de aquellos compuestos originalmente presentes en los alimentos. Sin embargo, su modesta biodisponibilidad ha despertado la posibilidad de contemplar otras rutas metabólicas mediante las cuales estos compuestos lleven a cabo su actividad biológica, ampliando la búsqueda de otros compuestos asociados a las formas originales. Con el objeto de considerar la distribución general de los compuestos fenólicos en el organismo humano, el metabolismo en el intestino grueso juega un papel central. Dicho interés se apoya en evidencias científicas que sostienen que los compuestos fenólicos se absorben de forma modesta y, por consiguiente, una porción considerable llega al colon sin metabolizar.

Los diferentes trabajos que conforman el núcleo principal de esta Tesis Doctoral se han focalizado en el estudio del metabolismo en el colon de los compuestos fenólicos más representativos de tres productos de origen vegetal. Los productos en cuestión fueron aceite de oliva enriquecido en sus propios compuestos fenólicos y en combinación con compuestos fenólicos presentes en el tomillo, granada y *Arbutus unedo* (madroños).

Diferentes ensayos incluyendo fermentaciones *in vitro* y tratamientos dietéticos con humanos se llevaron a cabo con el propósito de explorar qué ocurre con los compuestos fenólicos cuando llegan al colon. Como resultado de esto, se propusieron una serie de rutas metabólicas deducidas de las observaciones de los experimentos *in vitro*, que fueron posteriormente contrastadas con los datos obtenidos del análisis de heces humanas. Asimismo, el posible impacto que los compuestos fenólicos pueden tener sobre el

ecosistema intestinal se evaluó mediante el análisis del perfil metabólico y microbiano de heces obtenidas antes y después de la ingesta fenólica.

## RESUM

Els compostos fenòlics són una classe de fitoquímics que es troben àmplia però no uniformement distribuïts en el regne vegetal. Donades les seves propietats antioxidants, anti-inflamatories i anti-cancerígenes, aquests compostos han despertat gran interès en la comunitat científica com a possibles substàncies terapèutiques d'origen natural. Amb la finalitat de trobar associacions positives entre la ingesta de compostos fenòlics i la millora de marcadors de salut, molts esforços s'han invertit per definir el perfil fenòlic de molts productes.

Els compostos fenòlics normalment es consumeixen de forma oral a través d'aliments o suplements, per tant, el tracte gastrointestinal constitueix la principal via d'entrada a l'organisme. Al principi, molts treballs de recerca es van centrar a estudiar la biodisponibilitat d'aquells compostos originalment presents en els aliments. No obstant això, la seva modesta biodisponibilitat ha despertat la possibilitat de contemplar altres rutes metabòliques mitjançant les quals aquests compostos puguin a terme la seva activitat biològica, ampliant la cerca d'altres compostos associats a les formes originals. Amb l'objecte de considerar la distribució general dels compostos fenòlics en l'organisme humà, el metabolisme en l'intestí gruixut juga un paper central. Aquest interès es recolza en evidències científiques que sostenen que els compostos fenòlics s'absorbeixen de forma modesta i, per tant, una porció considerable arriba al còlon sense metabolitzar.

Els diferents treballs que conformen el nucli principal d'aquesta tesi doctoral s'han focalitzat en l'estudi del metabolisme en el còlon dels compostos fenòlics més representatius de tres productes d'origen vegetal. Els productes en qüestió van ser oli d'oliva enriquit en els seus propis compostos fenòlics i en combinació amb compostos fenòlics de la farigola, magrana i *Arbutus unedo* (arboç).

Diferents assatjos incloent fermentacions *in vitro* i tractaments dietètics amb humans es van dur a terme amb el propòsit d'explorar què ocorre amb els compostos fenòlics quan arriben al còlon. Com a resultat d'això, es van proposar una sèrie de rutes metabòliques deduïdes de les observacions dels experiments *in vitro*, que van anar posteriorment contrastades amb les dades obtingudes de l'anàlisi de femta humana. Així mateix, el possible impacte que els compostos fenòlics poden tenir sobre l'ecosistema intestinal es va

avaluar mitjançant l'anàlisi del perfil metabòlic i microbià de femta obtinguda abans i després de la ingesta fenòlica.

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# INTRODUCTION

# 1. An approach to the concept of phenolic compounds based on their occurrence, classification, distribution and properties

Dietary phenolic compounds are minority components of the plant kingdom. Approximately 8000 different phenolic compounds have been described in fruit, vegetables, leaves, roots, flowers, seeds and the products obtained from them, such as chocolate, and alcoholic (wine and beer) and non-alcoholic beverages (tea, coffee and juices). There is a growing recognition that these natural phytochemicals play an important role in the prevention of chronic diseases, which are considered subjects of study in an important number of scientific reports

## 1.1. Natural occurrence of phenolic compounds

Phenolic compounds include a broad spectrum of natural phytochemicals widely distributed in the plant kingdom. They are secondary metabolites derived from the shikimate pathway of plants that possess, in their chemical structure, at least one benzene (phenol) ring with a varied number of adjacent hydroxyl substituents (Tsao et al., 2010; Weaver et al., 1997). There is not a definitive consensus that explains the reason why phenolic compounds are synthesized by plants. The activity of the shikimate pathway increases in the event of environmental stress such as nutrient deprivation, ultraviolet light exposure, wounding and biological threat (herbivores, microorganisms and fungus) (Weaver et al., 1997). In addition, it has been suggested that phenolic compounds are involved in the symbiotic relationship between microorganisms and plants, favouring the development of beneficial microbiota (Weaver et al., 1997). All this seems to indicate that phenolic compounds play a relevant role in the protection of plants during their growth and development, ensuring their survival in hostile environments.

## 1.2. Classification of phenolic compounds

Despite phenolic compounds sharing common features, each of these compounds maintains its own unique characteristics, allowing their classification in different groups. Taking into consideration the phenolic part of the molecule, called aglycone, phenolic compounds could be organized into two big groups: flavonoids and non-flavonoids.

*Flavonoids*

This phenolic category includes those compounds containing aglycone skeleton, consisting of two phenolic rings (ring A and ring B) connected by a heterocyclic ring (ring C). The whole structure is commonly represented as $C_6$-$C_3$-$C_6$. Flavonoids are sub-classified into six sub-groups: anthocyanidins, flavan-3-ols, isoflavones, flavanones, flavones and flavonols (Beecher et al., 2003; Manach et al., 2004; Tsao et al., 2010). This classification is based on the hydroxylation (position 3), double bound (2,3-dihydro) and the presence of carbonyl group (position 4) of the C ring, as well as the binding position of B ring to C ring (see **Figure 1** and **Figure 2**).

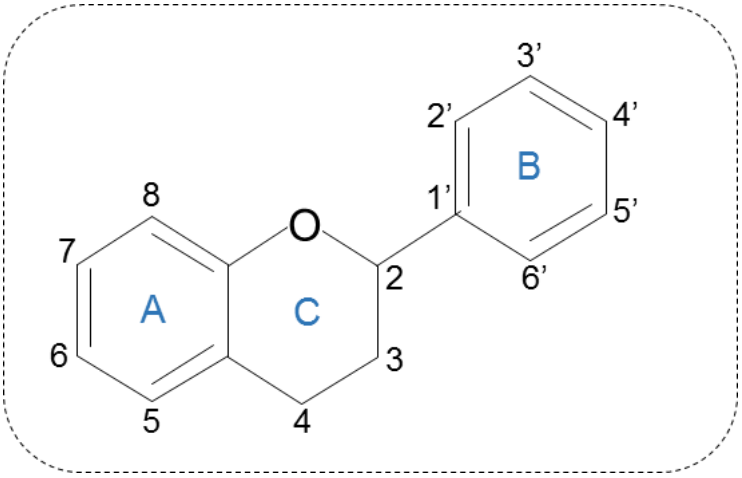


**Figure 1.** Schematic representation of the flavonoid aglycone

In food, flavonoids are normally combined with aliphatic and aromatic acids, methyl groups and/or glycosides to form numerous types of conjugates. The glycosyl anthocyanidins are called anthocyanins. In the particular case of flavan-3-ols, they can occur as monomers (catechin and epicatechin), monomers associated with gallic acid (epigallocatechin gallate) or in a varied combination of interconnected monomer units to form proanthocyanins (polymeric forms). On occasions, proanthocyanins are considered as an individual group called condensed tannins (Beecher et al., 2003).

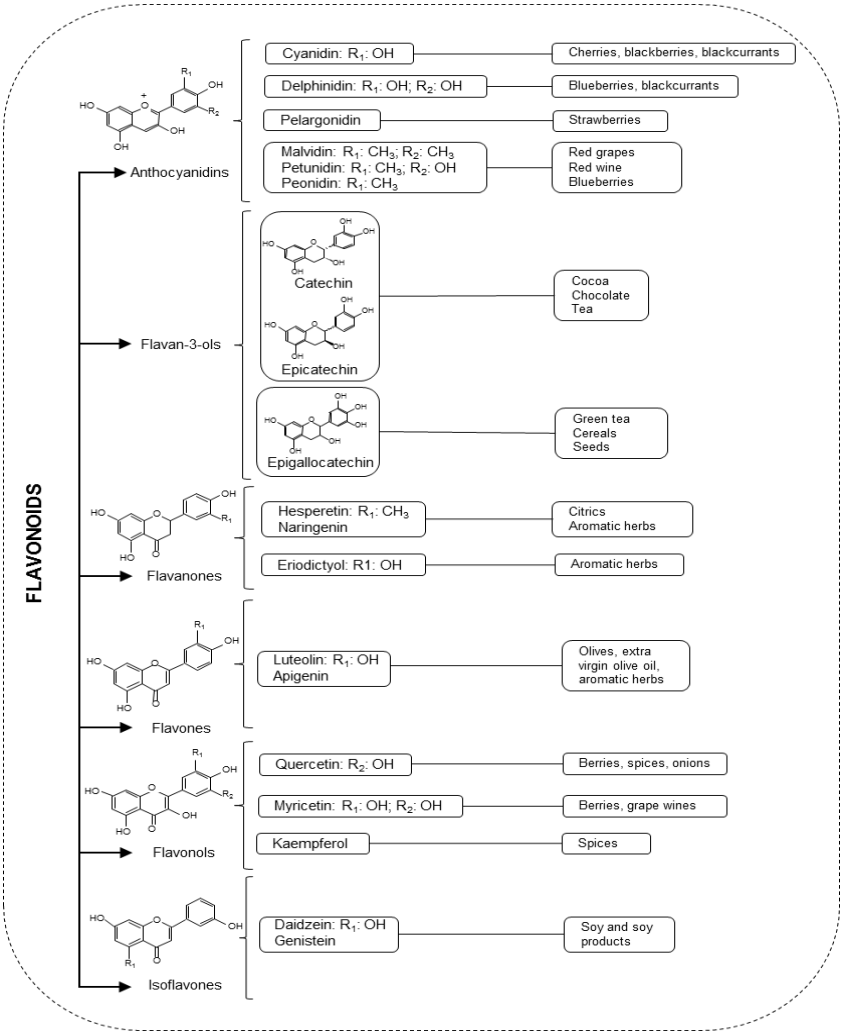


**Figure 2.** Description of flavonoid sub-groups including the most representative constituents as well as some examples of plant-products sources.

*Non-flavonoids*

This classification includes those compounds that possess a benzoic (phenol) ring but the phenolic skeleton does not respond to the $C_6$-$C_3$-$C_6$ chemical pattern. Phenolic acids (hydroxybenzoic and hydroxycinamic acids), phenolic alcohols, phenolic monoterpenes, lignans and stilbenes are some of the sub-groups considered as non-flavonoids. Within the hydroxybenzoic acids, gallic and ellagic acids can be found in food, forming more complex structures. These are called gallotannins and ellagitannins, respectively, and they are

derived from the linkage between different monomer units, which are normally interconnected by sugars or aromatic acids. Polymeric forms of gallic and ellagic acids are also known as hydrolyzable tannins (Beecher et al., 2003).

Hydroxycinamic acids include caffeic acid, ferulic acid, and coumaric acid, to mention some examples. Caffeic and ferulic acids are also found in vegetal sources as tartrate and quinic esters (e.g. chlorogenic acid is a quinic ester of caffeic acid). In addition, caffeic acid esterified with dihydroxyphenylactic acid forms rosmarinic acid. **Figure 3** shows a summary of the latter mentioned non-flavonoids with a brief description of the foods that contain considerable amounts of these compounds.

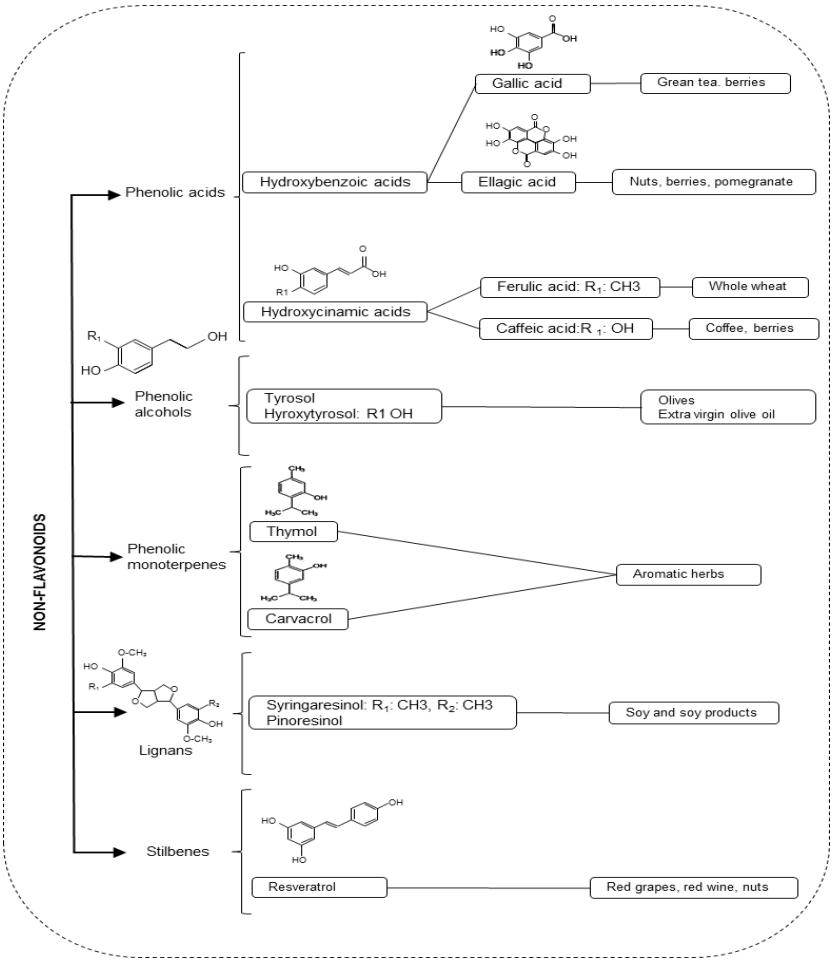


**Figure 3**. Classification of phenolic compounds belonging to the non-flavonoid group. Each sub-group includes an example of their most representative compounds as well as the plan-products sources.

## 1.3. Distribution of phenolic compounds in plant products

As an integral part of the human diet, an estimated average of 1-3 g of phenolic compounds is consumed per day through plant-derived products (Saura-Calixto et al., 2007; Scalbert et al., 2000). Of course, the amount and specific type of phenolic compounds ingested vary between populations and markedly depend on their dietary habits (Beecher et al., 2003; Brat et al., 2006; Manach et al., 2004; Scalbert et al., 2000). Phenolic compounds are widely but not uniformly distributed in the plant kingdom. Beyond the genetic predisposition, other factors, such as edaphoclimatic conditions and technical procedures during manufacturing and storage, determine the qualitative and quantitative profile of phytochemicals in plant-based foods (Brat et al., 2006; Manach et al., 2004; Mphahlele et al., 2014).

Certain phenolic compounds are widely present in many products, whereas others are limited to specific families or species (Manach et al., 2004; Scalbert et al., 2000; Williamson et al., 2005). Under this assumption, scientific interest can be focused on the study of the predominant individual phenolic compounds in a food or else on its content in a specific one. Red wine is a good example for a better understanding. This alcoholic beverage obtained from the fermentation of grapes has been extensively studied. Its interest is focused on the content of anthocyanins, flavan-3-ols and/or phenolic acids as major phenolic constituents (Scalbert et al., 2000) (**Table 1**). In addition, the presence of resveratrol, although in modest quantities, has been the subject of numerous publications, since this stilbene is found in red grapes in greater amounts than in other products (Scalbert et al., 2000) (**Table 1**).

Marked differences also exist among plant products in terms of total phenolic concentration. Based on this supposition, we could distinguish between low and high phenolic-content products. For example, a serving of melon provides less phenolic compounds than strawberries (Brat et al., 2006) (**Table 1**). However, more important than the total phenolic contribution is the frequency of dietary intake or the possibility of including the product as part of the regular diet.

**Table 1**. Phenolic concentration of fruit, vegetables and their derivatives products

| Product | Phenolic compounds | Concentration (mg/100 g) |
|---|---|---|
| *Fruits* | | |
| Blackberry | Cyanidin-3-*O*-glucoside | 138±37.1 |
| | Ellagic acid | 43.7±24.5 |
| | Epicatechin | 11.5±10.9 |
| | 3-caffeoyquinic acid | 4.53±0.59 |
| Strawberry | Pelargonidin-3-*O*-glucoside | 47.1±14.6 |
| | Catechin | 6.36±5.46 |
| | Ellagic acid glucoside | 2.85±1.12 |
| | Resveratrol | 0.35±0.00 |
| Orange | Hesperetin | 33.6±7.35 |
| | Naringenin | 11.2±0.64 |
| Melon | Lariciresinol | 4.40±0.00 |
| | Pinoresinol | 2.20±0.00 |
| Apple | 5-Caffeoyl quinic acid | 18.2±10.6 |
| (peeled) | Procyanidin trimer C1 | 7.02±3.47 |
| | Epicatechin | 6.72±4.50 |
| Apple | Procyanidin dimer B2 | 14.6±9.19 |
| (whole) | 5-Caffeoyl quinic acid | 13.4±11.3 |
| *Vegetables* | | |
| Broccoli | Kaempferol-3-*O*-sophoroside | 16.6±0.00 |
| (raw) | Quercetin-3-*O*-sophoroside | 6.50±0.00 |
| Onion (raw) | Quercetin-3,4'-*O*-diglucoside | 3.12±2.50 |
| | Quercetin-4'-*O*-glucoside | 2.25±1.80 |
| *Beverages* | | Concentration (mg/100 mL) |
| Red wine | Malvidin-3-*O*-glucoside | 9.97±5.48 |
| | Procyanidin dimer B3 | 9.47±4.29 |
| | Catechin | 6.81±6.24 |
| | Gallic acid | 3.59±2.71 |
| | Caffeoyl tartaric acid | 3.35±3.79 |
| | Resveratrol-3-*O*-glucoside | 0.62±0.65 |
| Coffee (filter) | 5-Caffeoyl quinic acid | 43.1±0.00 |
| | 4-Caffeoyl quinic acid | 19.0±0.00 |
| | 4-feruloyl quinic acid | 13.3±0.00 |
| Pomegranate | Punicalagin | 43.6±71.3 |
| juice | Ellagic acid glucoside | 3.97±3.77 |
| | Cyanidin-3-*O*-glucoside | 3.43±4.53 |
| *Others* | | Concentration (mg/100 g) |
| Black | Epicatechin | 70.4±29.5 |
| chocolate | Proanthocyanins tetramer | 53.8±20.1 |
| | Procyanidin dimer B2 | 36.5±11.7 |
| | Ferulic acid | 24.0±0.00 |
| Cellery (fresh) | Luteolin-7-O-(2-aplosyl-6-malonyl)-glu | 35.8±0.00 |
| | Apigenin-7-O-(6''-aplosyl-6-malonyl)-glu | 31.6±0.00 |
| Thyme (dried) | Rosmarinic acid | 829±260 |
| Extra virgin | 3,4-DHPEA-EDA | 25.2±26.3 |
| olive oil | 4-HPEA-EDA | 14.3±7.32 |
| | Oleuropein aglycone | 3.66±2.43 |
| | Apigenin | 1.17±1.28 |
| | Pinoresinol | 0.42±0.28 |
| Sunflower oil | Total phenolic content | 1.00±0.00 |

*3,4-DHPEA-EDA:* dialdehydic form of decarboxymethylelenolic acid linked to hydroxytyrosol*; 4-HPEA-EDA :* dialdehydic form of decarboxymethylelenolic acid linked to tyrosol. Source: *phenol-explorer.ue (access December 2015).*

This aspect could be connected to many examples. Olive oil is consumed as the main source of fat in the Mediterranean area, replacing refined oils commonly used in other countries. The frequency of olive oil intake provides an important daily contribution of phenolic alcohols, lignans and flavones, which are not present in other culinary fats (García et al., 2003) (**Table 1**). The regular consumption of beverages such as wine, coffee, tea, and juices is also an important phenolic source (Brat et al., 2006; Manach et al., 2004). In addition, phenolics are not distributed evenly throughout the food product. For example, the qualitative and quantitative phenolic profile is not the same in the outer and inner parts of the product (**Table 1**). In the case of fruits and some vegetables, the peel and seeds normally concentrate major polymerized substances and a higher phenolic concentration than the flesh (Scalbert et al., 2000).

In summary, the estimation of the average phenolic intake in a specific population requires the collection of detailed information, such as the variety of fruit or vegetable, frequency of intake, means of consumption (whole products, raw or cooked) and form and time of conservation. These aspects are not always included in food or dietary surveys. Deducing the correlation between biological effects and phenolic intake is quite difficult due to the variable aspects mentioned below. In addition, lack of solid conclusions regarding the amount and class of phenolic compounds necessary to intake to acquire beneficial effects hinders the elaboration of dietary recommendations.

Moreover, when selecting products with the aim of increasing phytochemicals intake, it is important to consider the overall composition of the food in order to limit the incorporation of other components such as alcohol (in wine and beer), sugar (when added to fruit juices), fat (nuts and olive oil), high density calories (chocolate and nuts) or stimulants (caffeine in coffee).

## 1.4. The properties of phenolic compounds

Based on the specific area of study, phenolic compounds may have several functions with different end objectives.

### Technological properties

The incorporation of phenolic compounds into food products that have experienced important losses or else are naturally deprived of phenolic constituents improves their microbiological quality and minimizes their oxidation (Li et al., 2012; Murcia et al., 2004). For example, a rancidity delay of meat and meat products, fish and fish products, refined oils, butter or margarine is achieved thanks to the addition of phenolic compounds, especially those inhibitors of phospholipids peroxidation (Murcia et al., 2004).

Phenolic-rich foodstuff has emerged as a therapeutic alternative in animal feeding. Supplementation with phenolic compounds improves animal systemic physiology and productivity (Liu et al., 2014; Starčević et al., 2015; Tedeschi et al., 2014). Moreover, other benefits, such as increase of shelf-life and antioxidant protection, are transferred to the end products, such as fresh meat or meat products, obtained from these animals (Di Trana et al., 2015; Liu et al., 2014; Ortuño et al., 2014; Starčević et al., 2015).

In addition, an innovative approach is the inclusion of phenolic-rich oils or extracts for antimicrobial and antioxidant applications. This allows an expansion of the shelf-life, and maintains the nutritional value and safety of packaged foods (Espitia et al., 2014; Perazzo et al., 2014). The purpose of increasing the general quality of food products using naturally occurring compounds is an attractive alternative to limit the use of synthetic preservatives (Murcia et al., 2004). This latter point is of particular importance if we take into consideration the latest trends in consumer choice, based on the increasing popularity of foods formulated with natural additives. Phenolic extracts for animal feeding or food antioxidants are usually derived from by-products generated by the food industry. This motivation offers an extra incentive, since it collaborates with good environmental practices through the recovery of industrial waste (Li et al., 2012). The main limitation of the use of phenolic compounds in food conservation is alteration of sensory perception, due to flavour transference (Lesschaeve et al., 2005).

## Sensorial properties

In the elaboration and commercialization of foods, one of the interests in the phenolic composition lies in the fact that phenols are responsible for the colour, bitterness, astringency and development of some flavours. These sensorial characteristics define the

general appearance and the texture in the mouth, two important factors that govern the choice of consumers (Lesschaeve et al., 2005).

Astringency and bitterness should be properly balanced because their high intensity would promote consumer rejection (Lesschaeve et al., 2005). Therefore, in some products, such as wine, for example, a note of astringency seems to be well perceived (Lesschaeve et al., 2005). Anthocyanins contribute with the red, blue and purple colour of fruits and vegetables. This attribute is really appreciable in berries, pomegranate, eggplant, red grapes, as well as their derived products (juices, jams and purees). Other particular colour shades are due to the transformation of phenolic compounds during technological processes, such as in the case of black tea and red wine (Cheynier et al., 2005; Wang et al., 2003).

### Biological properties

During the last few decades, increasing attention has been focused on the association between diet and health. Based on the results reported by epidemiological studies, heath authorities have advocated the importance of the intake of fruit and vegetables as a policy to reduce the incidence of chronic disease (Boeing et al., 2012; Nishida et al., 2004). Fruit and vegetables provide a wide range of phytochemicals, such as phenolic compounds, fibre, minerals and vitamins. Strictly speaking, phenolic compounds are not considered to be nutrients, since they are not indispensable for essential growth or maintenance of life, and phenolic-deprived diets do not cause deficiency-related diseases. Nevertheless, they have been recognized as protective agents to combat chronic disease (Cardona et al., 2013; Del Rio et al., 2013).

Chronic diseases or non-communicable diseases (NCDs), adopting the definition proposed by the Word Health Organization (WHO), are characterized by a slow or silent development. The most frequent causes are associated with genetic factors and/or continuous exposure to an unhealthy lifestyle. Nowadays, chronic diseases are the primary cause of death in developed countries and already raise concern in emerging societies. An important number of diagnostic cases include chronic conditions associated with cardiovascular disease (CVD) (heart attacks and strokes), cancer (especially colon and breast cancer), diabetes type 2, respiratory diseases and neurodegenerative alterations

(Alzheimer and Parkinson) (Boeing et al., 2012; Nishida et al., 2004). A cure is generally not possible for most of these diseases. Therefore, primary prevention and the maintenance of conditions under control by patients, as soon as the disease is diagnosed, are of pivotal importance.

Phenolic compounds exhibit a wide range of bioactive functions, among them antioxidant, anti-inflammatory and antiproliferative properties, which make them good weapons to fight against chronic diseases (**Figure 4**).

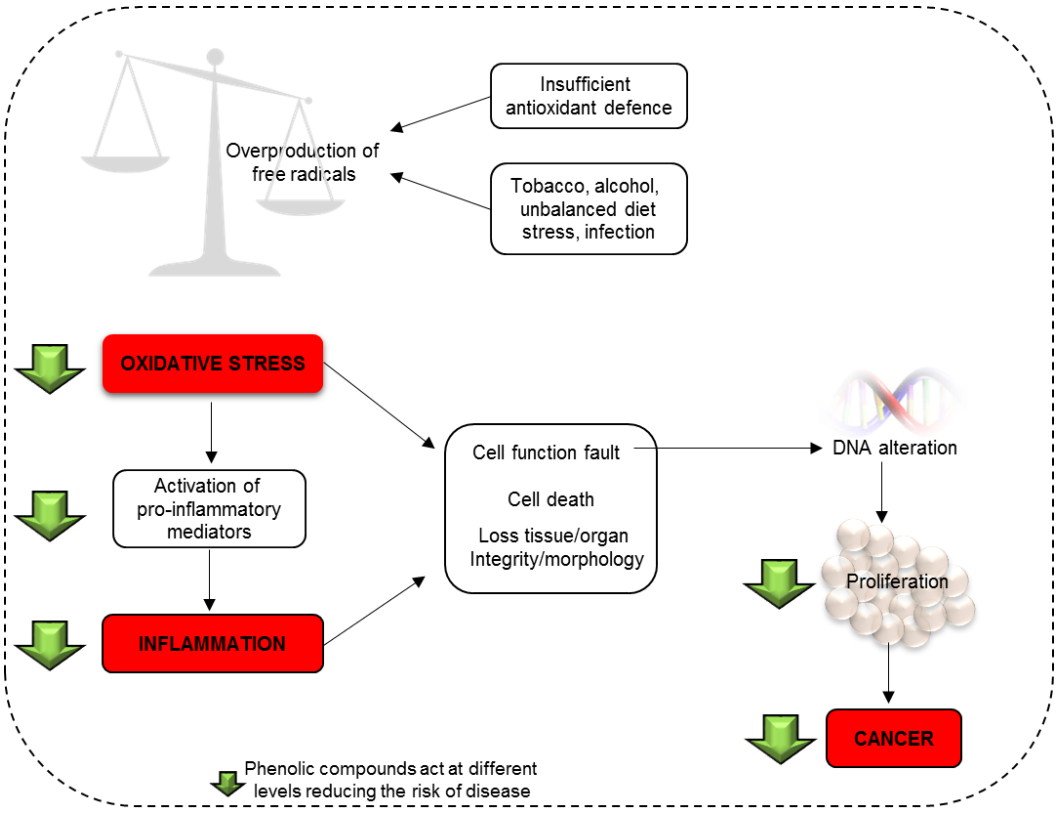


**Figure 4.** Graphic representation of the main biological activities described for phenolic compounds in human beings

*Antioxidant properties*

Free radicals are normal products of cell metabolism and, in basal amounts, they participate as molecular mediators in several physiological events (Görlach et al., 2015).

On the other hand, free radicals are very reactive species capable of attacking cellular components, including lipids (disruption of cellular membrane), proteins (alteration of structural and regulatory proteins) and nucleic acids (DNA damage) (Coates et al., 2007; Rezaie et al., 2007). Therefore, sustained exposure to over-physiological concentrations of free radicals compromises the structure and function of organs and tissues, increasing the risk of developing metabolic complications (Görlach et al., 2015; Rezaie et al., 2007).

Oxidative stress is the term essentially used to describe a situation of uncontrolled generation of free radicals and/or the reduced ability of the endogenous antioxidant machinery (superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPO)) to neutralize them (Rezaie et al., 2007). The antioxidant status of the organism can be reinforced by the intake of phenolic-rich foods (Murcia et al., 2004; Rezaie et al., 2007; Tsao et al., 2010). Their antioxidant properties are supported by the capacity of phenolic compounds to act as metal chelators and free radical scavengers (Tsao et al., 2010). Reduction of aberrations caused to the organism by free radicals is linked to the protection against diseases associated with oxidative stress, such as cardiovascular disease (Siti et al., 2015) and cancer (Coates et al., 2007; Hietanen et al., 1994).

*Anti-inflammatory properties*

Inflammation is a physiological response to external or internal injuries that could compromise the body's well-being. Inflammation is a very sophisticated biological process of signalization in which a series of mediators, transcription factors, protein kinases and enzymes take part. The inflammatory response may turn into a vicious circle of continued stimulation, resulting in its perpetuation. This phenomenon could bring structural and physiological changes to the affected tissue, resulting in the development of chronic diseases such as arteriosclerosis and inflammatory bowel disease (IBD) (Rezaie et al., 2007).

Free radicals are also involved, since they can activate several pathways involved in the stimulation and prolongation of the inflammatory response (Görlach et al., 2015; Rezaie et al., 2007). Nevertheless, phenolic compounds are not only considered as anti-inflammatory agents due to their ability to inhibit free radicals, but also to interfere with other specific biological events (Colombo et al., 2013). Most of these properties are still to be completely

elucidated. However, the capacity of phenolic compounds to modulate the activity of a wide range of enzymes and molecular pathways involved in inflammation has been proposed as the main mechanism of defence (Colombo et al., 2013). For example, the intake of phenolic-rich products has been associated with the improvement of markers associated with inflammation in cardiovascular disease, cancer, diabetes, intestinal environment and obesity (Cardona et al., 2013; Colombo et al., 2013; Del Rio et al., 2010; Martin et al., 2015), to give some examples.

*Antiproliferative properties*

The efficacy of phenolic compounds to prevent or treat cancer has been studied in several types of malignancies both *in-vivo* and *in-vitro* (Coates et al., 2007; Cardona et al., 2013). Oxidative stress and inflammation seem to play a relevant role in the initiation of carcinogenesis (Coates et al., 2007; Colombo et al., 2013). The antioxidant and anti-inflammatory properties of phenolic compounds might therefore reduce the risk of cancer development (**Figure 4**). Independent of the antioxidant and anti-inflammatory properties, evidence of chemopreventive and chemotherapeutic properties of diet phenolic compounds may also be achieved throughout cell cycle arrest, induction of cell death and inhibition of metastasis (Cardona et al., 2013; Coates et al., 2007). The anticarcinogenic effects of several phenolic compounds has been demonstrated in different cancer cells and animal models including prostate (Ma et al., 2015), breast (Kubatka et al., 2015) and colon (Coates et al., 2007).

## 2. Metabolism of phenolic compounds during gastrointestinal digestion

Phenolic compounds are normally consumed through the daily intake of foods and beverages of plant origin, but can also be ingested via dietary supplements (Kubatka et al., 2015). Whatever the vehicle, both forms include the gastrointestinal tract as the principal route of entry into the body. The human gastrointestinal tract includes a series of compartments with specific functions and physiological conditions to favour the digestion and absorption of food components, providing nutrients to the organism (Kararli et al., 1995). Digestion starts in the mouth and is completed in the stomach, small intestine (subdivided into duodenum, jejunum and ileum) and colon (cecum, ascendant, transversal and descendent colon) (**Figure 5**). The study of the phenomena occurring during digestion promotes the understanding and contributes to explaining the general metabolism and fate of phenolic compounds in living beings. Gastrointestinal digestion is a very sophisticated process where internal (enzymatic and microbial activities, pH, transit time) and external (chemical and physical characteristics of the phenolic compounds, dose and food composition) factors govern the stability, bioaccessibility and bioavailability of the food components (Kararli et al., 1995).

Bioaccessibility and bioavailability are different but closely related concepts. The former is understood as the liberation of phenolic compounds from the food matrix into the digestive juice (Saura-Calixto et al., 2007). The release and the subsequent solubilization in the intestinal lumen considerably increase the possibility of phenolic compounds being absorbed by the intestinal epithelium. On the other hand, bioavailability is referred to when phenolic compounds are absorbed by intestinal cells and pass into the bloodstream, becoming available for physiologic processes and/or storage in organs and/or tissues (Jackson et al., 1997). Certain phenolic compounds have been described as poorly absorbed molecules; thus, this non-absorbable fraction is a valuable nutritional resource for the gut microbiota (Kahle et al., 2005).

*In-vitro* gastrointestinal digestion models are usually used to evaluate the bioaccessibility of phenolic compounds in mouth, stomach and small intestine. Furthermore, to predict what happens in the colon with non-absorbed compounds, *in-vitro* fermentations are the model

of choice. These models imitate the conditions of the different parts of the gastrointestinal tract to achieve a more accurate approximation to the *in-vivo* situation (**Figure 5**).

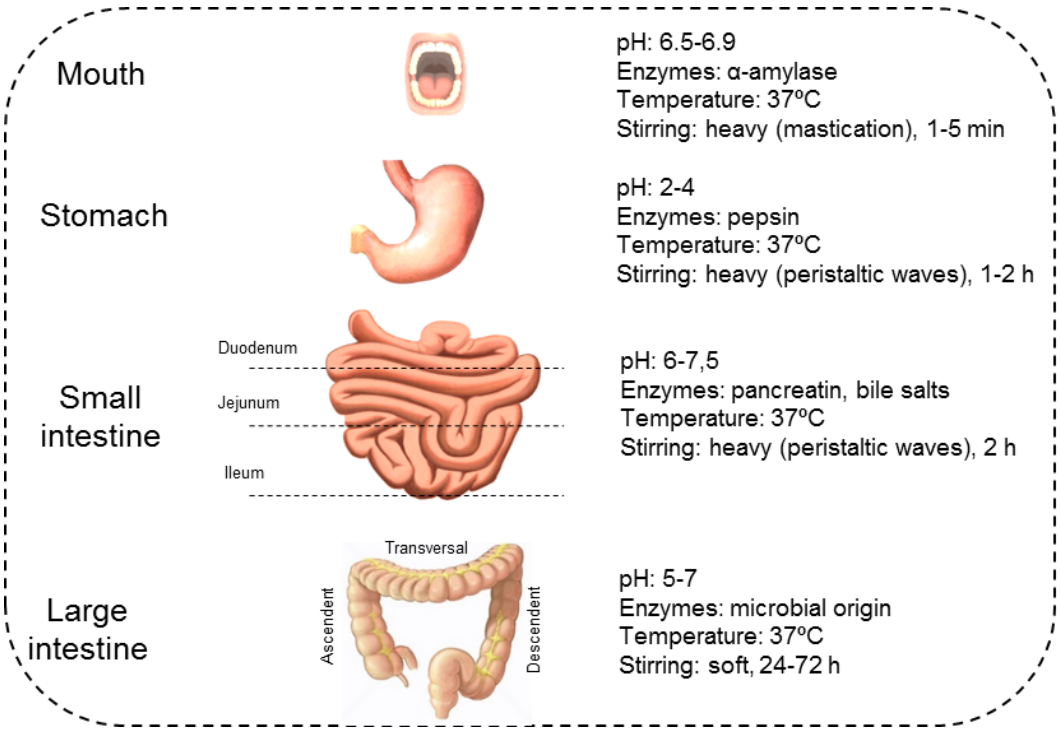


**Figure 5.** Physical and chemical conditions used in *in-vitro* digestion to represent the main compartments of the gastrointestinal tract involved in food digestion

## 2.1. Oral digestion

Digestion starts in the oral cavity with the mechanical action of mastication together with the activity of the salivary enzymes (α-amylases) (Hoebler et al., 1998). The oral digestion of phenolic compounds is not always considered in *in-vitro* bioavailability studies. This would be partially justified because they are minimally affected during the short time of artificial saliva exposure (Correa-Betanzo et al., 2014; Tenore et al., 2015). Therefore, the physical (change in particle size and viscosity of the food) and chemical (modifications of food matrix structure) changes in food, promoted by saliva and trituration, make it available for enzymatic degradation in the stomach and small intestine (Hoebler et al., 1998; Tenore et al., 2015). The changes in the food matrix after the oral step probably increase the

possibility of phenolic compounds liberation and solubilization in the intestinal juice. However, the incubation with artificial saliva does not contemplate the action of oral microbiota on phenolic compounds, which possesses the capacity to release aglycones from glycosylated phenolic structures usually present in plant-based foods (Kahle et al., 2011).

## 2.2. Gastric digestion

The digestion in the stomach is carried out at low pH in combination with mechanical action (peristaltic waves) and enzymatic activity (proteases), which increase the degree of food disintegration (Karali et al., 2005). During digestion, changes in the phenolic profile can occur in two ways: by modifications of the molecular structure (qualitative changes) or else by differences in the amount recovered with respect to the original product (quantitative changes). The impact of gastric digestion on the qualitative profile is more pronounced in certain polymeric forms, whereas simpler compounds have shown good stability (Bermudez-Soto et al., 2007; Borges et al., 2007; Colombo et al., 2013; Correa-Betanzo et al., 2015; Fernández et al., 2013; Kahle et al., 2005; Kahle et al., 2011; Lamothe et al., 2014; Ríos et al., 2002; Serra et al., 2010; Tenore et al., 2015). Among the polymeric forms, whereas ellagitannins were not affected (Colombo et al., 2013; Borges et al., 2007), the hydrolysis resistance of proanthocyanidins under acidic conditions seems to be related to their molecular characteristics. The hydrolysis of proanthocyanidins of cloudy apple juice (Kahle et al., 2011) and grape skin extract (Fernández et al., 2013) was major compared to the proanthocyanins present in grape seed extract (Fernández et al., 2013; Serra et al., 2010) and cocoa beverage (Ríos et al., 2002). Occasionally, disappearance of ellagitannins during gastric incubation was noticed; this was not attributable to their hydrolysis but to the complexation with the protein present in the food and, to a lesser extent, to digestive enzymes (Borges et al., 2007; Lamothe et al., 2014).

From a quantitative point of view, the results obtained from different *in-vitro* studies have shown that, under gastric conditions, most of the phenolic compounds are recovered in similar amounts to those already present in the initial product (Bermúdez-Soto et al., 2005; Correa-Betanzo et al., 2014; Lamothe et al., 2014; Serra et al., 2010; Tenore et al., 2015).

Some losses were reported for flavan-3-ols (Bermúdez-Soto et al., 2007; Tenore et al., 2015).

Moreover, food matrix characteristics and other food that may be ingested simultaneously with the phenolic source also affect the stability and release of phenolic compounds in the stomach, since physical properties of the food could determine access by digestive enzymes (Lamothe et al., 2014).

In synthesis, gastric digestion is important for two reasons. Firstly, it has been suggested that some phenolic compounds could be absorbed in the stomach (Borges et al., 2007; Kararli et al., 1995; Manach et al., 2004). Secondly, it determines the nature of food bolus that reaches the small intestine (duodenum), where the digestion process continues.

## 2.3. Intestinal digestion

The structural architecture and specific function convert the small intestine into the most suitable gastrointestinal segment to absorb a varied repertoire of food components. When the chyme (or digesta) passes from the stomach to the duodenum, the pH starts to increase gradually until reaching neutral to low alkaline values (Karali et al., 1995). The action of the secreted pool of digestive enzymes continues to promote the degradation of food, favouring the release of smaller components to the lumen (Karali et al., 1995).

*In-vitro* studies have shown that the stability of phenolic compounds is closely linked to the pH of the media, while enzymatic degradation seems to play a secondary role (Bermúdez-Soto et al., 2007; Correa-Betanzo et al., 2014; Kahle et al. 2011; Rubiò et al., 2014; Tenore et al., 2015). The amount of phenolic compounds recovered after simulated incubations reveals that their stability depends on their chemical resistance to the lumen environment (Correa-Betanzo et al., 2014; Bermúdez-Soto et al., 2007; Fernández et al., 2013; Kahle et al., 2011; Rubiò et al., 2014; Tenore et al., 2013; Tenore et al., 2015). On occasions, an increase in the concentration of phenolic compounds was observed due to reactions of hydrolysis of the more complex structures or to isomerization of monomers (Bermúdez-Soto et al., 2007; Coates et al., 2007; Colombo et al., 2013; Khale et al., 2011; Rubiò et al., 2014).

Different studies have shown that the nature of the food matrix influences the bioaccessibility of specific phenolic compounds. Thus, digestive enzymes have easier access to liquid and semi-liquid products (juices and smoothies), whereas solid matrices hampered the enzymes' accessibility (Saura-Calixto et al., 2007). Consequently, a solid food matrix might protect phenolic compounds that are more sensitive to the intestinal conditions by gradual liberation to the media (Lamothe et al., 2014; Saura-Calixto et al., 2007). In fact, the combination of a phenolic-rich source with other foods in the context of a complete diet favours the stability of phenolic compounds during digestion in comparison with when they are digested alone (in water) (Lamothe et al., 2014; Serra et al., 2010).

Once phenolic compounds are released into the digestive juice after digestion, they are susceptible to being absorbed. The chemical structure, which in turn determines the solubility and stability, has a profound impact on the degree of absorption of phenolic compounds (Bergmann et al., 2010; Rubiò et al., 2014; Williamson et al., 2005). For example, the type and position of conjugate moieties are likely to affect the bioavailability (Kahle et al., 2005). The mechanism of absorption also differs according to the compound. Whereas, for some, transcellular transport predominates, others preferably cross the epithelium by mediated paracellular transport (restricted by tight junctions) (Konishi et al., 2004) (**Figure 6**). For those compounds in which transcellular absorption takes place, the passage through the membrane can predominantly occur by passive diffusion or carried-mediated transport (Konishi et al., 2004; Lançon et al., 2004) (**Figure 6**).

Phenolic compounds are not always absorbed in the form in which they are present in the food. Previously, they can be deglucosylated in the lumen by lactase phlorizin hydrolase (LPH) or in the cytosol of intestinal cells by β-glucosidase (Bergmann et al., 2010, Kahle et al., 2005) (**Figure 6**). Once phenolic compounds have been absorbed, they undergo extensive intestinal, hepatic and renal phase I and II metabolism (glucuronidation, sulphatation, and methylation), these metabolites being the most abundant in biological fluids (Rubiò et al., 2014; Williamson et al., 2005). Nevertheless, it has been reported that some dimers and trimers (proanthocyanins), as well as anthocyanins, are absorbed and excreted intact (Serra et al., 2010). Additionally, some of these phenolic compounds that escape gastro-intestinal absorption reach the large intestine, becoming a potential source

of microbial substrate (Cardona et al., 2013; Colombo et al., 2013; Kahle et al., 2005; Kahle et al., 2006; Saura Calixto et al., 2007).

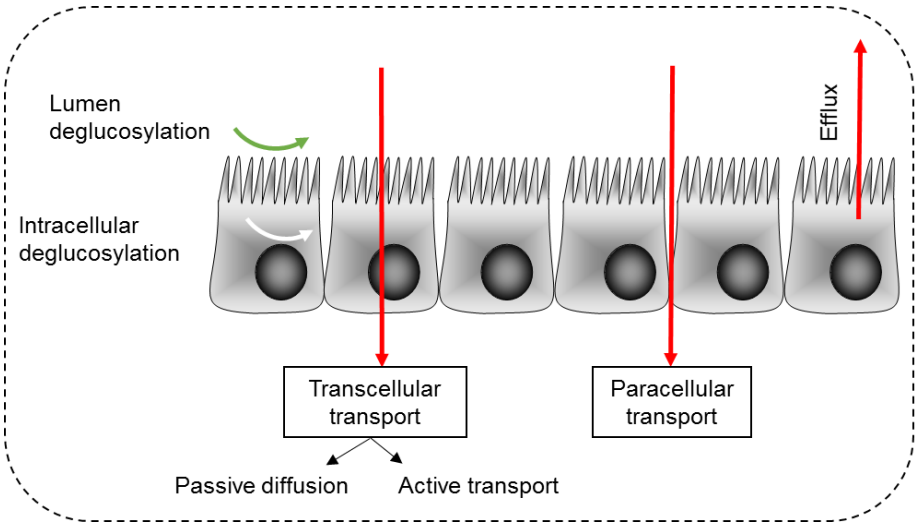


**Figure 6.** Graphic representation of the different mechanisms of intestinal trans-epithelial transport and deglucosylation of phenolic compounds

## 2.4. The large intestine

During the last few years, a huge body of information regarding the fate of phenolic compounds in the colon has been published. The main interest lies in the aspect that some phenolic compounds escape digestion and, consequently, reach the colon unmetabolized (Cardona et al., 2013; Colombo et al., 2013; Kahle et al., 2005; Kahle et al., 2006; Saura-Calixto et al., 2007). The major proportion of this phenolic fraction consists of non-absorbable compounds, completed by additional phenolic metabolites of phase II derived from the intestinal efflux and enterohepatic recirculation (Kahle et al., 2005; Kahle et al., 2006; Saura-Calixto et al., 2007) (**Figure 7**).

The microorganisms that colonize the large intestine are the main element responsible for phenolic metabolism. This complex and enormous population is commonly defined as gut microbiota. Its great variety confers a broad range of enzymes capable of transforming the native phenolic compounds into simpler metabolites (Cardona et al., 2013). This enzymatic

cocktail performs activity of phase I metabolism, such as depolymerization (proanthocyanins and ellagitannins), deconjugation (phase II metabolites), ring cleavage of flavonoids, deglycosylation, deserterification, deacetylation, dehydroxylation, demethylation, decarboxylation and oxidation, to mention some of the more commonly described degradation steps (Cardona et al., 2013; Williamson et al., 2010). These structural modifications produce changes in the physiological and physical properties of the native molecule. Thus, microbial metabolites do not necessarily share the same characteristics as their precursors (Colombo et al., 2013).

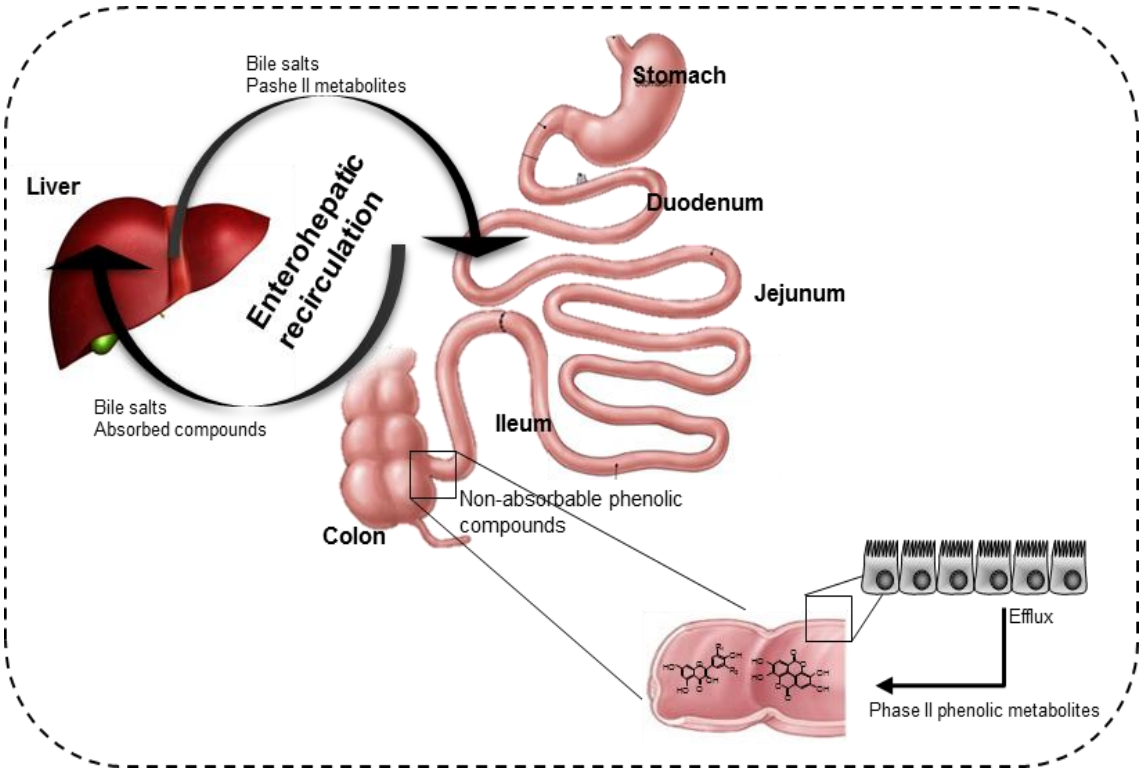


**Figure 7.** Different routes by which phenolic compounds reach the colon

The general bioaccessibility of phenolic species keeps increasing in the colon thanks to the microbial capacity to break down the linkage that maintains phenolic compounds associated with the food digesta in a way that the small intestine cannot (Saura-Calixto et al., 2007). The absorption efficiency of nutrients decreases in the colon compared with small intestine, but is partially promoted by its slower intestinal motility. It has been

demonstrated that microbial metabolites are absorbed and reach considerable systemic concentrations, in an even higher amount than their precursors (Cardona et al., 2013; Williamson et al., 2010). This prolonged time of residence is also responsible for the generation of a broad spectrum of phenolic metabolites. The composition of the lumen includes native compounds (present in the food), their products of early catabolism (initial metabolites) and a diverse mix of low molecular weight metabolites (Cardona et al., 2013; Cueva et al., 2015) generated from the subsequent microbial degradation of initial metabolites.

To fully understand the role of phenolic compounds in the gut and their possible involvement in local and systemic physiology, certain specific issues should be addressed (**Figure 8**):

- Deciphering the colonic metabolic pathways of phenolic compounds is a key to identifying the metabolites generated by microbial catabolism. After the identification, microbial metabolites should be studied to establish their potential bioactivity and hypothetical mechanisms of action.

- The time-degradation rate of phenolic compounds when they are in contact with gut microorganisms could explain the appearance of microbial metabolites in biological fluids at different time points. Pre-identification may encourage the inclusion of phenolic metabolites in future bioavailability and clinical studies.

- The identification of microbial metabolites as intermediates and end-products could be useful to establish biomarkers of consumption, treatment compliance and to correlate their presence with the improvement of health status. The selected biomarker (in faeces or in other fluids or tissue) should be sensitive to the variations in the intake of the dietary component of interest.

- The analysis of faeces or other biological fluids (urine or plasma) after a phenolic-rich dietary intervention helps to determine the average concentration that parent compounds and/or their microbial metabolites have in the intestine or at systemic level. This information is of vital importance to establish physiological concentration to be tested in future studies.

- The degree to which parent compounds and/or their metabolites could interfere with gut microbiota structure and activity. Moreover, the identification of specific bacteria involved in phenolic transformations could further provide valuable information about the potential role of phenolic compounds in the gut ecosystem.

- Different patterns of bioconversion of phenolic compounds in the colon by humans may be useful to make a link between healthy or at-risk populations. Factors such as the individual variability, physiological state, dose, and presence of other meal components when a phenolic source is studied should be considered to ensure success of therapeutic treatment for the major proportion of the population.

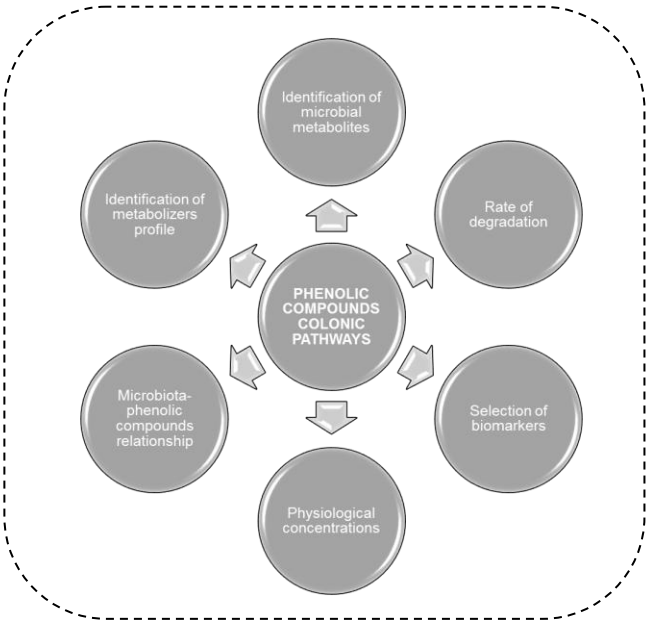


**Figure 8.** Information to be considered when colonic pathways of phenolic compounds are studied

# 3. Role of phenolic compounds in the large intestine

In early times, the large intestine was considered to be a segment of the gastrointestinal tract involved in water absorption and excretion of digestive waste (Karali et al., 1995). Gut was an “ignored organ”, and nowadays it is the central topic of numerous scientific studies. Phenolic compounds and/or their microbial metabolites are accumulated in the intestinal lumen for long residence time; hence, they may exert local protection against pro-oxidant, pro-inflammation and pro-carcinogenic events as well as impact on gut microbiota configuration and/or activity.

## 3.1. Possible implication of phenolic compounds in the large intestine health status

Several deleterious substances with potential pro-oxidant, pro-inflammatory and/or pro-carcinogenic effects enter or are generated daily in the alimentary canal, making the intestinal mucosa susceptible to functional and structural alterations (Coates et al., 2007; Rezaie et al., 2007). This hypothetical situation may be offset by the presence of luminal phenolic compounds, which could counteract the vulnerability to intestinal diseases. Phenolic compounds may act as antioxidants in the gut, providing local protection and preventing the racking of pro-oxidants from the intestine to the organism. Besides being pro-oxidant species, free radicals are also involved in the activation of pro-inflammatory mediators. When this occurs at intestinal level, it increases the risk of suffering the most common forms of inflammatory bowel disease (IBD), called ulcerative colitis and Crohn’s disease (Rezaie et al., 2007; Colombo et al., 2013). In turn, chronification of inflammation involves complex and interconnected mechanisms of molecular mediators, whose continued feedback increases the risk of colorectal cancers (Hagland et al., 2015; Martin et al., 2015; Seril et al., 2003).

The protective effects of phenolic compounds against intestinal alterations (inflammation and cancer) have been demonstrated in different experimental studies using cell and animal models (Cardona et al., 2013; Coates et al., 2007; Colombo et al., 2013; Martin et al., 2015; Williamson et al., 2010). In any case, to what extent dietary phenols and their microbial metabolites contribute to reducing intestinal oxidative stress, inflammation and

cancer is unknown, and this fact is mainly due to the limited data available derived from human studies (Colombo et al., 2013; Martin et al., 2015).

### 3.2. Phenolic-microbiota relationship

The large intestine is the body cavity that concentrates the most abundant and varied microbiota, reaching approximately $10^{12}$ bacteria per gram of intestinal content (Williamson et al., 2015). All this makes the gut a complex and multifunctional ecosystem where host and microorganism live in symbiosis. Whereas humans confer a suitable niche, providing access to nutrients from non-absorbed food (especially non-digestible carbohydrates and proteins), microorganisms participate in protective, immunologic and metabolic activities (Conlon et al., 2015; Donaldson et al., 2015; Hagland et al., 2015) (**Figure 9**). The gut microbiota protect the host, strengthening the intestinal barrier architecture and, through competitive exclusion (competing for food and physical space), controlling the overgrowth of pathogenic bacteria (Round et al., 2009). Gut inhabitants are aligned to the gut immunological system, ensuring it proper functioning and maturation (Round et al., 2009). In terms of metabolism, gut bacteria participate in several pathways, such as vitamin synthesis, optimization in the utilization of nutrients by fermentation of non-digested dietary compounds, and degradation of xenobiotics and endogenous compounds (Donaldson et al., 2015; Hagland et al., 2015).

Gut microorganism can negatively affect the host through either acute episodes (typical of foodborne illness) or chronic effects by the recurrent activation of pro-oxidant, pro-inflammatory and pro-carcinogenic pathways (Donaldson et al., 2015). On the other hand, other bacteria groups are considered positive for the host (Donaldson et al., 2015; Hagland et al., 2015). According to this premise, bacteria could roughly be classified under two categories: “beneficial” and “harmful”. The proper balance of microbial composition reinforces health status of the host and is normally called microbial homeostasis. On the contrary, disruption of microbial composition is known as dysbiosis and is associated with metabolic disorders (Round et al., 2009). Although abundance of bacteria in the alimentary tract is important, some authors have highlighted the fact that diversity may be the most relevant consideration to be taken into account in terms of the impact on gut ecology (Donaldson et al., 2015).

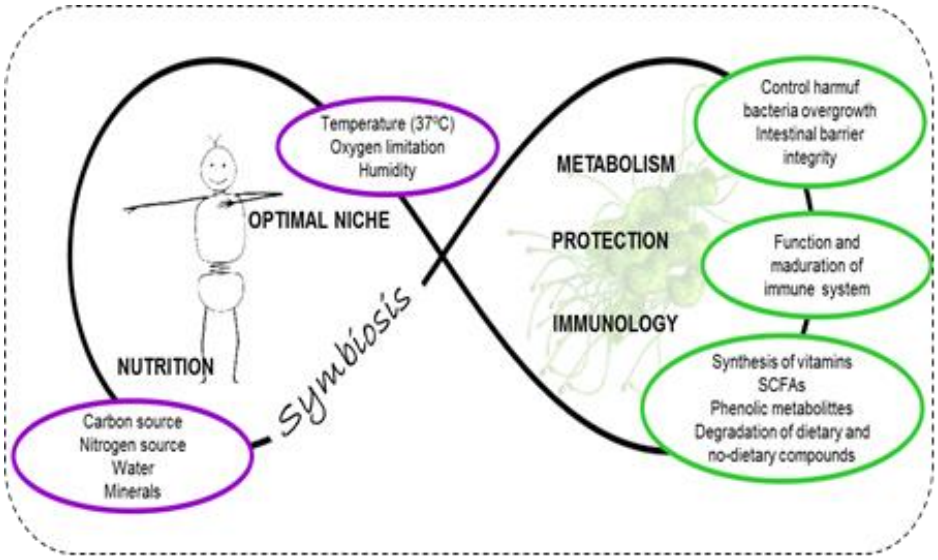


**Figure 9.** Symbiosis between human host and gut microbiota

It may be possible to promote the maintenance of microbial homeostasis with the intervention of phenolic-rich diets (Cardona et al., 2013; Colombo et al., 2013; Cueva et al., 2015). Phenolic compounds-microbiota relationship could be described as a two-way interaction. On the one hand, microorganisms transform the original compounds present in the food into related metabolites which could be subsequently utilized by secondary degraders, hence stimulating cross-feeding. Food phenolic compounds, in turn, may influence the reshaping of the microbiota population by favouring or hampering the growth of certain groups of microorganisms (Cardona et al., 2013; Cueva et al., 2015). It has been proposed that phenolic compounds and their microbial metabolites possess selectively antimicrobial properties, where beneficial bacteria are less affected (Cardona et al., 2013; Colombo et al., 2013).

The composition of the gut microbiota is a fundamental pillar of human health and its manipulation offers the possibility of maintaining general well-being, improving health and preventing disease (Colombo et al., 2013; Donaldson et al., 2015). Human studies in this area are scarce and the results obtained from different *in-vitro* and animal approaches are not conclusive (Cardona et al., 2013; Colombo et al., 2013; Cueva et al., 2015). Based on

this, it cannot be affirmed categorically that phenolic compounds are able to modulate gut microbiota toward a healthier composition. There is cumulative evidence that suggests that dysbiosis is involved in the development of several diseases, such as type 2 diabetes, obesity, intestinal diseases and hepatic and brain alterations (Donaldson et al., 2015). Therefore, there is a real need to establish the capacity of phenolic compounds to perform microbiota modulation, since they will be appropriate for therapeutic use.

## 3.3. Metabolic profile of the large intestine: implication of non-absorbed phenolic compounds

Colonic metabolism involves the interaction between available luminal substrates and gut microbiota. As a consequence of the colonic metabolism, a large number and type of compounds are formed, participating in the modulation of physiological, metabolic and immunologic pathways at gut level (Donaldson et al., 2015; Hagland et al., 2015; Krishnan et al., 2015; Nicholson et al., 2012; Tremaroli et al., 2012). Some of the colonic metabolites are beneficial for the host, but others may be conferring negative effects when they are present in high amounts (Donaldson et al., 2015; Nicholson et al., 2012; Tremaroli et al., 2012). The way in which phenolic compounds modify microbiota activity has not been completely described. Recent publications have proposed that phenolic compounds probably interfere with the enzymatic activity, with the availability of colonic substrates and with the bacteria development at gut level (Cueva et al., 2015). In this respect, the comprehension of the faecal metabolic pathways could be important to explain changes coinciding with regulation of the activity or growth of gut microorganisms, as well as to associate these changes with host metabolic and physiological alterations (Nicholson et al., 2012; Tremaroli et al., 2012). Among the bacteria fermentation products, those derived from carbohydrates, proteins, fats and sterols have probably been the most studied compounds (Cueva et al., 2015; Nicholson et al., 2012; Tremaroli et al., 2012).

*Short chain fatty acids (SCFAs)*

Short chain fatty acids (SCFAs) are the predominant end-products of bacterial fermentation of non-digested carbohydrates (poly and oligosaccharides and resistant starch) and proteins (Krishnan et al., 2015; Tremaroli et al., 2012). 90-95% of SCFAs is represented by acetic acid, propionic acid and butyric acid. Acetic acid is the predominant acid in the

faeces of healthy humans, followed by propionic and butyric acids. The proportion commonly found in the distribution of acetic, propionic and butyric acids is 60:20:20. The remaining fraction (5-10%) corresponds to isobutyric acid, isovaleric acid and valeric acid, called branched chain fatty acids. SCFAs are rapidly absorbed and metabolized by colonocytes, liver and muscle (Tremaroli et al., 2012). From among the SCFAs compounds, butyric acid has received more attention since it is the main source of energy for colonocytes and possesses selective anticarcinogenic and anti-inflammatory effects (Alcock et al., 2015; Hagland et al., 2015; Nicholson et al., 2012; Tremaroli et al., 2012). On the contrary, branched chain fatty acids (BCFAs), products of protein fermentation, are considered toxic for colonic cells (Macfarlane et al., 1992), which explains their major abundance in the faeces of individuals who have higher susceptibility to suffering from gut pathologies (Ou et al., 2012)

The production of SCFAs is an indicator of bacterial activity, and in some cases major production of acetic, propionic and/or butyric acids is accompanied by an increase in beneficial bacteria such as *Bifidobacterium* and *Lactobacillus* spp. (Blatchford et al., 2015; Cueva et al., 2015; Khodaei et al., 2016). Exploration of the effects that non-absorbed phenolic compounds may have on the bacterial production of SCFAs has also aroused the curiosity of several researchers, but a definitive argument has not been established in this aspect due to the differences observed between results. Phenolic compounds present in red wine (Cueva et al., 2015) and pomegranate extract (Bialonska et al., 2010) increase the *in-vitro* production of SCFA (especially butyrate), whereas those of aqueous passion fruit extract (da Silva et al., 2013) and isolated punicalagin (Bialonska et al., 2010) have shown the opposite effect. No modifications in SCFAs concentration was observed in rats after the administration of *trans*-resveratrol and quercetin (alone or in combination) (Etxeberria et al., 2015). The effects regarding these interactions seem to be confused, since the fibre of many plants is closely bound to phenolic compounds (Saura-Calixto et al., 2007). It is therefore difficult to discriminate whether SCFAs production results from the presence of fibre or is caused by phenolic compounds linked to fibre or the combination of both.

*Sterols*

Bile salts are metabolized in the liver from cholesterol and secreted into the intestinal lumen to facilitate the digestion of lipophilic compounds (fat-soluble nutrients and xenobiotics),

whereas diet and intestinal cellular turnover contribute with luminal cholesterol. Enzymes produced by certain gut microbial products catalyze the conversion of primary bile acids, cholic and chenodeoxycholic acids, into their respective secondary bile acids, lithocholic and deoxycholic acids (Hill et al., 1975; Nicholson et al., 2012). Microbial conversion of cholesterol generates mainly coprostanol and, to a lesser extent, coprostanone and cholestanone (Hill et al., 1975).

Whereas at physiological concentration the sterols and their microbial products are involved in the regulation of the pathways involved in the host metabolism, at higher amounts they are associated with anatomic and local function alterations related to the aetiology of colon cancer and other intestinal diseases (Nicholson et al., 2012; Tremaroli et al., 2012). High fat diets, especially those of animal origin, associated with metabolic alterations, increase the amount of bile acids and cholesterol in the colon lumen (Rafter *et al.* 1987).

*Proteins*

Every day, variable amounts of nitrogenous compounds of dietary and endogenous origins reach the colon. Protein-rich diets, particularly those containing red meat, increase gut toxicity as a consequence of an overproduction of microbial products derived from protein fermentation. These substances, also called putrefactive compounds, include ammonia, faecal phenolic (phenol and *p*-cresol) and indolic compounds (indol and skatole), sulphur compounds (hydrogen sulphide, methyl mercaptan, and dimethyl sulphide), branched chain fatty acids, and polyamides (putrescine, agmatine, cadaverine, tyramine, and histamine). Intestinal ammonia is generated through the microbial deamination of urea and amino acids (Smith et al., 1997, Vince et al., 1973). Phenolic and indolic compounds are mainly derived from the microbial catabolism of aromatic amino acids (phenylalanine, tyrosine, and tryptophan) (Smith et al., 1997). Sulphate-reducing bacteria produce sulphur metabolites as a product of anaerobic respiration during the catabolism of cysteine and methionine (Magee et al., 2000).

Elevated undigested luminal proteins could alter the intestinal ecology, stimulating the activity of nitrogen-degrading bacteria, which are considered to have detrimental effects on gut homeostasis (Nicholson et al., 2012; Tremaroli et al., 2012). The metabolism of

proteins, monitored by microbial products of their fermentation, also seems to be modified by the inclusion of phenolic-rich sources in the diet, as reported by *in-vitro* (Cueva et al., 2015) and *in-vivo* studies (Hara et al., 1995). The solution is not to eliminate the proteins from the diet, since they are essential nutrients for the human organism. However, a dietary recommendation based on control of the intake of proteins should be followed.

*Fat*

Unmetabolized saturated and unsaturated diet fats are partially transformed in the large intestine. The role of fats in the gut is still confusing, since there are no conclusive results. Reviews show that saturated and polyunsaturated fatty acids, especially omega-6, may cause dysbiosis and promote proinflammatory events, whereas omega-3 and monounsaturated fatty acids (present in olive oil) seem to protect against bacteria translocation and reduce the risk of inflammation (Alcock et al., 2015).

Choline takes part in cell membranes and, thus, red meat and eggs are probably the main dietary choline source. Trimethylamine is the end-product of the choline bacteria metabolism that is produced by intestinal microbes. The presence of this metabolite and its hepatic derivative has been associated with cardiovascular disease (Tremaroli et al., 2012).

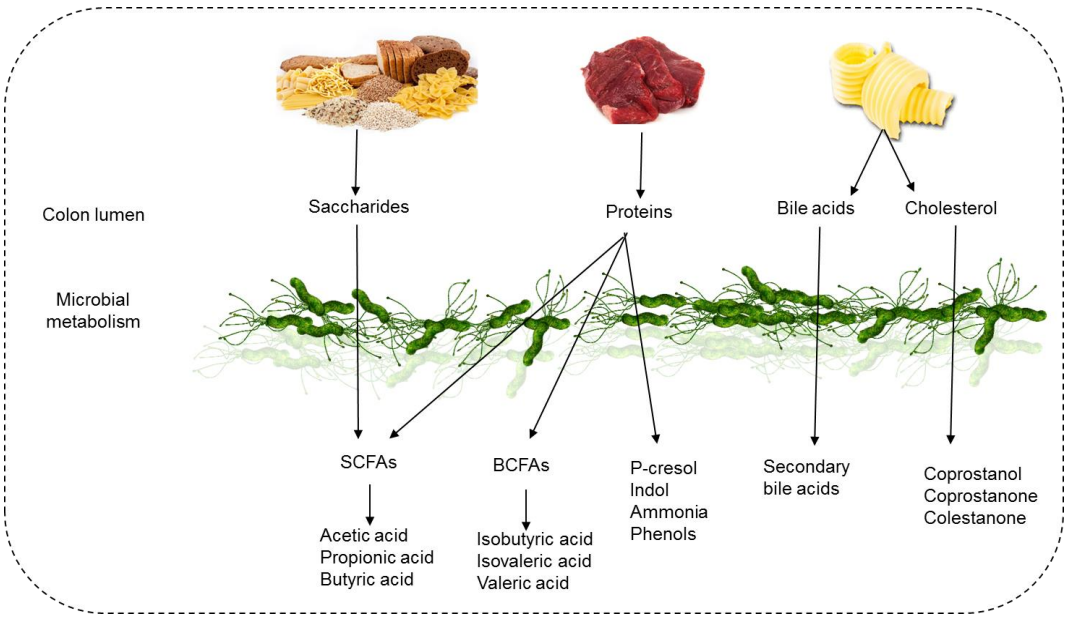


**Figure 10.** Microbial metabolism of carbohydrates (including fibre), proteins and faecal sterols in the large intestine

# 4. Metabolic fate of phenolic compounds in the large intestine.

## *4.1. In vitro* models to study the colonic fate of phenolic compounds

*In-vitro* models, including both enzymatic incubations and cell or tissue-based models, have gained a respectable position in preliminary studies to predict and interpret the behaviour of phenolic compounds in living beings. The main advantages that justify the use of these models are linked to the possibility of monitoring, under constant surveillance of environmental conditions, a large amount of samples with a relatively low investment in time and money, and the total absence of ethical conflicts. The main downside of *in-vitro* studies is that they do not fully imitate *in-vivo* systems. Certain parameters, such as enterohepatic recirculation, the continuous absorption (or efflux) by intestinal epithelium and the inclusion of microbiota associated with intestinal mucosa, are probably the most difficult to achieve in digestion models (Williamson et al., 2010).

The different options to study the microbial transformation of phenolic compounds in the gut are shown in **Figure 11**. *In-vitro* fermentations have been extensively used to obtain a closer insight into the colon metabolism where a phenolic source comes into contact with gut microorganisms (Williamson et al., 2010). The phenolic test includes pure or isolated compounds, phenolic-rich extracts, food or pre-digested material (Correa-Betanzo et al., 2014; Cueva et al., 2015) (**Table 2**). The pre-digested material can be supplied by intestinal content obtained from humans (ileostomy) (Langkilde et al., 2002) or from *in-vitro* gastrointestinal digestions (oral, gastric and/or duodenal steps) (Correa-Betanzo et al., 2014) (**Table 2**). In turn, *in-vitro* gastrointestinal digestions could be different depending on the incubation time, incubation temperature, pH, composition of buffers, and modality, which includes static or dynamic models. Static digestion is performed in closed containers (whether or not including agitation system) and the potential bioaccessibility of each phenolic compound is normally calculated after the centrifugation to separate the undigested fraction from the digested (soluble) fraction (Bermúdez-Soto et al., 2007; Correa-Betanzo et al., 2014; Kahle et al., 2011). The dynamic digestion mimics the transit time and introduces a mechanism that simulates the biodisponibility of compounds during intestinal passage (Coates et al., 2007; Rubiò et al., 2014).

To carry out colon-simulated fermentations, faeces from animals or humans are used as microbial inocula (Cueva et al., 2015). In addition, to avoid high variability and to identify the role of faecal microorganisms and/or their produced enzymes, phenolic subtracts have also been exposed to a defined community of faecal microbiota composed of individual bacteria or a bacteria-mix (Correa-Betanzo et al., 2014).

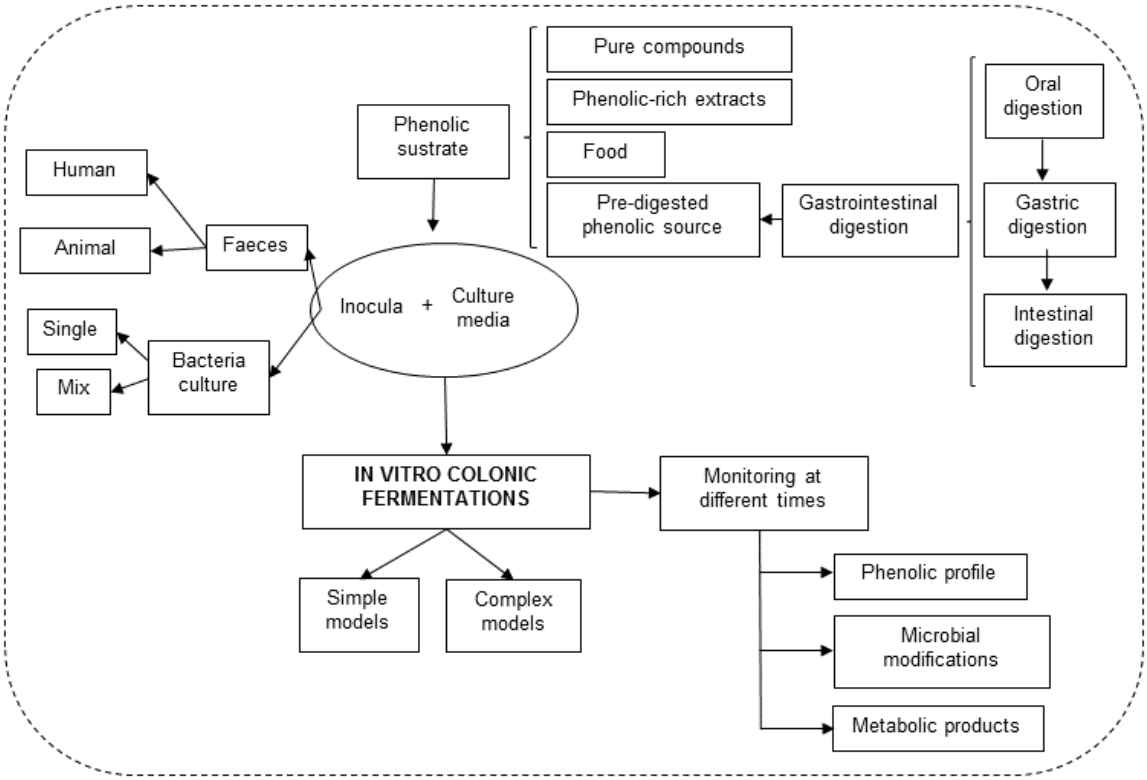


**Figure 11.** Scheme of the different substrates and models used for *in vitro* colonic fermentation of phenolic compounds

Except for the anaerobic conditions, the techniques of *in-vitro* incubations do not always respond to the same instrumental and methodological characteristics. Colonic fermentation can be performed using simple (reactors, vessels, tubes) or more complex multi-compartmental continuous systems. These consist of interconnected reactors that represent the different segments of the large intestine and, sometimes, oral, gastric and intestinal digestion steps are also included (Cueva et al., 2015). Microbial incubations

including simpler procedures are usually sufficient to study the colonic metabolism of phenolic compounds and their time-based bioconversions (**Table 2**).

**Table 2**. Description of *in vitro* fermentations using different phenolic compounds and their respective microbial metabolites.

| Phenolic source | Phenolic compounds | Microbial source | Fermentation time | Microbial metabolites | Reference |
|---|---|---|---|---|---|
| Pre-digested wild blueberries | Anthocyanins<br>Chlorogenic acid<br>Quercetin<br>Syringetin | Defined human community composed by 33 bacteria groups | 12 h (Anaerobic batch fermentation) | Syringic acid, rhamnetin, hippuric acid, cinnamic acid, protocatechuic, caffeic acid, kaempferol rhamnoside | **Correa-Betanzo et al., 2014** |
| Red wine | Catechin<br>Epicatechin<br>Gallic acid<br>Resveratrol | Human faeces | Computed controlled dynamic simulator including 5 steps of digestion (Stomach, intestine, colon) | Phenylpropanol, phenylvaerolactones, phenylvaleric acid, phenylpropionic acids, phenylacetic acids, benzoic acid, 3-O-methylgallic acid, pyrogallol, catechol, pyrocatechol, hippuric acid, p-coumaric acid | **Cueva et al., 2015** |
| Wine extract | Not defined | Human faeces | 0, 4, 18 and 24 h (Anaerobic batch fermentation) | 4-hydroxyphenylpropionic acid, thihydroxybenzoic acid, phloroglucinol, protocatechuic acid, acid, gallic acid, ferulic acid, caffeic acid | **Grün et al., 2008** |
| Freeze dried espresso coffee | Chlorogenic acids | Human faeces | 0, 1, 2, 3, 4, 6 h (Anaerobic tubes) | Caffeic acid, ferulic acid, dihydroxyphenylpropionic acid, dihydroferulic acid, hydroxyphenylpropionic acid, phenylpropionic acid, phenylacetic acid, benzoic acid, catechol, protocatechuic acid. | **Ludwig et al., 2013** |
| Pure compound | Trans-resveratrol | Human faeces | 0, 2, 4, 6, 8, 24, 48 (Vessels) | Lunularin<br>Dihydroresveratrol + lunularin<br>dihydroresveratrol | **Bode et al., 2013** |
| Pure compound | Hesperetin (1)<br>Naringenin (2)<br>Ferulic acid (3) | Human faeces | 0, 1, 2, 4, 6, 8, 24 h (Vessels) | (1)Hydroxymethoxyphenylpropionic acid,<br>dihydroxyphenylrpopionic acid,<br>hydroxyphenylpropionic acid,<br>phenylpropionic acid<br>(2)Hydroxyphenylpropionic acid, phenylpropionic acid<br>(3)methoxyhydroxyphenylpropionic acid,<br>hydroxyphenylpropionic acid | **Pereira-Caro et al., 2014** |

The most complex continuous models are probably best-adapted to observe the effects on the microbial population, including long-term studies that intend to imitate a continuous intake of phenolic compounds. Those dynamic models containing different vessels are also appropriate to predict the sites of the colon (ascendant, transversal or descendent) where fermentation predominates and how the primary metabolites generated are transported and transformed during faecal stream (Cueva et al., 2015). Nevertheless, multicompartment reactors are expensive and require more labour, economic investment and time for the *in-vitro* procedure than static models of *in-vitro* incubations.

Complementary to microbial metabolism, the exposure of test substance to cell culture models is used to study cellular transport (apparent bioavailability), metabolism, permeability (integrity of intestinal barrier) and molecular mechanisms modulation. For the bioavailability studies, transport mechanisms and intracellular metabolism, differentiated Caco-2 cells are often chosen (Konishi et al., 2004; Rubió et al., 2014), although other cell lines such as hepatocytes have also been used to study hepatic metabolism (Khale et al., 2011; Rubió et al., 2014) and cellular transport (Lançon et al., 2004).

To elucidate the regulation of molecular mechanisms by means of which phenolic compounds may exert biological activity (antioxidant, anti-inflammatory and anticarcinogenic effects), tumour and activated cells have been used (Coates et al., 2007; Colombo et al., 2013). In a simplified way, cell culture models consist of the exposure of cells to a certain amount of phenolic-source under defined environmental conditions and time periods (Coates et al., 2007; Colombo et al., 2013). Imperfections in cell culture models may occur when high concentration or pure standards or extracts are used, since the use of crude fruit or vegetable extracts or preparations may overestimate the biological activity and is not comparable with what occurs in the human organism. A more realistic approach to reach the adaptation to *in-vivo* situations could be the use of phenolic fractions obtained after *in-vitro* digestion or else from intestinal aspirations collected from humans or animals (Coates et al., 2007; Rubió et al., 2014).

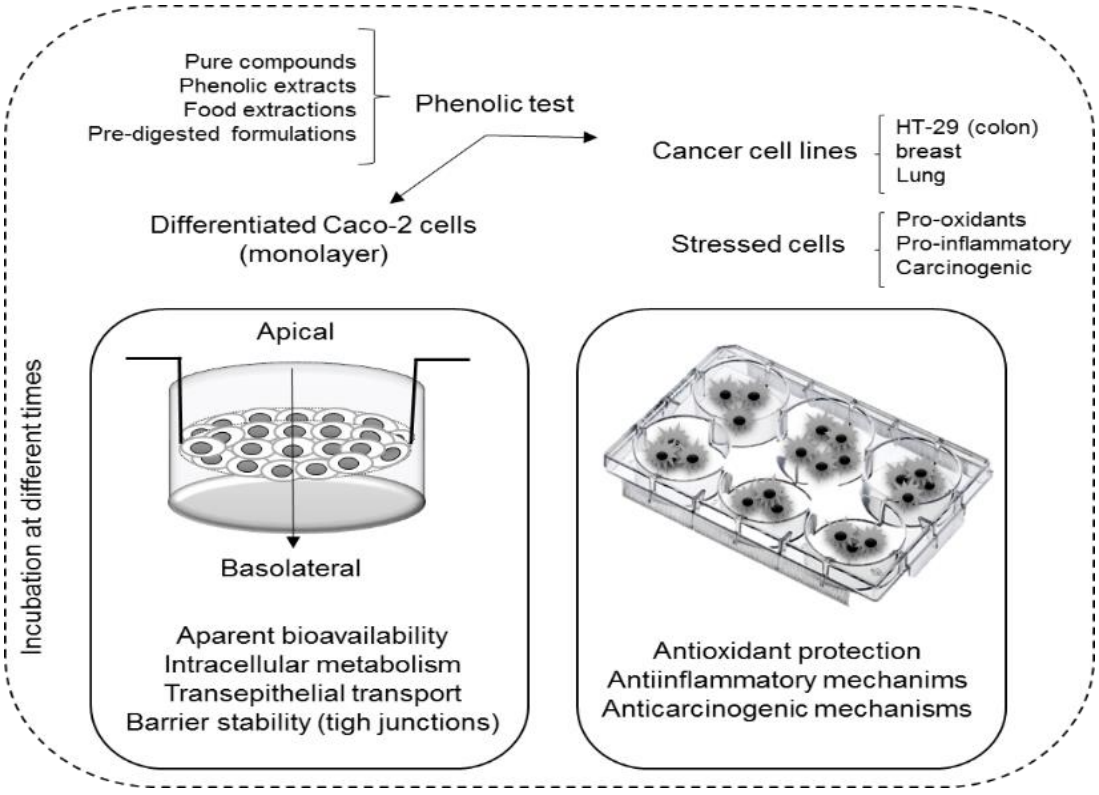


**Figure 12.** Cellular *in vitro* experiments used to study phenolic metabolism and/or molecular mechanisms

## *4.2. In vivo* studies to monitor the metabolism of phenolic compounds

*Human studies*

The scientific community is clamouring for further human trials to explore the role of phenolic compounds in the organism and to correlate their presence in biological fluids, such as plasma and/or urine, with the modulation of biomarkers of disease. Probably, the most popular design adopted to investigate the human colonic metabolism of phenolic compounds is through the analysis of biological fluids after an acute or sustained intake of a phenolic-rich source (Kahle et al., 2005) (**Table 3**). The main limitations related to human clinical studies are usually marked by economic, instrumental and infrastructural availability. Additionally, there are key points which differ among studies, such as the characteristics of the volunteers (health status, age, sex, and lifestyle), the type of biospecimen collected (urine, blood, faeces, breath, tissues, gastrointestinal content), the

phenolic source (food, extract or pure compounds) and the biomarkers to monitor the efficacy of the phenolic compounds studied (Williamson et al., 2005).

**Table 3**. Phenolic microbial metabolites detected in human biological fluids after the intake of different phenolic-rich sources.

| Phenolic source | Phenolic compounds | Ingestion | Time of treatment | Biological sample | Microbial metabolites | Reference |
|---|---|---|---|---|---|---|
| Mix extract of red wine and red grape juice | No defined | 800 mg/day total phenolic compounds | 4 weeks | Urine | 3 and 4 hydroxypehnylacetic acid, 3-hydroxyphenylpropionic acid, canillic acid, homovanillic acid, hippuric acid, syringic acid | **Grün et al., 2008** |
| | | | | Plasma | Different hydroxylation patterns of benzoic acid, hydroxyphenylpropionic acid, vanillic acid, homovanillic acid, methylgallic acid, ferulic acid, caffeic acid, syringic acid | |
| Green tea | No defined | 8 cup of tea (200 mL each) | Acute dose | Faeces | 3 and 4-hydroxybenzoic acid, 4-hydroxyphenylpropionic acid, 3,4-dihydroxymethylacetic acid, ferulic acid | |
| Nuts | Ellagitannins | 30 g/day for 3 days | 3 days | Urine | Urolithin A | **Tomás-Barberán et al., 2014** |
| Pomegranate extract | Ellagitannins | 4 capsules | Acute intake | Urine | Urolithin A+ Urolitin B + isourolithin A | |
| Pomegranate extract | Ellagitannins | 2 capsules | 2 days | Urine | None | |
| Red wine | Flavan-3-ols, anthocyanins, phenolic acids | 250 mL/day | 4 weeks | Faeces | Syringic acid, benzoic acid, phenylacetic acid, 3 and 4' phenylpropionic acids, phenylpropionic acid, 4-hydroxy-5-phenylvaleric acid, 3-O-methylgallic acid, vanillic acid, protocatechuic acid. | **Muñoz-Gonzales et al., 2013** |
| Supplement | *trans*-resveratrol | 0.5 mg/kg body weigh | Acute | Urine | Dihydroresveratrol<br>Mixed dihydroresveratrol + lunularin<br>Lunularin | **Bode et al., 2013** |

In the particular case of colonic metabolism studies, one of the aspects to be considered is to clarify how phenolic compounds reach the colon. For this, the analysis of ileum fluids, provided by ileostomy patients, offers valuable data regarding the qualitative and quantitative phenolic profile of the product leaving the small intestine (Kahle et al., 2005). Comparison of biological fluids (mainly urine and plasma) between ileostomy patients and volunteers with intact colon offers an overview of the potential prominence of colon metabolism in the human body.

To understand more about phenolic bioavailability and to have partial results about the tissue and organ exposure, the analysis of plasmatic and urinary phenolic concentrations is probably the predominant approach in human studies (Williamson et al., 2005). To study absorption both in the small intestine and in the colon, the most extensive approach is the phenolic analysis of samples at different collection time-points after a single or continuous administration of a phenolic dose (Khale et al., 2011; Williamson et al., 2005). The time lapse required for phenolic compounds to pass into the bloodstream depends on the intestinal absorption site. For example, those compounds detected within 0.5 h post-ingestion are considered to be absorbed into the stomach. Those compounds absorbed into the small intestine are normally detected within an hour, and the maximum concentration is usually less than 4 h. Phenolic compounds and/or phenolic metabolites that appear 4 h post-intake are generally considered to have been absorbed at colon level.

In the context of human bioavailability studies on phenolic compounds, blood (or plasma) and urine are probably the biological fluids most suitable to determine the phenolic metabolites concentration (Khale et al., 2011; Williamson et al., 2005). Although faeces are more accessible and easy to collect than blood samples, they have not been extensively analyzed after phenolic interventions. Faecal samples obtained before and after a dietary intervention offer the possibility to study local microbial and metabolic reactions to the treatment as well as resistance of phenolic compounds to the digestion process.

*Animal studies*

Certain approaches regarding colon metabolism under *in-vivo* conditions are impracticable in humans or require strict measures for their approval by Ethics Committees. To overcome these limitations, animal models can be a good alternative (Borges et al., 2007; Martin et al., 2015). Rodents and, on occasions, pigs have been used to study colonic fate and/or general metabolism of phenolic compounds (Borges et al., 2007; Espín et al., 2007). Beyond the possibility of diet and environmental control, animal models should be suitable to imitate, as far as possible, the human metabolism.

Studies with animals offer the alternative of understanding the colonic fate of phenolic compounds in a deeper manner, due to the possibility of studying phenolic deposition and distribution in organs and tissue (Borges et al., 2007). However, results derived from animal

studies are not always well-received by certain sectors of the scientific community. In terms of colonic studies, some researchers remain sceptical about the extrapolation of the effects observed on humans. The main argument put forward to explain this behaviour is the difference in microbiota composition between animals and humans (Lee et al., 2011).

Germ-free rodents have opened up an exceptional area that allows an understanding of the metabolic role of the colon, thus limiting differences between species. Germ-free animals born and grown in sterile conditions are hence a model that lacks colonic microbial metabolism. This makes it possible to explore the role of gut microbiota in the metabolism and fate of phenolic compounds through comparison with their conventional counterparts or with germ-free animals inoculated with selected microbial species (Schneider et al., 2000) or whole communities from humans (Tamura et al., 2009). Transplantation of human faeces to sterilized rodents offers advantages such as reducing the differences observed between humans and animals in terms of gut metabolism.

**Table 4.** Phenolic microbial metabolites detected in animal biological fluids after the intake of phenolic-rich sources

| **Phenolic source** | **Phenolic compounds** | **Animal model** | **Time of treatment** | **Sample** | Microbial metabolites | Reference |
|---|---|---|---|---|---|---|
| Pure compounds | Quercetin-3-glucoside | Germ free rats Germ free rats associated with 1 and 2 bacteria strains | 16 d | Faeces | 3,4-dihydroxyphenylacetic acid in rats with Eu. ramulus and both bacteria but not in germ-free and Ent. casseliflavus animals | **Schneider et al., 2000** |
| Pure compound Isolated compounds | Catechin Procianidin dimer B3 and trimer C2 Polymer fraction | Rats | 5d | Urine | Pheylvaleric acids, phenylpropionic acids, phenylacetic acids, benzoic acids, vanillic acid, hydroxyhippuric acid, cinnamic acids | **Gonthier et al., 2003** |

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# HYPOTHESIS AND OBJECTIVES

Phenolic compounds are a small fraction of phytochemicals of plant-based products. Despite their low concentration, phenolic compounds have generated great expectations as natural therapeutics, due to their antioxidant, anti-inflammatory and anticarcinogenic biological properties.

An important percentage of the phenolic compounds ingested through the diet survive gastrointestinal digestion and reach the large intestine unmetabolized. Once there, the local microbiota transforms the original compounds present in the food into simpler related metabolites that do not necessary share the same biological features as their precursors. As a result, a variable mixture of native phenolic compounds and their microbial products stay in the large intestinal lumen. The accumulation of this complex mixture of dietary phenols and their metabolites not only provides local antioxidant, anti-inflammatory and anticarcinogenic protection, but also could have indirect implications in host health through the modification of microbiota composition and/or its activity. In addition, the absorption of these compounds through the colon contributes to the bioavailability of food phenols and consequently to their potential systemic bioactivity.

The present Doctoral Thesis is framed in the context of two different projects: (i) the MEFOP project: *Metabolic Fate of Olive Oil Phenolic Compounds in Humans: Study of metabolic pathways and tissue distribution*. The global aim of the project was to assess the metabolic fate of olive oil phenolic compounds by using a nutrigenomic approach, and (ii) the INCOMES project: *Guide for the substantiation of health claims in foods: immune, cognitive functions and metabolic syndrome* (Collaborative project between Food Companies & Research Centres). The main goal of the Consortium INCOMES, co-funded by the Spanish Government, was to give free access to the results, mainly conclusions and guidelines intended to clarify methodologies for scientific support of health claims in foods/ingredients. Prior to the efficacy studies of the food bioactive compounds, it is necessary to perform nutrikinetic studies: from the simplest *in vitro* assays, recommended in early stages of the development of a functional food, to human intervention studies, which have been carried out in the framework of this Doctoral Thesis.

In an attempt to complete the information concerning the fate of phenolic compounds in the human body, the **hypothesis** of the Doctoral Thesis was based on the fact that the process that take place in the colon should not be ignored, since it is a site of relevant metabolic

activity. The phenolic-bacteria interaction could modify several aspects of the gut ecosystem that could affect the local and general health of the host.

In the context of the interest in the colonic metabolism of food bioactive compounds, the **main objective** of the present Doctoral Thesis was to study the colonic metabolic pathways of different phenolic groups from *in-vitro* to *in-vivo* approach, and the possible implications that phenolic compounds (or their microbial derivatives) may have in the large intestine ecosystem. For this, three phenolic sources containing a wide and complete range of phenolic groups were selected: virgin olive oil, pomegranate, and *Arbutus unedo* fruit.

Different specific objectives have been defined in order to achieve the general aim:

I. Characterize and quantify the phenolic compounds of olive oil enriched with its own phenolic compounds and in combination with thyme phenolic compounds, pomegranate products (juice, pulp and peel extract) and *Arbutus unedo* fruit.

II. Propose the colonic metabolic pathways of the most representative phenolic compounds present in phenol-enriched olive oils, pomegranate and *Arbutus unedo* through the application of *in-vitro* colonic fermentation.

III. Compare and define the behaviour of the phenolic compounds in the large intestine according to their individual chemical structure and the nature of their microbial derivatives.

IV. Study the *in-vivo* colonic metabolism in humans of the main phenolic compounds detected in phenol-enriched olive oils, pomegranate juice and *Arbutus unedo* fruit through the analysis of faeces after dietary interventions.

V. Assess, through the analysis of human faecal samples, the changes in the gut ecosystem (microbial and metabolic modifications) as a consequence of the lumen accumulation of phenolic compounds and/or their microbial metabolites promoted by the sustained intake of phenol-enriched olive oils and pomegranate juice.

VI. Compare the results obtained from *in-vitro* and *in-vivo* studies in order to establish whether the observations derived from *in-vitro* experiments are appropriate to predict the colonic metabolism of phenolic compounds in human beings

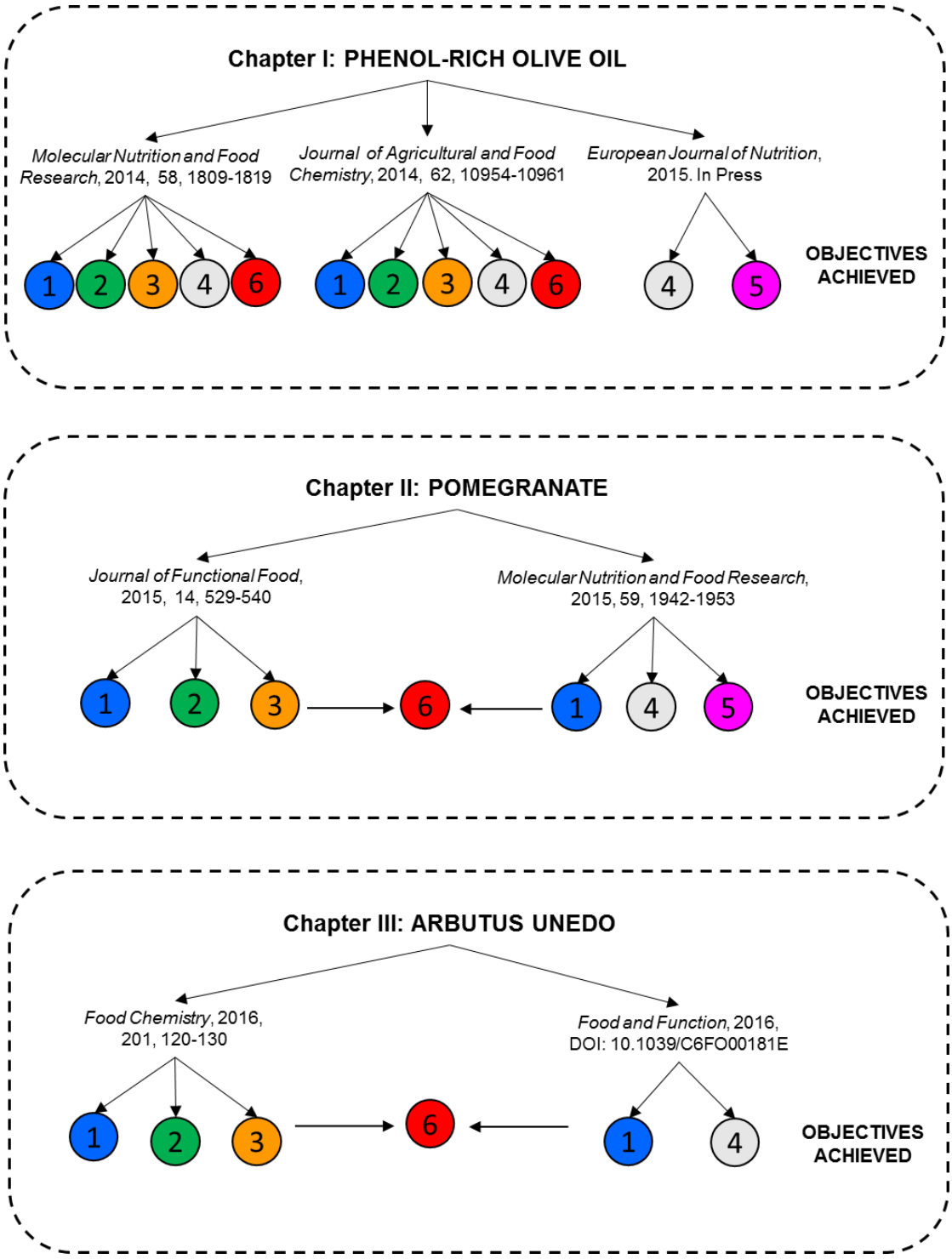


**Figure 13.** The proposed objectives achieved in each publication contained in this Doctoral Thesis

# RESULTS AND DISCUSSION

## **CHAPTER I**: PHENOL-RICH OLIV E OIL

**Publication I** Mosele et al.

*Molecular Nutrition and Food Research*, 2014, 58, 1809-1819.

**Publication II** Mosele et al.

*Journal of Agricultural and Food Chemistry,* 2014, 62, 10954-10961.

**Publication III** Martín-Peláez et al.

*European Journal of Nutrition*, 2015; 13. Article in Press.

**PUBLICATION I** Faecal microbial metabolism of olive oil phenolic compounds: in vitro and in vivo approaches

*Molecular Nutrition and Food Research*, 2014, 58, 1809-1819

RESEARCH ARTICLE

# FAECAL MICROBIAL METABOLISM OF OLIVE OIL PHENOLIC COMPOUNDS: IN VITRO AND IN VIVO APPROACHES

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**Scope:** In the present study, the individual colonic metabolism of the main components of the virgin olive oil phenolic fraction was evaluated by an in vitro model using human faecal microbiota. To assess differences in metabolism related to the molecular structure, four phenolic standards were selected, tyrosol, hydroxytyrosol, hydroxytyrosol acetate and oleuropein. After studying the in vitro colonic metabolism pathways of the individual phenols, the presence of their colonic metabolites was investigated in human faecal samples obtained before and after the sustained intake (3 weeks) of a daily dose of 25 mL of a phenol-enriched olive oil.
**Methods and results:** The in vitro colon fermentation of the four individual phenolic compounds revealed (i) an increase in phenolic acids, (ii) the stability of hydroxytyrosol and tyrosol and (iii) the high degradation of hydroxytyrosol acetate and oleuropein. Additionally, a moderate intake of a phenol-rich olive oil raised the concentration in human faeces of free hydroxytyrosol and phenylacetic and phenylpropionic acids.
**Conclusion:** The products of colonic catabolism of olive oil phenolic compounds could be good candidates for novel preventive strategies and open a promising line of research into the preventive action of olive oil phenols in colon and other bowel diseases.

**Keywords:**
Faecal microbial metabolism / Gut phenolic metabolites / Hydroxytyrosol / Metabolic pathways / Olive oil phenolic compounds

## 1. INTRODUCTION

Virgin olive oil is the most popular fat source in Mediterranean countries where it is consumed daily in moderate amounts [1]. Its production is based on mechanical procedures that imply the presence of minor bioactive substances in contrast with refined olive oil and other commercial oils [2]. Among these substances, the phenolic compounds have received much attention over recent years because they have properties related to a decreased risk and risk factors of chronic diseases such as cardiovascular disease [3] and certain cancers [4]. The phenolic fraction of virgin olive oil is made up of simple phenols, the most representative of which are the phenolic alcohols (tyrosol, hydroxytyrosol, hydroxytyrosol acetate) and complex phenols, such as oleuropein and ligstroside and their aglycone derivatives (secoiridoids) [5–7].

Several research groups have investigated the absorption, metabolism, distribution and excretion of olive oil phenols, especially tyrosol, hydroxytyrosol, secoiridoids (oleuropein and ligstroside) and their aglycones, under both in vitro and in vivo conditions [8–10]. These studies are useful for the knowledge they supply about the bioavailability and the mechanism of action of these compounds in the body and to understand how they could carry out their bioactive function.

The digestion process of the aforementioned phenolics has been widely studied, especially the phenomena in the upper gastrointestinal tract: (stomach and small intestine). Over recent years, the behaviour of phenolic compounds in the colon has attracted much attention because they may act as prebiotics, since they are able to reach the colon and be used by bacteria such as bifidobacteria [11], which exert beneficial effects. Also, microbial fermentation of phenolic compounds in the colon results in the release of low molecular weight metabolites that could influence host health [12]. Virgin olive oil phenols could reach the colon by different ways: non-absorbed food fraction, metabolites resulting from the enterohepatic recirculation or as a consequence of the basolateral-apical transport of intestinal cells [13]. For a first assessment of gut metabolism and to characterise microbial fermentation products of dietary polyphenols, many researchers opt for an in vitro fermentation model using faeces from human or animals as microbial inoculums. In vitro experiments have been extensively used to study colonic catabolism of polyphenols such as flavan-3-ols [14–16], estilbenes [12], lignans and isoflavones [17, 18].

The main limitation of in vitro fermentation procedures is that they do not mimic the in vivo conditions but are useful for studying the metabolic fate of the compounds in the colon because it is possible to follow their performance over time and they are good models to complement with in vivo studies.

Despite the relevant information in the literature related with the olive oil polyphenols, the study of their colon fate has yet to be considered. To the best of our knowledge, only two studies have been focused on the colon metabolism of oleuropein, one in vitro [9] and the other from a study in rats [19], but there is no information about the most characteristic olive oil phenols as hydroxytyrosol, tyrosol and hydroxytyrosol-acetate colonic pathways. In this context, the aim of this work is to study the individual colonic metabolism of the main components of the virgin olive oil phenolic fraction through an in vitro model using human faecal microbiota. To assess differences in metabolism related to the molecular structure, four phenolic standards were selected, these being tyrosol, hydroxytyrosol, hydroxytyrosol acetate and oleuropein. After studying the in vitro colonic metabolism pathways of the four individual phenolics, the presence of their colonic metabolites was investigated in human faecal samples obtained before and after the sustained intake of a daily dose of 25 mL of a phenol-enriched olive oil during 3 weeks.

## 2 MATERIALS AND METHODS

### 2.1. In vitro colonic fermentation

Faecal samples were collected from three healthy volunteers who followed normal dietary habits and declared they had not ingested antibiotics for at least 3 months prior to the sample collection. The fermentation medium was carbonate–phosphate buffer and this was prepared according to Durand et al. [20] by mixing (g/L): 9.240 $NaHCO_3$, 3.542 $Na_2HPO_4{\cdot}2H_2O$, 0.470 NaCl, 0.450 KCl, 0.227 $Na_2SO_4{\cdot}10H_2O$, 0.055 $CaCl_2$ (anhydrous), 0.100 $MgCl_2{\cdot}6\ H_2O$, 0.400 urea with 10 mL of trace element solution containing (mg/L) 3680 $FeSO_4{\cdot}7H_2O$, 1159 $MnSO_4{\cdot}H_2O$, 440 $ZnSO_4{\cdot}7H_2O$, 120 $CoCl_2{\cdot}6H_2O$, 98 $CuSO_4{\cdot}5H_2O$ and 17.4 $Mo7(NH_4)6O_24{\cdot}4H_2O$. The medium was adjusted to pH 7.0 using hydrochloric acid. Before its use, the culture medium was maintained in anaerobic chamber for 48 h, using gas generator bags ($CO_2$) to generate anaerobic atmosphere. The anaerobic environment was continuously monitored by anaerobic indicator strips (Becton Dickinson, MD, USA). Standards of tyrosol and oleuropein were purchased from Extrasynthese (Genay, France). Hydroxytyrosol and hydroxytyrosol acetate were purchased from Seprox Biotech (Madrid, Spain). 1,2-dihydroxy benzoic acid, phenylacetic acid, 4-hydroxybenzoic acid, 2-phenylpropionic acid, 2-(4'–hydroxyphenyl) acetic acid, 2-(3'-hydroxyphenyl)acetic acid, 3,4-dihydroxybenzoic acid, 2-(3',4'-dihydroxyphenyl) acetic acid, 3-(4'-hydroxyphenyl)propionic acid and caffeic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA).

The in vitro colonic fermentation method used in this study was adapted from Serra et al. [21]. For each volunteer, 15 g of fresh faeces were homogenised 60 s in a stomacher bag with 300 mL of culture medium to make 5% faecal slurry.

After homogenization, the faecal slurry was filtered and left to stand for 30 min. After this time, 10 mL were added to 15 mL disposable tubes. After the addition of 5 µmol of the standard aqueous solution (phenol standard final concentration 500 µM), the disposable tubes were incubated in anaerobic chambers at 37 ºC in an orbital shaker (60 strokes/min) in order to mimic colon conditions (anaerobic conditions were controlled by anaerobic indicator strips). The high initial concentration (500 µM) of the parent molecule was chosen in order to determine, at low concentration levels, the generated metabolites being possible to establish the colonic fermentation pathways for each molecule. Samples were collected at 0, 2, 6, 12, 24 and 48 h of incubation. Aliquots of the fermented faecal samples (500 µL) were collected from each time/tube and stored immediately at −80 ºC until the chromatographic analysis. All the fermentation samples were incubated in triplicate. Parallel to the fermentation samples, a control (faecal slurry without phenolic substrate) was prepared for each fermentation time point to identify the metabolite signals no generated by the phenolic standards added to the fermentation tubes. Also, incubations of the phenolic standards without faecal inoculation (blank 1) and with heat-inactivated faecal slurry (blank 2) were incubated at each time points (0, 2, 6, 12, 24 and 48 h) to take into account possible chemical degradation of the phenolic compounds.

**2.2. Human intervention study: In vivo model**

Faecal samples from ten subjects were used to determine olive oil phenolic metabolites identified in the in vitro colonic fermentation model. The faecal samples were obtained before and after the consumption (25 mL/day) of phenol-rich olive oil (**Table 1**) for a period of 3 weeks. Preceding the intervention there was a 2-week wash-out period where common olive oil (blend of refined and a small percentage of virgin olive oil) was the main fat consumed. To avoid an excessive intake of phenolic compounds during the intervention period, participants were advised to limit the consumption of phenol-rich foods. Faecal samples from each volunteer were collected after the wash-out period (pre-olive oil intake) and at the end of the 3 weeks of the intervention study (post-olive oil intake).

**Table 1.** Hydroxytyrosol derivatives composition of ingested phenol-enriched olive oil

| Compound | mg/kg oil |
|---|---|
| Hydroxytyrosol | 8.5 ± 0.6 |
| Hydroxytyrosol acetate | 33.4 ± 2.2 |
| 3,4-DHPEA-EDA | 269.3 ± 14.8 |
| 3,4-DHPEA-EA | 28.5 ± 2.5 |
| Total hydroxytyrosol derivatives | 339.8 |
| Tyrosol | 4.4 ± 0.4 |
| *p*-DHPEA-EDA | 11.4 ± 1.2 |
| *p*-DHPEA-EA | 9.9 ± 0.7 |
| Total tyrosol derivates | 25.7 |

3,4-DHPEA-EDA: hydroxytyrosol linked to the dialdehydic form of elenolic acid
3,4-DHPEA-EA: hydroxytyrosol linked to elenolic acid *p*-DHPEA-EDA: tyrosol linked to the dialdehydic form of elenolic acid
*p*-DHPEA-EA: tyrosol linked to elenolic acid

The subjects gave their written informed consent before participation (Ethics Committee for Human Research of the IMIM-Hospital del Mar, Barcelona, Spain, CEIC-PSMAR 2009/3347/I). Freshly voided faecal samples were collected by the volunteers into a sterile pot and brought to the laboratory under anaerobic conditions within 2 h after defecation, immediately mixed in a phosphate buffer (PBS; final concentration 10% of faecal sample) and centrifuged for 5 min at 13000 rpm. The supernatant was divided into aliquots of 500 µL and stored at −20ºC until chromatographic analysis.

**2.3 Metabolite analysis**

**2.3.1 Sample pre-treatment**

Samples of in vitro colonic fermentations and the supernatant, obtained from the faeces of the human intervention study (500 µL), were slowly thawed in ice, transferred into a disposable tube and acidified with 60 µL of hydrochloric acid (37%) to inactivate the microbiota. A liquid–liquid extraction was carried out twice by adding 5 mL of ethyl acetate and centrifuged (10 min, 9000 rpm, at room temperature). The supernatants of both centrifugations were combined and evaporated under nitrogen flow at 30ºC. The residue was reconstituted with 500 µL of water/acetonitrile/methanol (86:12:2, v/v/v), filtered with a 0.22 µm nylon syringe filter and transferred into chromatographic vials to be analysed.

**2.3.2. Chromatographic analysis**

The chromatographic analysis of tyrosol, hydroxytyrosol, hydroxytyrosol acetate and oleuropein was performed with a Waters Acquity Ultra-PerformanceTM LC coupled to MS/MS (UPLC-ESI-MS/MS) system (Waters, Milford, MA, USA), equipped with a binary pump system (Waters). The chromatographic column was an Acquity BEH C18 (100 × 2.1 mm i.d.) with a 1.7 µm particle size (Waters). A binary mobile phase with a gradient elution was used. Eluent A was Milli-Q water/acetic acid (99.8:0.2, v/v) and eluent B was ACN. The gradient was performed as follows: 0–2.10 min, 3% B; 2.10–7 min, 3–7% B; 7–25 min, 7–40% B; 25–25.10 min, 40–100% B; 25.10–27.10 min, 100–3% B; 27.10–30 min, 3% B isocratic. The flow rate was 0.4 mL/min and the injection volume was 2.5 µL. The MS/MS analyses were carried out on a triple quadrupol detector mass spectrometer (Waters) equipped with a Z-spray electrospray interface. The analyses were done in the negative ion mode and the data were acquired with the selected reaction monitoring mode. The MS/MS parameters were as follows: capillary voltage, 3 kV; source temperature, 150 ºC; cone gas flow rate, 80 L/h and desolvation gas flow rate, 800 L/h; desolvation temperature, 400 ºC. Nitrogen (>99% purity) and argon (99% purity) were used as the nebulising and collision gases, respectively. The cone voltages and collision energies were optimised for each analyte by injection of each standard compound in a mixture of acetonitrile/water (50/50, v/v) at a concentration of 10 mg/L. Two transitions were studied for each compound. The most abundant transition was used for quantification, while the second most abundant was used for confirmation purposes. The dwell time established for each transition was 30 ms. Data acquisition was carried out with the MassLynx v 4.1 software.

1,2-Dihydroxybenzene, phenylacetic acid, 4-hydroxybenzoic acid, 2-phenylpropionic acid, 2-(4'-hydroxyphenyl)acetic acid, 2-(3'-hydroxyphenyl)acetic acid, 3,4-dihydroxybenzoic acid, hydroxytyrosol, tyrosol, hydroxy-tyrosol acetate, oleuropein, 2-(3',4' -dihydroxyphenyl) acetic acid, 3-(4'-hydroxyphenyl)propionic acid were quantified by using its own calibration curve. Elenolic acid was quantified using the caffeic acid calibration curve and oleuropein aglycone was quantified using the oleuropein calibration curve.

**2.4. Statistical analysis**

From in vitro study, each sample was analysed in triplicate (three replicates by three volunteers; $n$ = 9) and data are presented as mean values ± standard deviation ($n$ = 9) minus the concentration of the compound in the control (faecal culturemedium without phenolic compound). From the in vivo study, the differences in the concentration of metabolites in human faecal samples between day 0 (before the intake of phenol-rich olive oil) and day 21 (after the intake of phenol-rich olive oil during 3 weeks) were analysed by ANOVA using STAT-GRAPHICS Plus 5.1. The level of significance was $p$ 0.05.

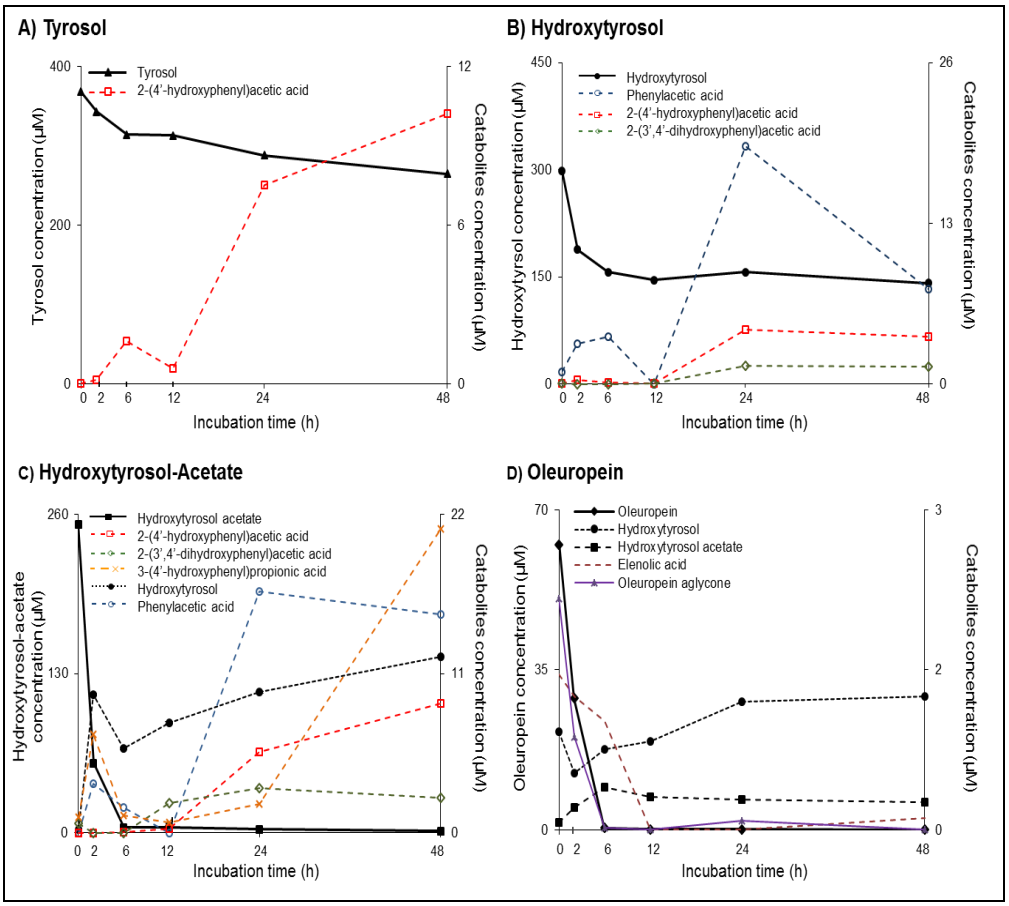
## 3 RESULTS AND DISCUSSION

### 3.1 Kinetics of colonic metabolism in in vitro fermentation

Changes in the precursor phenolic compounds tyrosol, hydroxytyrosol, hydroxytyrosol acetate and oleuropein as well the formation of the microbial metabolites, were monitored during the in vitro colon fermentation (from 0 to 48 h). Three independent fermentations were carried out using three human faecal samples provided by three healthy volunteers (H1, H2 and H3). The results correspond to the average concentration (three

replicates from three volunteers) at each fermentation time (0–48 h). The amounts of the phenolic compounds were corrected for the endogenous levels of the phenolic metabolites detected in the control fermentations of the respective donor faeces (faecal culture medium without phenolic compound; Sections 2 and 2.2).

**Figure 1.** (A) Conversion of tyrosol (solid line) during in vitro colon fermentation and generation of 2-(4''-hydroxyphenyl)acetic acid (dotted line). (B) Conversion of hydroxytyrosol (solid line) during in vitro colon fermentation and generation of phenylacetic acid, 2-(4'-hydroxyphenyl)acetic acid and 2-(3',4''-dihydroxyphenyl)acetic acid (dotted lines). (C) Conversion of hydroxytyrosol acetate (solid line) during in vitro colon fermentation and generation of hydroxytyrosol, phenylacetic acid, 2-(4''-hydroxyphenyl)acetic acid, 2-(3'',4''-dihydroxyphenyl)acetic acid, 3(4''-hydroxyphenyl)-propionic acid (dotted lines). (D) Conversion of oleuropein (solid line) during in vitro colon fermentation and generation of hydroxytyrosol, hydroxytyrosol acetate, elenolic acid and oleuropein aglycone (dotted lines).




To monitor the possible non-microbial degradation of the phenolic compounds during incubation, two controls were included: culture medium with phenolic compound but without faecal inoculums (blank 1), and faecal culture medium with heat-inactivated microbiota incubated with phenolic compound (blank 2). When these two controls were analysed, in blank 1 more degradation of

phenolic compounds was observed than in blank 2. Tyrosol, hydroxytyrosol, hydroxytyrosol acetate and oleuropein showed an important degree of degradation when they were incubated in culture medium (pH 7.0) without faecal inoculums (blank 1) with a practically absent generation of metabolites. This suggests that these phenolic compounds were chemically degraded as a consequence of incubation conditions. Chemical degradation of olive oil phenols was also observed in other studies in digestive mediums at pH 6.5 [22]. However, when olive oil phenols were fermented in a faecal medium with heat-inactivated microbiota (blank 2), the parent compound reached concentrations similar to those in samples at 0 h. These suggest that faecal matrix could protect olive oil phenols from chemical degradation and put in evidence that the metabolism of parent compound is also due to the action of colon microbiota. Kinetic studies of tyrosol, hydroxytyrosol, hydroxytyrosol acetate and oleuropein are presented in **Fig. 1A–D**, respectively (tables with complete data are presented in Supporting Information). The four phenolics studied showed modifications in their concentration during the first period of incubation (0–6 h) being the more unstable hydroxytyrosol acetate (**Fig. 1C**) and oleuropein (**Fig. 1D**). In contrast, tyrosol (**Fig. 1A**) and hydroxytyrosol (**Fig. 1B**) showed a partial degradation until 6 h to remain stables until 48 h of fermentation. Similar stability has also been observed with other low molecular weight phenols, such as gallic acid, after in vitro colonic fermentation by using rat [23] or human [24] faecal inoculums.

**Figure 2** shows the colonic pathways of tyrosol, hydroxytyrosol and hydroxytyrosol acetate. When tyrosol was fermented, 2-(4'-hydroxyphenyl)acetic acid was the only fermentation product detected and its concentration was greater than 10 μM after 48 h of incubation (**Fig. 1A**). On the other hand, more effective catabolism was observed when hydroxytyrosol was incubated (**Fig. 1B**). Three metabolites, 2-(3',4'-dihydroxyphenyl)acetic acid, 2-(4'-hydroxyphenyl)acetic acid (common metabolite obtained from the metabolism of tyrosol) and phenylacetic acid (**Fig. 2**) were identified. Phenylacetic acid was the main end catabolite through the fermentation in all the samples, reaching a peak at 24 h. Low recovery of parent compounds through the initial time of fermentation may be related to their low solubility and/or bond to another compounds presented in the fermentation matrix. Moreover, low recovery of metabolites compared with the rate of degradation of parent compounds hydroxytyrosol and tyrosol could be a caused of chemical degradation, as we observed when we analysed blank 1.

When hydroxytyrosol acetate was fermented, a rapid transformation into hydroxytyrosol was observed (**Fig. 1C**). The metabolism of hydroxytyrosol acetate resulted in a high number of catabolites (**Fig. 2**), with 2-(3',4'-dihydroxyphenyl)acetic acid, 2-(4'-hydroxyphenyl) acetic acid and phenylacetic acid, being common to hydroxytyrosol metabolism. Besides these common metabolites, some others, such as 3-(4'-hydroxyphenyl) propionic acid was only detected in the hydroxytyrosol acetate fermentation medium and reached their maximum concentration at 48 h (**Fig. 1C**). As a general trend, tyrosol, hydroxytyrosol and hydroxytyrosol acetate showed a rise in the production of phenylacetic and phenylpropionic acids and their hydroxylated forms in faecal culture mediums over up to 48 h of fermentation, reaching their maximum concentration between 12 and 48 h. These phenolic acids were also reported after fermentation of other types of flavonoids [23–28].

**Figure 2** shows the common metabolic pathways of the proposed routes for the colonic metabolism of tyrosol, hydroxytyrosol and hydroxytyrosol acetate. The first reaction of catabolism for the three phenolic compounds is probably due to a microbial oxidation of the primary alcohol that transforms the original molecule into hydroxylated phenylacetic acids. There are two microbial enzymes involved in the oxidation of alcohols in the colon, namely alcohol dehydrogenase and aldehyde dehydrogenase [29]. The first one transforms the phenol alcohol into phenylaldehyde while the second one, which has also been reported as a possible pathway of flavonols and flavones [30], transforms, in general terms, hydroxyphenylacetaldehyde and dihydroxyphenyl acetaldehyde into hydroxyphenylacetic acid and dihydroxyphenyl acetic acid, respectively.

In this research, hydroxytyrosol was the main end

product of hydroxytyrosol acetate as a consequence of a first hydrolysis of the molecule (**Fig. 2**), probably due to the action of microbial esterases that cleave the ester bond from the molecule. Despite oscillations, a constant increase in the concentration of hydroxytyrosol over the fermentation time was observed (**Fig. 1C**). Non-substract-specific demethylases have been described in human faeces [31]. These may be involved in the demethylation of hydroxytyrosol acetate to produce 2-(3',4'-dihydroxyphenyl)acetic acid as an intermediate product or they may be produced in parallel by the oxidation of the molecules of hydroxytyrosol yielded by the hydrolysis of hydroxytyrosol acetate, as described above in the in vitro fermentation of hydroxytyrosol. Subsequent dehydroxylations of 2-(3',4'-dihydroxyphenyl)acetic acid caused the appearance of 2-(4'-hydroxyphenyl)acetic and phenylacetic acids (**Fig. 2**). Colon bacteria dihydroxylation is an extended activity in the colon where a wide range of substances undergo the loss of one or more hydroxyl groups [32]. In this research, 3-(3'-hydroxyphenyl)propionic acid and 3-(4'-hydroxyphenyl)propionic acid (**Fig. 2**) were also detected as microbial metabolites of hydroxytyrosol acetate (in the figure is only shown 3-(4'-hydroxyphenylpropionic) acid). However, we were unable to establish the reaction involved in this transformation (**Fig. 2**).

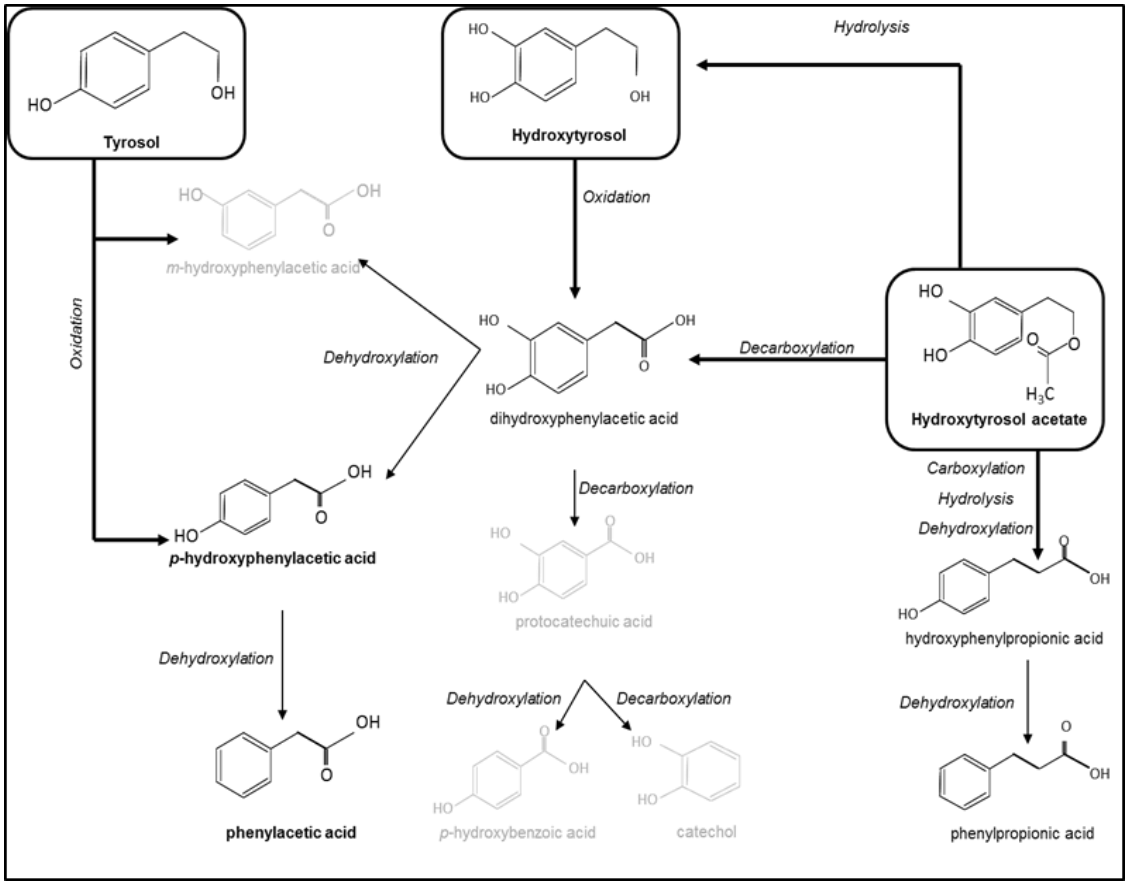



**Figure 2.** Proposed colonic pathway of tyrosol, hydroxytyrosl and hydroxytyrosol acetate. Compounds marked in bold were detected in the fermentation medium between 0 and 48 h. Compounds marked in grey were not detected in the fermentation medium at any time.

The final metabolites, 4'-hydroxybenzoic acid and 1,2-dihydroxybenzene (marked in grey), described in the metabolic fate of tyrosol, hydroxytyrosol and hydroxytyrosol acetate (**Fig. 2**) were not detected during 48 h of fermentation. This may indicate that dihydroxylation is the preferential catabolic route of microorganisms present in human faeces. However, 4-hydroxybenzoic acid appears in urine after flavan-3-ols intake but it was absent from the in vitro fermentation samples of these flavonoids suggesting that this compound is a product of the post absorption of faecal metabolites [27]. 2-(3'-hydroxyphenyl)acetic acid was detected at very low quantities supporting the idea of the sensitivity of the hydroxyl group in third position to be degraded. Alternatively, it may have been rapidly transformed to release phenylacetic acid into the medium, a metabolite absent in tyrosol fermentation (**Fig. 2**). Oleuropein is an ester of hydroxytyrosol and elenolic acid linked to glucose present in the olive fruit. During the virgin olive oil extraction process, this glycoside phenolic is hydrolysed by endogenous glucosidases releasing the aglyconic forms (secoiridoids) and the simple phenols, such as hydroxytyrosol and tyrosol. Oleuropein was degraded significantly during the first period of incubation (**Fig. 1D**). This instability was also observed by Corona et al. when oleuropein was fermented in the presence of human faecal inoculums [9]. **Figure 3** shows the colonic pathway of oleuropein. Fermentation of oleuropein yielded hydroxytyrosol as the main metabolite increasing its concentration progressively during the 48 h of incubation (**Fig. 1D**). Hydroxytyrosol was also detected when oleuropein was fermented in the presence of human faeces together with two other metabolites that could not be identified [9]. In our study, besides hydroxytyrosol, we detected hydroxytyrosol acetate, elenolic acid and oleuropein aglycone (**Fig. 3**) probably formed as consequence of the hydrolytic transformation of oleuropein. Interestingly, hydroxytyrosol acetate that might be produced as a consequence of the oleuropein aglycone hydrolysis, contrary to when it was fermented alone, remained in the medium for the 48 h of incubation. A similar stability was observed when caffeic and chlorogenic acids (caffeic acid linked to quinic acid) were fermented [24].

As it is shown in **Fig. 1D**, oleuropein was rapidly deglycosylated to oleuropein aglycone. This compound was subsequently hydrolysed to elenolic acid and hydroxytyrosol, probably by microbial esterase activity. Previous studies have shown that oleuropein and its aglycone were degraded by the enzyme mentioned below [33].

In our study, elenolic acid and oleuropein aglycone were detected at initial times of incubation at low amounts, decreasing to trace levels at 12 h until there were no negligible amounts at 24 h of incubation (**Fig. 1D**).

A recent study has been reported that bacterial strains of *Lactobacillus*, *Bifidobacterium* and *Enterococcus*, common inhabitant of the human colon, were capable of hydrolysing oleuropein, releasing hydroxytyrosol as the main product. However, only two strains were capable of producing oleuropein aglycone [34]. These results suggest that specific bacteria could be involved in the production of oleuropein aglycone and its rapid degradation by esterase enzymes could justify its low concentration in the oleuropein fermentation medium in our study.

The microbial origin of the aforementioned metabolites was already demonstrated herein by in vitro fermentation. Interestingly, in the in vitro fermentation, 2-(3'-hydroxyphenyl)acetic acid, although it was proposed in the metabolic fate of tyrosol (**Fig. 2**) and hydroxytyrosol (**Fig. 2**), was detected at very low concentrations in the fermentation medium.

Colonic microbial fermentation products from polyphenol-rich foods and other types of dietary ingredients, such as proteins [35], produce a wide range of phenolic acids, especially phenylacetic and phenylpropionic acids. However, specific metabolites may be characteristic of a specific food source or polyphenol class. For example, the production of phenyl-γ-valerolactones and phenyl-valeric acid is typical when flavan-3-ols are fermented [27]. In our study, a highest production of phenylacetic and phenylpropionic acids was found when olive oil polyphenols were fermented. Nevertheless, the presence of hydroxytyrosol and other oleuropein metabolites could be the consumption marker of olive oil phenols in faeces.

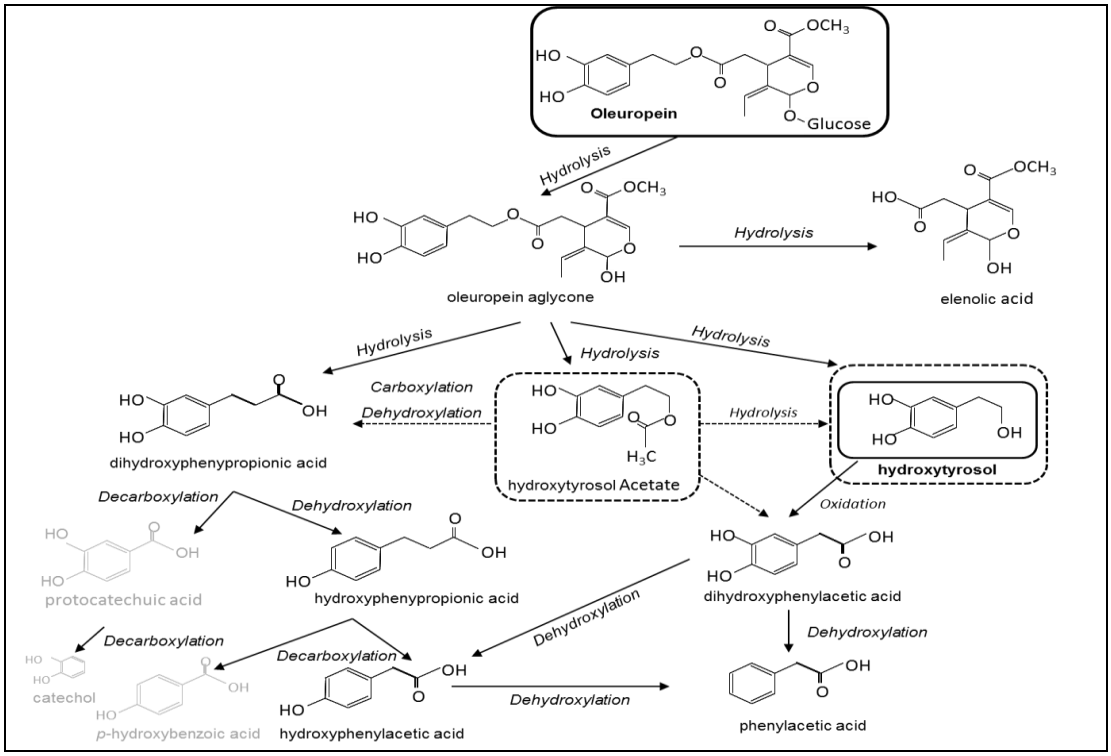



**Figure 3.** Proposed colonic pathway of oleuropein. Compounds marked in bold were detected in the fermentation medium between 0 and 48 h. Compounds marked in grey were not detected in the fermentation medium at any time.

### 3.2 Human intervention study: In vivo model

In this work, an in vitro colon fermentation and an in vivo intervention study were combined. We considered the in vitro experiment to elucidate the main colon metabolites of olive oil polyphenols to facilitate the exploratory analysis of human faecal samples in an in vivo study. The phenolic metabolites in faecal samples from the ten volunteers were determined after the wash-out period (prior to olive oil intake) and after 21 days of moderate intake of 25 mL/day of a phenol-rich olive oil (post-olive oil intake) and the results obtained are shown in **Fig. 4**. After 3 weeks, an increase in the concentration of phenylacetic acid, 2-(4'-hydroxyphenyl)acetic acid, 2-(3'-hydroxyphenyl)acetic acid, 3-(4'-hydroxyphenyl)-propionic acid, and the total phenol content obtained by the sum of all the phenolic metabolites quantified was observed (**Fig. 4A**) but the differences in the samples between the pre- and post-olive oil intakes were not statistically significant. The microbial origin of the aforementioned metabolites was already demonstrated herein by in vitro fermentation. Interestingly, in the in vitro fermentation, 2-(3'-hydroxyphenyl)acetic acid, although it was proposed in the metabolic fate of tyrosol (Fig. 2) and hydroxytyrosol (Fig. 2), was detected at very low concentrations in the fermentation medium. However, an important increase in this metabolite in human faeces was observed after the intake of olive oil (**Fig. 4A**).

To the best of our knowledge, there are no data regarding the effects of olive oil intake on the colonic phenol metabolites in human intervention studies; but the intake of other phenol-rich foods has showed changes in their concentration in faeces. For example, an increase of phenolic acids was observed after the intake of raspberries [36] and orange juice [37], similarly to the results observed in our study. This common catabolism of

different phenolic groups could explain the presence of these phenolic acids in human faeces obtained before the phenol-rich olive oil intake (**Fig. 4A**). It is not possible to ascertain whether or not a small portion of these metabolites were derived from the catabolism of olive oil phenolic compounds. However, the intervention period with the phenol-rich olive oil during 3 weeks resulted in an increase in the concentrations of these phenolic acids in human faeces.

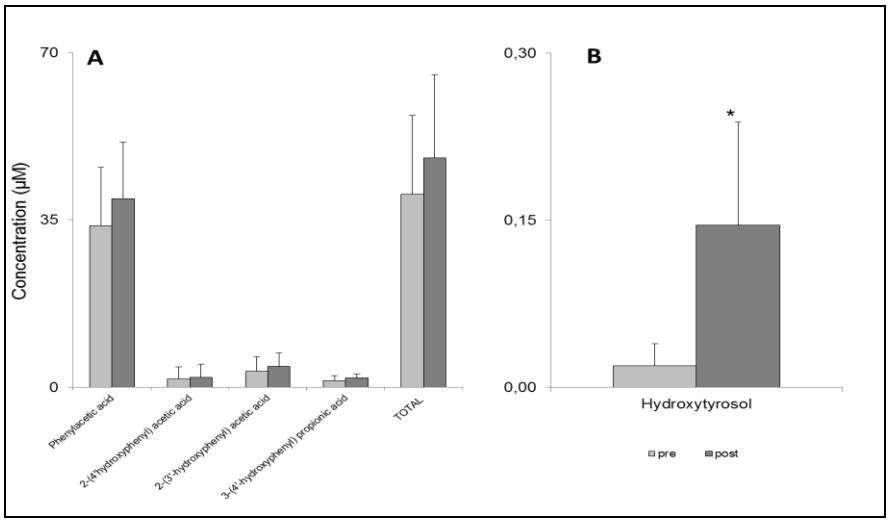



**Figure 4.** (A) Phenylacetic acid: PhelAA, 2-(4'-hydroxyphenyl)acetic acid: 2–4'-OH-PhelAA, 2-(3'-hydroxyphenyl)acetic acid: 2–3'-OH-PhelAA, 3-(4'-hydroxyphenyl)propionic acid: 3–4'-OH-PhelPA and the total; and (B) hydroxytyrosol metabolites in human faecal samples before (pre-) and after (post-) intake of phenol-enriched olive oil. The asterisk (*) indicates the mean value is significantly different from the baseline concentration ($p < 0.05$).

Of special interest is the significant increase in the concentration of free hydroxytyrosol after the intake of phenol-rich olive oil ($p < 0.05$; **Fig. 4B**). Besides low concentrations of free hydroxytyrosol being detected in the faeces of the all participants in the study, the increase in its concentration after 3 weeks of phenol-rich olive oil intake could be used as a biomarker of virgin olive oil consumption (**Fig. 4B**). Therefore, while a rapid absorption occurred in the small intestine [8], and eventual losses could occur during intestinal transit [22], a small proportion of hydroxytyrosol and its derivatives still pass into the large intestine [38]. The presence of low but significant amounts of free hydroxytyrosol in faeces in the present work could be related with the high stability of hydroxytyrosol observed in the in vitro study. In relation to other olive oil phenols, tyrosol and hydroxytyrosol acetate were detected in higher amounts in two and three faecal samples, respectively, while oleuropein was negligible in all the faecal samples analysed (data not shown) after the 3 weeks intervention period. Detection of tyrosol in only two of ten faecal samples may be related with the low quantities of tyrosol and ligstroside aglycone compared with the concentration of hydroxytyrosol and oleuropein derivatives in the phenol-rich olive oil used in the intervention study (**Table 1**).

Oleuropein was not detected in any of the faecal samples probably due to its low concentration in the olive oil and its instability during digestion. As we have observed in the in vitro experiment, oleuropein aglycone and elenolic acid were detected in the fermentation medium during the first 12 h of fermentation at low concentration (**Fig. 1D**), despite the high amount of oleuropein submitted to fermentation. In a recent in vivo

study, the parent compound oleuropein and three metabolites, homovanillic acid, elenolic acid and oleuropein aglycone, were detected in rat faeces collected over 24 h after an acute oral dose of oleuropein [19]. However, in our study, homovanillic acid was not detected in human faeces. This discrepancy could be explained by differences in the gut metabolic responses between rat and human. For example, homovanillic acid has been detected when catechins were fermented with rat faeces as inoculums [21] but has not been found in catechin fermentation with human faeces [26]. The presence of methylated metabolites in plasma after olive oil intake has been noticed before [10], suggesting that homovanillic acid in rat faeces may be a product of the basolateral-apical efflux back into the intestinal lumen or by the enterohepatic recirculation of the absorbed phenolics. The differences in phenolic metabolite types or concentration can reflex inter-individual variation response to polyphenol consumption.

Phenolic luminal compounds might provide additional benefits to the colonic epithelium. The aqueous phase of the colon content, namely faecal water, interacts directly with the colonic epithelium. Consequently, the type of substances solubilised could condition the development of colon diseases. In addition, faecal water has been reported to be a highly variable bioactive material, whose composition could be influenced by the diet, thus modifying its antioxidant power. Current epidemiological and experimental studies support the beneficial role of dietary polyphenols in several gastrointestinal diseases, including intestinal bowel disease (ulcerative colitis and Crohn's disease) and colon cancer. For example, a dietary supplement with virgin olive oil enriched with phenol extract reduced the severity and extent of progressive chronic colonic damage in mice by reducing intestinal inflammatory mediators and cell proliferation [41]. Moreover, there is clear evidence that olive oil intake contributes to a fall in the incidence of colorectal cancer [4]. For example, faecal water activity has been proposed as a risk biomarker of colon cancer [39] because its genotoxicity could be responsible for the DNA cell damage that may contribute to an enhanced risk of colorectal cancer [42, 43]. Fatty acid synthetase expression appears to play an important role in the growth and pathogenesis of colon carcinoma [44]. Notarnicola et al. have reported that hydroxytyrosol suppresses the expression of fatty acid synthetase, as well as its activity, and significantly inhibits cell proliferation through a proapoptotic effect in colon adenocarcinoma HT29 cells in an in vitro assay [45]. Growth inhibition of colon cancer HT29 and SW480 due to the presence in the cytoplasm of oleuropein derivatives and its cell metabolites was noticed by Fernandez-Arroyo et al. when these cells were treated with olive oil polyphenol extracts [46]. These observations, together with our data on the increase of some phenolic metabolites in human faecal samples after the intake of a phenol-rich olive oil, support the view that the prevention of colon disease exerted by olive oil could be ascribed to its content of phenolic compounds.

## 4. CONCLUDING REMARKS

To sum up, the scientific data on beneficial effects of olive oil polyphenols, together with our findings, provide additional information regarding the gut metabolism of polyphenols. The in vitro colon fermentation of the four individual phenols revealed (i) an increase in phenolic acids, (ii) the stability of hydroxytyrosol and tyrosol and (iii) the high degradation of hydroxytyrosol acetate and oleuropein in a faecal culture medium. Additionally, a moderate intake of a phenol-rich olive oil raised the concentration in human faeces of phenylacetic and phenylpropionic acids; although their concentration increase was not statistically significant. Of special interest is the significant increase in the concentration of free hydroxytyrosol after the intake of phenol-rich olive oil ($p < 0.05$). These products of catabolism of olive oil phenolic compounds could be good candidates for novel preventive strategies and opening a promising line of research into the preventive action of olive oil phenols in colon and other bowel diseases. Nevertheless, more studies focused in the investigation of the roll of olive oil phenols in the large intestine are needed to strengthen the knowledge concerning the re-lationship between these compounds and human

intestinal health.

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**ADDITIONAL INFORMATION**

**Table 1.** Metabolites detected during *in vitro* fermentation of hydroxytyrosol. Results are expressed as the mean of the three individual replicates SD for each of the incubation times studied. H1, H2 and H3 correspond to the faeces of the three donors.

| Compound (M) | Faecal sample | Incubation time (h) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 2 | 6 | 12 | 24 | 48 |
| Hydroxytyrosol | H1 | 324±16.4 | 173±11.9 | 149±15.9 | 135±9.67 | 164±12.1 | 144±4.17 |
| | H2 | 248±25.2 | 214±62.2 | 158±22.4 | 166±7.37 | 151±4.53 | 163±0.94 |
| | H3 | 322±53.7 | 177±7.91 | 161±9.97 | 135±21.42 | 154±9.81 | 116±4.94 |
| 2-(3',4'-dihydroxyphenyl) acetic acid | H1 | n.d. | n.d. | n.d. | n.d. | n.d. | 0.60±0.27 |
| | H2 | 0.05±0.07 | n.d. | n.d. | 0.13±0.06 | 2.19±0.06 | 0.21±0.23 |
| | H3 | n.n | n.d. | n.d. | n.d. | 2.25±0.02 | 3.48±0.06 |
| 2-(4'-hydroxyphenyl) acetic acid | H1 | 0.07±0.07 | 0.10±0.2 | n.d. | n.d. | 2.81±4.77 | 6.16±4.13 |
| | H2 | n.d. | 0.80±0.15 | n.d. | 0.06 | 2.48±1.32 | 3.80±2.57 |
| | H3 | 0.02±0.16 | n.d. | 0.38±0.21 | 0.10 | 7.94±2.7 | 1.61±0.9 |
| 2-(3'-hydroxyphenyl) acetic acid | H1 | 0.23±0.07 | 2.11±3.41 | n.d | n.d. | 1.28±0.23 | 0.07±0.79 |
| | H2 | n.d | 1.20±0.09 | 0.16±0.19 | n.d. | n.d | 1.19±0.43 |
| | H3 | 0.32±1.98 | n.d | n.d | 0.47 | 0.91±1.13 | n.d |
| Phenylacetic acid | H1 | 1.17±3.88 | n.d. | 3.29±8.49 | n.d. | 36.6±13.9 | 10.1±9.88 |
| | H2 | 1.64±2.48 | 3.88±1.04 | 4.64±4.69 | n.d. | 11.2±1.71 | 12.9±12.0 |
| | H3 | 0.10±2.18 | 5.95±18.7 | 3.59±5.19 | n.d. | 9.87±19.43 | n.d |
| 4-hydroxybenzoic acid | H1 | 0.19±0.33 | n.d. | n.d. | n.d. | 0.84±0.39 | 0.13±0.03 |
| | H2 | n.d. | n.d. | n.d. | n.d. | n.d | n.d |
| | H3 | n.d. | 2.39±4.13 | n.d. | n.d. | n.d | 0.07±0.23 |
| Protocatecuic acid | H1 | 0.07±0.02 | n.d | n.d. | n.d. | 0.55±0.47 | 0.11±0.35 |
| | H2 | n.d | 0.20±0.06 | n.d. | n.d. | 0.05±0.06 | n.d. |
| | H3 | 0.01±0.04 | n.d | n.d. | 0.07±0.06 | 0.23±0.02 | n.d. |
| Catechol | H1 | 0.01±0.02 | n.d | n.d. | n.d. | 0.240.09 | n.d |
| | H2 | n.d. | 0.02±0.04 | n.d. | n.d. | n.d | 0.08±0.06 |
| | H3 | n.d. | n.d | n.d. | n.d. | 0.03±0.06 | n.d |
| 3-(4'-hydroxyphenyl) propionic acid | H1 | n.d. | n.d | n.d. | n.d. | 1.05±2.53 | 6.13±3.29 |
| | H2 | n.d. | 53.3±17.6 | n.d. | n.d. | n.d. | 129±6.86 |
| | H3 | n.d. | 0.59±2.36 | 2.44±0.88 | 3.25±0.08 | 5.00±10.8 | n.d |
| Phenylpropionic acid | H1 | n.d. | 1.30±9.09 | n.d. | n.d. | 4.07±15.57 | 0.16±9.49 |
| | H2 | n.d. | n.d. | n.d. | n.d. | 10.4±5.66 | 12.7±5.05 |
| | H3 | n.d. | n.d. | 2.01±4.35 | 5.74±3.21 | 5.46±20.8 | n.d |

Data are expressed as mean in µmol/L.
Mean values were calculated subtracting to the average value of the three replicates the control content of the respective substance.

**Table 2.** Metabolites detected during *in vitro* fermentation of hydroxytyrosol acetate. Results are expressed as the mean of the three individual replicates SD for each of the incubation times studied. H1, H2 and H3 correspond to the faeces of the three donors.

| Compound (M) | Faecal sample | Incubation time (h) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 2 | 6 | 12 | 24 | 48 |
| Hydroxytyrosol acetate | H1 | 127±84.7 | 35.2±3.71 | 3.33±2.00 | 2.36±2.72 | 2.56±4.43 | n.d |
| | H2 | 228±98.3 | 122±42.0 | 9.40±2.45 | 6.28±1.08 | 2.53±2.42 | n.d |
| | H3 | 236±66.8 | 13.4±6.39 | n.d | 5.15±3.17 | 3.76±0.98 | 4.13±0.52 |
| Hydroxytyrosol | H1 | 64.8±40.9 | 80.2±10.7 | 64.1±10.6 | 68.1±59.2 | 87.2±13.4 | 152±2.12 |
| | H2 | 61.8±28.1 | 123±25.9 | 64.1±16.8 | 81.8±46.9 | 75.0±12.5 | 123±27.9 |
| | H3 | 204±23.4 | 135±31.9 | 78.6±6.94 | 194±63.6 | 106±14.4 | 157±16.0 |
| 2-(3',4'-dihydroxyphenyl) acetic acid | H1 | n.d. | n.d. | n.d. | n.d | 1.98±0.01 | 0.60±0.16 |
| | H2 | n.d. | n.d | n.d | 4.11±0.64 | 5.31±0.65 | 6.13±0.76 |
| | H3 | 1.99±0.03 | n.d | n.d | 1.98±0.04 | 2.01±0.04 | 0.58±0.08 |
| 2-(4'-hydroxyphenyl) acetic acid | H1 | n.d | n.d. | 0.08±0.05 | n.d. | 10.4±9.95 | n.d |
| | H2 | n.d. | n.d | n.d. | 0.20±0.04 | n.d | 10.7±1.67 |
| | H3 | n.d | n.d | n.d | 0.66±0.21 | 6.28±4.15 | 16.1±1.56 |
| 2-(3'-hydroxyphenyl) acetic acid | H1 | n.d | 0.10±0.68 | 0.89±1.34 | n.d | 0.63±0.11 | 0.11±0.15 |
| | H2 | n.d. | n.d. | 0.21±0.33 | 0.08±0.16 | n.d | n.d |
| | H3 | n.d | 4.26±0.95 | 0.56±0.39 | 0.250.15 | 0.13±0.73 | 0.27±0.52 |
| Phenylacetic acid | H1 | n.d | 10.1±6.99 | 5.26±13.9 | n.d | 50.1±18.09 | n.d |
| | H2 | n.d | n.d | n.d | n.d | n.d | n.d |
| | H3 | n.d | n.d | n.d | n.d | n.d | 45.3±3.47 |
| Protocatechuic acid | H1 | n.d | n.d. | 0.38±0.19 | Tr | 0.41±0.04 | Tr |
| | H2 | n.d | 0.14±0.05 | 0.11±0.04 | 0.13±0.09 | Tr | 0.38±0.18 |
| | H3 | Tr | n.d. | Tr | Tr | Tr | 0.18±0.15 |
| Catechol | H1 | n.d. | n.d | n.d | n.d | Tr | n.d |
| | H2 | n.d | Tr | n.d | n.d | n.d | n.d |
| | H3 | n.d | n.d | n.d | n.d | n.d | n.d |
| 4-hdroxybenzoic acid | H1 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| | H2 | n.d | n.d | n.d. | n.d | n.d | n.d |
| | H3 | n.d | n.d | n.d. | n.d | n.d | n.d |
| 3-(4'-hydroxyphenyl) propionic acid | H1 | n.d | n.d | n.d | n.d | 1.99±0.37 | 21.1±0.94 |
| | H2 | 2.85±2.82 | 20.5±3.88 | n.d. | 0.85±1.38 | n.d. | 13.2±10.0 |
| | H3 | n.d | n.d | n.d | n.d | n.d | 15.1±0.42 |
| Phenylpropionic acid | H1 | n.d | 0.73±4.11 | 6.27±14.3 | n.d | 60.3±12.3 | 6.27±16.4 |
| | H2 | n.d | 3.23±2.1 | n.d | n.d | n.d | 4.51±10.7 |
| | H3 | n.d | n.d | 2.95±1.44 | 0.11±0.99 | n.d | 30.0±3.78 |

Data are expressed as mean in µmol/L.
Mean values were calculated subtracting to the average value of the three replicates the control content of the respective substance.

**Table 3.** Metabolites detected during *in vitro* fermentation of **oleuropein**. Results are expressed as the mean of the three individual replicates. SD for each of the incubation times studied. H1, H2 and H3 correspond to the faeces of the three donors.

| Compound (M) | Faecal Sample | Incubation time (h) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 2 | 6 | 12 | 24 | 48 |
| Oleuropein | H1 | 71.6±0.35 | 43.3±2.88 | 14.7±3.04 | 0.16±0.18 | 0.11±0.05 | Tr |
| | H2 | 53.3±2.54 | 37.4±0.82 | 0.84±0.11 | 0.16±0.08 | 0.20±0.02 | Tr |
| | H3 | 62.3±12.79 | 5.54±5.56 | n.d. | n.d. | Tr | Tr |
| Hydroxytyrosol | H1 | 3.32±0.83 | 11.3±2.78 | 14.6±0.80 | 20.1±1.4 | 33.2±3.21 | 36.7±9.09 |
| | H2 | 36.8±6.88 | 15.8±1.95 | 19.8±2.05 | 25.4±4.98 | 30.5±3.09 | 29.1±6.20 |
| | H3 | 23.65±9.43 | 10.1±0.39 | 15.5±1.47 | 12.6±5.03 | 20.6±1.27 | 21.8±7.95 |
| Hydroxytyrosol acetate | H1 | 3.31±2.92 | n.d | 7.60±0.41 | 5.66±0.58 | 5.77±1.97 | 6.41±0.82 |
| | H2 | n.d. | 6.28±0.52 | 7.60±2.22 | 7.47±1.6 | 6.04±0.67 | 3.56±0.52 |
| | H3 | n.d. | 8.38±1.16 | 11.2±2.65 | 8.66±2.86 | 7.81±1.58 | 8.35±2.45 |
| Oleuropein aglycone | H1 | 0.46±0.10 | 1.35±0.32 | 0.40±0.03 | n.d | 0.25±0.00 | n.d |
| | H2 | 3.26±0.53 | 1.27±0.31 | Tr | n.d | n.d | n.d |
| | H3 | 2.73±0.64 | n.d. | n.d. | n.d. | n.d. | n.d. |
| Elenoic acid | H1 | 1.08±0.02 | 1.20±1.35 | 1.04±0.03 | n.d | n.d | 0.34±0.59 |
| | H2 | 1.68±0.26 | 1.32±0.06 | 1.03±0.01 | n.d | n.d | n.d. |
| | H3 | 1.61±0.10 | 1.24±0.02 | 1.01±0.01 | n.d. | n.d. | n.d. |
| 3-(3',4'-dihydroxyphenyl) propionic acid | H1 | n.d. | n.d. | Tr | 0.38±0.05 | 0.54±0.08 | 1.75±0.6 |
| | H2 | n.d. | Tr | n.d. | 0.10±0.01 | 0.57±0.14 | 0.83±0.27 |
| | H3 | Tr | Tr | 0.79±0.09 | 0.10 | Tr | 0.16±0.15 |
| 3-(4'-hydroxyphenyl) propionic acid | H1 | n.d. | 0.13±0.61 | 0.32±0.38 | 1.77±3.2 | 0.44±0.77 | n.d. |
| | H2 | n.d. | n.d. | n.d. | 0.83±0.15 | 2.24±0.57 | 0.43±0.43 |
| | H3 | n.d. | n.d | n.d | 1.49±2.26 | 0.25±0.39 | 0.77±0.9 |
| Coumaric acid | H1 | 0.11±0.11 | 1.48±0.09 | 2.36±0.28 | n.d | n.d | n.d |
| | H2 | 3.01±1.51 | 3.50±0.26 | 3.77±0.21 | 1.67±0.53 | Tr | n.d |
| | H3 | 1.54±1.02 | 1.26±0.08 | n.d. | n.d. | n.d. | n.d. |
| Protocatechuic acid | H1 | 0.18±0.02 | 0.40±0.08 | 0.44±0.04 | 0.16±0.18 | 0.20±0.02 | 1.17±0.45 |
| | H2 | 0.41±0.23 | 0.33±0.05 | 0.38±0.06 | 0.28±0.11 | 0.26±0.12 | Tr |
| | H3 | 16.8±27.7 | 0.35±0.02 | 0.47±0.05 | 0.27±0.10 | 0.31±0.15 | 0.36±0.19 |
| 2-(3'-hydroxyphenyl) acetic acid | H1 | n.d. | n.d. | 0.10±0.2 | n.d. | 0.73±0.08 | 1.37±0.33 |
| | H2 | 0.34±0.50 | n.d. | 0.42±0.23 | n.d. | n.d. | n.d. |
| | H3 | n.d. | n.d. | n.d. | 0.68±1.31 | 0.40±1.21 | 1.98±2.26 |
| 4-hydroxybenzoic acid | H1 | n.d. | n.d. | n.d. | n.d. | n.d. | Tr |
| | H2 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| | H3 | n.d. | n.d. | n.d. | Tr | n.d. | Tr |

Data are expressed as mean in µmol/L.

Mean values were calculated subtracting to the average value of the three replicates the control content of the respective substance

**Table 4. M**etabolites detected during *in vitro* fermentation of tyrosol. Results are expressed as the mean of the three individual replicates. SD for each of the incubation times studied. H1, H2 and H3 correspond to the faeces of the three donors.

| C'ompound (M) | Fecal sample | Incubation time (h) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 2 | 6 | 12 | 24 | 48 |
| Tyrosol | H1 | 337±28.1 | 309±8.39 | 288±20.3 | 279±19.9 | 274±0.25 | 255±3.52 |
| | H2 | 332±44.4 | 308±9.59 | 314±27.1 | 287±23.7 | 234.9861.39 | 210.457.93 |
| | H3 | 435±9.07 | 413±16.2 | 351±65.8 | 365±8.26 | 351±5.06 | 329±19.9 |
| Hydroxytyrosol | H1 | 0.90±0.04 | 0.93±0.04 | 1.01±0.05 | 1.02±0.01 | 0.89±0.19 | 0.72±0.0 |
| | H2 | 0.82±0.02 | 0.85±0.03 | 1.03±0.06 | 1.03±0.06 | 0.82±0.07 | 0.76±0.01 |
| | H3 | 1.22±0.13 | 1.29±0.05 | 1.26±0.07 | 1.40±0.02 | 1.39±0.06 | 1.09±0.03 |
| 2-(4'-hydroxyphenyl) acetic acid | H1 | n.d | 0.01±0.2 | 4.13±0.24 | 1.40±0.34 | 20.6±4.19 | 16.1±1.10 |
| | H2 | n.d | n.d | n.d | n.d | 1.99±5.78 | 0.23±2.60 |
| | H3 | n.d | 0.47±0.18 | 0.71±0.52 | 0.40±2.30 | n.d | 14.4±1.80 |

Data are expressed as mean in µmol/L.

Mean values were calculated subtracting to the average value of the three replicates the control content of the respective substance

**PUBLICATION II** Study of the catabolism of thyme phenols combining in vitro fermentation and human intervention

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JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

# STUDY OF THE CATABOLISM OF THYME PHENOLS COMBINING IN VITRO FERMENTATION AND HUMAN INTERVENTION

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ABSTRACT: The gut metabolism of four thyme phenolics (monoterpenes thymol and carvacrol, rosmarinic acid, and eriodictyol) was evaluated in vitro. After the in vitro transformations of the individual phenols had been studied, the presence of their microbial metabolites was investigated in human feces collected before and after a sustained intake (3 weeks) of 25 mL/day of a thyme phenol-enriched olive oil. Results of in vitro fermentation showed low degradation of thymol and carvacrol. By contrast, large catabolism was noted when rosmarinic acid and eriodictyol were fermented, yielding hydroxyphenylpropionic acid as the main metabolite. In accordance with these results, after the in vivo intervention with thyme phenol-enriched olive oil, an increase in the concentration of hydroxyphenylpropionic and phenylpropionic acids was observed in human feces, confirming the effective in vivo microbial degradation of rosmarinic acid and eriodictyol. Carvacrol was detected in fecal samples at trace levels, suggesting that monoterpenes are well absorbed in the upper part of the gastrointestinal tract.

KEYWORDS: colonic metabolites, in vitro colonic fermentation, thyme phenols, thyme

## 1. INTRODUCTION

The consumption of aromatic herbs is increasing worldwide because of their particular characteristics, highly valued by the food and pharmaceutical industries. They are commonly used in cooking as flavoring agents and for their antifungal, antimicrobial, and antioxidant properties, capable of delaying undesirables changes in food,[1,2] with the added advantage of being positively perceived by consumers due to their "natural" origin. Furthermore, their consumption is associated with anti-inflammatory, anti-hypertensive, and anti-cancer properties.[3−5] Thyme (*Thymus vulgaris*) is one of the most used aromatic herbs, particularly in the Mediterranean countries. Its beneficial effects have been attributed to a wide range of chemical compounds such as phenolic compounds with terpenoid origin (thymol and carvacrol), flavonoids (quercetin, eriocitrin, luteolin, eriodictyol, and apigenin), and phenolic acids (rosmarinic acid and caffeic acid).[6] The bioavailability of phenolic compounds has been extensively studied, particularly focused on their absorption, distribution, and deposition in organs and tissues, plasma concentration, and urine excretion.[7,8] Because a large portion of phenolic compounds are not absorbed in the small intestine, recent studies are more focused on the polyphenol−gut microbiota interactions and the gut microbial bioconversion capability, as it has a substantial effect on the phenolic compounds'

bioavailability. Local microbiota converts the original compounds in the unabsorbed fraction of the digesta to simple phenols with similar or even greater bioactivity than their precursors.[9] These changes in the metabolome may have great implications for the gut health[10] and, if absorbed, beneficial effects throughout the body.[11]

In vitro fermentation models, using animal or human feces as inocula, have been carried out in previous studies to assess the gut metabolism of different types of phenolic compounds.[12,13]

However, these in vitro experiments cannot reproduce some phenomena that take place in in vivo conditions, such as the enterohepatic recirculation (for compounds that enter the enterohepatic cycle) or the water and nutrient absorption. Therefore, a multidisciplinary approach that combines well-established in vitro and in vivo studies is essential for understanding the nature and extent of the different metabolic processes of food bioactive compounds. This approach may be very useful to predict which compounds could reach the molecular targets and exert health effects.

There is limited information about human interventions with aromatic herbs or their bioactive compounds in the literature. Most of the in vivo studies related to monoterpenes have been carried out in the veterinary area due to the promising overall efficacy of aromatic herbs for the benefit of animals and the quality of animal-derived products. Some authors have even proposed these naturally occurring substances as substitutes for antibiotic growth promoters.[14] In a preliminary study conducted by our group, the bioaccessibility and phase II metabolism of thyme phenols were assessed by an in vitro digestion model combined with Caco-2 and HepG-2 cell models.[15] The results of this study showed different metabolic profiles depending on the phenolic compound. Whereas eriodictyol appeared in both its sulfated and glucuronidated forms after cell incubation, naringenin was only conjugated into its glucuronide form. To complement these studies, it becomes essential to study the microbial conversion of thyme phenolic compounds and the elucidation of their colonic pathways. In this sense, the main aim of the present study was to provide more complete, comprehensive, and convincing results about the gut metabolism of thyme phenolic compounds by using a combination of in vitro and in vivo experiments. For the in vitro fermentation study, the most representative compounds from different subclasses of thyme phenols were selected: (i) the monoterpene thymol and its isomer carvacrol; (ii) the flavonoid eriodictyol; and (iii) rosmarinic acid, a phenolic acid occurring in many common aromatic herbs from the Lamiaceae group. After studying the in vitro gut metabolism pathways of the aforementioned thyme phenols, we investigated their colonic metabolites in human fecal samples obtained before and after a 3 week intervention with a daily administered dose of 25 mL (23 g) of a thyme phenol-enriched olive oil (TPEOO).

## 2. MATERIALS AND METHODS

### 2.1. In Vitro Colonic Fermentation

Fecal samples were collected from three healthy volunteers who followed normal dietary habits and declared that they had not taken antibiotics for at least 3 months prior to the sample collection. The fermentation medium was carbonate− phosphate buffer. This was prepared according to a previously described method[16] by mixing (all in g/L) 9.240 $NaHCO_3$, 3.542 $Na_2HPO_4{\cdot}2H_2O$, 0.470 NaCl, 0.450 KCl, 0.227 $Na_2SO_4{\cdot}10H_2O$, 0.055 $CaCl_2$ (anhydrous), 0.100 $MgCl_2{\cdot}6H_2O$, and 0.400 urea with 10 mL/L of added trace element solution (in mg/L) 3680 $FeSO_4{\cdot}7H_2O$, 1159 $MnSO_4{\cdot}H_2O$, 440 $ZnSO_4{\cdot}7H_2O$, 120 $CoCl_2{\cdot}6H_2O$, 98 $CuSO_4{\cdot}5H_2O$, and 17.4 $Mo_7(NH_4)_6O_24{\cdot}4H_2O$). Before its use, the culture medium was maintained in an anaerobic chamber for 48 h, using gas generator bags ($CO_2$) (Becton Dickinson, Sparks, MD, USA) to remove the oxygen. The anaerobic conditions were continuously monitored by using anaerobic indicator strips (Becton Dickinson). Standards of thymol, rosmarinic acid, and eriodictyol were purchased from Extrasynthese (Genay, France), whereas carvacrol was purchased from Sigma-Aldrich (St. Louis, MO, USA). For the selection of the phenolic compounds to carry out the in vitro experiments, we took into consideration the availability of commercial standards and the

occurrence of these substances in the TPEOO.

The in vitro fermentation procedure used in this study was adapted from the method proposed by Serra and co-workers.[17] For each volunteer, 15 g of fresh feces was homogenized for 60 s in a stomacher with 300 mL of prereduced culture medium to obtain a 5% fecal slurry. Following the homogenization, the fecal slurry was filtered and left to stand for 30 min. Once done, 10 mL was taken and added to 15 mL disposable tubes. After the addition of the standard (phenol standard final concentration 500 μM for thymol, carvacrol, and eriodictyiol, and 200 μM for rosmarinic acid), the disposable tubes were incubated in anaerobic chambers at 37 °C in an orbital shaker (60 strokes/min) to mimic colon conditions (anaerobic conditions were controlled by anaerobic indicator strips).

**Table 1**. Phenolic Composition of Thyme Phenol-Enriched Olive Oil (TPEOO)[a]

| Compound | mg phenol/kg oil | |
|---|---|---|
| Rosmarinic acid | 16.3 | ±1.2 |
| Flavonoids | | |
| Eriodictyol | 6.9 | ±0.5 |
| Luteolin | 8.5 | ±0.8 |
| Apigenin | 3.8 | ±0.2 |
| Naringenin | 7.9 | ±0.8 |
| Thymusin | 48.6 | ±3.6 |
| Xanthomicrol | 21.3 | ±2.2 |
| 7-methylsudachitin | 21.1 | ±3.4 |
| Monoterpenes | | |
| Thymol | 25.4 | ±2.1 |
| Carvacrol | 9.1 | ±0.8 |

[a]Values are means ± SD (n = 5)

The high initial concentrations (500 and 200 μM) of the parent compounds were chosen to determine more clearly the microbial metabolites generated, even at relatively low concentrations. Aliquots of the fermented fecal samples (500 μL) were collected at 0, 2, 6, 12, 24, and 48 h of incubation and stored immediately at −80 °C until the chromatographic analysis. All of the fermentation samples were incubated in triplicate. In parallel, a control sample (fecal slurry without addition of phenolic standard) was prepared for each fermentation time. Also, the phenolic standards without feces (blank 1) were incubated at each time (0, 2, 6, 12, 24, and 48 h) to take into account the possible chemical degradation of phenolic compounds.

### 2.2. Human Intervention Study

Fecal samples from 10 volunteers were used to determine thyme phenolic metabolites identified in the in vitro fermentation model. The feces were obtained before and after the consumption (25 mL/day) of a thyme phenol-enriched olive oil (TPEOO) over a period of 3 weeks. The TPEOO was prepared by adding a thyme phenolic extract to an olive oil according to the methodology described by Rubió and co-workers.[18] The main phenolic compounds that were transferred from thyme to the olive oil were flavonoids, such as eriodictyol, thymusin, naringenin, and xanthomicrol, phenolic acids, such as rosmarinic acid, and the monoterpenes thymol and carvacrol (**Table 1**).

Prior to the TPEOO intervention, there was a 2 week wash-out period, in which common olive oil (a blend of refined olive oil and a small percentage of virgin olive oil) was the main fat consumed. To avoid an excessive intake of phenolic compounds during the intervention period, the participants were advised to limit the intake of phenol-rich foods. Fecal samples from each volunteer were collected after the wash-out period (pre TPEOO intake) and at the end of the 3 week intervention (post TPEOO intake). The subjects gave their written informed consent before participation (Ethics Committee for Human Research of the IMIM-Hospital del Mar, Barcelona, Spain; CEIC-PSMAR 2009/3347/I). Freshly voided fecal samples were collected by the volunteers into a sterile pot and brought to the laboratory under anaerobic conditions within 2 h of defecation, immediately mixed in a phosphate buffer (PBS; final concentration 10% of fecal sample), and centrifuged for 5 min at 13000 rpm. The supernatant was divided into aliquots of 500 μL and stored at −20 °C until chromatographic analysis of phenol metabolites.

### 2.3. Treatment of Fecal Samples and Chromatographic Analysis of Thyme Metabolites

The in vitro fermentation samples and the supernatants obtained from the human intervention study were slowly thawed in ice, transferred into a disposable tube (15 mL), and

acidified with 60 µL of HCl to inactivate the microbiota. A liquid−liquid extraction was carried out by adding 5 mL of ethyl acetate, and after shaking for 10 min in a vortex, samples were centrifuged (10 min, 9000 rpm, at room temperature). The supernatants resulting from two ethyl acetate extractions were combined and evaporated under nitrogen flow at 30 °C. The dry residue was reconstituted with water/acetonitrile/methanol (86:12:2, v/v/v), filtered with a 0.22 µm nylon syringe filter, and transferred into chromatographic vials for analysis. The chromatographic analysis of rosmarinic acid, eriodictyol, and their metabolites and thymol and carvacrol metabolites was performed with a Waters Acquity Ultra-Performance liquid chromatography (UPLC-ESI-MS/MS) system (Waters, Milford MA, USA), according to the previously described method.[19] The tandem mass spectrometry (MS/MS) analyses were carried out on a triple-quadrupole detector (TQD) mass spectrometer (Waters) equipped with a Z-spray electrospray interface. The analyses were done in the negative ion mode, and the data were acquired with the selected reaction monitoring mode (SRM). The MS/MS parameters were as follows: capillary voltage, 3 kV; source temperature, 150 °C; cone gas flow rate, 80 L/h; desolvation gas flow rate, 800 L/h; desolvation temperature, 400 °C. Nitrogen (>99% purity) and argon (99% purity) were used as the nebulizing and collision gases, respectively. The cone voltages and collision energies were optimized for each analyte by injection of each standard compound in a mixture of acetonitrile/water (50:50, v/v) at a concentration of 10 mg/L. Two transitions were studied for each compound. The most abundant transition was used for quantification, whereas the second most abundant was used for confirmation purposes. The dwell time established for each transition was 30 ms. Data acquisition was carried out with MassLynx v 4.1 software. Catechol, phenylacetic acid, 4-hydroxybenzoic acid, 3-phenylpropionic acid, 2-(4′-hydroxyphenyl)acetic acid, 2-(3′-hydroxyphenyl)acetic acid, 4-coumaric acid, protocatechuic acid, 2-(3′,4′-dihydroxyphenyl) acetic acid, 3-(4′-hydroxyphenyl)propionic acid, 3-(3′,4′-dihydroxyphenyl)-propionic, caffeic acid, rosmarinic acid, and eriodictyol were quantified using the calibration curves of their respective standards.

Additionally, all samples were subjected to gas chromatography (GC) analyses to identify and quantify the volatile monoterpene thymol and carvacrol in their native forms. These compounds were first extracted from the in vitro fermentation samples and from the human fecal supernatants to the TPEOO intervention by hexane. For this purpose, 500 µL of sample was mixed with 1 mL of hexane and shaken during 1 h and then centrifuged for 10 min at 9000 rpm at room temperature. The volatile material collected from the super-natant was analyzed by gas chromatography (GC) (Agilent 7890A series) coupled to a flame ionization detector (FID) using a capillary column TRB-5SM (Teknocroma, Barcelona, Spain). The column temperature was programmed at 90 °C for 1 min, increased by 15 °C/ min to 270 °C, and then maintained at 270 °C for 10 min (total run time of 25 min). Helium was the carrier gas (1 mL/min). Injector temperature was 250 °C, the detector temperature was 280 °C, and 1 µL of the solution was injected into the GC-FID system. Thymol and carvacrol were identified by comparing the retention times to those for the authentic standards and quantified by using the calibration curve of the respective standard.

### 2.4. Statistical Analysis

Each sample from the in vitro experiment was analyzed in triplicate (three replicates from three volunteers; n = 9), and the data are presented as the mean values of all samples at each time (n = 9 per each time) minus the concentration of the compound in the control (fecal culture medium without phenolic compound). In the in vivo study, to compare the baseline with the post-treatment concentration values of the metabolites in human fecal samples, the ANOVA analysis was employed using STAT-GRAPHICS Plus 5.1 software (Manugistics Inc., Rockville, MD, USA). The level of significance was 0.05.

## 3. RESULTS AND DISCUSSION

### 3.1. Kinetics of Thyme Phenols. Colonic Metabolism in in Vitro Fermentation.

Changes in the precursor compounds (thymol, carvacrol, rosmarinic acid, and eriodictyiol) during the in vitro fermentation were monitored from 0 to 48 h. Results are reported as the average of three independent fermentations for each human fecal sample, obtaining nine replicates for each fermentation time (individual values are shown in **Tables 1−4** of the **Supporting Information**). The amounts of the phenolic compounds and their metabolites were corrected for the endogenous levels detected in the control fermentations (fecal culture medium without phenolic compound)

### 3.2. Monoterpenes: Thymol and Carvacrol.

On the basis of the volatile nature of thymol and carvacrol, these compounds were analyzed by GC, whereas colonic metabolites formed during in vitro fermentation were analyzed by liquid chromatography (UPLC-MS/MS). The kinetics of the generated metabolites after thymol and carvacrol fermentation is shown in **Figure 1**, panels A and B, respectively. In general, carvacrol and thymol showed a similar behavior during the 48 h incubation, possibly due to the closely related chemical structure. Both compounds were poorly degraded and remained relatively stable until the end-time of the fermentation, indicating the nonexistent or low activity of gut microbiota in terms of monoterpene utilization. However, some metabolites were formed during the in vitro fermentation of thymol (**Figure 1A**): (i) phenylacetic acid, which reached its maximum concentration (16.91 μM) at 24 h of incubation; (ii) 2-(4′-hydroxyphenyl)acetic acid with a maximum recovery (15.39 μM) also at 24 h of incubation. Furthermore, (iii) phenylpropionic acid and (iv) 3-(4′-hydroxyphenyl)propionic acid reached the maximum concentration at 48 h (11.20 μM) and at 24 h (5.14 μM) of incubation, respectively. Interestingly, low concentrations of carvacrol were found at different times during the thymol fermentation (**Supporting Information Table 1**) but were also detected in thymol standard samples incubated without fecal slurry (blank 1), which may indicate that carvacrol is generated by the medium conditions as a consequence of the isomerization of thymol.

Similar results were observed when carvacrol was fermented. During the fermentation the main metabolites that appeared were (i) phenylpropionic acid (10.13 μM) and (ii) phenylacetic acid (7.43 μM), both by the end of fermentation. After the fermentation of carvacrol, (iii) 2-(4′-hydroxyphenyl)acetic acid and (iv) 3-(4′-hydroxyphenyl)propionic acid wer also detected. Both phenolic acids reached their maximum concentrations (2.78 and 2.77 μM, respectively) at 48 h of fermentation.

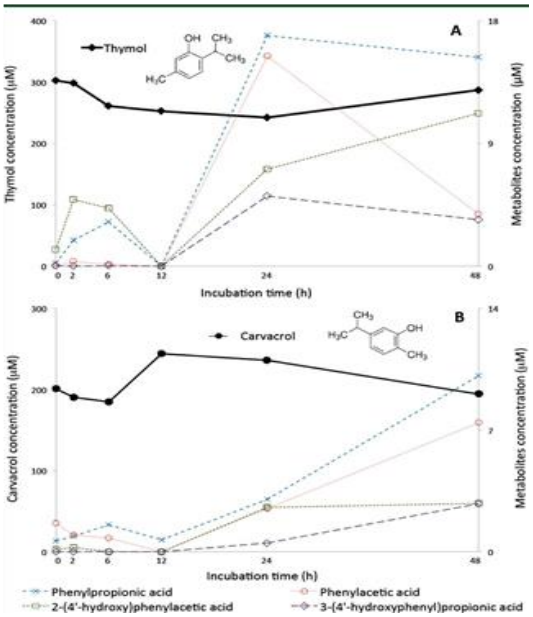


**Figure 1**. (A) Thymol concentration (solid line) and metabolite generation (dotted line) during in vitro fermentation. (B) Carvacrol concentration (solid line) and metabolite generation (dotted line) during in vitro fermentation

In vitro fermentation of monoterpenes using animal intestinal content as inocula also showed low microbial activity.[20,21] Michiels and co-workers have reported that thymol and carvacrol were degraded by 29% after the fermentation of both compounds using piglet cecal as inocula, but no degradation products were specified.[20] Varel and co-workers also observed high stability of thymol and carvacrol after anoxic swine waste fermentation, recovering close to 95% of both monoterpenes at 62 days.[21] Therefore, our results confirm that phenol terpenes remain stable during the fermentation, probably due to the complex carbon skeleton that may hamper their microbial

degradation. To the best of our knowledge, there are no data available in the literature about thymol and carvacrol metabolism in human bowel. However, cholesterol and bile acids, which also have a terpenoid lipid structure, are daily exposed to gut microbiota, and their bacterial transformations have been extensively documented in humans.

The reduction of the C7 double bond of the cholesterol molecule,[22] the 7α-dehydroxylation, and the side chain degradation of functional groups in the case of bile acids are typically caused by bacteria activity, but in any case cholesterol, bile acids, and their metabolites suffer further degradation.[23] Similarly, our results suggest that thymol and carvacrol (phenolic terpenes) may be recalcitrant molecules to biodegradation in the human large intestine, probably as a consequence of the low number of functional groups and/or the lack of intestinal bacteria specialized in their metabolism. Therefore, the appearance of phenolic acids, especially within 24 h of incubation (**Figure 1**), may be explained by the loss of bacteria cell integrity due to the modification of membrane permeability, which could induce the release of endogenous metabolites into the culture medium. Linked to this, Thapa and co-workers[1] studied the effects of different essential oils and their pure compounds, including thymol, on pathogenic and commensal intestinal bacteria. The results showed that phenolic monoterpenes were the most active class of antibacterial agents and caused a dose-dependent loss of cell integrity in Gram-positive bacteria, probably by modifying the permeability of cellular membranes, thus promoting the loss of ATP and intracellular metabolites or enzymes.

Therefore, in our study the release of endogenous microbial material could explain the presence of different phenylacetic and phenylpropionic acids in the thyme and carvacrol fermentation media.

### 3.3. Rosmarinic Acid and Eriodictyol

Analyses of the fermentation samples of rosmarinic acid and eriodictyol revealed a full degradation of the parent compounds and a high generation of microbial products over the incubation time (0−48 h). The kinetics of the rosmarinic acid fermentation showed that this compound was insignificant at 12 h of incubation (**Figure 2A**). The first intermediate microbial metabolite derived from the hydrolysis of rosmarinic acid was caffeic acid, which reached its maximum concentration at 6 h (17.9 μM) (**Figure 2A**) before declining to trace levels at 48 h of fermentation. The gut metabolic pathway of rosmarinic acid is proposed in **Figure 3**. A previous study has also revealed a rapid transformation of rosmarinic acid when it was fermented in pure cultures of *Lactobacillus johnsonii,*[24] a type of lactic acid bacteria (LAB) commonly found in the human gut.[25] Degradation of rosmarinic acid seems to be mediated by microbial chlorogenate esterases.[24]

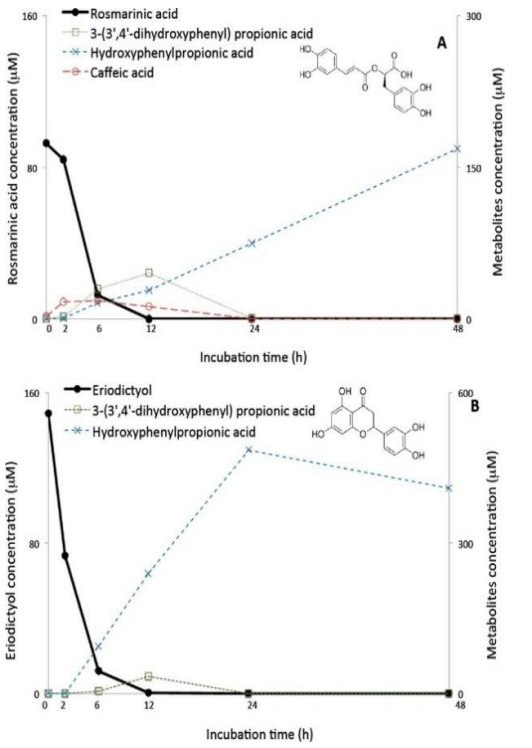


**Figure 2**. (A) Rosmarinic acid concentration (solid line) and metabolite generation (dotted line) during in vitro fermentation. (B) Eriodictyol concentration (solid line) and metabolite generation (dotted line) during in vitro fermentation

The second intermediate metabolite detected in the fecal culture medium was 3-(3′,4′-dihydroxyphenyl)propionic acid, probably formed

as a result of the microbial transformation of caffeic acid (**Figure 3**) by the reduction of the aliphatic double bond of the second ring of the molecule. 3-(3′,4′-dihydroxyphenyl)propionic acid reached its highest peak of concentration at 12 h (24.2 μM) coinciding with the decrease in the concentration of caffeic acid in the fermentation medium (**Figure 2A**). An isomer of 3-(4′-hydroxypehyl)propionic acid was identified as the main metabolite formed by the fermentation of rosmarinic acid, and this reached the highest concentration in the medium (168.2 μM) at 48 h of fermentation, parallel to the decrease in the concentration of its precursor, 3-(3′,4′-dihydroxyphenyl)propionic acid. The generation of hydroxyphenylpropionic acid probably came from the 4 dehydroxylation of 3-(3′,4′-dihydroxyphenyl) propionic acid, but we were not able to confirm this information. Caffeic acid is a hydroxycinammic acid widely present in foods, in both free and conjugated forms (normally with quinic acid to form chlorogenic acid). Microbial fermentation of free or conjugated caffeic acid has been studied, and our findings seem to be in line with previous works in which 3-(3′-hydroxyphenyl) propionic acid was recovered as the main bacteria metabolite.

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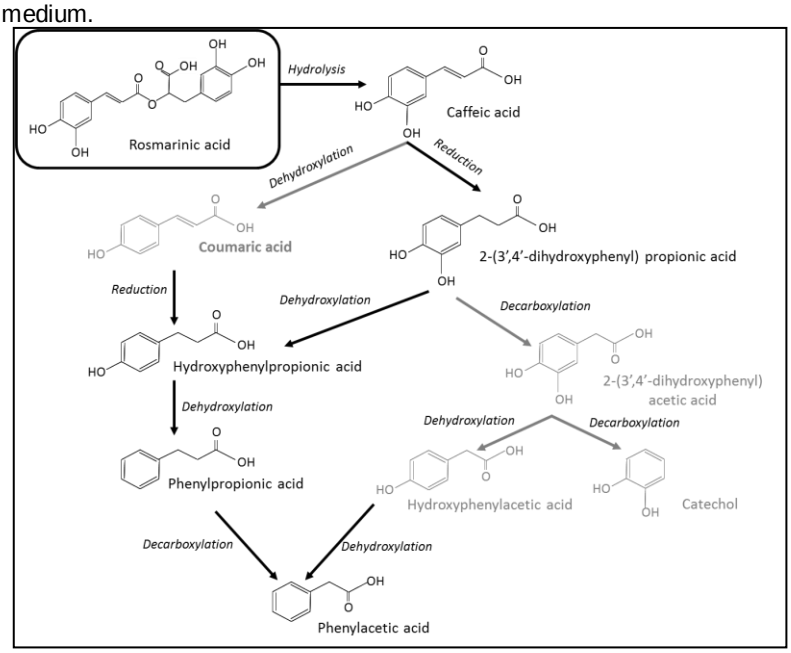
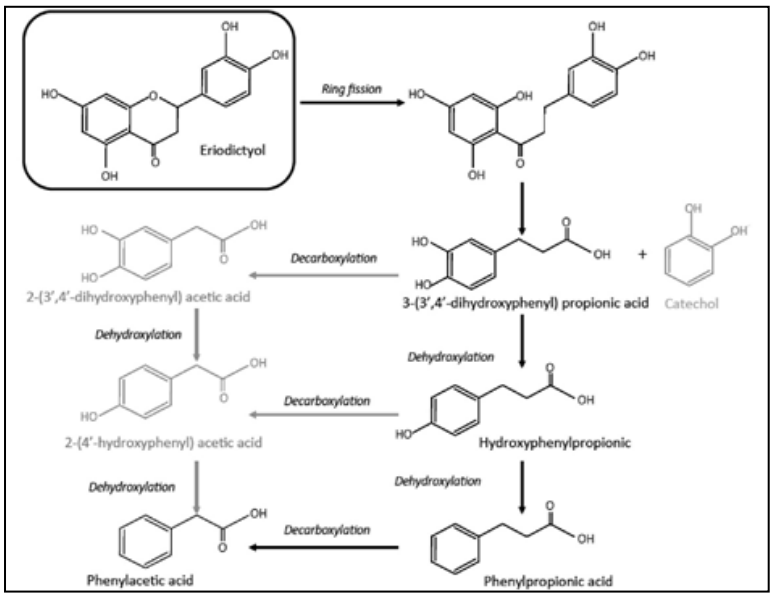
**Figure 3.** Proposed colonic pathway of rosmarinic acid. Compounds in bold were detected in the fermentation medium between 0 and 48 h. Compounds in gray were detected as minor metabolites in the fermentation medium.




Chemically, rosmarinic acid is an ester of caffeic acid and 3-(3′,4′-dihydroxyphenyl)lactic acid. As a consequence of its hydrolysis, two molecules are released into the medium, but both yield hydroxyphenylpropionic acid as the final metabolite, which could explain the high molar ratio between the parent compound and its formed metabolite. In the proposed rosmarinic acid colonic pathway (**Figure 3**), other metabolites such as the no hydroxylated form of phenylpropionic acid, coumaric acid, benzoic acid, and catechol have been described as other possible final metabolites. However, the low and variable amounts of these metabolites detected

during the fermentation suggest that they could be attributed to other food precursors, as was reported after an in vitro fermentation of coffee polyphenols.[13] With regard to the colonic metabolism of eriodictyol, the kinetic response of the generated microbial metabolites is shown in **Figure 2B**. Complete degradation of eriodictyiol before 48 h of fermentation has been seen in all of the samples analyzed, indicating high microbial activity. Total degradation of eriodictyol has also been reported in previous works using *Eubacterium ramulus*[26] and *Clostridium orbiscindens*[27] as inocula. On the basis of the different metabolic compounds detected at different collection times, we proposed a hypothetical colon degradation pathway for eriodictyol (**Figure 4**). Heterocyclic C-ring cleavage of eriodictyol was the first evidence of microbial action, yielding 3-(3′,4′-dihydroxyphenyl) propionic acid as an intermediate metabolite probably derived from the B-ring fragment. 3-(3′,4′-dihydroxyphenyl)propionic acid reached its maximum concentration (12.9 μM) at 12 h and declined thereafter (**Figure 2C**). Dehydroxylation of 3-(3′,4′-dihydroxyphenyl)propionic acid produced an isomer of hydroxyphenylpropionic acid, which represents the main phenolic end product. There was a progressive increase in the concentration of hydroxyphenylpropionic acid over the time course of fermentation with the maximum peak of concentration at 24 h (344.4 μM). These results are in line with those reported by other authors, who also detected 3-(3′,4′-dihydroxyphenyl)propionic acid as a fermentation product of eriodictyol.[26,27] However, no hydroxyphenylpropionic acid was described, probably because the experiments were carried out with two types of bacteria, *E. ramulus* and *Cl. orbiscinders,* that may not have dehydroxylation activity.

**Figure 4.** Proposed colonic pathway of eriodictyol. Compounds in bold were detected in the fermentation medium between 0 and 48 h. Compounds in gray were detected as minor metabolites in the fermentation medium

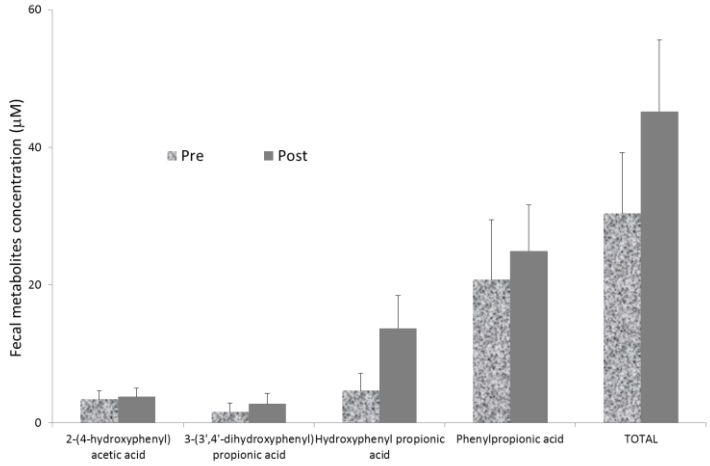



### 3.4. Human Intervention Study.

The impact of human intestinal microbiota on the transformation of thyme phenols was investigated in 10 volunteers after the daily consumption of 25

mL of TPEOO over 3 weeks. The feces of the volunteers were analyzed at baseline and after the intervention period to elucidate eventual changes in the profile of the microbial metabolites. Although we observed that thymol and carvacrol were stable over 48 h during the in vitro incubation, only carvacrol was quantified in feces from one of the volunteers after the intake of TPEOO (data not shown). Nevertheless, an increment in the instrumental signal corresponding to the chromatographic peak of carvacrol (Treatment of Fecal Samples and Chromatographic Analysis of Thyme Metabolites under Material and Methods) was observed in 7 of the 10 fecal samples analyzed, although the carvacrol concentration was below to the limit of quantification (data not shown). This is in line with Anderson and co-workers, who failed to detect the aforementioned monoterpenes in pig feces after the intake of a thyme-rich diet.[28]

**Figure 5**. Phenolic metabolites detected in human fecal samples before (pre-) and after (post-) intake of thyme phenol-enriched olive oil (TPEOO).




In a similar study, only <10% of limonele was detected in the feces of animals (dog and guinea pig) after a high dose, whereas a high recovery was observed in urine.[29] In a preliminary human intervention study conducted by our research group in which hyperlipidemic adults consumed thyme-enriched olive oil, a high recovery of thymol metabolites (main glucuronide conjugates) in 24 h urine was observed.[30] The findings of the present survey, together with the previous reported data regarding the urinary excretion of mono-terpenes,[18] support the hypothesis that phenol monoterpenes are effectively absorbed in the upper part of the gastrointestinal tract, and consequently the trace levels of carvacrol found in fecal samples actually represent only nonabsorbed remnantsWith regard to rosmarinic acid and eriodictyol, these compounds were not detected in the human fecal samples as their native structure after the sustained intake of TPEOO, which is in line with the complete degradation of these compounds observed in the in vitro study. In accordance with the in vitro fermentation, increased in the phenolic acids concentrations of 3-(3′,4′-dihydroxyphenyl) propionic acid, hydroxyphenylpropionic acid (the same isomer of acid reported in the in vitro experiments), 3-phenylpropionic acid, and 2-(4′-hydroxyphenyl)acetic acid were detected in human fecal samples after the intake of TPEOO. 3-(3′,4′-dihydroxyphenyl)propionic acid and hydroxyphenylpropionic acid were identified in the in vitro colon fermentation study as the intermediate and main metabolites of rosmarinic acid (**Figure 3**) and eriodictyol (**Figure 4**).

However, the increase in the concentrations of

these catabolites was not statistically significant ($p > 0.05$) (**Figure 5**). Similarly, other in vivo studies also noted an increment in the concentration of hydroxyphenylpropionic acid in feces after the intake of grape seed extract and cranberries. Microbial metabolites produced in the large intestine could carry out their bioactive activity at a local level (intestinal lumen) or, if absorbed, they could be distributed by blood circulation to organs and tissues where they might accumulate or be detoxified by metabolizing enzymes (primarily in the liver) before being excreted in the urine.[8,12] Bioactivity assays using human or animal cell lines have made extensive use of phenolic microbial catabolites to elucidate their possible mechanisms of action, and experiments have been widely reviewed by Del Rio and co-workers[33] and Rodriguez-Mateos and co-workers,[34] From this recent literature it is apparent that polyphenols and itheir microbial catabolites do not act simply as antioxidants in vivo and that their diverse effects are, in most instances, based on more complex and specific modes of action, mainly related to cancer and cardiovascular diseases. In this sense, microbial catabolites derived from phenylacetic and phenylpropionic acids, which were also detected in large amounts in fecal samples following TPEOO intake, were proposed as anti-inflammatory agents.[35] Additional experiments were carried out with the advance glycation end products (AGEs) model and pointed to the glucose-mediated pathway as a likely site of action of several microbial catabolites derived from hydroxyphenylacetic acid. These minor changes in phenol metabolite concentrations observed in feces after the sustained intake of thyme phenols through TPEOO (**Figure 5**) could indicate a high rate of absorption of thyme phenolic compounds in the upper part of the gastrointestinal tract. There is in vitro evidence that suggests that minor constituents of herb and spice extracts could enhance the rate of absorption of hydrophilic compounds present in the lumen of the small intestine, thus increasing paracellular transport by the transient opening of tight junctions.[37,38]

Recent in vitro studies have proposed that aromatic herbs could inhibit the growth of colon cancer cells,[39] which suggests that these compounds might have antitumoral effects in contact with colon mucosa. Also, a promising use of aromatic herbs as natural antimicrobials of gut pathogens was proposed.[1] However, in our study the thyme phenolic compounds (thymol, carvacrol, rosmarinic acid, or eriodictyol) in their native structures were not detected in human feces.

In conclusion, the results of the present study provide very useful information regarding the gut metabolism of thyme phenols. Although the monoterpenes carvacrol and thymol were poorly metabolized by gut microbiota in the in vitro model fermentation, neither of these compounds, except for one volunteer, was quantified in human feces after the sustained intake (3 weeks) of a daily dose of 25 mL of TPEOO. These results support the idea suggested by other authors that thymol and carvacrol are well absorbed during small intestine passage. In contrast, rosmarinic acid and the flavonoid eriodictyol were extensively metabolized during in vitro colonic fermentation, yielding hydroxyphenylpropionic acid as the main metabolite. In accordance with these results, after the sustained intake of TPEOO, neither rosmarinic acid nor eriodictyol was detected in human feces, observing at the same time an increase in the concentration of hydroxyphenylpropionic and phenylpropionic acids, confirming the effective microbial degradation for these compounds. The most likely effective absorption in the upper part of the gastrointestinal tract, together with the intense colonic metabolism of other representative compounds of thyme phenolic fraction (rosmarinic acid and eriodictyol), opens the possibility of the application of technologic resources, such as microencapsulation, to facilitate the arrival of this type of compound to the colon. Further research is needed to understand the physiological effects of thyme phenols in the gut and their implications when thyme is used as such (dry leaf) or as an essential oil for food flavoring.

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**ADDITIOINAL INFORMATION**

**Table 1.** In vitro fermentation of thymol and generation of microbial metabolites

| Compound (μM) | Fecal sample | Incubation time (h) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 2 | 6 | 12 | 24 | 48 |
| *Thymol* | H1 | 463±94.4 | 499±59.9 | 267±105 | 267±89.1 | 212±94.7 | 172±40.4 |
| | H2 | 234±10.1 | 203±166 | 255±162 | 276±25.4 | 257±29.9 | 450±143 |
| | H3 | 209±98.5 | 192±135 | 261±244 | 214±57.4 | 257±34.0 | 238±87.6 |
| Carvacrol | H1 | n.d. | 0.81±0.04 | n.d. | 1.3±2.61 | 0.69±0.24 | n.d. |
| | H2 | n.d | 0.33±0.07 | 0.07±0.22 | n.d. | n.d. | n.d |
| | H3 | n.d | n.d. | 0.2±0.38 | 0.29±0.76 | 0.76± | n.d |
| 2-(3',4'-dihydroxyphenyl) acetic acid | H1 | n.d. | n.d. | Tr | n.d | n.d. | Tr |
| | H2 | n.d. | n.d | n.d | n.d. | 1.06±0.09 | 1.50±0.02 |
| | H3 | n.d. | n.d | n.d | n.d. | n.d. | n.d. |
| 2-(4'-hydroxyphenyl) acetic acid | H1 | Tr | 0.75±0.10 | 0.31±0.08 | n.d | n.d | 11.8±7.89 |
| | H2 | 0.23±0.02 | 4.35±3.74 | 0.95±0.27 | n.d | n.d | n.d |
| | H3 | n.d | 0.70±0.40 | n.d | n.d | 51.5±5.30 | n.d |
| Phenylacetic acid | H1 | 0.65±5.15 | 1.58±0.78 | 7.64±6.06 | n.d | n.d | 44.7±42.3 |
| | H2 | n.d | n.d | 2.06±0.89 | n.d | n.d | n.d |
| | H3 | n.d | 4.09±1.91 | n.d | n.d | 50.7±4.08 | 1.21±5.44 |
| 3-(4'-hydroxyphenyl) propionic acid | H1 | 0.53±0.96 | 3.13±5.42 | 2.93±3.96 | n.d | 2.89±2.10 | 9.70±8.96 |
| | H2 | 0.64±0.05 | 0.97±0.23 | 16.17±5.47 | Tr | 0.25±0.15 | 6.26±0.67 |
| | H3 | n.d | 54.5±46.8 | n.d | n.d | 13.1±5.64 | 6.10±4.47 |
| Phenylpropionic acid | H1 | 3.59±2.09 | n.d | 8.25±4.06 | n.d | n.d | n.d |
| | H2 | n.d | n.d | 4.48±1.66 | n.d | 21.3±50.0 | 15.3±34.1 |
| | H3 | n.d | 14.7±10.9 | n.d | n.d | n.d | 18.3±7.49 |

**Table 2.** In vitro fermentation of carvacrol and generation of microbial metabolites

| Compound (μM) | Fecal sample | Incubation time (h) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 2 | 6 | 12 | 24 | 48 |
| *Carvacrol* | H1 | 217±56.4 | 176±19.5 | 175±19.2 | 234±6.28 | 243±3.82 | 221±19.4 |
| | H2 | 247±4.13 | 180±15.4 | 201±26.9 | 243±4.58 | 235±5.58 | 178±8.36 |
| | H3 | 137±20.6 | 215±56.5 | 179±8.83 | 256±53.1 | 229±10.1 | 185±21.2 |
| 2-(3',4'-dihydroxyphenyl) acetic acid | H1 | n.d. | 0.22±0.36 | 0.15±0.05 | 0.06±0.02 | n.d. | n.d. |
| | H2 | 0.24±0.14 | 0.15±0.09 | n.d | n.d. | 0.53±0.13 | 0.38±0.10 |
| | H3 | 0.13±0.12 | 0.08±0.03 | 0.08±0.06 | n.d. | n.d. | n.d. |
| 2-(4'-hydroxyphenyl) acetic acid | H1 | n.d. | 1.21±1.05 | 0.15±0.16 | n.d. | n.d. | 4.56±1.28 |
| | H2 | 0.92±0.13 | 0.08±0.11 | n.d. | n.d. | 0.37±0.10 | 1.25±0.39 |
| | H3 | 0.13±0.05 | 0.19±0.12 | n.d. | n.d. | 7.41±2.08 | 2.70±0.47 |
| Phenylacetic acid | H1 | 1.05±0.69 | 2.21±4.98 | 2.36±1.40 | n.d. | n.d. | 9.65±2.02 |
| | H2 | 1.20±0.14 | n.d. | n.d. | n.d. | n.d. | n.d. |
| | H3 | 2.69±0.62 | 0.66±1.01 | n.d. | n.d. | 7.44±1.64 | 12.6±8.29 |
| 3-(4'-hydroxyphenyl) propionic acid | H1 | 0.14±0.22 | n.d. | 1.35±0.67 | 0.25±0.16 | n.d. | 0.68±1.54 |
| | H2 | 15.3±0.18 | 4.06±1.34 | n.d. | n.d. | 43.2±12.2 | 16.2±7.84 |
| | H3 | 0.07±0.18 | 0.97±0.27 | n.d. | n.d. | 2.60±0.76 | 9.52±1.16 |
| Phenylpropionic acid | H1 | 0.06±1.07 | 1.43±1.82 | 3.96±0.50 | 2.08±0.80 | 3.84±1.40 | 22.8±1.33 |
| | H2 | n.d. | 0.13±0.49 | 0.31±1.55 | n.d. | n.d. | n.d. |
| | H3 | 1.78±1.02 | 1.16±0.40 | 0.37±1.18 | n.d. | 5.22±0.85 | 7.60±2.56 |

Data are expressed as mean in μmol/L. Mean values were calculated subtracting to the average value of the three replicates the control content of the respective substance. n.d.: not detected. Below the limit of detection Tr.: traces. Below the limit of quantification

**Table 3.** In vitro fermentation of rosmarinic acid and generation of microbial metabolites

| Compound (μM) | Fecal sample | Incubation time (h) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 2 | 6 | 12 | 24 | 48 |
| *Rosmarinic acid* | H1 | 72.7±10.6 | 74.7±4.18 | 14.9±2.24 | n.d. | n.d. | n.d. |
| | H2 | 61.95±25.03 | 51.4±13.6 | 10.3±1.68 | n.d | n.d | n.d |
| | H3 | 143±15.3 | 126±15.4 | 12.6±2.84 | n.d | n.d | n.d |
| Caffeic acid | H1 | 2.11±0.71 | 24.7±4.18 | 33.2±2.24 | 17.9±7.54 | n.d | 0.09±0.18 |
| | H2 | 2.68±0.27 | 11.0±1.77 | 20.4±1.87 | 17.8±1.94 | 0.04±0.05 | 0.01±0.03 |
| | H3 | 2.09±1.04 | 15.1±1.00 | 0.10±0.02 | 0.08±0.07 | n.d | 0.13±0.02 |
| *p*-cumaric acid | H1 | n.d | n.d. | n.d. | n.d. | n.d. | n.d |
| | H2 | 0.91±0.01 | 0.05±0.07 | 0.56±0.04 | 0.40±0.04 | n.d | 0.90± |
| | H3 | n.d | n.d | n.d. | n.d | n.d. | n.d. |
| 3- (3', 4'-dihydroxyphenyl) propionic acid | H1 | 0.03±0.01 | 0.13±0.02 | 15.8±0.87 | 36.2±5.68 | 0.33±0.19 | 0.06±0.14 |
| | H2 | 0.16±0.02 | 0.05±0.01 | 7.18±0.86 | 28.7±2.73 | n.d | 0.15±0.06 |
| | H3 | n.d | 3.19±0.27 | 24.1±1.10 | 7.80±4.86 | 0.48±0.10 | 0.15±0.06 |
| Hydroxyphenylpropionic acid | H1 | n.d. | n.d. | 1.48±0.32 | 6.45±4.54 | 67.9±5.97 | 166.±24.0 |
| | H2 | n.d | n.d | 2.75±1.01 | 0.19±0.05 | 55.7±7.57 | 132±13.0 |
| | H3 | n.d | 2.25±0.87 | 45.9±3.42 | 78.2±6.59 | 101±6.25 | 207±15.9 |
| Phenylpropionic acid | H1 | 1.09±3.40 | n.d. | n.d | n.d | 4.09±6.02 | 18.8±12.3 |
| | H2 | 5.47±0.31 | n.d | 0.63±3.69 | n.d | n.d | n.d |
| | H3 | n.d | n.d | n.d | 3.00±1.11 | n.d | 3.38±1.47 |
| 2-(3',4'-dihydroxyphenyl) acetic acid | H1 | 0.04±0.08 | 2.26±0.03 | 0.30±0.07 | 2.52±0.06 | 0.31±0.04 | 0.24±0.05 |
| | H2 | 0.26±0.05 | 0.10±0.03 | 0.09±0.01 | 0.03±0.03 | 1.14±0.36 | 1.14±0.38 |
| | H3 | n.d | 2.09±0.05 | 0.12±0.02 | 2.22±0.09 | 3.04±0.27 | 2.18±0.01 |
| 2-(4'-hydroxyphenyl) acetic acid | H1 | 0.13±0.08 | 0.02±0.02 | 0.14±0.06 | 0.51±0.22 | 0.46±0.17 | 0.95±0.47 |
| | H2 | 1.07±0.09 | n.d | 0.32±0.13 | 0.26±0.06 | 0.13±0.01 | 7.30±1.25 |
| | H3 | n.d, | n.d | 1.41±0.68 | 0.63±0.22 | 7.59±1.07 | 10.4±1.86 |
| Phenylacetic acid | H1 | n.d. | 0.89±0.45 | n.d. | n.d. | 7.60±3.48 | 5.73±6.77 |
| | H2 | 1.14±0.38 | n.d. | 0.41±1.83 | n.d. | 0.43±2.60 | 14.7±1.81 |
| | H3 | 0.03±1.79 | n.d. | n.d. | n.d. | 13.4±11.50 | 13.7±5.29 |

Data are expressed as mean in μmol/L. Mean values were calculated subtracting to the average value of the three replicates the control content of the respective substance. n.d.: not detected. Below the limit of detection Tr.: traces. Below the limit of quantification

**Table 4.** In vitro fermentation of eriodictyol and generation of microbial metabolites

| Compound (μM) | Fecal sample | Incubation time (h) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 2 | 6 | 12 | 24 | 48 |
| *Eriodyctyol* | H1 | 138±22.1 | 78.0±5.80 | 14.2±0.73 | 1.30±0.41 | 0.49±0.43 | n.d. |
| | H2 | 163±35.3 | 51.3±22.10 | 11.7±3.33 | 0.98±0.47 | n.d. | n.d. |
| | H3 | 149±43.7 | 73.2±32.1 | 12.0±1.58 | 0.50±0.44 | n.d. | n.d. |
| 3- (3', 4'-dihydroxyphenyl) propionic acid | H1 | n.d. | n.d. | 2.27±3.29 | 1.57±0.18 | 23.9±9.23 | n.d. |
| | H2 | n.d. | 0.05±0.02 | 0.38±0.12 | 3.09±0.17 | 19.5±5.04 | 0.07±0.05 |
| | H3 | n.d. | n.d. | 4.53±3.27 | 34.2±3.08 | n.d. | 0.05±0.05 |
| Hydroxyphenylpropionic acid | H1 | n.d. | n.d. | n.d. | 15.6±40.1 | 152±19.7 | 370±25.8 |
| | H2 | n.d. | n.d. | n.d. | 1.07±0.12 | 396±50.2 | 356±43.1 |
| | H3 | n.d. | 0.04±0.10 | 95.0±32.2 | 240±15.3 | 485±19.7 | 409±25.0 |
| Phenylpropionic acid | H1 | 3.75±2.38 | 1.19±4.45 | n.d. | 3.41±1.96 | n.d. | n.d. |
| | H2 | n.d. | n.d. | n.d. | 1.08±2.25 | n.d. | n.d. |
| | H3 | n.d. | n.d. | n.d. | 1.55±0.66 | 19.7±5.46 | 19.4±4.93 |
| 2-(3',4'-dihydroxyphenyl) acetic acid | H1 | 0.01±0.01 | n.d. | n.d. | 2.07±0.02 | n.d. | n.d. |
| | H2 | n.d. | 0.01 | n.d. | n.d. | 0.07±0.03 | n.d. |
| | H3 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 2-(4'-hydroxyphenyl) acetic acid | H1 | n.d. | n.d. | 2.57±0.92 | n.d. | n.d. | n.d. |
| | H2 | n.d. | n.d. | n.d. | n.d. | 1.11±0.10 | 1.53±0.45 |
| | H3 | n.d. | n.d. | n.d. | n.d. | n.d. | 0.79±0.40 |
| Phenylacetic acid | H1 | 2.24±2.34 | 1.41±1.43 | n.d. | 1.36±0.81 | n.d. | n.d. |
| | H2 | 0.87±0.83 | n.d. | n.d. | n.d. | n.d. | 31.6±10.5 |
| | H3 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |

Data are expressed as mean in μmol/L. Mean values were calculated subtracting to the average value of the three replicates the control content of the respective substance. n.d.: not detected. Below the limit of detection Tr.: traces. Below the limit of quantification

**PUBLICATION III** Effect of virgin olive oil and thyme phenolic compounds on blood lipid profile: implications of human gut microbiota

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ORIGINAL CONTRIBUTION

# EFFECT OF VIRGIN OLIVE OIL AND THYME PHENOLIC COMPOUNDS ON BLOOD LIPID PROFILE: IMPLICATIONS OF HUMAN GUT MICROBIOTA

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**ABSTRACT**

*Purpose*. To investigate the effect of virgin olive oil phenolic compounds (PC) alone or in combination with thyme PC on blood lipid profile from hypercholesterolemic humans, and whether the changes generated are related with changes in gut microbiota populations and activities.

*Methods* A randomized, controlled, double-blind, cross-over human trial (*n* = 12) was carried out. Participants ingested 25 mL/day for 3 weeks, preceded by 2-week washout periods, three raw virgin olive oils differing in the concentration and origin of PC: (1) a virgin olive oil (OO) naturally containing 80 mg PC/kg, (VOO), (2) a PC-enriched virgin olive oil containing 500 mg PC/kg, from OO (FVOO), and (3) a PC-enriched virgin olive oil containing a mixture of 500 mg PC/kg from OO and thyme 1:1 (FVOOT). Blood lipid values and faecal quantitative changes in microbial populations, short chain fatty acids, cholesterol microbial metabolites, bile acids, and phenolic metabolites were analysed.

*Results* FVOOT decreased seric ox-LDL concentrations compared with pre-FVOOT, and increased numbers of bifidobacteria and the levels of the phenolic metabolite protocatechuic acid compared to VOO ($P < 0.05$). FVOO did not lead to changes in blood lipid profile nor quantificative changes in the microbial population analysed but increased the corpostanone compared to FVOOT ($P < 0.05$), and the levels of the faecal hydroxytyrosol and dihydroxyphenylacetic acids, compared with pre-intervention values and to VOO, respectively ($P < 0.05$).

*Conclusion*. The ingestion of a PC-enriched virgin olive oil, containing a mixture of olive oil and thyme PC for 3 weeks, decreases blood ox-LDL in hypercholesterolemic humans. This cardio-protective effect could be mediated by the increases in populations of bifidobacteria together with increases in PC microbial metabolites with antioxidant activities.

**Keywords** Bifidobacteria · Gut microbiota · ox-LDL · Cholesterol · Phenolic compounds · Prebiotic · Virgin olive oil.

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Abbreviations

| | |
|---|---|
| CVD | Cardiovascular diseases |
| FC | Flow cytometry |
| FISH | Fluorescence in situ hybridization |
| FSC | Forward scatter detector |
| FVOO | Phenolic compounds-enriched virgin olive oil containing 500 mg phenolic compounds/kg, from olive oil |
| FVOOT | Phenolic compounds-enriched virgin olive oil containing a mixture of 500 mg phenolic compounds/kg, from olive oil and thyme, 1:1 |
| MD | Mediterranean diet |
| PC | Phenolic compounds |
| PCA | Protocatechuic acid |
| SCFA | Short chain fatty acids |
| SSC | Side scatter detector |
| VOO | Virgin olive oil naturally containing 80 mg of phenolic compounds/kg |

## 1. INTRODUCTION

Adherence to the Mediterranean diet (MD) has shown to be cardio-protective [1]. Consumption of virgin olive oil (OO), the main fat of the MD, has demonstrated a relevant influence on its beneficial effects [2]. Besides oleic acid, the phenolic fraction of virgin OO also contributes to the health effects associated with virgin OO consumption [3]. Its antioxidant and antiinflammatory activities, acting as pathway and gene expression modulators [4], may explain such health properties. However, the mechanisms by which virgin olive oil PC influence cardiovascular disease (CVD) risk factors are not fully understood. Recent insights indicate that gut microbiota plays an important role in CVD and represents a realistic therapeutic target [5]. Some of the reported gut microbiota-related mechanisms by which gut bacteria could influence CVD risk factors, such as the presence of abnormal levels of blood lipids [6], could be one or a combination of the following: (1) involvement in cholesterol synthesis through generation of short chain fatty acids (SCFA) which are generated from microbial fermentation of undigested substrates, either increasing blood total cholesterol (i.e. acetic) or decreasing it (i.e. propionic, butyric) [7]; (2) reduction in the amount of cholesterol available for re-absorption from the intestine either by transforming gut cholesterol to insoluble metabolites and, thereby, its uptake from the gut [8] or by incorporating cholesterol into the microbial cellular membrane [9, 10]; (3) deconjugation of bile salts in the gut, generating insoluble primary bile acids which are excreted in faeces [11], which lead to cholesterol expenditure in the liver in order to synthesize new bile acids; (4) generation of bioactive metabolites in the gut with cardio-protective properties. Diet appears to critically influence both, the relative abundance of different gut microorganisms and their metabolic output. In this sense, high intake of PC from different sources appears to regulate some CVD risk factors [12, 13], through the modulation of microbial populations and activities [14], as many plants PC are not totally absorbed and become available for microbiota utilization as an energy source, which has an impact on nutrient bioavailability and host metabolism. Regarding virgin olive oil PC, recent studies have demonstrated that they are able to reach the gut, being transformed by gut microbiota [15]. It has also been shown that the bioaccessibility of virgin olive oil PC can be increased by combining virgin olive oil PC with other PC sources (i.e. thyme) [16, 17]. Since the interaction of virgin olive oil phenolic compounds with gut microbiota and its involvement in CVD risk remains to be elucidated, the aim of our study was to investigate the effect of a sustained consumption of virgin olive oil PC, alone or in combination with thyme PC on blood lipid levels in hypercholesterolemic subjects, and whether this effect is mediated by gut microbiota-related mechanisms.

## 2. MATERIAL AND METHODS

### 2.1. Study subjects and design

The present study included a subsample of 12 hypercholesterolemic (total cholesterol >200 mg/dL) adults (5 females and 7 males) aged 46–67 years from the VOHF (Virgin Olive Oil and HDL Functionality) study. The VOHF study was a randomized, controlled, double-blind, crossover clinical trial with 33 hypercholesterolemic volunteers, aged 35–80 years. Exclusion criteria

included the following: BMI > 35 kg/m$^2$, smokers, athletes with high physical activity (>3000 kcal/day), diabetes, multiple allergies, intestinal diseases, or other disease or condition that would worsen adherence to the measurements or treatments.

**Table 1.** Chemical characterization of the olive oils used in the study

| Composition[a] | Olive oils | | |
|---|---|---|---|
| | VOO | FVOO | FVOOT |
| Phenolic compounds (mg/25 mL) | | | |
| Hydroxytyrosol | 0.01 ± 0.00 | 0.21 ± 0.02 | 0.12 ± 0.00 |
| 3,4-DHPEA-AC | n.d. | 0.84 ± 0.06 | 0.39 ± 0.04 |
| 3,4-DHPEA-EDA | 0.04 ± 0.00 | 6.73 ± 0.37 | 3.43 ± 0.29 |
| 3,4-DHPEA-EA | 0.26 ± 0.04 | 0.71 ± 0.06 | 0.36 ± 0.03 |
| *Total hydroxytyrosol derivates* | 0.30 | 8.49 | 4.30 |
| *p*-Hydroxybenzoic acid | n.d. | 0.02 ± 0.00 | 0.06 ± 0.00 |
| Vanillic acid | n.d. | 0.07 ± 0.00 | 0.13 ± 0.01 |
| Caffeic acid | n.d. | 0.00 ± 0.00 | 0.06 ± 0.00 |
| Rosmarinic acid | n.d. | n.d. | 0.41 ± 0.03 |
| *Total phenolic acids* | – | 0.09 | 0.65 |
| Thymol | n.d. | n.d. | 0.64 ± 0.05 |
| Carvacrol | n.d. | n.d. | 0.23 ± 0.02 |
| *Total monoterpenes* | – | – | 0.86 |
| Luteolin | 0.04 ± 0.00 | 0.18 ± 0.02 | 0.21 ± 0.02 |
| Apigenin | 0.02 ± 0.00 | 0.06 ± 0.00 | 0.10 ± 0.00 |
| Naringenin | n.d. | n.d. | 0.20 ± 0.02 |
| Eriodictyol | n.d. | n.d. | 0.17 ± 0.01 |
| Thymusin | n.d. | n.d. | 1.22 ± 0.09 |
| Xanthomicrol | n.d. | n.d. | 0.53 ± 0.06 |
| 7-Methylsudachitin | n.d. | n.d. | 0.53 ± 0.09 |
| *Total flavonoids* | 0.06 | 0.23 | 2.95 |
| Pinoresinol | 0.05 ± 0.00 | 0.12 ± 0.00 | 0.10 ± 0.05 |
| Acetoxipinoresinol | 2.47 ± 0.19 | 3.66 ± 0.31 | 3.24 ± 0.28 |
| *Total lignans* | 2.52 | 3.78 | 3.34 |
| Fat-soluble micronutrients (mg/25 mL) | | | |
| α-Tocopherol | 3.27 ± 0.01 | 3.40 ± 0.02 | 3.44 ± 0.01 |
| Lutein | 0.05 ± 0.00 | 0.06 ± 0.00 | 0.06 ± 0.00 |
| β-Cryptoxanthin | 0.02 ± 0.00 | 0.03 ± 0.00 | 0.02 ± 0.00 |
| β-Carotene | 0.01 ± 0.00 | 0.02 ± 0.00 | 0.02 ± 0.00 |
| Fatty acids (relative area %) | | | |
| Palmitic acid | 11.21 | 11.20 | 11.21 |
| Stearic acid | 1.92 | 1.92 | 1.92 |
| Arachidic acid | 0.36 | 0.36 | 0.36 |
| Behenic acid | 0.11 | 0.11 | 0.11 |
| *Total saturated* | 13.75 | 13.74 | 13.75 |
| Palmitoleic acid | 0.70 | 0.70 | 0.69 |
| Oleic acid | 76.74 | 76.83 | 76.75 |
| Gadoleic acid | 0.27 | 0.27 | 0.27 |
| *Total monounsaturated* | 77.71 | 77.80 | 77.72 |
| Linoleic acid | 7.43 | 7.36 | 7.43 |
| Timnodonic acid | 0.36 | 0.36 | 0.35 |
| Linolenic acid | 0.43 | 0.43 | 0.43 |
| *Total polyunsaturated* | 8.22 | 8.15 | 8.22 |

PC and fat-soluble micronutrients are expressed as mean ± SD of mg in 25 mL oil/day. The acidic composition is expressed as relative area percentage. *n.d.* non-detected. VOO, 80 mg/kg of phenolic compounds (PC) from olive oil; FVOO, 500 mg/kg of PC from olive oil; FVOOT, 250 mg/kg of PC from olive oil and 250 mg/kg from thyme.[a] 3,4-DHPEA-AC, 4-(acetoxyethyl)-1,2-dihydroxybenzene; 3,4-DHPEA-EDA, dialdehydic form of elenolic acid linked to hydroxytyrosol; 3,4-DHPEA-EA, oleuropein aglycone

The study was conducted at IMIM-Mar Insitute of Medical Research (Spain) from April 2012 to September 2012. Participants ingested 25 mL/day for 3 weeks, preceded by 2 week washout

periods, of three raw virgin olive oils differing in the concentration and origin of phenolic compounds (PC): (1) a virgin olive oil naturally containing 80 mg PC/kg (VOO), (2) a PC-enriched virgin olive oil containing 500 mg PC/kg, from olive oil (FVOO), and (3) a PC-enriched virgin olive oil containing a mixture of 500 mg PC/kg from olive oil and thyme, 1:1 (FVOOT). Participants were randomized to one of three orders of administration (Order 1: FVOO, FVOOT, and VOO, Order 2: FVOOT, VOO, and FVOO, Order 3: VOO, FVOO, and FVOOT). Elaboration of PC-enriched olive oils (i.e. FVOO and FVOOT) is described by Rubió et al. [18]. Full phenolic composition of the three oils is presented in **Table 1**. The random allocation sequence was generated by a statistician, participant enrolment was carried out by a researcher, and participants' assignment to interventions according to the random sequence was done by a physician. Due to the fact that all participants received each one of the three oils, restrictions such as blocking were unnecessary. In order to avoid an excessive intake of PC other than those provided by the intervention's oils, participants were asked to limit the consumption of rich-polyphenol food and dietary data were recorded by 3-day dietary record at baseline and before and after each intervention period. The corresponding 25 mL bottles of the corresponding oil for each day of consumption were provided to the participants at the beginning of each intervention period. The participants were instructed to return the bottles in order to register the amount of the intervention oil consumed. Subjects with <80 % of treatment adherence (≥5 full oil containers returned) were considered non-compliant for the dietary intervention. Blood at fastIng state (of at least 10 h) and faecal samples were collected before and after each intervention period. All participants provided written informed consent, and the local institutional ethics committees approved the protocol (CEIC-IMAS 2009/3347/I). The trial was registered with the International Standard Randomized Controlled Trial register (www.con-trolled-trials.com; ISRCTN77500181).

**2.2. Dietary adherence**

Twenty-four-hour urine was collected at the start of the study and before and after each treatment. Urine samples were stored at −80 °C prior to use. We measured urinary hydroxytyrosol sulphate and thymol sulphate as biomarkers of adherence to the type of OO ingested in urine by ultra-HPLC–ESI–MS/MS [19]. A 3-day dietary record was administered to the participants at baseline and before and after each intervention period. A nutritionist personally advised participants to replace all types of habitually consumed raw fats with the oils provided, and to limit their polyphenol-rich food consumption.

**2.3. Serum lipid profile analysis**

Total and HDL cholesterol, and triglyceride concentrations were measured by using standard enzymatic automated methods. When triglyceride concentrations were <300 mg/dL, LDL cholesterol was calculated by using Friedewald's formula. Oxidized LDL was determined with an ELISA procedure that employed the murine monoclonal antibody mAb-4E65 (Mercodia AB).

**2.4. Faecal sample collection and pre-analytical treatment**

For faecal collection, participants were given a set containing: a sterile pot, a w-zip plastic pouch (AN0010 W, Oxoid, Basingstoke, UK), two anaerobic sachets (Anaero-Gen Compact AN0025, Oxoid), and one anaerobic indi-cator (BR0055, Oxoid). Freshly voided faecal samples were collected by the volunteers in the sterile pot and kept under anaerobic conditions by introducing them into the plastic pouch, together with the anaerobic sachets and the anaerobic indicator. In order to avoid changes in microbial populations, faecal samples were brought to the laboratory within 2 h after defecation.

For quantitative analysis of gut microbiota, faeces were diluted with sterile 0.1 M, pH 7.0, phosphate-buffered saline (PBS, Sigma-Aldrich Co. LLC., St. Louis, USA) (1:10, w/v), mixed in a Stomacher 400 (Seward, Thetford, Norfolk, UK) for 2 min and faecal slurries homogenized. After centrifugation (1300*g*, 3 min), hexane (Sigma-Aldrich, UK) was added to the faecal homogenate supernatant (4:1, v/v), mixed by inversion for 2 min and removed

after centrifugation (15,500*g*, 5 min) and evaporation. Pellets were washed in 1 mL of filtered sterile PBS and centrifuged (15,500*g*, 5 min). Afterwards, pellets were diluted in 375 µL of PBS and fixed in ice-cold 4 % (w/v) paraformaldehyde (PFA) (1:4, v/v) for 4 h at 4 °C. PFA was discarded after centrifugation (15,500*g*, 5 min) and washed twice in 1 mL of sterile PBS.

### 2.5. Quantification of faecal microbiota by FISH-FC

Bacterial hybridizations were based on the method described by Massot-Cladera et al. [20] with some modifications. Briefly, 5 µL of fixed cell suspensions were centrifuged at 15,500*g* for 5 min. 30 µL of a mixture (1:10, v/v) of synthetic oligonucleotide probes (50 ng/µL) targeting specific diagnostic regions of 16S rRNA and labelled with the fluorescent Cy3 dye (Ato291 [21], Bac303 [26], Bif164 [27], Chis150 [22], Erec482 [22], Fprau645 [23], Lab158 [24], Prop853 [25] and Rrec584 [25]), plus pre-heated hybridization buffer (0.9 M NaCl, 20 mM Tris–HCl pH 8, 0.01 % sodium dodecyl sulphate), were added to the pellets, homogenized, and incubated in a thermocycler for 4 h at each specific probe hybridization temperature, in the dark. After hybridization, samples were washed by adding 2 mL of a mixture of preheated wash buffer (0.9 M NaCl, 20 mM Tris–HCl, pH 8) where 0.04 % of 6-diamidino-2-phenylindole dihydrochloride (50 ng/µL; Sigma-Aldrich) was added, for 15 min at each specific probe hybridization temperature in the dark. Washed samples were then centrifuged at 15,500*g* for 5 min. Pellets were homogenized in 200 µL of PBS solution and kept in the dark at 4 °C overnight. Immediately before the flow cytometry analysis, 30 µL of Commercial Flow CheckTM Fluorospheres (Beckman Coulter, Inc. FL, USA) were added to the samples in order to calculate total counts of bacteria.

An LSRFortessa flow cytometer (Becton–Dickinson, New Jersey, USA) was used for bacteria quantifications. The flow cytometer parameters were adjusted for bacterial counts. Bacteria morphology was selected according to their FSC/SSC signal. For this purpose, selected bacteria (Leibniz-Institut DSMZ collection, Germany) representative of each bacterial group hybridized by each probe (*Collinsella aerofaciens* for Ato291; *Bacteroides caccae* for Bac303; *Bifidobacterium bifidum* for Bif164; *Clostridium acetobutyl-icum* for Chis150; *Clostridium saccharolyticum* for Erec482; *Faecalibacterium praustnizii* for Fprau645; *Lactobacillus plantarum* for Lab158; *Megasphaera elsdenii* for Prop853; *Roseburia intestinalis* for Rrec584) were grown, fixed, and hybridized as described above. Bacteria hybridized with Cy3-labelled probes were detected using a yellow and green laser (561 nm filter), and DAPI bacteria stained DNA was detected using violet one (405-nm filter). Both lasers worked at 50-mW power. An acquisition gate of 2500 fluorospheres was established. Analysis was performed using the FACS-Diva software version 6.1.2 (Becton–Dickinson). Microbiota composition results were expressed as the log faecal cells/g dry faeces for each sample.

### 2.6. Analysis of faecal SCFA

For the analysis of SCFA, freeze-dried samples were diluted 10-fold with milli-Q water and centrifuged, first at 1800*g* for 5 min and afterwards at 8784*g* for 4 min at 4 °C. Supernatants were filtered through a 0.22 µm filter and subjected to GC analysis (Agilent 7890A Series, Santa Clara, EEUU) using a capillary BP-21 column (SGE, Cromlab SL, Barcelona, Spain) (30 m, 0.25 mm, 0.25 µm) coupled to a flame ionization detector (FID) [28]. 4-methyl valeric acid (Sigma-Aldrich) was used as internal standard. Concentrations of SCFA were calculated from calibration curves using standard solutions with known concentrations of acetic, propionic, butyric, isobutyric, isovaleric, and valeric acids (Sigma-Aldrich). Results were expressed as µmol/g dry faeces.

### 2.7. Analysis of faecal cholesterol microbial metabolites (neutral sterols) and bile acids

Freeze-dried milled faeces were diluted in milli-Q water (0.1:4, w/v) and homogenized. A volume of 400 µL of faecal homogenate was used for the extractions, following the method described by Santas et al. [29] with some modifications. For cholesterol and its microbial metabolites, sterol

mixtures were prepared as calibrators. 5-α-Cholestane (10 µg, Sigma-Aldrich) was used as internal standard. Samples were hydrolysed with 1 mL of NaOH (1 N, in ethanol) for 1 h at 70 °C. After cooling the tubes at room temperature, 0.5 mL of water was added and tubes were sonicated for 5 min. After two extractions with cyclohexane (3 mL each), mixed organic phases were evaporated under a 15-psi nitrogen stream at 30 °C.

For bile acid analysis, bile acid mixtures were prepared. 5-β-Cholanic acid (Sigma-Aldrich) was used as internal standard. Samples were hydrolysed with 1 mL NaOH 1 N at 70 °C for 1 h. After cooling the tubes, liquid–liquid extraction with 3 mL of *tert*-butylmethyl ether was done twice. Organic phases were further cleaned with 2 mL of NaCl 1 %. The organic phase was evaporated under a 15-psi nitrogen stream at 30 °C.

Derivatization of both sterols and bile acids was car-ried out by addition of 50 µL of *N*-methyl-bis (trifluoro-acetamide)/$NH_4I$/2-mercaptoethanol (1000/2/6) (Mach-erey–Nagel, Düren, Germany) and dry heated at 60 °C for 20 min. A gas chromatograph (6890 N; Agilent Tech-nologies, Wilmington, DA, USA) equipped with a mass selective detector (5973 Network, AT) and an autosampler injector (7683 series, AT) was used for analysis and performed in a 100 % methylsiloxane column (Agilent Ultra 1) in all cases. After derivatization, both neutral sterols and bile acids were quantified with their respective standards, expressed as µmol/g and µmol/10 g dry faeces, respectively.

### 2.8. Analysis of faecal phenolic microbial metabolites

For PC metabolite analysis, freeze-dried faeces (0.1 g) were mixed with 1 mL of milli-Q water. Samples were shaken for 30 min and centrifuged (13,200*g*, 10 min, 4 °C). Supernatants were centrifuged (13,200*g*, 10 min, 4 °C) and filtered through a membrane (0.22 µm pore size) and transferred to chromatographic vials. PC metabolites analysis was performed as previously described [15, 17] and was quantified using the calibration curve of their respective standard. PC metabolites concentration results were expressed as µmol/100 g dry faeces.

### 2.9. Sample size

The sample size for this study was calculated with the free software GRANMO (http://www.imim.cat/ofertadeserveis/ software-public/granmo/) by selecting 80 % power (5 % α level) to detect a 0.4 log10/g dry faeces difference between treatments in the primary outcome variable (Bif164 counts/g of dry faeces), with a standard deviation of treatment differences <0.48 (log10 scale).

### 2.10. Statistical analysis

Normality of continuous variables was assessed with normal probability plots and the Shapiro–Wilk test. Non-normally distributed variables were log transformed previous to the analysis. Paired *t* test was used for intra-intervention comparisons. Adjusted general linear mixed models with a period-by-treatment interaction term were used for inter-intervention comparisons, given results as adjusted means. $P \leq 0.05$ was considered significant. Statistical analyses were performed with an R software version 2.11.1.

## 3. RESULTS

### 3.1. Compliance

Urine compliance markers (hydroxytyrosol sulphate and thymol sulphate) indicated good adherence to the oil interventions (**Table 1** of **Supplementary**). The three intervention oils were well tolerated by all participants, and no adverse events were reported.

### 3.2. Blood lipid profile

Serum concentrations of cholesterol, triglycerides, HDL cholesterol, and LDL cholesterol did not present any statistical change with any of the dietary interventions (data not shown). Oxidized LDL concentrations decreased after FVOOT intervention compared to pre-FVOOT values ($P = 0.049$) (**Fig. 1**).

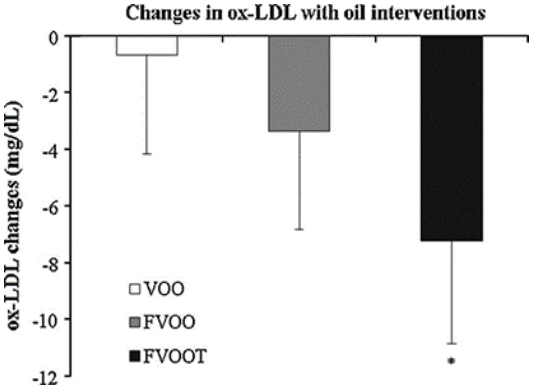


**Fig. 1** Changes in serum oxidized LDL (after–before interventions values) with olive oil interventions. Values are given as means of mg/dL and SE *bars* $n = 12$ subjects. *$P < 0.05$, for the differences within the same olive oil intervention.

### 3.3. Analysis of quantitative changes in gut microbiota

Quantitative changes in faecal microbiota before and after interventions are presented in **Table 2**. Consumption of virgin olive oil PC alone (FVOO) did not have a significant effect on bacterial counts. Only a decrease in numbers of *Clostridium cluster IX* (Prop853) compared to VOO intervention was observed, although this decrease did not reach statistical significance ($P = 0.066$).

When virgin olive oil PC were combined with thyme PC (FVOOT), the numbers of the bacterial groups hybridized by Bif164 probe (most *Bifidobacterium spp* and *Paras-cardovia denticolens*) significantly increased compared to VOO ($P = 0.044$). PC combination also increased the numbers of *Roseburia–Eubacterium rectale* group (Rrec584 probe) compared to FVOO intervention, but this increment did not reach statistical significance ($P = 0.085$).

**Table 2.** Bacterial enumerations determined by FISH-flow cytometry in faecal samples collected before (B) and after (A) each olive oil intervention

| Probe | | VOO | FVOO | FVOOT | $P^a$ | | |
|---|---|---|---|---|---|---|---|
| | | | | | VOO–FVOO | VOO–FVOOT | FVOO–FVOOT |
| Ato291 | B | 8.80 ± 0.09 | 8.86 ± 0.11 | 8.72 ± 0.10 | | | |
| | A | 8.78 ± 0.09 | 8.64 ± 0.09 | 8.72 ± 0.09 | 0.319 | 0.947 | 0.293 |
| Bac303 | B | 8.68 ± 0.38 | 8.87 ± 0.42 | 8.64 ± 0.40 | | | |
| | A | 8.75 ± 0.39 | 8.73 ± 0.39 | 8.73 ± 0.40 | 0.423 | 0.917 | 0.373 |
| Bif164 | B | 8.33 ± 0.25 | 8.29 ± 0.27 | 8.14 ± 0.26 | | | |
| | A | 8.10 ± 0.25 | 8.11 ± 0.26 | 8.32 ± 0.26 | 0.818 | 0.044 | 0.073 |
| Fprau645 | B | 8.90 ± 0.06 | 9.03 ± 0.07 | 8.96 ± 0.06 | | | |
| | A | 8.90 ± 0.06 | 8.92 ± 0.06 | 8.93 ± 0.06 | 0.420 | 0.831 | 0.558 |
| Lab158 | B | 8.30 ± 0.20 | 8.43 ± 0.22 | 8.24 ± 0.21 | | | |
| | A | 8.44 ± 0.20 | 8.32 ± 0.21 | 8.27 ± 0.21 | 0.145 | 0.512 | 0.427 |
| Prop853 | B | 8.81 ± 0.08 | 8.99 ± 0.10 | 8.81 ± 0.09 | | | |
| | A | 8.98 ± 0.08 | 8.79 ± 0.08 | 8.80 ± 0.08 | 0.066 | 0.338 | 0.364 |
| Rrec584 | B | 8.74 ± 0.08 | 8.81 ± 0.10 | 8.63 ± 0.09 | | | |
| | A | 8.76 ± 0.09 | 8.61 ± 0.09 | 8.79 ± 0.09 | 0.303 | 0.461 | 0.085 |

Values are given as adjusted means of log10 bacteria/g dry faeces ± SE; $n = 12$ subjects
VOO, 80 mg/kg of phenolic compounds (PC) from olive oil; FVOO, 500 mg/kg of PC from olive oil; FVOOT, 250 mg/kg of PC from olive oil and 250 mg/kg from thyme. [a]*P* values for inter-dietary intervention comparison.

### 3.3. Analysis of changes in faecal microbial activities

The analysis of the main SCFA generated by gut microbial fermentation (acetic, butyric, propionic, and branched acids) did not show significant changes with any of the interventions (**Table 3**).

**Table 3.** Faecal short chain fatty acids (SCFA), neutral sterols, and bile acids determined before (B) and after (A) each olive oil intervention.

| | | Olive oil interventions | | | *P*[b] | | |
|---|---|---|---|---|---|---|---|
| | | VOO | FVOO | FVOOT | VOO –FVOO | VOO –FVOOT | FVOO –FVOOT |
| SCFA (µmol/g df) | | | | | | | |
| Acetic | B | 195 (102, 372) | 144 (73.8, 281) | 148 (80.5, 272) | | | |
| | A | 178 (94.0, 337) | 168 (94.5, 294) | 160 (74.7, 341) | 0.364 | 0.547 | 0.772 |
| Butyric | B | 64.3 (26.2, 157) | 42.4 (19.7, 91.3) | 44.4 (21.1, 93.5) | | | |
| | A | 58.0 (22.2, 151) | 52.6 (25, 109) | 42.3 (16.8, 106) | 0.264 | 0.854 | 0.347 |
| Propionic | B | 62.9 (32.6, 121) | 48.5 (24.0, 97.9) | 48.0 (23.9, 96.4) | | | |
| | A | 50.8 (26.7, 96.7) | 51.7 (26.9, 99.3) | 48.6 (21.0, 113) | 0.333 | 0.444 | 0.858 |
| Branched[a] | B | 28.2 (14.7, 54.0) | 25.8 (16.8, 39.7) | 25.3 (14.8, 43.0) | | | |
| | A | 24.6 (14.2, 42.7) | 29.9 (17.7, 50.6) | 23.4 (15.0, 36.4) | 0.117 | 0.751 | 0.207 |
| Neutral sterols (µmol/g df) | | | | | | | |
| Cholesterol | B | 4.18 (2.06, 8.50) | 2.17 (0.29 16.1) | 4.57 (1.72, 12.2) | | | |
| | A | 3.61 (1.28, 10.2) | 3.33 (0.72, 15.4) | 3.53 (1.28, 9.73) | 0.176 | 0.798 | 0.103 |
| Coprostanol | B | 89.0 (38.8, 204) | 79.7 (17.9, 354) | 110 (42.1, 285) | | | |
| | A | 74.0 (27.0, 203) | 111 (29.4, 423) | 116 (39.0, 348) | 0.266 | 0.622 | 0.559 |
| Cholestanone | B | 0.21 (0.01, 6.99) | 0.07 (0.01, 0.33) | 0.17 (0.02, 1.74) | | | |
| | A | 0.28 (0.01, 6.74) | 0.11 (0.03, 0.35) | 0.21 (0.03, 1.37) | 0.723 | 0.904 | 0.631 |
| Coprostanone | B | 0.73 (0.25, 2.16) | 0.39 (0.04, 4.03) | 1.02 (0.25, 4.12) | | | |
| | A | 0.78 (0.17, 3.62) | 0.82 (0.22, 2.97) | 0.75 (0.23, 2.43) | 0.155 | 0.440 | 0.028 |
| Bile acids (mmol/10 g df) | | | | | | | |
| Chenodeoxycholic | B | 2.42 (0.14, 40.8) | 2.39 (0.37, 15.3) | 1.68 (0.18, 15.5) | | | |
| | A | 2.78 (0.23, 33.3) | 4.54 (0.99, 20.9) | 1.55 (0.34, 7.15) | 0.493 | 0.776 | 0.319 |
| Cholic | B | 1.10 (0.16, 7.68) | 0.65 (0.14, 3.05) | 0.64 (0.09, 4.35) | | | |
| | A | 1.22 (0.24, 6.18) | 1.46 (0.27, 7.76) | 0.99 (0.17, 6.00) | 0.356 | 0.666 | 0.621 |
| Lithocholic | B | 9.07 (1.73, 47.7) | 11.0 (2.15, 56.2) | 8.67 (1.47, 51.1) | | | |
| | A | 9.81 (3.21, 30.0) | 19.0 (7.09, 51.0) | 11.6 (4.05, 33.1) | 0.407 | 0.721 | 0.644 |
| Isolithocholic | B | 2.34 (0.52, 10.5) | 3.98 (0.72, 21.9) | 2.10 (0.25, 17.8) | | | |
| | A | 2.17 (0.48, 9.81) | 3.42 (1.18, 9.89) | 2.80 (0.90, 8.79) | 0.895 | 0.556 | 0.450 |
| Deoxycholic | B | 32.8 (11.4, 94.7) | 34.4 (12.0, 99.0) | 27.9 (3.37, 230) | | | |
| | A | 40.0 (11.4, 139) | 54.7 (20.0, 149) | 48.2 (8.15, 285) | 0.604 | 0.519 | 0.870 |

Values are given as adjusted means and CI; *n* = 12
25 mL/day extra virgin olive oil containing: VOO, 80 mg/kg of phenolic compounds (PC) from olive oil; FVOO, 500 mg/kg of PC from olive oil; FVOOT, 250 mg/kg of PC from olive oil and 250 mg/kg from thyme
*df* dry faeces

[a] Sum of isobutyric, isovaleric, and valeric acids
[b] *P* values for inter-dietary intervention comparisons.

Their relative amounts remained constant along the trial irrespective of the intervention (54–58, 15–18, 16–18, and 8–10 %, respectively).

From the faecal concentrations of cholesterol and microbial cholesterol metabolites (coprostanol, coprostanone, cholestanone) analysed, only coprostanone changed with the dietary interventions, by increasing with FVOO compared to FVOOT ($P$ = 0.028) (**Table 3**). Changes in concentrations of faecal bile acids after dietary interventions did not reach statistical significance (**Table 3**). However, the relative proportion of

isolithocholic acid, referred to the total of faecal bile acids analysed, decreased after FVOO compared with pre-FVOO [from 6.29 % CI (5.81, 6.80) to 3.47 % CI (3.20, 3.75); *P* = 0.020]. The analysis of faecal phenolic metabolites is presented in **Table 4**. Hydroxytyrosol increased and tended to increase after FVOO (*P* = 0.034) and FVOOT (*P* = 0.064), respectively. FVOO increased dihydroxyphenylacetic acid compared to VOO (*P* = 0.014). Protocatechuic acid with FVOOT compared to VOO (*P* = 0.003). Two unknown phenolic metabolites increased after FVOO compared with VOO (*P* = 0.027 and *P* = 0.042, respectively.

**Table 4** Phenolic metabolites determined by LC in faecal samples collected before (B) and after (A) each olive oil intervention

| Phenolic metabolite | | VOO | FVOO | FVOOT | *P*[a] | | |
|---|---|---|---|---|---|---|---|
| | | | | | VOO–FVOO | VOO–FVOOT | FVOO-FVOOT |
| Hydroxytyrosol | B | 0.19 (0.02, 2.28) | 0.15 (0.02, 1.13) | 0.08 (0.01, 1.16) | | | |
| | A | 0.16 (0.02, 1.50) | 0.74 (0.10, 5.76)** | 0.33 (0.01, 10.3)* | 0.108 | 0.140 | 0.928 |
| Dihydroxypheny | B | 46.6 (39.0, 55.6) | 30.1 (25.3, 35.7) | 30.5 (25.3, 36.7) | | | |
| lacetic acid | A | 23.3 (19.1, 28.3)** | 40.9 (34.4, 48.7) | 30.6 (25.5, 36.8) | 0.014 | 0.092 | 0.435 |
| Hydroxyphenylacetic | B | 31.8 (24.6, 41.1) | 18.7 (14.6, 23.9) | 23.7 (17.9, 31.5) | | | |
| acid | A | 14.3 (10.5, 19.4)* | 17.9 (14.0, 23.0) | 28.7 (22.0, 37.6) | 0.226 | 0.129 | 0.708 |
| Phenylacetic acid | B | 247 (236, 262) | 234 (222, 247) | 232 (219, 246) | | | |
| | A | 206 (194, 219) | 263 (249, 277) | 203 (192, 215) | 0.194 | 0.818 | 0.281 |
| Dihydroxyphenyl- | B | 0.01 (0.00, 0.02) | 0.00 (0.00, 0.01) | 0.00 (0.00, 0.02) | | | |
| propionic acid | A | 0.01 (0.00, 0.05) | 0.00 (0.00, 0.01) | 0.01 (0.00, 0.02) | 0.690 | 0.617 | 0.913 |
| Hydroxyphenyl | B | 102 (56.9, 185) | 42.2 (23.2, 76.8) | 36.8 (19.5, 69.4) | | | |
| -propionic acid | A | 65.6 (33.4, 128.6) | 33.1 (18.6, 58.7) | 39.1 (21.2, 72.1) | 0.796 | 0.534 | 0.703 |
| Phenylpropionic acid | B | 165 (149, 184) | 122 (111, 136) | 128 (114, 143) | | | |
| | A | 143 (127, 161) | 138 (125, 153) | 117.8 (106, 131) | 0.394 | 0.847 | 0.510 |
| Rosmarinic acid | B | 2.60 (1.40, 4.83) | 1.80 (1.01, 3.23) | 2.76 (1.57, 4.87) | | | |
| | A | 2.83 (1.41, 5.68) | 1.85 (1.13, 3.04) | 2.22 (1.29, 3.82) | 0.938 | 0.684 | 0.733 |
| Protocatechuic acid | B | 0.55 (0.03, 9.15) | 0.11 (0.00, 3.01) | 0.07 (0.00, 1.45) | | | |
| | A | 0.04 (0.00, 0.68)** | 0.11 (0.00, 3.41) | 0.10 (0.01, 1.80) | 0.008 | 0.003 | 0.652 |
| Coumaric sulphate | B | 2.92 (1.74, 4.89) | 3.96 (2.40, 6.53) | 2.93 (1.64, 5.24) | | | |
| acid | A | 1.50 (0.83, 2.70) | 4.46 (2.70, 7.37) | 3.81 (2.22, 6.54) | 0.288 | 0.231 | 0.842 |
| Caffeic acid | B | 1.94 (1.68, 2.24) | 2.18 (1.89, 2.51) | 2.41 (2.07, 2.81) | | | |
| | A | 2.74 (2.35, 3.19) | 2.68 (2.32, 3.09) | 2.16 (1.87, 2.49) | 0.247 | 0.597 | 0.110 |
| Ferulic acid | B | 1.01 (0.61, 1.67) | 0.58 (0.40, 0.85) | 0.89 (0.52, 1.52) | | | |
| | A | 1.81 (1.10, 2.97) | 0.93 (0.65, 1.31) | 1.30 (0.81, 2.09) | 0.881 | 0.817 | 0.924 |
| Hydroxyphenyl- | B | 2.52 (1.77, 3.60) | 4.94 (3.48, 7.00) | 3.93 (2.55, 6.06) | | | |
| valerolactone | A | 3.61 (2.32, 5.63) | 3.42 (2.41, 4.84) | 7.80 (5.39, 11.3) | 0.401 | 0.718 | 0.231 |
| M1[b] | B | 141 (104, 192) | 106 (78.0, 144) | 127 (92.7, 173) | | | |
| | A | 106 (77.8, 146) | 135 (99, 183) | 127 (93, 172) | 0.027 | 0.236 | 0.295 |
| M2 | B | 1.61 (1.18, 2.20) | 1.00 (0.73, 1.37) | 1.40 (1.01, 1.95) | | | |
| | A | 0.88 (0.63, 1.25)* | 1.50 (1.11, 2.04) | 1.58 (1.15, 2.18) | 0.042 | 0.152 | 0.559 |

Values are given as adjusted means of µmol/100 g dry faeces and CI; *n* = 12 subjects VOO, 80 mg/kg of phenolic compounds (PC) from olive oil; FVOO, 500 mg/kg of PC from olive oil; FVOOT, 250 mg/kg of PC from olive oil and 250 mg/kg from thyme. * 0.05 < *P* < 0.1; ** *P* ≤ 0.05 for intra-dietary intervention differences [a] *P* values for inter-dietary intervention comparisons. [b] M1: unknown metabolite. $(M–H)^-$ = 187 m/z. $MS^2$ fragments = 125, 117, 89 m/z; M2: unknown metabolite. $(M–H)^-$ = 243 m/z. $MS^2$ frag-ments = 227, 207, 119 m/z

## 4. DISCUSSION

The aim of this study was to elucidate whether the possible cardio-protective effects of a sustained consumption of virgin olive oil phenolic compounds (PC) alone or in combination with thyme PC are mediated by changes in gut

microbiota populations and metabolic activities, in hyper-cholesterolemic humans. The combination of olive oil and thyme PC exerted a cardio-protective effect, by decreasing blood levels of ox-LDL. The antioxidant activity of some microbial phenolic metabolites such as hydroxytyrosol and protocatechuic acid, generated after gut microbial fermentation of phenolic compounds contained in FVOOT, could be involved in the observed effects on ox-LDL. After oxidation, LDL becomes more toxic and plays a primary role in the development and progression of atherosclerosis [30]. It has been reported that oxidation of LDL decreases with increasing phenolic content of olive oil [3, 31, 32]. In fact, the European Food Safety Authority approved a claim concerning the benefits of olive oil polyphenols for the protection of LDL from oxidation [33]. We observed quantitative significant changes in gut microbiota only when virgin olive oil PC were ingested together with thyme PC, by increasing *Bifidobacterium* group numbers. The gut microbial usage of virgin olive oil and thyme PC has been recently reported in a linear non-controlled study, by Mosele et al. [15, 17]. They observed the generation of PC metabolites after microbial transformation of virgin olive oil and thyme parental PC, concluding that some of the investigated parental PC were able to reach the gut, afterwards being transformed by gut microbiota. Increases in gut bifidobacteria have been reported with other sources of phenolic compounds such as wine [34], wild blueberry [35], pomegranate peel [36] and cocoa [7]. However, this is the first time that a potential bifidogenic effect is reported for a combination of virgin olive oil and thyme PC. Since prebiotic is defined as “a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health” [37], a potential prebiotic activity of the combination of PC in FVOOT is suggested and should be further investigated.

Since recent studies in animals and humans have shown improvements in blood lipid profile with the ingestion of bifidobacteria and lactobacilli mixtures [38, 39], the increase in *Bifidobacterium* could be responsible at least in part for the decrease in ox-LDL levels observed with the ingestion of FVOOT.

Regarding blood cholesterol levels, hardly any effects were observed on the cholesterol-lowering mechanisms investigated. This is in accordance with the lack of changes reported in blood cholesterol levels. First, we investigated whether microbial usage of PC was able to generate changes in SCFA production, related to cholesterol synthesis. In our study, neither faecal amounts of acetic, propionic, and butyric acids nor their relative amounts changed with any of the interventions. Grapefruit PC have been shown to increase amounts of SCFA in rats [40], whereas black tea and red wine PC decreased them in vitro [41]. The lack of effects observed in our study could be due to the fact that, contrary to what occurs in an in vitro system, SCFA generated are also absorbed in vivo, making difficult to observe any difference at faecal level. Different results could also indicate that the influence on the microbial generation of SCFA depends on the PC source. The second mechanism analysed was the insolubilization of gut cholesterol by its transformation into non-soluble metabolites by gut microbiota. Gut bacteria are able to metabolize cholesterol by two pathways [11]. One of them transforms cholesterol directly into coprostanol and the other implies the transformation of cholesterol into cholestanone, coprostanone, and finally coprostanol. Few studies have investigated this gut microbiota-mediated blood cholesterol-lowering mechanism after dietary interventions with PC [42]; most of them focusing on changes in total faecal cholesterol or total faecal lipids. In our study, faecal concentrations of cholesterol remained constant with the dietary interventions besides the increase in coprostanone observed with FVOO. This could be due to the fact that we performed faecal analysis in the whole faecal residue, containing bacteria that could be also either assimilating cholesterol or including it in the bacterial cell wall or both. The absence of any impact on faecal cholesterol could be also related to the PC source. Whereas PC sources such as horseradish [42], apple skin [43] and sesame flour [44] have been shown to increase faecal cholesterol in murine models, other sources such as peanut skin [45] or oolong tea [46] decreased or had no effect on faecal cholesterol levels.

The third mechanism studied was the increase in liver cholesterol expenditure due to microbial generation of insoluble bile acids in the gut. Bile acids are synthesized in the liver from cholesterol, conjugated and excreted to the biliary system. From them, 200 to 800 mg/day passes to the colon. Probiotic bacteria, such as bifidobacteria, encode bile salt hydrolase (BSH) enzymes, which deconjugate bile salts [47]. Deconjugated bile acids are not absorbed and are excreted in faeces. As synthesis of new bile acids rises in compensation, blood cholesterol levels fall [14]. In our study, faecal concentrations of primary bile acids did not change with any of the interventions. This is in concurrence with other authors [48] who were not able to detect any effect of black tea PC on faecal bile acids in humans. Other PC sources such as peanuts, hazelnut skin, and sesame flour [44, 49] have shown increases in bile acid excretion in murine models. Different sources of PC would have a different effect on BSH producing bacteria. In our study, the increase in populations of bifidobacteria with FVOOT, although significant, could have been not enough to significantly increase the concentrations of deconjugated bile acids. A novel bile acid-related mechanism has been published recently [50], which suggest that *Lactobacillus* could reduce blood cholesterol by lowering absorption of fat from the intestine via FXR activation by deconjugated bile acids absorbed from the intestine. Nevertheless, in the present work, no decrease in the systemic cholesterol has been observed and further studies are needed to elucidate the mechanisms and their degree of involvement.

Besides the absence of changes in the amounts of faecal bile acids, we found a potential detoxificating effect of FVOO, which reduced the relative proportions of the toxic isolithocholic acid. Deconjugated forms are the substrate for 7-dehydroxylation, which generates toxic secondary bile acids. Consumption of common dietary polyphenols has shown to reduce toxic faecal deoxycholic acid and lithocholic acids in rats [51]. Probiotic bacteria are not capable of dehydroxylate deconjugated bile salts [52], and so the majority of the breakdown products of BSH activity by a probiotic strain may be precipitated and excreted in faeces. Only certain strains of *Clostridium* and *Eubacterium spp.* have been shown to possess dehydroxylating activity [53]. In our study, the decrease in the relative proportions of isolithocholic acid could be related to the decrease in populations of *Clostridium cluster IX* observed with FVOO.

The last mechanism investigated in the present study was related to the microbial generation of bioactive PC metabolites in the gut. It has been demonstrated that bioactivity of some microbial metabolites from undigested phenolic compounds is physiologically more relevant on CVD risk than the native form present in the diet. Some representative examples are enterolignans from lignans [54] and protocatechuic acid (PCA) from flavonoids [55]. PCA promotes reverse cholesterol transport in mice [56] and inhibits LDL oxidation [57], suggesting a remarkable antiatherogenic effect. These and other microbial phenolic metabolites with antioxidant activity, such as hydroxytyrosol, generated after gut microbial fermentation of phenolic compounds ingested with the oils, can be further absorbed and enter into the blood stream. In our study, faecal PCA increased in FVOOT compared to VOO, which could be due to the microbial transformation of PCA precursors, vanillic and *p*-hydroxybenzoic acids, present in FVOOT and absent in VOO. We found also increase in hydroxytyrosol after FVOO and FVOOT, which could be due to microbial transformation of OO secoiridoids and demonstrates a high stability of hydroxytyrosol in the gut, which is considered to have the highest antioxidant power compared to other olive polyphenols [58]. The increase in the levels of free hydroxytyrosol in faeces after FVOO and FVOOT ingestion confirms early observations suggesting that a fraction goes via faeces [59] and recent observations in a parallel in vivo trial [15]. In this regard, although the increase in hydroxytyrosol with FVOOT did not reach statistical significance, it could be behind, in combination with the increase in PCA, the decrease in LDL oxidation observed after the FVOOT intervention.

Although effects on gut microbiota and ox-LDL were observed with the combination of both sources of PC (olive oil and thyme, FVOOT), it is not clear the relevance of each PC source in the

results obtained. The lack of effects observed in FVOO would not necessary mean that these PC are not able to exert an effect on gut microbiota growth and metabolism. It would be probably due to the high amount used (500 ppm in FVOO compared to 250 ppm in FVOOT), which could exert an inhibitory effect on gut microbiota growth and metabolism. Furthermore, another advantage of the combination of both PC sources is that the pro-oxidant activity observed in with PC-rich foods containing unique PC sources could be eliminated with the use of complementary PC sources; a functional oil with complementary antioxidants (FVOOT), according to their structure/activity relationship, could be a suitable option to obtain PC's beneficial effects avoiding those harmful ones [60].

In conclusion, the ingestion of a PC-enriched virgin olive oil, containing a mixture of olive oil and thyme PC for 3 weeks, decreases blood ox-LDL in hypercholesterolemic humans. This cardio-protective effect could be mediated by the increases in populations of bifidobacteria together with increases in PC microbial metabolites with antioxidant activities such as protocatechuic acid and hydroxytyrosol. The specific growth stimulation of bifidobacteria in human gut suggests for the first time a potential prebiotic activity of an olive oil enriched in virgin olive oil and thyme PC.

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**ADDITIONAL INFORMATION**

**Table 1**. Compliance markers before (B) and after (A) each olive oil intervention[a]

| Compound | | VOO | FVOO | FVOOT | *P*[b] | | |
|---|---|---|---|---|---|---|---|
| | | | | | *VOO-FVOO* | *VOO-FVOOT* | *FVOO-FVOOT* |
| Hydroxytyrosol | B | 5.26±4.87 | 7.12±4.06 | 5.35±4.76 | | | |
| sulfate | A | 8.95±5.05 | 14.3±5.54* | 10.1±8.40* | 0.210 | 0.709 | 0.371 |
| Thymol sulfate | B | 141±144 | 72.5± 16 | 219±386 | | | |
| | A | 74.6±132 | 106±121 | 804±370* | 0.466 | <0.001 | <0.001 |

Values are given as adjusted means of µmol/24h ± SE; n=12 subjects
[a)] 25 mL/day extra virgin olive oil containing: VOO, 80 mg/kg of phenolic compounds (PC) from olive oil; FVOO, 500mg/kg of PC from olive oil; FVOOT, 250 mg/kg of PC from olive oil and 250 mg/kg from thyme.
[b)] *P* values for inter-interventions comparisons.
*P<0.05 for intra-intervention comparison

## **CHAPTER II**: POMEGRANATE

**PUBLICATION IV** Mosele et al.

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**PUBLICATION V** Mosele et al.

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**PUBLICATION IV** Application of in vitro gastrointestinal digestion and colonic fermentation models to pomegranate products (juice, pulp and peel extract) to study the stability and catabolism of phenolic compounds.

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# APPLICATION OF *IN VITRO* GASTROINTESTINAL DIGESTION AND COLONIC FERMENTATION MODELS TO POMEGRANATE PRODUCTS (JUICE, PULP AND PEEL EXTRACT) TO STUDY THE STABILITY AND CATABOLISM OF PHENOLIC COMPOUNDS

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**Abstract**

Pomegranate fruit contains a wide range of phenolic compounds that have been related to several health benefits. The stability of pomegranate phenols during digestion was tested by the application of *in vitro* gastrointestinal digestion (GID) to different pomegranate products: juice (PJ), pulp (PP) and peel extract (PE). The resulting non-absorbable fractions were submitted to *in vitro* colonic fermentation with human faeces to monitor the generation of microbial metabolites. During the duodenal step, we observed a low stability of anthocyanins and flavonoids and an important release of ellagic acid, especially after PE digestion. The poor potential absorption of the studied phenolic compounds led to their high exposure to colonic metabolism. After colonic fermentation, PE appeared to be the best source of microbial substrates leading to a larger generation of gut microbial catabolites in terms of absolute amounts. We suggest that using PE might be a good strategy to enrich food products with potential health benefits in the prevention of chronic diseases.

*Keywords: In vitro* gastrointestinal digestion *In vitro* fermentation Microbial metabolites Phenolic compounds Pomegranate

## 1. INTRODUCTION

The daily intake of fruits and vegetables is associated with a lower risk of cancer, cardiovascular diseases, metabolic syndrome and other degenerative diseases, as well as with the maintenance of optimal body functions (Boeing et al., 2012). Therefore, recent trends propose diet management as a useful strategy to reduce the incidence and progression of chronic diseases (Akbaraly et al., 2011). The pomegranate (*Punica granatum L.*) is an ancient and highly distinctive fruit which, in addition to its historical uses, is found in several medicinal systems for a variety of diseases. The high phenolic content of pomegranate when compared to other fruits, along with its anti-inflammatory and antiobesity properties (Viladomiu, Hontecillas, Lu, & Bassaganya-Riera, 2013), has increased the interest in investigating its potential nutraceutical and functional food applications Ellagitannins, also known as hydrolyzable tannins, correspond to the main pomegranate phenolic fraction (Fischer, Carle, & Kammerer, 2011; Madrigal-Carballo,

Rodriguez, Krueger, Dreher, & Reed, 2009), and an important number of studies have focused on their metabolism, absorption and excretion (Espín et al., 2007; García-Villalba, Beltrán, Espín, Selma, & Tomás-Barberán, 2013; González-Barrio, Borges, Mullen, & Crozier, 2010; Tomás-Barberán, García-Villalba, González-Sarrías, Selma, & Espín, 2014). These studies indicate that hydrolyzable tannins and ellagic acid are poorly absorbed, and therefore they reach the large intestine, where they are largely metabolized by local microbiota. The resulting catabolites have been identified as urolithins, considered as microbial metabolites with potential bioactivity. Other types of phenolic compounds present in pomegranate are flavonoids, such as anthocyanins and flavan-3-ols, phenolic acids and lignans (Mena et al., 2012), which may also contribute to the beneficial properties of pomegranate. However, little is known about the digestion stability and metabolism of these minor phenolic compounds of pomegranate (Pérez-Vicente, Gil-Izquierdo, & García-Viguera, 2002), suggesting that a large percentage of this phenolic fraction also reaches the colon and is submitted to microbial transformations.

Polyphenol-rich foods have gained a key position in the attempt to include new food ingredients that offer potential therapeutic properties. Health claims associated with pomegranate intake have stimulated the production of pomegranate-based products. Commercial juices have emerged as an alternative to fresh fruit consumption due to their practical use and close similarity in composition and sensory characteristics to the natural fruit. Pomegranate peel extract has also been subject of numerous studies and its potential use as a nutraceutical or functional food ingredient is well documented (Akhtar, Ismail, Fraternale, & Sestili, 2015). Faced with this variety of pomegranate-derived products, it is important to consider the different food matrices. Food matrix will determine the stability during gastric and intestinal digestion and will affect the intact proportion of phenolic compounds that reach the colon or that will potentially be absorbed.

In view of all the above, in the present study, an *in vitro* gastrointestinal digestion (GID) model was used to evaluate the stability and the potential bioaccessibility of phenols from pomegranate in three different pomegranate products: juice (PJ), pulp (PP) and peel extract (PE). The non-digested fraction resulting from *in vitro* digestion was then submitted to *in vitro* fermentation. The main objective was to determine the effect of different pomegranate products submitted to digestion on phenol recovery after GID and the nature of the catabolites formed after colonic fermentation. Results might be used in the development of future food formulations, representing a prior step in functional food development to select the most suitable delivery system

## 2. MATERIALS AND METHODS

### 2.1. Chemicals and reagents

Ultrapure water was obtained from a Milli-Q water purification system (Millipore Corp., Bedford, MA, USA). Alpha-amylase, pepsin, pancreatin, bile salts, $KH_2PO_4$ and $Na_2HPO_4{\cdot}2H_2O$ were obtained from Sigma Aldrich (St. Louis, MO, USA). Acetoni-trile, methanol, ethanol (all HPLC-grade), hydrochloric acid (37%), glacial acetic acid (99.8%), $NaHCO_3$, KCl, $CaCl_2$, $MgCl_2{\cdot}6H_2O$, $FeSO_4{\cdot}7H_2O$, $MnSO_4{\cdot}H_2O$, $ZnSO_4{\cdot}7H_2O$, $CoCl_2{\cdot}6H_2O$, $Mo(NH_4)_6O_{24}{\cdot}4H_2O$ were purchased from Pancreac Quimica S.A. (Barcelona, Spain) and $CuSO_4{\cdot}5H_2O$, NaCl, $Na_2SO_4{\cdot}10H_2O$ from Scharlau S.L. (Barcelona, Spain).

For the identification and quantification of pomegranate phenolic compounds, different commercial standards were used. Ellagic acid, punicalagin, *p*-coumaric acid, vanillic acid and kaempferol were purchased from Fluka (Buchs, Switzerland); protocatechuic acid, catechin, epicatechin, *p*-hydroxybenzoic acid, catechol, and ferulic acid from Sigma Aldrich; caffeic acid, rutin, myricetin, quercetin, cyanidin-3-*O*-glucoside and cyanidin-3,5-*O*-diglucoside from Extrasynthese (Genay, France). Gallic acid and malic acid were purchased from Panreac (Barcelona, Spain) and pinoresinol and secolariciresinol were purchased from Arbonova (Turku, Finland). Standards of urolithin A (dihydroxyurolithin), urolithin B (hydroxyurolithin) and urolithin C (trihydroxyurolithin) were kindly provided by Dr. Thasana from Chulabhorn Research Institute, Laksi, Bangkok, Thailand (Nealmongkol,

Tangdenpaisal, Sitthimonchai, Ruchirawat, & Thasana, 2013). These standard phenolic compounds were prepared in methanol

### 2.2. Pomegranate products preparation

The pomegranate fruits (Punica granatum *L*. Mollar de Elche cv.) (growing area of Alicante, Spain) were purchased from a local market in Lleida (Catalonia, Spain) in November 2013. Fruits were washed and manually peeled. The pomegranate juice (PJ) was prepared by blending the entire arils without the peel using a commercial blender with an incorporated filter (Molinex, France) in which pulp and intact seeds were retained. The pomegranate pulp (PP), which represented the whole pomegranate fruit intake, was prepared by blending the entire arils without any filtration. Thirty grams of fresh PJ and PP were aliquoted under nitrogen flux in dark bottles and immediately stored at −80 °C until the *in vitro* study. Part of PJ and PP were freeze-dried and maintained also at −80 °C for the chromatographic analysis. The residual peels resulting from the PJ and PP preparation were used to obtain the pomegranate peel extract (PE). Peels were dried (40 °C) overnight, reduced to small pieces and powdered using a domestic grinder (particle size <0.5 mm). The PE was obtained by accelerated solvent ex-traction ASE 100 (Dionex, Voisins le Bretonneaux, France). An aqueous solution containing 50% ethanol (50:50, v/v) was selected as extraction solvent after optimizing the extraction conditions. The resulting extract was rotary-evaporated to eliminate the ethanol, freeze-dried and stored in dark bottles at −80 °C for two weeks.

### 2.3. Simulated in vitro gastrointestinal digestion (GID)

The *in vitro* digestion procedure was adapted from the method proposed by Ortega, Reguant, Romero, Macià, and Motilva (2009), which consisted of three-steps to mimic the digestive process in the mouth, stomach (gastric digestion) and small intestine (dynamic duodenal digestion) with a slight variation in the time course of the gastric incubation (1 h in the present study) (**Fig. 1**). Briefly, the method consisted of a continuous-flow dialysis system performed with a dialysis tube through which the duodenal mixture flowed by using a peristaltic pump with a flow rate of 1 mL/min and a phosphate buffer solution (pH 7.4) covering the dialysis tub. Distilled water was pumped from a bath through the water jacket to keep the system's temperature around 37 °C. After the dynamic duodenal digestion two fractions were collected, the "IN" and the "OUT" fractions. IN is considered as the non-absorbable fraction that reaches the colon, while the OUT represents the absorbable fraction. Each *in vitro* digestion was performed in the absence of light and in triplicate. Samples obtained from the different digestion steps were lyophilized and stored at −80 °C in dark bottles until the time of their chromatographic analysis.

To evaluate the effect of pomegranate product on the polyphenol stability during digestion, a percentage of variation (% var) was calculated for each phenol compound. This index provides the amount of each phenol (μmols) present in the complete digesta after gastric or duodenal (IN + OUT) digestions in relation to the amount (μmols) quantified per gram of pomegranate product submitted to *in vitro* digestion.

### 2.4. In vitro colonic fermentation by human microflora

Fresh faecal samples were collected from three healthy adults (25–35 years) who declared no gastrointestinal diseases and no intake of antibiotics at least 3 months before the beginning of the study. Faeces were collected 2 h maximum from defecation and maintained at 4 °C in anaerobic conditions until the moment of the experiment. The fermentation medium was a carbonate–phosphate buffer and was prepared according to Durand, Dumay, Beaumatin, and Morel (1988). The medium was adjusted to pH 6.5 using HCl and reduced in an anaerobic chamber for 48 h prior to the fermentation. A 0.5:10 (w/v) dilution of the faecal samples with the anaerobic buffer was prepared and homogenized in a stomacher (1 min). The resulting faecal suspension was filtered, distributed in disposable tubes (10 mL/tube/incubation time) and 0.1 g of the IN fraction collected after the duodenal

digestion of PJ, PP and PE were added. The mixtures were fermented at 37 °C with continuous shaking (60 rpm) under anaerobic conditions. In parallel, two different controls were conducted under the applied conditions: (i) the IN fraction was incubated in buffer solution without faeces to determinate possible chemical degradation, and (ii) the faecal suspension was incubated without the IN fraction as a negative control. All incubations were performed in triplicate and both the samples and the controls were collected at 0, 2, 8, 24, 48 and 72 h. The tubes obtained at each time of fermentation were freeze-dried and stored at −80 °C until their analysis (**Fig. 1**).

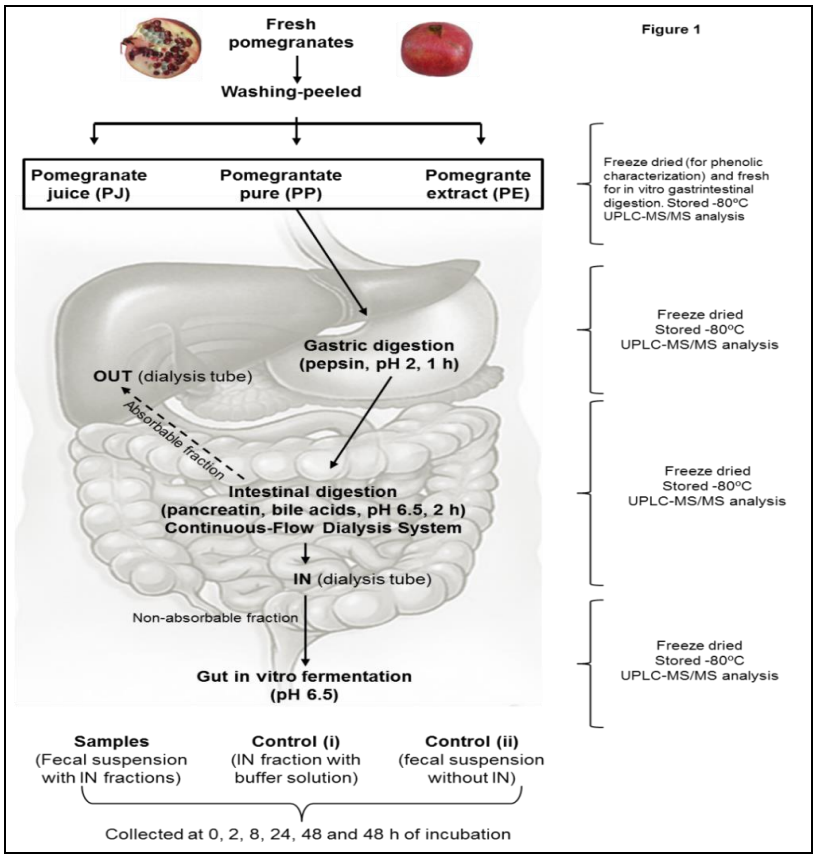



**Figure 1** – Graphic representation of the *in vitro* procedure carried out with the different pomegranate products (PJ: juice, PP: pulp and PE: peel extract)

### 2.5. Sample preparation

Freeze-dried PJ, PP and PE (0.5 g) were mixed with 8 mL of methanol/HCl/water (79.9:0.1:20, v/v/v), shaken during 30 min, centrifuged (13,200 *g*, 10 min, room temperature), filtered (0.22 µm pore size) and dispensed in chromatographic vials. For the analysis of digestion and fermentation samples, freeze-dried samples were homogenized in 10 volumes of methanol/HCl/water (79.9:0.1:20, v/v/v) following the same conditions as the pomegranate products.

### 2.6. Chromatographic analysis

The analysis of the phenolic fraction of PJ, PP, PE, the digestion and fermentation products were performed by using a Waters Acquity Ultra-Performance™ liquid chromatography system (Waters, Milford, MA, USA), equipped with a

binary pump system (Waters). The chromatographic column was an Acquity BEH C18 (100 mm × 2.1 mm i.d.) with a 1.7 mm particle size (Waters). A binary mobile phase with a gradient elution was used. For the analysis of anthocyanins, eluent A was Milli-Q water/acetic acid (90:10, v/v) and eluent B was acetonitrile. The gradient was performed as follows: 0–10 min, 3%B; 10–10.1 min, 3–25%B; 10.1–11 min, 25–80%B; 11–11.1 min, 80–80%B; 11.1– 12.5 min, 80–3%B. In the case of the other phenolic compounds, the eluent A was Milli-Q water/acetic acid (99.8:0.2, v/v) and eluent B was acetonitrile. The gradient was performed as follows: 0–5 min, 5%B; 5–10 min, 5–10%B; 10–18 min, 10–12.4%B; 18–23 min, 12.4–28%B; 18–23 min, 28–70%B; 23–25 min, 70%B; 25–27 min, 70–5%B and 27–30 min, 5%B. For the analy-sis of all the phenolic compounds, the flow rate was 0.4 mL/min and the injection volume was 2.5 µL. The tandem mass spectrometry (MS/MS) analysis was carried out on a triple quadrupole detector (TQD) mass spectrometer (Waters) equipped with a Z-spray electrospray interface. The analysis was done in the positive ion mode for anthocyanins and urolithins and in the negative ion mode for the rest of phenolic compounds and the data were acquired with the selected reaction monitoring mode (SRM). The MS/MS parameters were as follows: capillary voltage, 3 kV; source temperature, 150 °C; cone gas flow rate, 80 L/h and desolvatation gas flow rate, 800 L/h; desolvatation temperature, 400 °C Nitrogen (>99% purity) and argon (99% purity) were used as nebulizing and collision gases, respectively. Cone voltages and collision energies were optimized for each analyte by injection of each standard compound in a mixture of acetonitrile/water (50:50, v/v) at a concentration of mg/L. Two transitions were studied for each compound. The most abundant transition was used for quantification, while the second most abundant was used for confirmation purposes. The dwell time established for each transition was 30 ms. Data acquisition was carried out with the MassLynux v 4.1 Soft-ware (**Supplementary Table S1** shows the SRM transition, cone voltage and energy collision used for quantification. In this table it is also specified the standards used for quantification). So, malic acid, *p*-hydroxybenzoic acid, protocatechuic acid, *p*-coumaric acid, vanillic acid, gallic acid, caffeic acid, ferulic acid, quercetin, kaempferol, catechin epicatechin, ellagic acid, myricetin, pinoresinol, secolariciresinol, dimer $B_2$, rutin, punicalagin, cyanidin-3-*O*-glucoside and cyanidin-3,5-*O*-diglucoside were quantified using their own calibration curve. The phenol-glycoside compounds were tentatively quantified using the calibration curves of their respective aglycones. Syringaldehyde was tentatively quantified with the calibration curve of gallic acid. Syringic and chlorogenic acid were tentatively quantified as caffeic acid equivalents. Pinocembrin, naringenin, phloretin and eriodyctiol were tentatively quantified as quercetin equivalents. Ellagitannins were tentatively quantified as ellagic acid equivalents and the anthocyanins glucoside and diglucoside were tentatively quantified as cyanidin-3-*O*-glucoside equivalents and cyanidin-3,5-*O*-diglucoside equivalents, respectively. Urolithins tetrahydroxyurolithin and pentahydroxyurolithins were tentatively quantified as trihydroxyurolithin equivalents (Data regarding LOD and LOQ of each compound are available in **Supplementary Table S2).**

## 3. RESULTS AND DISCUSSION

### 3.1. Phenolic composition of pomegranate products

Phenolic composition of PJ, PP and PE is presented in **Table 1**. Data are expressed as µmols of phenolic compound per gram of lyophilized pomegranate product submitted to *in vitro* digestion. PE presented the highest total phenolic concentration (155 ± 21.4 µmol/g of PE) ahead of PJ and PP, which presented similar concentrations (1.41 ± 0.43 and 1.17 ± 0.08 µmol/g of PJ and PP, respectively). Ellagitannins and free and glycosylated ellagic acid were the main phenolic compounds in the three pomegranate products studied, followed by anthocyanins in PJ or flavonoids in the case of PP and PE. Differences in the phenolic composition between PP and PJ could be attributed to the manufacturing process of both

products. In this respect, the presence of pulp and seeds in PP could be associated with the presence of a higher concentration of certain phenolic compounds.

### *3.2.* Stability of pomegranate phenolic compounds during in vitro gastric step

In order to test the stability of each individual phenol during gastric digestion, their concentration was measured after digestion, expressing the results as µmols of each phenolic compound per gram of pomegranate product and as percentage of variation (% var) between the initial phenolic concentration and the concentration detected after digestion (**Table 1**).

**Table 1** – Pomegranate juice (PJ), pomegranate pulp (PP) and pomegranate extract (PE) initial phenolic composition and their modification duodenal steps. Values are expressed as µmol of phenolic compound present per g of PJ, PP and PE. (% var) represents the difference bet original product and after the gastric digestion and between the moles present in the original product and the sum of phenolic compound fractions (expressed as percentage).

| | Phenolic compound group | Concentration (µmol/g) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | PJ | | | PP | | | PE | | | |
| COMPOSITION | Free and glycosylated ellagic acid | 1.41 ± 0.43 | | | 1.17 ± 0.08 | | | 155 ± 21.4 | | | |
| | Ellagitannins | 3.23 ± 0.12 | | | 2.07 ± 0.41 | | | 282 ± 34.6 | | | |
| | Free and glycosilated gallic acid | 0.07 ± 0.01 | | | 0.16 ± 0.01 | | | 3.12 ± 0.25 | | | |
| | Anthocyanins | 0.43 ± 0.01 | | | 0.28 ± 0.01 | | | 0.11 ± 0.01 | | | |
| | Phenolic acids | 0.23 ± 0.01 | | | 0.35 ± 0.02 | | | 2.05 ± 0.14 | | | |
| | Flavonoids | 0.17 ± 0.01 | | | 0.79 ± 0.01 | | | 29.4 ± 4.21 | | | |
| | Lignans | 0.03 ± 0.00 | | | 0.04 ± 0.00 | | | 0.23 ± 0.01 | | | |
| | *Total* | 5.57 ± 0.54 | | | 4.86 ± 0.85 | | | 472 ± 46.3 | | | |
| | | | % var | | | % var | | | % var | | |
| GASTRIC DIGESTION | Free and glycosylated ellagic acid | 1.71 ± 0.45 | +21.7 | | 1.51 ± 0.26 | +29.1 | | 265 ± 29.3 | +71.0 | | |
| | Ellagitannins | 2.87 ± 0.10 | −11.1 | | 1.90 ± 0.55 | −7.97 | | 269 ± 32.9 | −4.71 | | |
| | Free and glycosylated gallic acid | 0.05 ± 0.01 | −24.4 | | 0.06 ± 0.01 | −16.7 | | 2.59 ± 0.25 | −17.1 | | |
| | Anthocyanins | 0.45 ± 0.01 | +4.39 | | 0.28 ± 0.01 | +1.75 | | 0.11 ± 0.01 | −6.31 | | |
| | Phenolic acids | 0.21 ± 0.01 | −9.08 | | 0.31 ± 0.01 | −11.3 | | 1.69 ± 0.25 | −17.3 | | |
| | Flavonoids | 0.14 ± 0.01 | −13.8 | | 0.66 ± 0.01 | −16.4 | | 21.5 ± 2.85 | −26.7 | | |
| | Lignans | 0.03 ± 0.00 | −5.53 | | 0.04 ± 0.00 | −11.9 | | 0.21 ± 0.01 | −10.5 | | |
| | Total | 5.46 ± 0.52 | −1.69 | | 4.75 ± 0.85 | −0.13 | | 560 ± 62.4 | +18.6 | | |
| | | Concentration (µmol/g) | | | | | | | | | |
| | | PJ | | | PP | | | PE | | | |
| DUODENAL DIGETION | Phenolic compounds group | OUT | IN | % var (IN + OUT) | OUT | IN | % var (IN + OUT) | OUT | IN | % var (IN + OUT) | |
| | Free and glycosylated ellagic acid | 0.07 ± 0.04 | 2.56 ± 0.24 | +86.6 | 0.04 ± 0.01 | 2.30 ± 0.35 | +100 | 21.9±2.91 | 553±80 | +271 | |
| | Ellagitannins | n.d. | n.d. | −100 | n.d. | 0.31 ± 0.04 | −84.9 | 14.9±2.72 | 38.2±4.71 | -81.2 | |
| | Free and glycosylated gallic acid | 0.01 ± 0.00 | 0.05 ± 0.01 | −5.39 | 0.02 ± 0.00 | 0.07 ± 0.00 | +29.3 | 1.31±0.17 | 2.71±0.38 | -28.7 | |
| | Anthocyanins | 0.04 ± 0.00 | 0.09 ± 0.01 | −70.0 | 0.03 ± 0.00 | 0.05 ± 0.01 | −69.5 | 0.01±0.00 | 0.07±0.01 | -28.1 | |
| | Phenolic acids | 0.11 ± 0.01 | 0.18 ± 0.01 | +28.6 | 0.13 ± 0.00 | 0.28 ± 0.01 | +18.1 | 0.39±0.01 | 1.09±0.01 | -27.6 | |
| | Flavonoids | 0.01 ± 0.00 | 0.06 ± 0.00 | −60.9 | 0.01 ± 0.00 | 0.19 ± 0.01 | −76.3 | 5.21±1.83 | 6.80±1.83 | -59.1 | |
| | Lignans | n.d. | 0.03 ± 0.00 | +6.78 | n.d. | 0.05 ± 0.00 | +7.98 | 0.01±0.00 | 0.35±0.04 | +59.4 | |
| | Total | 0.24 ± 0.04 | 2.97 ± 0.24 | -42.2 | 0.22 ± 0.01 | 3.25 ± 0.36 | -27.2 | 43.7±5.64 | 602±105 | +36.9 | |

n.d.: not detected (below LOD. Data regarding LOD of each phenolic compound are available in Supplementary Table S2)

To a greater or lesser extent, all the individual pomegranate phenolic groups were modified after acidic incubation. Recovery of ellagic acid and its glycosides after gastric digestion varied depending on the type of pomegranate product, observing a greater release in the case of PE (+71.0%) compared to PJ (+21.7%) and PP (+29.1%). Parallel to this generation of free and glycosylated ellagic acid, we observed a decrease in the ellagitannin concentration. However, the reduction of ellagitannins (% var) did not correspond to the ellagic acid generation. Considering our results and the low degree of ellagitannin hydrolysis at pH 3 observed by Tuominen and Sundman (2013), the extra amount of free and glycosylated ellagic acid recovered could be explained by the sum of both: ellagitannins degradation and the release of ellagic acid from the food matrix during *in vitro* gastric digestion. Accordingly, higher recovery of total phenolic compounds was observed after gastric digestion of pomegranate extract (Sengul, Surek, & Nilufer-Erdil, 2014). In another study, a slight modification in total phenolic content was noticed after the acidic incubation of a wild chokeberry commercial juice (Bermúdez-Soto, Tomás-Barberaán, & García-Conesa, 2007).

After gastric digestion, anthocyanins were slightly increased in PJ (+4.39 %) and PP (+1.75 %), whereas in the case of PE they were slightly reduced (−6.31 %) (**Table 1**). Accordingly, previous studies reported that anthocyanins from red cabbage and wild blueberry juice were stable under acidic gastric conditions (Correa-Betanzo et al., 2014; McDougall, Fyffe, Dobson, & Stewart, 2007), which was attributed to the low pH that would favour the stability of these molecules (Mazza & Brouillard, 1978). With regards to phenolic acids, flavonoids and lignans, all of them showed a slight decrease in their concentration after the gastric digestion of the three pomegranate products, varying from −5.53 to −26.7 % (**Table 1**). In relation to these compounds, previous studies have noticed low stability of phenolic acids under gastric incubation (Kamiloglu & Capanoglu, 2013; Vallejo, Gil-Izquierdo, Paez-Vicente, & Gasrcía-Viguera, 2004), while flavonoids (Bouayed, Deußer, Hoffmann, & Bohn, 2012; Vallejo et al., 2004) and lignans (Eeckhaut et al., 2008) were not affected under the same conditions. These discrepancies might be related to different chemical structures of the phenolic compounds studied, the food matrix characteristics or the *in vitro* conditions of digestion. Vallejo et al. (2004), for example, noticed different degrees of acidic degradation depending on the phenolic acid type after gastric digestion of broccoli inflorescence.

### 3.5. Stability of pomegranate phenolic compounds during duodenal in vitro digestion step

Results from the duodenal step are shown in **Table 1**. Under the intestinal conditions, an important decrease in the total phenolic content was observed in PJ (−42.2 %) and PP (−27.2 %). In contrast, after PE digestion the total phenolic content increased by 36.9%, mainly due to the large amounts of ellagic acid released (+271 %). In previous studies, generation of ellagic acid from hydrolysis of ellagitannins was also observed in the lumen of pigs fed with oak acorns (Espín et al., 2007).

Accordingly, almost double amounts of ellagic acid were detected after a middle alkaline incubation of dried pomegranate peel extract during 3 h (Liu, Li, Hu, & Zhao, 2013). Punicalagin (a pomegranate ellagitannin) was quickly hydrolyzed, yielding ellagic acid in a cell culture medium both in absence and in presence of Caco-2 cells, supporting that alkaline pH promotes ellagitannin breakdown (Larrosa, Tomás-Barberán, & Espín, 2006). The major increase in the concentration of ellagic acid after the gastrointestinal incubation of PE observed in the present study may be linked to its liberation from the complex phenols present in the food matrix. Unlike the juice and pulp, PE appears to be a more complex matrix that might contain greater amounts of non-extractable ellagitannins linked to cell wall constituents (polysaccharides and protein) or trapped within the food matrix. This non-extractable phenolic fraction cannot be detected in the original test foods with the common extraction procedures with organic solvents (Saura-Calixto, 2012), and although they are mainly hydrolyzed in the colon, some

ellagitannins could have also been hydrolyzed during digestion under acidic (gastric phase) and alkaline (duodenal phase) conditions resulting in the concentration increase of ellagic acid in the digesta. These results suggest that pomegranate peel could be considered as a source of great interest to obtain pomegranate phenolic extracts.

Together with the release of ellagic acid during intestinal digestion, an elevated liberation of gallic acid was also expected, as it is also part of the ellagitannin structure. However, no important increase of gallic acid was observed after digestion (**Table 1**), which could be explained by the reported instability of gallic acid under alkaline conditions (Liu et al., 2013) or the possible generation of ellagic acid from monogalloyl derivatives (Tuominen & Sundman, 2013). In contrast to the gastric step, anthocyanins underwent an important decrease after duodenal digestion, being less intense than the reduction in PE (**Table 1**). Accordingly, different degrees of instability of anthocyanins under alkaline conditions were previously described in red cabbage and wild blueberry pulp (Correa-Betanzo et al., 2014; McDougall et al., 2007).

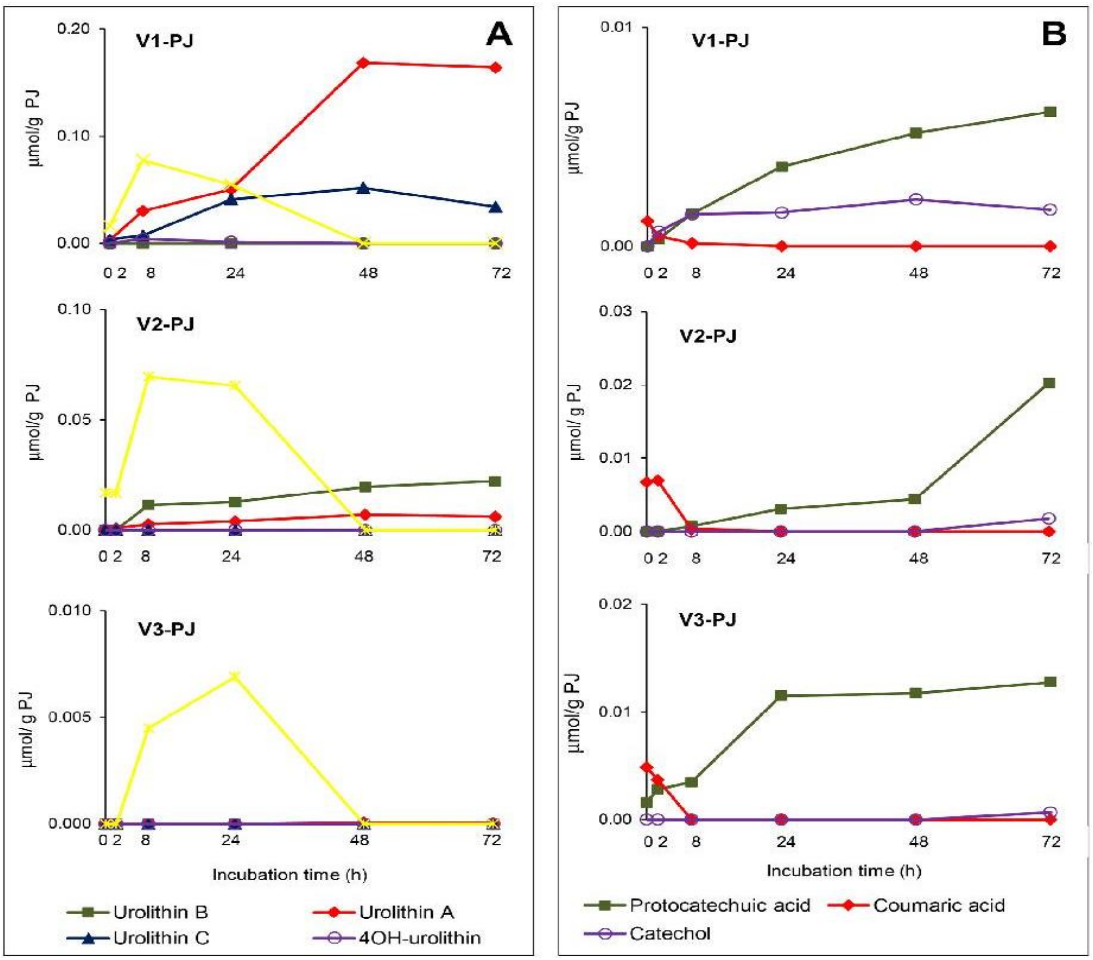


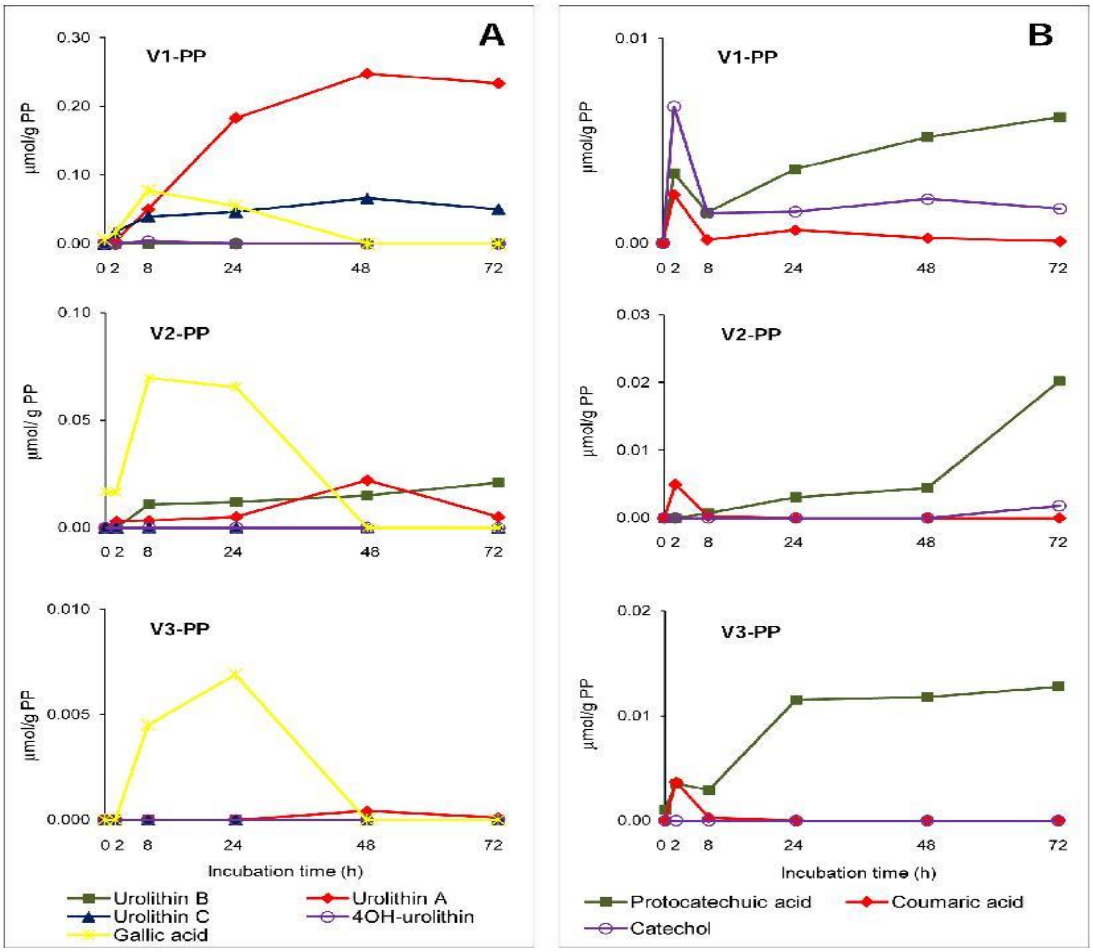

**Figure. 2** – Microbial metabolites generation during the *in vitro* fermentation of non-digested fraction of PJ (pomegranate juice). (A) Time course of urolithins and gallic acid generation and (B) time course of catechol, coumaric acid and protocatechuic acid generation

The higher stability of anthocyanins when PE was digested could be explained by the food matrix composition, which could play a protective role as some components of the peel, such as soluble and insoluble fibre, could interact with anthocyanins during the digestion process, increasing their stability. In a previous study, a higher recovery of anthocyanins was observed in whole fruits in relation to liquid products, indicating the importance of the food matrix in their stability during digestion (Parada & Aguilera, 2007). The decreasing trend in the concentration of flavonoids after the duodenal step was in accordance with Vallejo et al. (2004), who also detected important losses of these compounds after a simulated in vitro intestinal digestion of broccoli inflorescence.
On the other hand, a greater recovery of lignans was observed specially in the PE, which was also reported after in vitro gastrointestinal digestion of a flax seed complex (Eeckhaut et al., 2008). Regarding the absorbable fraction (OUT fraction in the dialysis tube), in general our results revealed that the concentration of pomegranate phenolics detected was low (**Table 1**) compared with the initial amounts presented in the pomegranate products. These results indicate a low potential bioavailability of pomegranate phenolic compounds, which are in accordance with previous in vivo studies. A study by Espín et al. (2007) with Iberian pigs after a continued intake of ellagitannin rich-food (fresh acorns from Quercus ilex and Quercus suber) showed that under in vivo conditions ellagic acid is released from ellagitannins and then ellagic acid is gradually metabolized in the intestine, and finally transformed into urolithins, with no detection of ellagitannins in plasma and urine. In another study in which healthy humans consumed 300 g of raspberries, low concentrations of anthocyanins and ellagic acid were detected in urine from 0 to 7 h after ingestion, indicating low absorption rate of these compounds (González-Barrio et al., 2010). of ellagic acid and ellagitannins (Cerdá, Periago, Espín, & Tomás-Barberán, 2005; García-Villalba et al., 2013), in our study urolithins were not detected in controls (i) and (ii) (Section 2.4), indicating their specificity as microbial metabolites. The efficiency of the faecal microbiota from the three volunteers to generate urolithins from ellagic acid and ellagitannins after the *in vitro* fermentation of PJ (**Fig. 2A**) and PP (**Fig. 3A**) was highly variable; however, these quantitative differences were reduced after PE incubation (**Fig. 4A**). Volunteer 1 was the most effective urolithin producer, reaching maximum concentrations of urolithin A (dihydroxyurolithin) after fermentation of the IN fraction of PJ (0.17 μmols/g PJ) (numerical data are available in **Supplementary Table S3**) and PP (0.25 μmols/g PP) (numerical data are available in **Supplementary Table S4**). Differences between volunteers were minimized after fermentation of the IN fraction of PE, and in this case the concentrations of urolithin A were from 10 to 15 μmols/g PE (72 h) (**Fig. 4A**) (numerical data are available in **Supplementary Table S5**).

### 3.4. In vitro colonic fermentation of pomegranate phenolic compounds

After *in vitro* digestion of the three pomegranate products, the respective IN fractions (non-absorbable) were submitted to *in vitro* colonic fermentation using human faeces inoculum from three healthy volunteers. The generation of phenolic catabolites was monitored at different incubation times: 0, 2, 8, 24, 48 and 72 h (**Fig. 1**). After analyzing the fermentation media, a wide range of phenolic catabolites was detected. Because of the quantitative and qualitative differences observed among volunteers, the microbial metabolite generation during the *in vitro* fermentation of non-digested fraction of PJ (**Fig. 2**) (numerical data are available in **Supplementary Table S3**), PP (**Fig. 3**) (numerical data are available in **Supplementary Table S4**) and PE (**Fig. 4**) (numerical data are available in **Supplementary Table S5**) was presented individually for each volunteer. In line with other studies that investigated the microbial conversion

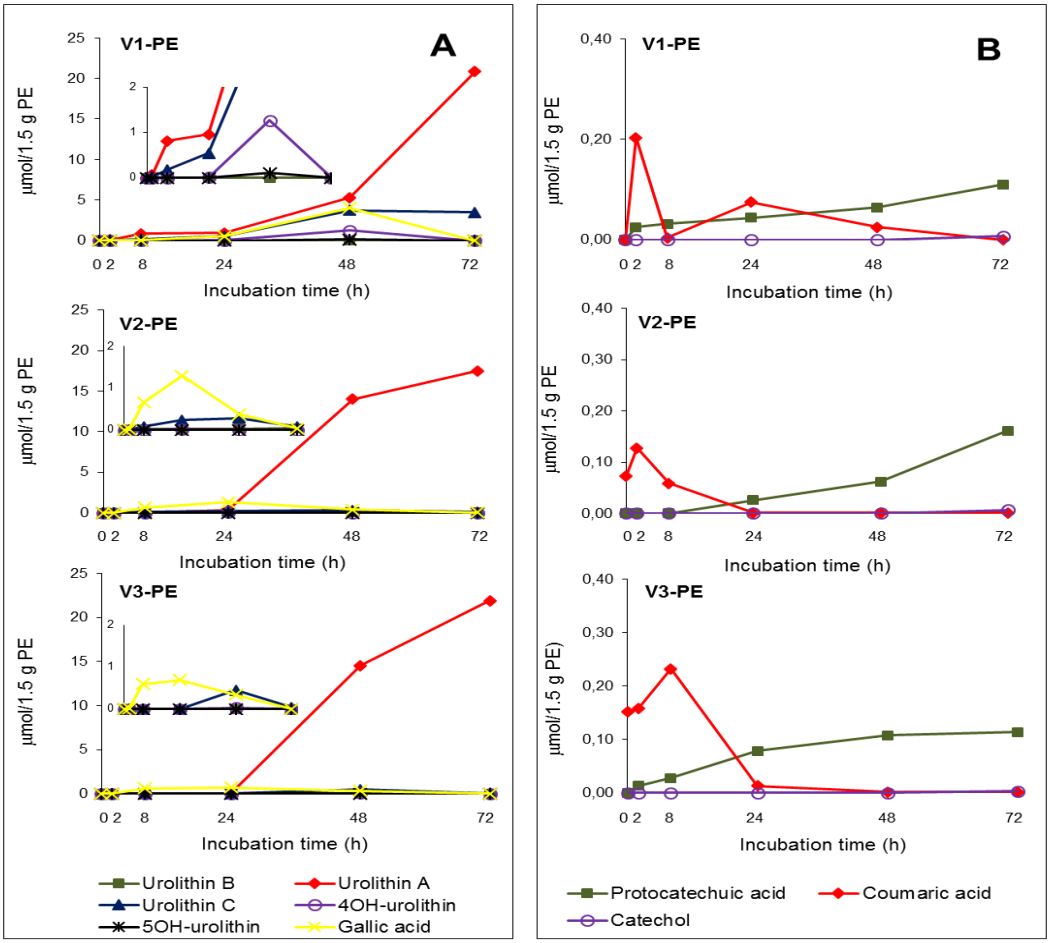
**Figure. 3** – Microbial metabolites generation during the *in vitro* fermentation of non-digested fraction of PP (pomegranate pulp).(A) Time course of urolithins and gallic acid generation and (B) time course of catechol, coumaric acid and protocatechuic acid generation




Regarding other urolithins, urolithin B (hydroxyurolithin) was only produced by volunteer 2, being the main metabolite detected in PJ and PP faecal suspensions. On the other hand, tri-and tetrahydroxyurolithins were detected in faecal suspension of volunteer 1 after fermentation of the three pomegranate products and after PE fermentation in volunteers 2 and 3. Pentahydroxyurolithin was detected only in the faecal suspensions of volunteer 1 after fermentation of PE. These results confirm that colonic microflora is critically involved in the interindividual variability of the excretion of microbial metabolites (Tomás-Barberán et al., 2014). In the case of PE faecal suspensions (**Fig. 4A**), this variability was markedly lower, which could be due to the enhanced urolithin excretion by the low urolithin producer (volunteer 3). Another explanation could be the saturation caused by the higher microbial substrate or its products (urolithins) that could probably hamper the transformation of ellagic acid to urolithins. Previous studies have proposed the metabolic colonic pathway of ellagitannins and ellagic acids in which penta-, tetra- and trihydroxyurolithins were described as intermediate products of major final metabolite urolithins A and B (García-Villalba et al., 2013). In the present study, volunteer 3 produced very small amounts of urolithin A after the incubation of PJ (**Fig. 2A**) and PP (**Fig. 3A**) and no type of urolithin was detected until 48 h of fermentation, which suggests that, apart from

higher amounts of precursors, more time is needed to start the microbial conversion. Thus, the transit time might also be a crucial factor in determining the colonic metabolic profile, which varies considerably depending on individual characteristics (age, sex, health status) (McClements, Decker, & Park, 2009).

**Figure 4** – Microbial metabolites generation during the *in vitro* fermentation of non-digested fraction of PE (pomegranate peel extract). (A) Time course of urolithins and gallic acid generation and (B) time course of catechol, coumaric acid and protocatechuic acid generation




In relation to other pomegranate phenolic catabolites identified, gallic acid and catechol were not detected in controls (i) and (ii), indicating their specificity as microbial metabolites of pomegranate, similarly to urolithins. Although detected in control (i), coumaric and protocatechuic acid concentration increased after incubation. In contrast to the generation of urolithins, catechol, gallic acid, coumaric acid and protocatechuic acid production did not show considerable interindividual variability, which is probably related to the non-specific bacteria needed for the precursor's degradation, as required for the conversion of ellagic acid into urolithins (Selma, Beltrán, García-Villalba, Espín, & Tomás-Barberán, 2014).

Due to the simple structure of these phenolic acids and catechol, these catabolites could be formed by the degradation of multiple phenolic precursors (Dall'Asta et al., 2012). Indeed, one could be a product of the other (Cueva et al., 2013; González-Barrio, Edwards, & Crozier, 2011). Despite this aparent complex scenario, evidence exists that may help to determine their origin. Gallic acid could be generated by the hydrolysis of ellagitannins (Liu et al., 2013), but also by the breakdown of glycosylated gallic acid present in the IN fraction of pomegranate products. The gradual increase in its concentration observed during the first period of incubation (0 to 48 h) of the three pomegranate products studied could indicate the hydrolysis of ellagitannins and the liberation of gallic acid from the food matrix to the fermentation media as a consequence of the microbial activity (Saura-Calixto et al., 2010). The microbial dehydroxylation of gallic acid might be the explanation for protocatechuic acid generation, although this phenolic metabolite was also proposed as a product of anthocyanins (González-Barrio et al., 2011) and flavonoid degradation (Sánchez-Patán, Monagas, Moreno-Arribas, & Bartolomé, 2011). In addition, catechol has previously been suggested as a product of protocatechuic acid bacteria metabolism (González-Barrio et al., 2011). In regard to coumaric acid, its generation could result from the early degradation of flavonoids (Sánchez-Patán et al., 2011) present in high concentration in the IN fractions of PE (**Table 1**). Taking into account the initial concentration of anthocyanins and flavonoids in the IN fractions of the three pomegranate products (**Table 1**), the generation of higher amounts of gallic acid, catechol, coumaric acid and protocatechuic acid after PE fermentation could be attributed to the flavonoid metabolism while, in the case of PJ and PP, anthocyanins seem to be the main precursors for the generation of these phenolic metabolites

## 4. CONCLUSION

Results of the present study showed that differences in the stability of pomegranate phenolic compounds during *in vitro* digestion may not be only associated with the initial phenolic composition, but also with the food matrix. After the duodenal step, most of the pomegranate phenolic compounds remained in the IN fraction (non-absorbable), indicating a poor potential absorption and consequently a high exposure of these compounds to colonic metabolism. Results of *in vitro* colonic fermentation with human faeces inoculum revealed that urolithins (mainly urolithin A), and different simple phenols such as catechol, and gallic, coumaric and protocatechuic acids, were the main microbial metabolites after pomegranate products were submitted to *in vitro* digestion. Our results confirm that, depending on the colonic microflora of each individual, high or low ellagitannin metabolism can occur and, thus, a high or low amount of urolithin derivatives can be formed. This should be taken into account in intervention studies with ellagitannins containing foodstuffs because, depending on the individual microflora, a different biological activity could be expected. Among the three pomegranate products, pomegranate peel extract (PE) appeared to be the best source of microbial substrates at colonic level, leading to a larger generation of microbial catabolites in terms of absolute amounts. Therefore, the use of pomegranate peel extracts obtained as a sub-product of the pomegranate juice industry could be a good strategy to enrich or fortify pomegranate products or other fruit-based products that would allow the enhancement of the pomegranate's therapeutic effect, especially for those subjects with a low capacity to produce urolithins.

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**ADDITIONAL INFORMATION**

**Table 1**. Microbial metabolites generation during PJ in vitro fermentation. Results are expresed in umol/1 g of PJ for each fermentation time (0, 2, 8, 24, 48 and 72 h)

| Compound | Volunteer | 0 h | 2 h | 8 h | 24 h | 48 h | 72 h |
|---|---|---|---|---|---|---|---|
| 5-OH urolithin | | | | | | | |
| | 1 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| | 2 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| | 3 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 4-OH urolithin | | | | | | | |
| | 1 | n.d. | tr | 0.01±0.00 | 0.06±0.01 | 0.08±0.01 | 0.05±0.01 |
| | 2 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| | 3 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 3-OH urolithin | | | | | | | |
| | 1 | n.d. | n.d. | tr | tr | n.d. | n.d. |
| | 2 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| | 3 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 2-OH urolithin | | | | | | | |
| | 1 | n.d. | tr | 0.05±0.00 | 0.07±0.00 | 0.25±0.04 | 0.25±0.06 |
| | 2 | n.d. | tr | tr | tr | 0.01±0.00 | 0.01±0.00 |
| | 3 | n.d. | n.d. | n.d. | n.d. | tr | tr |
| OH urolithin | | | | | | | |
| | 1 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| | 2 | n.d. | n.d. | 0.02±0.00 | 0.02±0.00 | 0.03±0.00 | 0.03±0.00 |
| | 3 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Gallic acid | | | | | | | |
| | 1 | 0.01±0.00 | 0.02±0.00 | 0.12±0.01 | 0.08±0.01 | n.d. | n.d. |
| | 2 | 0.02±0.00 | 0.02±0.00 | 0.10±0.01 | 0.10±0.01 | n.d. | n.d. |
| | 3 | n.d. | n.d. | 0.01±0.00 | 0.01±0.00 | n.d. | n.d. |
| Protocatechuic acid | | | | | | | |
| | 1 | n.d. | tr | tr | 0.01±0.00 | 0.01±0.00 | 0.01±0.00 |
| | 2 | n.d. | n.d. | tr | tr | 0.01±0.00 | 0.03±0.00 |
| | 3 | tr | tr | tr | 0.02±0.00 | 0.02±0.00 | 0.02±0.00 |
| Coumaric acid | | | | | | | |
| | 1 | tr | tr | tr | n.d. | n.d. | n.d. |
| | 2 | 0.01±0.00 | 0.01±0.00 | tr | n.d. | n.d. | n.d. |
| | 3 | 0.01±0.00 | 0.01±0.00 | n.d. | n.d. | n.d. | n.d. |
| Catechol | | | | | | | |
| | 1 | n.d. | tr | tr | tr | tr | tr |
| | 2 | n.d. | n.d. | n.d. | n.d. | n.d. | tr |
| | 3 | n.d. | n.d. | n.d. | n.d. | n.d. | tr |

Data are expressed as mean in µmol/L. Mean values were calculated subtracting to the average value of the three replicates the control content of the respective substance.
n.d.: not detected. Below the limit of detection
tr.: traces. Below the limit of quantification

**Table 2**. Microbial metabolites generation during PP in vitro fermentation. Results are expresed in umol/1 g of PP for each fermentation time (0, 2, 8, 24, 48 and 72 h)

| Compound | Volunteer | 0 h | 2 h | 8 h | 24 h | 48 h | 72 h |
|---|---|---|---|---|---|---|---|
| 5-OHurolithin | | | | | | | |
| | 1 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| | 2 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| | 3 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 4-OH urolithin | | | | | | | |
| | 1 | n.d. | n.d. | tr | tr | tr | n.d. |
| | 2 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| | 3 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 3-OH urolithin | | | | | | | |
| | 1 | n.d. | 0.03±0.00 | 0.06±0.00 | 0.07±0.01 | 0.1±0.01 | 0.08±0.01 |
| | 2 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| | 3 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 2-OH urolithin | | | | | | | |
| | 1 | n.d. | 0.01±0.00 | 0.07±0.00 | 0.27±0.00 | 0.37±0.04 | 0.35±0.06 |
| | 2 | n.d. | n.d. | n.d. | 0.01±0.00 | 0.03±0.00 | 0.01±0.00 |
| | 3 | n.d. | n.d. | n.d. | n.d. | tr | tr |
| OH urolithin | | | | | | | |
| | 1 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| | 2 | n.d. | n.d. | 0.02±0.00 | 0.02±0.00 | 0.02±0.00 | 0.03±0.00 |
| | 3 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Gallic acid | | | | | | | |
| | 1 | 0.01±0.00 | 0.02±0.00 | 0.12±0.01 | 0.10±0.01 | n.d. | n.d. |
| | 2 | 0.02±0.00 | 0.02±0.00 | 0.10±0.01 | 0.10±0.01 | n.d. | n.d. |
| | 3 | n.d. | n.d. | 0.01±0.00 | 0.01±0.00 | n.d. | n.d. |
| Protocatechuic acid | | | | | | | |
| | 1 | n.d. | tr | tr | 0.01±0.00 | 0.01±0.00 | 0.01±0.00 |
| | 2 | n.d. | n.d. | tr | tr | 0.01±0.00 | 0.03±0.00 |
| | 3 | tr | tr | tr | 0.02±0.00 | 0.02±0.00 | 0.02±0.00 |
| Coumaric acid | | | | | | | |
| | 1 | n.d. | tr | tr | tr | tr | tr |
| | 2 | n.d. | 0.01±0.00 | tr | n.d. | n.d. | n.d. |
| | 3 | tr | 0.01±0.00 | tr | n.d. | n.d. | n.d. |
| Catechol | | | | | | | |
| | 1 | n.d. | 0.01±0.00 | tr | tr | tr | tr |
| | 2 | n.d. | n.d. | n.d. | n.d. | n.d. | tr |
| | 3 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |

Data are expressed as mean in µmol/L. Mean values were calculated subtracting to the average value of the three replicates the control content of the respective substance.
n.d.: not detected. Below the limit of detection
tr.: traces. Below the limit of quantification

**Table 3**. Microbial metabolites generation during PE in vitro fermentation. Results are expresed in umol/1 g of PE for each fermentation time (0, 2, 8, 24, 48 and 72 h)

| Compound | Volunteer | 0 h | 2 h | 8 h | 24 h | 48 h | 72 h |
|---|---|---|---|---|---|---|---|
| 5-OH urolithin | | | | | | | |
| | 1 | n.d. | n.d. | n.d. | n.d. | 0.11±0.04 | n.d. |
| | 2 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| | 3 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 4-OH urolithin | | | | | | | |
| | 1 | n.d. | n.d. | n.d. | 0.01±0.00 | 1.26±0.46 | n.d. |
| | 2 | n.d. | n.d. | n.d. | 0.02±0.00 | n.d. | n.d. |
| | 3 | n.d. | n.d. | n.d. | n.d. | 0.03±0.00 | n.d. |
| 3-OH urolithin | | | | | | | |
| | 1 | n.d. | 0.02±0.00 | 0.18±0.01 | 0.54±0.02 | 3.74±0.35 | 3.45±0.29 |
| | 2 | n.d. | n.d. | 0.08±0.01 | 0.23±0.01 | 0.29±0.01 | 0.08±0.01 |
| | 3 | n.d. | n.d. | n.d. | 0.02±0.00 | 0.45±0.03 | 0.04±0.00 |
| 2-OH urolithin | | | | | | | |
| | 1 | n.d. | 0.07±0.00 | 0.82±0.08 | 0.96±0.09 | 5.32±0.5 | 20.84±0.68 |
| | 2 | n.d. | 0.01±0.00 | 0.04±0.00 | 0.31±0.04 | 14.01±1.50 | 17.51±0.82 |
| | 3 | n.d. | n.d. | n.d. | n.d. | 14.54±0.75 | 21.89±1.65 |
| OH urolithin | | | | | | | |
| | 1 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| | 2 | n.d. | n.d. | 0.04±0.00 | 0.11±0.01 | 0.33±0.02 | 0.12±0.01 |
| | 3 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Gallic acid | | | | | | | |
| | 1 | n.d. | 0.02±0.00 | 0.02±0.00 | 0.55±0.02 | 4.03±0.68 | n.d. |
| | 2 | n.d. | 0.01±0.00 | 0.66±0.01 | 1.30±0.05 | 0.37±0.02 | 0.04±0.00 |
| | 3 | n.d. | 0.01±0.00 | 0.60±0.04 | 0.70±0.04 | 0.35±0.03 | n.d. |
| Protocatechuic acid | | | | | | | |
| | 1 | n.d. | 0.03±0.00 | 0.03±0.00 | 0.04±0.00 | 0.06±0.00 | 0.11±0.01 |
| | 2 | n.d. | n.d. | n.d. | 0.03±0.00 | 0.06±0.00 | 0.16±0.01 |
| | 3 | n.d. | 0.01±0.00 | 0.03±0.00 | 0.08±0.00 | 0.11±0.01 | 0.11±0.01 |
| Coumaric acid | | | | | | | |
| | 1 | n.d. | 0.20 | n.d. | 0.08±0.00 | 0.03±0.00 | n.d. |
| | 2 | 0.07 | 0.13±0.01 | 0.06±0.00 | n.d. | n.d. | n.d. |
| | 3 | 0.15±0.01 | 0.16±0.01 | 0.23±0.02 | 0.01±0.00 | n.d. | n.d. |
| Catechol | | | | | | | |
| | 1 | n.d. | n.d. | n.d. | n.d. | n.d. | 0.01±0.00 |
| | 2 | n.d. | n.d. | n.d. | n.d. | n.d. | 0.01±0.00 |
| | 3 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |

Data are expressed as mean in µmol/L. Mean values were calculated subtracting to the average value of the three replicates the control content of the respective substance.
n.d.: not detected. Below the limit of detection
tr.: traces. Below the limit of quantification

**PUBLICATION V** Effect of daily intake of pomegranate juice on fecal microbiota and feces metabolites from healthy volunteers.

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# EFFECT OF DAILY INTAKE OF POMEGRANATE JUICE ON FECAL MICROBIOTA AND FECES METABOLITES FROM HEALTHY VOLUNTEERS

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**Scope:** The purpose of the study was to evaluate the effect, regarding the metabolic and microbial profile of feces, of diet supplementation of healthy adults with pomegranate juice (PJ).
**Methods and results:** Twelve healthy adults were recruited to the study, which consisted of the intake of 200 mL/day of PJ during 4 weeks. Feces were collected before and after the supplementation with PJ. Metabolites (phenolic catabolites, short-chain fatty acids, and fecal steroids) and microbial profile were analyzed at baseline and at 4 weeks. Fecal phenolic metabolites, 3-phenylpropionic acid, catechol, hydroxytyrosol, and urolithin A, showed a significant increase in their concentration after supplementation with PJ. Among fecal steroids, parallel to the significant increase of cholesterol concentration, a significant decrease of coprostanol was observed. Although no significant changes in the microbiota profile were observed, different relationships between initial microbiota and the metabolites produced were found. Catechol showed positive and negative correlation with *Oscillospora* and *Paraprevotella* genera, respectively, and 3-phenylpropionic acid was positively correlated with *Odoribacter* genus.
**Conclusion:** Inclusion of PJ in the diet did not significantly alter the gut microbiota composition in healthy adults, but the individual bacterial composition could contribute to the generation of potential health-promoting phenolic metabolites.

**Keywords:** Fecal steroids / Microbiota / Phenolic compounds / Pomegranate / Short-chain fatty acids

## 1. INTRODUCTION

Gut microbiota represents the largest microbial environment of the human organism and its composition and interplay with the host are closely connected to healthy conditions [1]
Intestinal microbiota markedly depends on diet and numerous nutritional interventions have been shown to selectively modify specific bacterial groups [2,3]. Although some studies have been conducted on the effect of dietetic fiber as prebiotic [3], little is known about the ability of other components of fresh fruit, such as phenolic compounds, which are also important contributors to modulate the gut microbiota composition. Some studies have revealed that gut microbiota is able to transform intact phenolic compounds into bioactive metabolites and the accumulation of these microbial metabolites may be a key factor to promote changes in the gut ecosystem, including modifications of microbial population [4–6]. In general, human studies evidence that dietary polyphenols may contribute to the maintenance of intestinal health by preserving the gut microbial balance through the stimulation of the growth of beneficial bacteria (i.e. *Lactobacilli* and *Bifidobacteria*) and the inhibition of pathogenic bacteria, exerting prebiotic-like effects [5, 7]. Therefore, the analysis of fecal composition not only provides valuable information regarding the unabsorbed diet components, but also clarifies whether the functional stability of the gut

ecosystem could undergo modifications after dietary interventions.

Apart from the study of the gut microbiota modulation through diet, recent studies have also revealed that fecal sterols might exert a much wider range of biological activities than initially recognized [8]. In this sense, it has been established that secondary metabolites of fecal sterols produced by the gut microbiota may promote health or favor disease development depending on the quantity and type produced. Specifically, increased concentrations of secondary bile acids (BAs) and cholesterol microbial metabolites in feces are involved in colorectal carcinogenesis [8, 9].

Therefore, it is interesting to study whether the fecal excretion of these metabolites can be modulated into a healthier profile through dietary intervention, as has already been demonstrated for phenolic compounds in rats [10]. On the other hand, the presence of other microbial products formed from non-digestible carbohydrates (dietary fiber), such as short chain fatty acids (SCFAs) [11], could serve as a protector factor of gastrointestinal disease, limiting the generation of pathogenic bacteria as well as promoters of the growth of beneficial bacteria [12], and maintain optimal bowel balance [11, 13].

Pomegranate is one of the most polyphenol-rich fruits with many associated health benefits such as antiinflammatory [14], anticarcinogenic [15] and cardioprotective effects [16] and are mainly attributed to its phenolic content [17]. Ellagitannins and anthocyanins are the most representative phenolic groups, followed by variable amounts of lignans, flavonoids, and phenolic acids [18, 19].

Most of these compounds are poorly absorbed in the small intestine and reach the large intestine, where they are subjected to catabolic transformations, being the urolithins derived from ellagic acid the most studied microbial phenolic metabolites [19, 20]. The microbial metabolites are thus likely to be responsible for the health benefits of pomegranate rather than the original compounds. However, to date no human studies have revealed whether pomegranate polyphenols could possess specific qualities to modulate the bacterial gut population and other steroid metabolites related to colonic health.

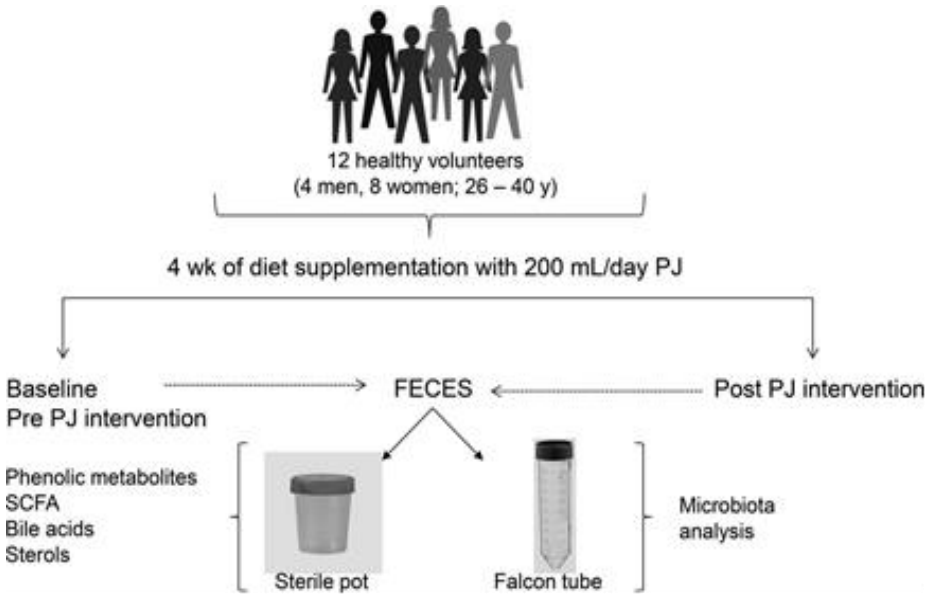



**Figure 1.** Graphic representation of the study design and sample collection

The purpose of the present study was to evaluate possible beneficial effects regarding the microbial and metabolic profile of feces from healthy adults after the addition of pomegranate juice (PJ; 200 mL/day) to the habitual diet during a period of 4 weeks.

Changes in the microbial phenolic metabolites, SCFAs, and fecal steroids were evaluated. The modulation of the intestinal microbial population after the intervention was also evaluated, studying the relationship between possible changes in microbial metabolite profile and microbial composition for each individual. To our knowledge, this is the first study that evaluates the microbiota profile of healthy volunteers and the phenol metabolic profile of human feces after a PJ intervention.

## 2. MATERIALS AND METHODS

### 2.1. Pomegranate juice

The pomegranate fruits (*Punica granatum* L. *Mollar de Elche* cv.; growing area of Alicante, Spain) were purchased from a local market in Lleida (Catalonia, Spain). Fruits were washed and manually peeled. The PJ was prepared by blending the entire arils without the peel using a commercial blender with an incorporated filter (Molinex, France) in which pulp and intact seeds were retained. The juice was immediately divided into daily doses of 200 mL in amber glass bottles and stored at −20°C, and on the day of consumption the juice was defrosted at 4°C. The phenolic composition analysis was carried out by using Ultra-performance LC MS/MS methodology on the basis of the protocol of Mosele et al. [19]. The average phenolic content in the daily intake of 200 mL of PJ was: 878.9 mg ellagic acid and ellagitannins, 41.5 phenolic acids and its derivates, 38.0 anthocyanins, 4.21 lignans, 3.39 flavonols, 1.19 flavan-3-ols, 0.50 flavanones, and minor phenols as syringaldehyde and phloretin glucoside; **Supporting Information Table 1**). Two hundred milliliters of PJ contributed approximately 968 mg/day of phenolic compounds to the habitual diet of the volunteers. Values correspond to the average concentration of different aliquots ($n$ = 5) collected over the 4 weeks of juice preparation

### 2.2. Study design

The diet intervention study was approved by the Ethical Committee of Clinical Research of Arnau Vilanova University Hospital, Lleida, Spain (Approval Number: CEIC-1326). Twelve healthy adults (4 men and 8 women, aged 26–40, BMI < 25 kg/m$^2$) were recruited to participate in the study. Inclusion criteria included healthy men and women aged between 26 and 40 years, not taking antibiotics at least 3 months before the study and reported to be in good health conditions before and during the study. After successful completion of screening, participants provided informed consent, including authorization to use their feces for analysis of microbiota and metabolites. Participants were instructed to maintain their regular diet, with the exception of probiotic consumption, in order to elucidate the gut metabolic profile and changes in microbiota population in a conventional diet complemented with the daily intake of PJ. They were also asked to complete a 3-day dietary record before and during the 4 weeks of PJ supplementation period which included 1 day of the weekend in order to consider all the dietary habits of volunteers. Mean total kilocalories of the volunteer diets and their composition in proteins, carbohydrates, and fat were estimated using Spanish food composition sheets (www.bedca.net), and the total intake of phenolic compounds was estimated using the information supplied by www.phenolexplored.eu. No significant differences were detected in energy, carbohydrate, protein, fat, and phenolic compound intake between baseline and the study period (see **Supporting Information Table 2**).

Before the intervention period with PJ, participants received detailed instructions to collect fecal samples and were provided with two different containers: a sterile pot and a falcon tube containing 10 mL of RNAlater® solution (Life Technologies). The volunteers were asked to transfer approximately 10 g of fresh feces from the sterile pot to the falcon tube immediately after defecation. Weekly, each volunteer received a box with seven bottles containing the daily PJ dose. During the intervention period, the volunteers consumed 200 mL/day of PJ with breakfast during

4 weeks. Each volunteer collected the feces sample in fasted state before (Pre; day 0) and after (Post; day 28) the intervention period. The same day of collection, fecal samples were lyophilized and stored at −80°C until the chromatographic analysis to determinate phenolic microbial metabolites, SCFAs, and fecal steroids (BAs and sterols). The feces preserved in falcon tubes, also stored at −80°C, were used for the analysis of microbiota composition

### 2.3. Phenolic quantification in feces

Lyophilized feces (0.1 g) were mixed in 1 mL of MeOH/HCl/$H_2O$ (79.9:0.1:20, v/v/v) and centrifuged (8784 × *g*, 5 min, 4°C) after 15 min of shaking. The supernatant was collected and re-centrifuged under the same conditions. The resulting supernatant was filtered (0.22 µm filter pore size) and analyzed by ultra-high LC coupled to MS/MS (**Supporting Information Method).** All phenolic compounds present in the PJ and their potential catabolites formed by microbiota fermentation were studied. To avoid differences in the water content of feces, depending on the individual, the results are expressed in mg or ng of compound per gram of dry feces.

**Table 1.** Changes in the phenolic compounds and their metabolites in human feces before (Pre) and after (Post) the supplementation period (4 weeks) with pomegranate juice (200 mL/day)

| Compound | Pre | Post | *n* (%) |
|---|---|---|---|
| Urolithins (mg/g dry feces) | | | |
| Urolithin D (tetrahydroxy-urolithin) | n.d. | t.r. | 1 (8) |
| Urolithin C (trihydroxy-urolithin) | 0.07 ± 0.05 | 0.69 ± 0.31 | 6 (50) |
| Urolithin A (dihydroxy-urolithin) | 7.00 ± 6.74 | 35.9 ± 11.3* | 8 (67) |
| Isourolithin A (dihydroxy-urolithin) | n.d. | 0.57 ± 0.57 | 1 (8) |
| Urolithin B (monohydroxyl-urolithin) | 2.23 ± 2.02 | 9.47 ± 5.74 | 3 (23) |
| Anthocyanins (ng/g dry feces) | | | |
| Cyanidin-*O*-3,5-diglucoside | n.d. | 112 ± 87.5 | 3 (25) |
| Cyanidin-*O*-3-glucoside | 0.89 ± 0.89 | 27.5 ± 14.7 | 9 (70) |
| Cyanidin pentoside | n.d. | 4.35 ± 2.98 | 3 (25) |
| Phenolic acids (mg/g dry feces) | | | |
| Phenylpropionic acid | 5.80 ± 3.33 | 10.98 ± 3.69* | 8 (67) |
| Protocatechuic acid | 0.13 ± 0.03 | 0.18 ± 0.03 | 9 (75) |
| Other phenolic compounds (mg/g dry feces) | | | |
| Catechol | 0.02 ± 0.01 | 0.05 ± 0.01** | 10 (83) |
| Hydroxytyrosol | 0.06 ± 0.01 | 0.13 ± 0.03* | 10 (83) |
| Unidentified metabolites (mg/g dry feces) | | | |
| Unkown: $m/z^-$ 333 $MS^2$ 285, 259 | 1.25 ± 0.63 | 54.4 ± 16.8** | 12 (100) |
| Total phenolic metabolites (mg/g dry feces) | 16.6 ± 7.24 | 112 ± 19.1** | 12 (100) |

a) All values are means (*n* = 12) ± SE. Compared with corresponding Pre-value, **p* < 0.05, ***p* < 0.01. n.d., no detected; t.r., traces level.

*n* is number of volunteers who presented an increase in the concentration for each compound after pomegranate juice, and the percentage of the total 12 volunteers who presented an increase for each compound

### 2.4. Determination of SCFAs in feces

Lyophilized feces (0.1 g) were mixed with 1 mL of acidified aqueous solution (1% phosphoric acid) containing 4-methyl valeric acid (Sigma-Aldrich, St. Louis, MO, USA) as internal standard (IS, final concentration 500 µM). Samples were shaken for 15 min and centrifuged (10 min, 1800 × *g*, 4°C). Before filtration (0.22 µm pore size filter), the supernatants were centrifuged (4 min, 8784 × *g*, 4°C) once more.

The analysis of acetic, propionic, butyric, isobutyric, isovaleric, and valeric acids was performed by GC (Agilent 7890A Series) using a

capillary BP-21 column (30 m, 0.25 mm, 0.25 m; SGE, Cromlab SL, Barcelona, Spain), coupled to a flame ionization detector (FID). The column temperature was programed at 90°C, rising by 15°C/min until it reached 150°C, then 5°C/min to 170°C, and then 20°C/min to 240°C, and maintained 3 min (total run time 14.5 min). Helium was the carrier gas (1 mL/min). Injection was carried out with a split injector (1:100) at 220°C, detector temperature was 250°C, and 1 L of the solution was injected into the GC/FID system. Identification of the SCFAs was carried out according to the retention time of standard compounds (acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, and isovaleric acid; Sigma-Aldrich) and their quantification was determined with reference to the peak side of IS (4-methyl valeric acid; **Supporting Information Fig. 1**). All samples were analyzed in duplicate.

### 2.5. Analysis of fecal steroids: BAs and sterols

Silylation of sterols and BAs was carried out simultaneously. For this, 100 L of pyridine and 100 L of *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (both from Sigma-Aldrich) were added to a vial containing 10 mg of feces and two ISs (1000 µM of 5α-cholestane and 500 µM 5β-cholanic acid; Sigma-Aldrich) and then maintained during 30 min at 60°C. After silylation, samples were centrifuged 10 min at 8784 × *g* at room temperature. The supernatants were analyzed by GC system (Agilent 7890A) coupled to an FID. Samples were injected into a capillary column DB-1 (30 m × 0.25 mm × 0.25 i.d.) and the simultaneous quantification of sterols and BAs was performed as follows: the column temperature was set at 240–290°C (20°C/min) and maintained 2 min; 290–295°C (1°C/min) and 295–310°C (25°C/min) maintained during 5 min, total run time 16.1 min. The temperature of the injector was 280°C, the volume of injection was 1 L and the FID temperature was 200°C. Peak identification was based on comparison of retention times with reference compounds. All quantifications were performed using calibration curves generated from different know concentrations of commercial standards. For fecal sterols quantifications, commercial standards of 5α-cholestane (IS), cholesterol, coprostanol, cholestanol, and cholestanone acid (Sigma-Aldrich) were used. In the case of fecal BAs commercial standards of 5β-cholanic acid (IS), cholic acid, deoxycholic acid, chenodeoxycholic acid, and lithocholic acid (Sigma-Aldrich) were used (**Supporting Information Fig. 2**).

**Table 2.** Changes in the SCFAs composition in human feces before (Pre) and after (Post) the supplementation during 4 weeks with pomegranate juice (200 mL/day)[a)].

| | Concentration (mg/g dry feces) | | Molar ratio (% mol of total) | |
|---|---|---|---|---|
| SCFAs | Pre | Post | Pre | Post |
| Acetic acid | 11.98 ± 1.78 | 13.53 ± 1.70 | 57.48 ± 1.24 | 57.63 ± 1.80 |
| Propionic acid | 5.05 ± 0.82 | 4.94 ± 0.64 | 19.19 ± 1.47 | 17.15 ± 0.90 |
| Butyric acid | 5.05 ± 1.38 | 5.37 ± 1.01 | 15.31 ± 1.31 | 18.08 ± 0.96 |
| Isobutyric acid | 0.63 ± 0.09 | 0.68 ± 0.10 | 2.46 ± 0.29 | 2.14 ± 0.38 |
| Isovaleric acid | 1.05 ± 0.14 | 0.93 ± 0.19 | 3.37 ± 0.46 | 2.68 ± 0.60 |
| Valeric acid | 0.73 ± 0.12 | 0.82 ± 0.12 | 2.19 ± 0.25 | 2.35 ± 0.42 |
| Total | 24.49 ± 4.00 | 26.26 ± 3.17 | | |

a) All values are means ($n$ = 12) ± SE

### 2.6. Fecal microbiota composition analysis

#### 2.6.1. DNA purification, amplicons, and sequencing

Fecal samples stored in RNAlater® were treated following Vázquez et al. [21]. Briefly, the samples were diluted with 5 mL of PBS solution. To remove fecal debris, the samples were centrifuged

at 600 × *g* at 4°C for 2 min and then the supernatant was centrifuged at 25 364 × *g* for 5 min to pel-let the cells. Total DNA was extracted from bacterial pellet with QIAamp® DNA Stool Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. A region of the 16S rRNA gene was amplified by PCR with the universal primers E8F (5‐ -AGAGTTT GATCMTGGCTCAG-3‐) and 530R (5‐ -CCGCGGCKGCTGGCAC-3‐) using the sample-specific Multiplex Identifier (MID) for pyrosequencing, and attached to the 454 Roche adaptors. The amplified region comprises hyper-variable regions V1, V2, and V3. PCR was run under the following conditions: 95° for 2 min followed by 28 cycles of 95° for 30 s, 52° for 1 min and 72° for 1 min and a final extension step at 72° for 10 min. After purification using NucleoFast® 96 PCR Clean-Up Kit (Macherey-Nagel), the PCR products were pooled and directly pyrosequenced using a Roche GS FLX sequencer and Titanium chemistry in the Centre for Public Health Research (FISABIO-Salud Publica,´ Valencia, Spain). All sequences were deposited in the public European Nucleotide Archive server under accession number PRJEB7859.

**Table 3.** Changes in the bile acids (BA) and sterols composition in human feces before (Pre) and after (Post) the supple-mentation during 4 weeks with pomegranate juice (200 mL/day)

| Compound | Concentration (mg/g dry feces) | |
|---|---|---|
| | Pre | Post |
| Primary BA | | |
| Cholic acid | 0.11 ± 0.07 | 0.19 ± 0.13 |
| Chenodeoxycholic acid | 0.09 ± 0.03 | 0.15 ± 0.06 |
| Total | 0.20 ± 0.08 | 0.33 ± 0.18 |
| Secondary BA | | |
| Lithocholic acid | 1.54 ± 0.20 | 1.55 ± 0.21 |
| Desoxycholic acid | 1.99 ± 0.40 | 1.82 ± 0.35 |
| Total | 3.53 ± 0.57 | 3.37 ± 0.50 |
| Primary + secondary BA | 3.73 ± 0.61 | 3.70 ± 0.55 |
| Sterols | | ± |
| Cholesterol | 1.88 ± 0.53 | 5.60 ± 1.56* |
| Colestanol | 0.54 ± 0.12 | 0.51 ± 0.19 |
| Coprostanol | 20.33 ± 4.32 | 13.9 ± 4.12* |
| Total | 22.7 ± 4.32 | 19.8 ± 3.48 |

a) All values are means ($n$ = 12) ± SE; compared with correspond-ing Pre-value, *$p$ < 0.05

#### 2.6.2. Composition, biodiversity, and interactions of fecal microbiota

16S rRNA gene reads with low-quality score (<20 out of 40 quality units assigned by the 454) and short read lengths (<170 nt) were removed. Potential chimeras were also re-moved from the remaining sequences using uchime in Qiime v1.8 pipe line [22]. Usearch tool in Qiime software was used to denoise data and to create the operational taxonomic unit (OTU) clusters (97%). Taxonomic information of the 16S rDNA sequences was obtained by comparison with the Ribosomal Database Project-II [23], using the pick_otus_through_otu_table.py pipe line avail-able in QIIME v1.8.0 software. We considered only annotations that were obtained with a bootstrap value greater than 0.8, leaving the assignation at the last well-identified level and consecutive levels as unclassified (uc). To statistically assess the effect of the PJ on the bacterial composition, the Permutational Multivariate Analysis of Variance Using Distance Matrices (Adonis) was applied, as implemented in

the Vegan package of the R software (Avail-able from: http://CRAN.R-project.org/package=vegan and http://www.R-project.org/. Accessed October 14, 2013).

The association between the microbiota before PJ intervention period (Pre-treatment) and metabolite production was determined by applying a generalized linear regression model, using glmnet function in the "glmnet" R package [24]. The models were estimated by setting the genus frequencies before PJ as the predictor matrix, transformed by the Arcsin square root transformation, and the increment of the metabolites as the response vector. The Least Absolute Shrinkage and Selection Operator was used as the regression method to penalize the absolute size of the regression coefficients. The performance of the predictive model was validated by means of the *k*-mean cross-validation using cv. glmnet function in the "glmnet" R package. Those bacterial taxa that gave a good fit within the linear regression model were validated using the Spearman correlation index. Those genera that matched both criteria were selected as bacteria involved in the metabolite production

#### 2.6.3. Functional prediction

Functional prediction was performed using the PICRUSt pipeline [25]. Usearch software was used to cluster the OTU sequences at 97% of identity, using the GreenGenes OTU database (version 13.5) as reference. OTUs were normalized by copy number using the normalize_by_copy_number.py script (version 1.0.0), and the functional predictions were estimated running the script predict_metagenomes.py, taking the KEGG database as reference. The resulting metagenomic prediction was then entered into the HUMAnN pipeline [26] in order to determine the abundance and coverage of putative microbial pathways. In the present study, we only predict the functional composition of all the OTUs whose taxonomic an-notation includes *Oscillospira*, *Odoribacter,* or *Paraprevotella* genera.

### 2.7. General statistical analysis

The results were presented as mean values ± SE. Paired Student's *t*-test was used to analyze changes of phenolic compounds and their metabolites, SCFAs, BA, sterols, and tocopherol, in the feces before and after PJ intake. The biodiversity Shannon index was estimated with the Kruskal–Wallis rank sum test. The *p*-values in the correlation analysis were adjusted using the Benjamini–Hochberg correction.

## 3 RESULTS

### 3.1. Effect of PJ intake on phenolic metabolite profile in human feces

Changes in some phenolic metabolite amounts were assessed in fecal samples of all volunteers before and after PJ intervention during 4 weeks (Table 1). It is most remarkable that five types of urolithins with different hydroxyl substitutions were detected in variable concentrations and high individual variability was observed in the fecal metabolism of pomegranate phenols. Urolithin A ($p < 0.05$) was the most abundant urolithin detected in larger amounts in eight out of 12 subjects, while isourolithin A (dihydroxyisourolithin) was quantified in the feces of one subject after the PJ intervention. Three volunteers produced higher amounts of urolithin B (hydroxyurolithin), especially in the isourolithin A producers. The excretion of urolithin C (trihydroxyurolithin) was detected in feces of six volunteers and urolithin D (tetrahy-droxyurolithin) only in one volunteer. In three volunteers no class of urolithins was detected. Apart from urolithins, the phenolic acids 3-phenylpropionic acid ($p < 0.05$), catechol ($p < 0.01$), and hydroxytyrosol ($p < 0.05$) were also detected in significantly higher concentrations after the PJ intervention.

Native anthocyanins from PJ were detected with a large variability in concentrations (**Table 1**). Cyanidin 3-*O*-glucoside was present in nine out of 12 volunteers after the intake of PJ (Post) while cyanidin pentoside and cyanidin 3,5-*O*-diglucoside were detected in the feces of the same three volunteers. The detection of an unknown compound ($m/z^-$ 333, $MS^2$ 285, 259) in the feces of all volunteers is noteworthy, a significant increase of this metabolite being observed following the PJ intake ($p < 0.01$). Neither ellagic acid nor ellagitannins were recovered in their

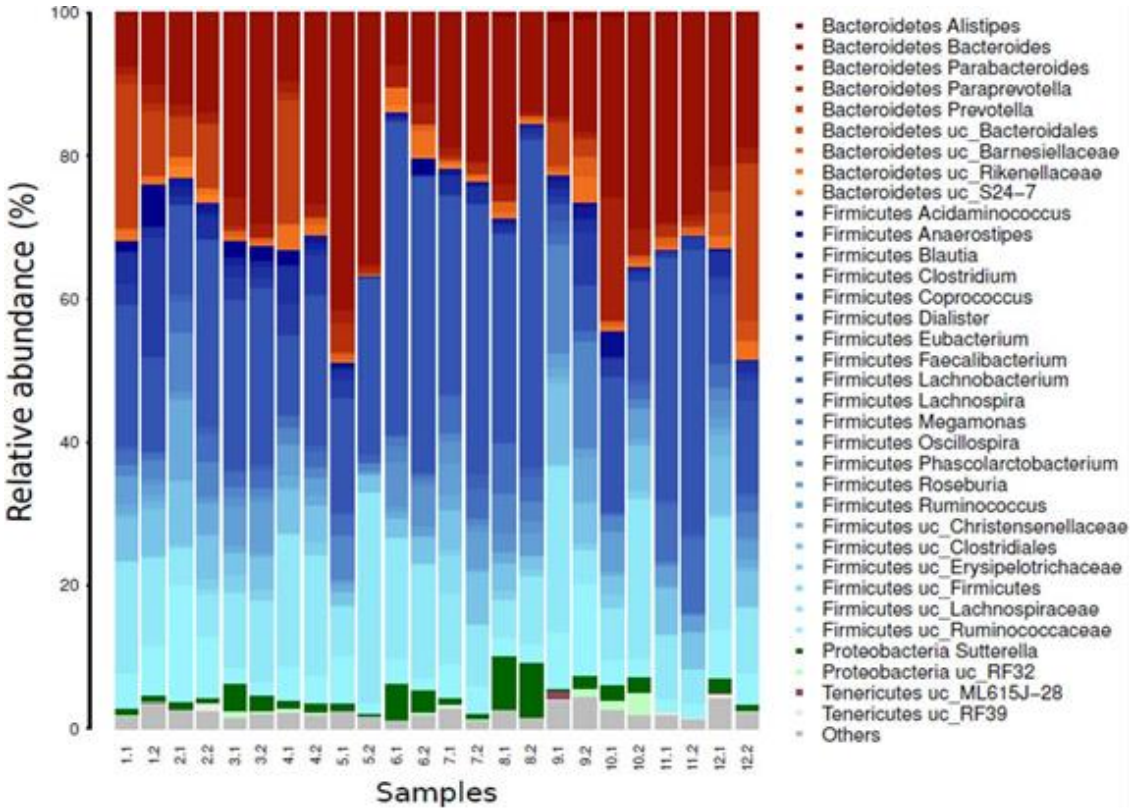
native form present in PJ in any of the feces analyzed.

### 3.2. Effect of PJ intake on SCFAs and steroid (BAs and sterols) composition in feces

Results of SCFAs (acetic, propionic, butyric, isobutiric, valeric, and isovaleric acids) concentrations are reported in **Table 2.** No significant statistical differences were observed between Pre and Post PJ intake in the concentration of total and individual SCFAs (mg/g dry feces). However, when the data were normalized to molar ratio (percentage of individual SCFAs in relation to total SCFAs) differences were observed between Pre and Post PJ intake.

Changes in fecal BA concentration are shown in **Table 3**. Although no significant changes were observed after the PJ consumption period, an increasing trend of primary BA was observed. Regarding the fecal sterols, cholesterol increased its concentration in feces ($p < 0.05$) and the concentration of coprostanol suffered a significant reduction ($p < 0.05$; Table 3).

**Figure 2.** Fecal microbiota composition in the individuals analyzed before and after PJ. The phylum and the genus level are shown for the most abundant bacterial groups (>1%)




### 3.3. Effect of PJ intake on gut microbiota composition

Despite some intraindividual differences, the bacterial composition of all the fecal samples, before and after PJ intervention during 4 weeks, was rather homogeneous since the distribution of genus abundance was similar (Shannon index, $p$ value = 0.386). *Bacteroides* (20.372 ± 9.128%)

and *Faecalibacterium* (23.138 ± 12.279%) were the most abundant genera. We also found that commensal members of intestinal community, such as *Prevotella* (3.913 ± 6.76%), *Oscillospira* (3.482±4.075%), *Lachnospira* (2.99 ± 2.571%), *Roseburia* (2.615 ±1.964%), *Parabacteroides* (2.398 ± 3.345%), *Ruminococcus* between metabolites produced and initial microbiota (Pre PJ), we performed a generalized linear regression model analysis. We identified three genera that showed significant correlation with two phenolic metabolites detected in feces. Catechol was positively correlated with *Oscillospira* genus and negatively correlated with *Paraprevotella* genus, while 3-phenylpropionic acid showed a positive correlation with *Odoribacter* genus (Table 4). Both phenolic metabolites presented a significant increase after the PJ supplementation (4 weeks). These three genera, *Oscillospira*, *Paraprevotella,* and *Odoribacter*, were low abundant in the initial microbiota (4.021 ± 4.245%, 0.821 ± 1.174%, and 0.123 ± 0.169%).

**Table 4.** Significant interactions between bacterial genera and phenolic metabolites in feces

| Phenolic metabolite | Genus | GLM coefficient | Spearman *rho* coefficient | *p*-Value | *q*-Value[a)] |
|---|---|---|---|---|---|
| Catechol | *Paraprevotella* | −6.809 | −0.822 | 0.001 | 0.012 |
| Catechol | *Oscillospira* | 3.297 | 0.685 | 0.012 | 0.087 |
| 3-phenylpropionic acid | *Odoribacter* | 2.179 | 0.643 | 0.0278 | 0.093 |

*q*-Value is the *p*-value adjusted using the Benjamini–Hochberg correction. GLM, generalized linear regression model

**Table 5.** Metabolic pathways related to the cathecol in *Oscillospira* and *Paraprevotella*

| | *Oscillospira* | | *Paraprevotella* | |
|---|---|---|---|---|
| Pathways | Abundance (%) | Coverage (%) | Abundance (%) | Coverage (%) |
| ko00361: Chlorocyclohexane and chlorobenzene degradation | 0.0497 ± 0.0485 | 5.701755 ± 1.5193 | 0.0343 ± 0.0339 | 5.2631 ± 2.8132 |
| ko00362: Benzoate degradation | 0.4946 ± 0.1152 | 12.0000 ± 0.0000 | 0.1347 ± 0.1021 | 8.9999 ± 4.0158 |
| ko00621: Dioxin degradation | 0.3339 ± 0.0948 | 7.6923 ± 0.0000 | 0.0112 ± 0.0234 | 5.7692 ± 7.9622 |
| ko00624: Polycyclic aromatic hydrocarbon degradation | 0.2293 ± 0.0489 | 2.9412 ± 0.0000 | 0.1718 ± 0.1274 | 3.3088 ± 1.0398 |
| ko00626: Naphthalene degradation | 0.6160 ± 0.0604 | 13.6905 ± 1.3902 | 0.3072 ± 0.2328 | 16.0714 ± 3.3064 |
| ko00627: Aminobenzoate degradation | 0.2922 ± 0.1044 | 7.3446 ± 0.8345 | 0.1308 ± 0.0980 | 7.86128 ± 2.5255 |

The relative abundance is expressed as percentage based on the total pathways predicted. The coverage represents the percentage of the pathway that has been found in the prediction.

For *Paraprevotella*, we obtained a functional prediction in eight of the 12 samples, the same metabolic pathways being represented. Table 5 shows the relative abundance and coverage of each pathway. The 3-phenylpropionic acid could be involved in three KEGG pathways: phenylalanine metabolism, degradation of aromatic compounds, and microbial metabolism in diverse environments. In the *Odoribacter* genome, we found only the phenylalanine metabolism pathway with a relative abundance and coverage of 0.3456 ± 0.0675 and 17 ± 8.00, respectively.

To determine if the metabolic pathways involved in the production of these phenolic metabolites are present in these members of fecal microbiota, we obtained a prediction of metagenome functional content from each genera using bioinformatic

PICRUSt and HUMAnN tools [25, 26]. Thus, for *Oscillospira*, the functional category "Xenobiotics Biodegradation and Metabolism" contained the different pathways (benzoate degradation, naphthalene degradation, aminobenzoate degradation, polycyclic aromatic hydrocarbon degradation, dioxin degradation, and chlorocyclohexane and chlorobenzene degradation) where cathecol could be synthesized.

## 4 DISCUSSION

To our knowledge, this is the first study that evaluates the interaction between the gut microbiota composition, the nature of the biotransformation products of pomegranate polyphenols, and the modulation of other fermentation products such as fecal steroids and SCFAs. Regarding the fecal microbial metabolism of pomegranate polyphenols, the main metabolites detected in feces after the intervention period with PJ were urolithins, which is in accordance with previous works where the predominant fecal metabolites after ellagitanninrich food administration were urolithins [20]. Besides that, other phenolic metabolites such as 3-phenylpropionic acid and catechol have been described for the first time in human feces after pomegranate consumption which could be products from the anthocyanin colonic metabolism, as previously described in an in vitro colonic fermentation of a red wine extract [27] and cyanidin [28]. Hydroxytyrosol, which has normally been associated with the intake of virgin olive oil phenols [29], presented a significant increase in feces after PJ consumption in the present study. In a previous study, tyrosol appeared as a phenolic catabolite after an in vitro colonic fermentation of raspberry anthocyanins [28], so we hypothesize that hydroxytyrosol could also derive from pomegranate anthocyanins colonic metabolism. Related to the native structures of anthocyanins present in PJ, an important increase in their concentration in feces was observed after the intervention period with PJ, which indicated that these phenolic compounds could resist the digestion conditions and reach the large intestine. As demonstrated in the present study, urolithins, together with the phenolic acid metabolites and the non-degraded anthocyanins, are able to persist in the intestinal lumen and their contact with the gut epithelium and other compounds from diet could modulate some of the mechanisms involved in intestinal diseases The biological mechanisms proposed for these phenolic catabolites have been previously described, being the maintenance of adequate organic antioxidant status, the increase in antiinflammatory defense and the protection against intestinal pathologies [30,31]. Remarkable activities associated with antioxidant effects against radical oxygen species have been noticed for urolithins [32, 33], catechol [34], hydroxytyrosol [35], and cyanidin [36]. The capacity of phenolic compounds to regulate the signaling pathways, such as nuclear factor-kappa B (NF-kB) and mitogen-activated protein kinases, responsible for the regulation of proinflammatory modulators, has been the central topic in recent studies [36–39]. In the case of the fecal phenolic catabolites of pomegranate, urolithins A and C were effective in the inhibition of cytokine-induced proinflammatory markers TNF-alfa and IL-6, respectively, in THP-1 cell line-derived macrophages [38]. In addition, urolithin A was also effective at reducing the levels of PGE2, PAI-1, and IL-8 in parallel with the inhibition of colon fibroblast migration and monocyte adhesion to fibroblast [39]. Catechol also showed a reduction in the expression of adhesion molecules in a microvascular endothelial cell line [40], in addition to NF-κB factor inhibition [41]. Cytokine-stimulated human HT-29 cells treated with cyanidin 3-glucoside also reduce the production of the proinflammatory mediators as well as the amounts of activated STAT1 [42]. Several studies based on rodents induced inflammatory bowel disease (IBD) highlighted the properties of rich-ellagic acid [43] and hydroxytyrosol [44] based diets, observing a reduction in intestinal inflammation promoters and decreasing colon epithelial damage. All these data suggest that, apart from the classical urolithins derived from pomegranate, other fecal metabolites described in the present study, such as phenolic acids or intact anthocyanins, might also be potential candidates to keep down the inflammatory response, especially in IBD pathogenesis.

As inflammation is a manifestation of oxidative stress and the intensity and duration of the inflammation process were also linked to the development of cancer [45, 46], the phenolic compounds detected in feces in our study could play a role in the prevention of the proliferation of cancer cells by control of oxidative stress and inflammation episodes. In this sense, several in vitro studies have described specific anticarcinogenic effects of urolithin A [47], catechol [48], and hydroxytyrosol [49]. Molecular mechanisms, such as inhibition of COX-2 in the case of 3-phenylpropionic acid [50] and down regulation of AP-1 and NF-κB activity by anthocyanins [36], were proposed as chemopreventive activity. We hypothesize that a broad spectrum of the latter-mentioned functions could occur, not only due to urolithins, but as a consequence of the synergistic combination of all the microbial phenolic metabolites detected after PJ intake in our study. The sustained intake of PJ could also potentiate their effects, increasing the time of their exposure to gut epithelium, with a consequent restriction of the oxidative damage and limiting the inflammatory episodes, contributing to reducing the risk of colon cancer and IBD development as well as to achieving remission of symptoms.

There is limited information regarding the ability of dietary components, including phenolic compounds, to influence the gut microbial population. To address this, we investigated the influence of pomegranate intake on the composition of human fecal microbiota. Only one study has been performed in animals suggesting that pomegranate polyphenols can be very active at the gut level, modulating the gut microbial population [51]. Contrary to this study, we showed, using high-throughput sequencing, that a 4 weeks daily ingestion of 200 mL of PJ that represented a daily intake of around 968 mg of pomegranate phenols (**Supporting Information Table 1**) did not significantly modify the composition of the individual gut microbiota in healthy humans. In another study, no differences in fecal microbiota profile were detected after consumption of a probiotic-enriched yogurt [52]. These results are in good agreement with our observations. This may be related to the microbial resilience reported under normal physiological conditions, which contributes to maintaining homeostasis in healthy adults [53]. Moreover, Espin et al. [54] observed the conversion of ellagic acid to urolithins in the jejunum of pigs fed with oak acorns (rich source of ellagitannins), associated with special ellagic acid converter microbial groups situated in upper segments of the gastrointestinal tract. Due to the difficulty in obtaining internal human samples, microbial analyses are normally carried out in feces, whose bacteria profile may differ from the microbial population of the intestinal mucosa and small intestine segments [55]. This could explain that no relation between the bacteria profile and urolithin production was inferred in our study.

However, we identified catechol that was positively correlated with *Oscillospora* genus and negatively correlated with *Paraprevotell*a, while 3-phenylpropionic acid showed a positive correlation with *Odoribacter* genus. Since none of these genera are abundant members of the fecal microbiota, they were poorly described in human studies and scarce information is available in terms of reference genomes. However, by bioinformatics we detected several pathways involved in the production of catechol in *Oscillospira*. Thus, the positive correlation between this genus and catechol in feces indicates that *Oscillospira* could be involved in the conversion of pomegranate phenolics into catechol based on metabolic predictions and, therefore, volunteers presenting higher proportions of this specific genus could also present higher concentrations of catechol. Despite the fact that *Paraprevotella* presented the same pathway profile as *Oscillospira*, we found a negative correlation between the fecal catabolite and this genus. This result may indicate differences in enzymatic com-position within the catechol pathways involved. Thus, this bacterium could transform the phenolic compounds from PJ in a different manner, yielding other products. Furthermore, *Ododribacter* genus was positively correlated with 3-phenylpropionic acid. We found that this compound is only synthesized in the phenylalanine metabolism pathway, as occurred in *Odoribacter splanchnicus*, whose genome has been recently sequenced [56]. Further studies are

needed to obtain direct evidence of metabolic capabilities of these genera.

The modulation observed in the sterols profile and the slight changes in BAs and SCFAs might mean that pomegranate phenolic compounds are not directly involved in their formation or excretion rate but may interfere with the activity of some microbial enzyme, as has been reported after fiber fermentation [57]. This may explain the differences among molar ratios of butyrate, less conversion of secondary BAs, and less conversion of cholesterol to coprostanol. According to recent studies [57], these changes in the propor-tions and profile of fecal SCFAs, BAs, and sterols could contribute to the health status of the gut. Butyric acid, the main energy source of intestinal cells, supports metabolic health by preventing the development of abnormal intestinal cells as well as by the selective induction of apoptosis of cancer cells [58]. High fecal secondary BAs and coprostanol enhance the lumen toxicity, which increased the incidence of colorectal cancer, and it is therefore expected that the change in the profile of these compounds after PJ intake could potentially exert protective action against colon cancer.

In conclusion, although the inclusion of PJ in the diet did not significantly alter the gut microbiota composition, specific genera, as *Oscillospira* and *Odoribacter,* could contribute to the generation of potential health-promoting phenolic metabolites in the individuals that harbor these bacteria. Besides that, we found that the inclusion of PJ in the diet modulates the concentration in feces of fecal metabolites of interest, as SCFAs, sterols, and secondary BAs. Further research should be undertaken to unravel the bacterial mechanisms involved in metabolite production. The comprehensive identification of bacteria groups associated with certain food components, such as the case of phenolic compounds, is a pending task and must be considered essential to establish dietetic recommendation, functional food design, or provide information to develop a therapeutic target to prevent or treat intestinal diseases.

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**ADDITIONAL INFORMATION**

**Table 1.** Phenolic composition of pomegranate juice (PJ)[1]

| Phenolic compounds | Cumulative daily intake (mg / 200 mL PJ) |
|---|---|
| **Ellagic acid and ellagitannins (Total)** | **878.9 ± 75.13** |
| Ellagic acid | 2.28 ± 0.44 |
| Ellagic acid pentoside | 48.21 ± 4.15 |
| Ellagic acid deoxyhexoside | 80.69 ± 7.18 |
| Ellagic acid hexoside | 144.04 ± 10.29 |
| Valoneic acid bilactone | 9.91 ± 1.37 |
| Dehydrogalloyl HHDP hexoside | 3.16 ± 0.52 |
| Galloyl HHDP glucoside | 39.18 ± 4.52 |
| *Bis*-HHDP glucoside | 78.50 ± 6.08 |
| Digalloyl HHDP glucoside | 12.95 ± 2.26 |
| Ellagic acid derivate | 75.36 ± 5.04 |
| Punigluconin | 61.42 ± 6.05 |
| 2-O-galloylpunicalin | 5.42 ± 1.07 |
| *Tris*-galloyl HHDP glucose isomer | 21.25 ± 2.18 |
| Punicalagin A, B, isomer | 86.95 ± 7.82 |
| Di-(HHDP galloyl glucoside) pentoside | 0.48 ± 0.05 |
| Digalloyl tri-HHDP diglucoside isomer | 87.27 ± 2.15 |
| Brevifolin carboxylic acid | 3.04 ± 0.77 |
| Galloyl HHDP gluconate isomer | 97.59 ± 9.51 |
| Punicalin | 21.15 ± 3.68 |
| **Phenolic acids and its derivates (Total)** | **41.5 ± 5.56** |
| Malic acid | 6.94 ± 1.19 |
| *p*-hydroxybenzoic acid | 0.13 ± 0.16 |
| Hydroxybenzoic acid hexoside | 1.12 ± 0.17 |
| Protocatechuic acid | 0.03 ± 0.03 |
| Protocatechuic acid hexoside | 0.17 ± 0.27 |
| Protocatechuic acid pentoside | 0.20 ± 0.01 |
| Citric acid | 12.4 ± 1.01 |
| Chlorogenic acid glucoside | 0.03 ± 0.01 |
| Gallic acid | 0.20 ± 0.05 |
| Gallic acid glucoside | 2.24 ± 0.68 |
| Gallic acid diglucoside | 0.04 ± 0.04 |
| *p*-Coumaric acid | 0.02 ± 0.01 |
| Coumaric acid glucoside | 1.10 ± 0.20 |
| Coumaric acid derivate | 3.68 ± 0.13 |
| Caffeic acid glucoside | 2.75 ± 0.68 |
| Vanillic acid hexoside | 1.60 ± 0.22 |
| Vanillic acid derivate | 6.06 ± 0.45 |
| Ferulic acid hexoside | 2.13 ± 0.08 |
| Ferulic acid derivate | 0.47 ± 0.00 |
| Ferulic acid hexoside derivate | 0.21 ± 0.16 |
| **Anthocyanins (Total)** | **38.0 ± 16.2** |
| Cyanidin-3-O-glucoside | 1.29 ± 0.20 |
| Cyanidin-3,5-O-diglucoside | 26.7 ± 7.8 |

| | |
|---|---|
| Cyanidin pentoside | 0.02 ± 0.00 |
| Cyanidin pentoside hexoside | 0.05 ± 0.02 |
| Delphinidin-3-O-glucoside | 0.03 ± 0.02 |
| Delphinidin-3,5-O-diglucoside | 0.13 ± 0.03 |
| Pelargonidin-3-O-glucoside | 0.41 ± 0.33 |
| Pelargonidin-3,5-O-diglucoside | 9.30 ± 7.80 |
| Pelargonidin pentoside hexoside | 0.01 ± 0.01 |
| (epi)afzelechin cyanidin hexoside | 0.01 ± 0.00 |
| **Lignans (Total)** | **4.21 ± 0.45** |
| Pinoresinol | 0.64 ± 0.08 |
| Secoisolariciresinol glucoside | 2.65 ± 0.10 |
| Cyclolariciresinol | 0.92 ± 0.27 |
| **Flavonols (Total)** | **3.39 ± 1.07** |
| Kaempferol glucoside | 0.62 ± 0.43 |
| Kaempferol rutinoside | 0.23 ± 0.08 |
| Kaempferol coumaroyl glucoside | 0.28 ± 0.34 |
| Mirycetin rhamnoside | 0.01 ± 0.00 |
| Mirycetin glucoside | 0.21 ± 0.14 |
| Quercetin rutinoside (rutin) | 0.01 ± 0.00 |
| Quercetin coumaroyl glucoside | 0.01 ± 0.00 |
| Syringetin glucoside | 2.02 ± 0.07 |
| **Flavan-3-ols (Total)** | **1.19 ± 0.64** |
| Catechin | 0.31 ± 0.19 |
| Epicatechin | 0.28 ± 0.11 |
| Dimer | 0.47 ± 0.26 |
| Trimer | 0.13 ± 0.08 |
| **Flavanones (Total)** | **0.50 ± 0.10** |
| Naringenin | 0.19 ± 0.01 |
| Eriodictyol glucoside | 0.11 ± 0.07 |
| **Phenolic aldehydes** | |
| Syringaldehyde | 0.38 ± 0.11 |
| **Dihydrochalcones** | |
| Phloretin glucoside | 0.20 ± 0.02 |
| **Total phenolic compounds** | **968.00 ± 99.36** |

[1] All values are means ± SDs (n=5)
HHDP: hexahydroxydiphenic acid

**Table 2.** Calculated daily nutrient composition of the volunteers' diets before (Pre) and after (Post) the 4 wk of the pomegranate juice (PJ) supplementation (n=12)[1].

| Diet basic composition | Pre | Post |
|---|---|---|
| Total energy (kcal) | 2263 ± 522 | 2263 ± 522 |
| Total protein (g) | 88.1 ± 22.2 | 88.1 ± 22.2 |
| Total carbohydrates (g) | 243 ± 37.3 | 243 ± 37.2 |
| Fiber (g) | 27.2 ± 9.7 | 27.23 ± 9.72 |
| Total fat (g) | 84.1 ± 47.4 | 84.08 ± 47.38 |
| Saturated fat | 34.5 ± 14.2 | 34.53 ± 14.16 |
| Monounsaturated fat (g) | 39.5 ± 14.0 | 39.48 ± 14.01 |
| Polyunsaturated fat (g) | 10.9 ± 3.7 | 10.94 ± 3.68 |
| Cholesterol (mg) | 333 ± 215 | 333.3 ± 215.1 |
| Total phenolic compounds (mg) | 1927 ± 346 | 1928 ± 345 |

[1] All values are means SDs

**Methods**

**Chromatographic analysis of phenolic metabolites in feces**

The chromatographic analysis of phenol metabolites was performed by a Waters Acquity Ultra-Performance™ liquid chromatography system (Waters, Milford, MA, USA), equipped with a binary pump system (Waters, Milford, MA, USA). The chromatographic column was an Acquity BEH C18 (100 mm x 2.1 mm i.d.) with a 1.7 µm particle size (Waters, Milford, MA, USA). A binary mobile phase with a gradient elution was used. For the analysis of anthocyanins, eluent A was Milli-Q water:acetic acid (90:10, v/v) and eluent B was acetonitrile. The flow-rate was 0.4 mL/min. The gradient was performed as follows: 0-10 min, 5-35%B; 10-10.1 min, 35-80%B; 10.1-11 min, 80%B isocratic; 11-11.1 min, 80-5%B; 11.1-12.5 min, 5%B isocratic. In the case of the other phenolic compounds, the eluent A was Milli-Q water:acetic acid (99.8:0.2, v/v) and eluent B was acetonitrile. The flow rate was 0.3 mL/min. The gradient was performed as follows: 0-5 min, 5-10%B; 5-10 min, 10-12.4%B; 10-18 min, 12.4-28%B; 18-23 min, 28-100%B; 23-25.5 min, 100 %B isocratic; 25.5-27 min, 100-5%B; 27-30 min, 5%B isocratic. The injection volume was 2.5 µL. The tandem mass spectrometry (MS/MS) analysis were carried out on a triple quadrupole detector (TQD) mass spectrometer (Waters, Milford, Ma, USA) equipped with a Z-spray electrospray interface. The analysis was done in the positive ion mode for anthocyanins and urolithins and in the negative ion mode for the rest of phenolic compounds and the data was acquired with the selected reaction monitoring mode (SRM) (Table 1SM). The MS/MS parameters were as follows: capillary voltage, 3 kV; source temperature, 150ºC; cone gas flow rate, 80 L/h and desolvatation gas flow rate, 800 L/h; desolvatation temperature, 400ºC Nitrogen (>99% purity) and argon (99% purity) were used as nebulizing and collision gases, respectively. Cone voltages and collision energies were optimized for each analyte by injection of each standard compound in a mixture of acetonitrile/water (50:50, v/v) at a concentration of 10 mg/L. Two transitions were studied for each phenolic compound. The most abundant transition was used for quantification, while the second most abundant was used for confirmation purposes. The dwell time established for each transition was 30 ms. Data acquisition was carried out with the MassLynux v 4.1 software. The concentration of each compound was measured by using an external calibration curve produced with the use of authentic standards (Table 1SM).

Ellagic acid, punicalagin, *p*-coumaric acid, vanillic acid, kaempferol, *p*-hydroxyphenylacetic acid, phenylpropionic acid, 3-(4-hydroxyphenyl)propionic acid and 3-(2,4-dihydroxyphenyl)propionic acid were purchased from Fluka (Buchs, Switzerland); protocatechuic acid, catechin, epicatechin, *p*-hydroxybenzoic acid, catechol, ferulic acid, 3,4-dihydroxyphenylacetic acid, phenylacetic acid and hippuric acid from Sigma Aldrich (St. Louis, MO, USA); caffeic acid, rutin, myricetin, quercetin, dimer $B_2$, hydroxytyrosol, tyrosol, cyanidin-3-*O*-glucoside and cyanidin-3,5-*O*-diglucoside from Extrasynthese (Genay, France). Gallic acid and malic acid were purchased from Panreac (Barcelona, Spain) and pinoresinol and secolariciresinol were purchased from Arbonova (Turku, Finland). Standards of urolithin A (dihydroxy urolithin), urolithin B (hydroxy urolithin) and urolithin C (trihydroxy urolithin) were kindly provided by Dr. Thasana from Chulabhorn Research Institute, Laksi, Bangkok (Nealmongkol et al., 2013*). These standard phenolic compounds were prepared in methanol.

*p*-hydroxybenzoic acid, protocatechuic acid, *p*-coumaric acid, vanillic acid, gallic acid, caffeic acid, ferulic acid, quercetin, kaempferol, catechin epicatechin, ellagic acid, myricetin, pinoresinol, seicolariciresinol, dimer $B_2$, rutin, punicalagin, cyanidin-3-*O*-glucoside and cyanidin-3,5-*O*-diglucoside were quantified using their own calibration curve. The phenol-glycoside compounds were tentatively quantified using the calibration curves of their respective aglycones. Syringaldehyde was tentatively quantified with the calibration curve of gallic acid. Chlorogenic acid was tentatively quantified as caffeic acid. Pinocebrin, naringenin, phloretin and eriodictyol were tentatively quantified as quercetin.

(*) Nealmongkol P, Tangdenpaisal K, Sitthimonchai S, Ruchirawat S, Thasana N. Cu(I)-mediated lactone formation in subcritical water: A benign synthesis of benzopyranones and urolithins A-C. *Tetrahedron* 2013;69:9277-83

Ellagitannins were tentatively quantified as ellagic acid and the anthocyanins glucoside and diglucoside were tentative quantified as cyanidin-3-*O*-glucoside and cyanidin-3,5-*O*-diglucoside, respectively. Urolithins tetrahydroxy urolithin and pentahydroxy urolithins were tentatively quantified as trihydroxy urolithins.

**Table 1 Method.** Optimized SRM conditions and commercial standards used for the quantification analysis of the studied phenolic compounds and their colonic fermentation products in feces.

| Phenolic compound | MW (g/mol) | SRM quantification | | Standard used for quantification |
|---|---|---|---|---|
| | | Transition | Cone voltage (V) / Collision energy (eV) | |
| **Anthocyanins** [1] | | | | |
| Pelargonidin-3-*O*-glucoside | 434 | 433 > 271 | 40 / 20 | Cyanidin-3-*O*-glucoside |
| Pelargonidin pentoside | 434 | 403 > 271 | 40 / 15 | Cyanidin-3-*O*-glucoside |
| Pelargonidin-3,5-*O*-diglucoside | 594 | 595 > 271 | 40 / 30 | Cyanidin-3,5-*O*-diglucoside |
| Pelargonidin pentoside hexoside | 564 | 565 > 271 | 40 / 30 | Cyanidin-3-*O*-glucoside |
| (epi)afzelechin pelargonidin hexoside | 704 | 705 > 543 | 40 / 20 | Cyanidin-3-*O*-glucoside |
| (epi)gallocatechin pelargonidin hexoside | 736 | 737 > 575 | 40 / 30 | Cyanidin-3-*O*-glucoside |
| Cyanidin pentoside | 418 | 419 > 287 | 40 / 20 | Cyanidin-3-*O*-glucoside |
| Cyanidin-3-*O*-glucoside | 448 | 449 > 287 | 40 / 20 | Cyanidin-3-*O*-glucoside |
| Cyanidin-3,5-*O*-diglucoside | 610 | 611 > 287 | 40 / 30 | Cyanidin-3,5-*O*-diglucoside |
| Cyaniding pentoside hexoside | 580 | 581 > 287 | 40 / 35 | Cyanidin-3-*O*-glucoside |
| Cyanidin rutinoside | 594 | 595 > 287 | 40 / 25 | Cyanidin-3-*O*-glucoside |
| Delphinidin-3-*O*-glucoside | 464 | 465 > 303 | 40 / 20 | Cyanidin-3-*O*-glucoside |
| Delphinidin-3,5-*O*-diglucoside | 626 | 627 > 303 | 40 / 30 | Cyanidin-3,5-*O*-diglucoside |
| (epi)afzelechin cyanidin hexoside | 720 | 721 > 559 | 40 / 25 | Cyanidin-3-*O*-glucoside |
| **Phenolic acids and its derivates** [2] | | | | |
| Malic acid | 134 | 133 > 115 | 20 / 10 | Malic acid |
| *p*-hydroxybenzoic acid | 138 | 137 > 93 | 30 / 15 | *p*-hydroxybenzoic acid |
| Hydroxybenzoic acid hexoside | 300 | 299 > 137 | 40 / 15 | *p*-hydroxybenzoic acid |
| Protocatechuic acid | 154 | 153 > 109 | 45 / 15 | Protocatechuic acid |
| Protocatechuic acid hexoside | 316 | 315 > 153 | 45 / 15 | Protocatechuic acid |
| Protocatechuic acid pentoside | 286 | 285 > 153 | 40 / 25 | Protocatechuic acid |
| Citric acid | 192 | 191 > 111 | 40 / 10 | Malic acid |
| Chlorogenic acid | 516 | 515 > 353 | 60 / 15 | Caffeic acid |
| Gallic acid | 170 | 169 > 125 | 35 /10 | Gallic acid |
| Gallic acid glucoside | 332 | 331 > 169 | 35 / 15 | Gallic acid |
| Gallic acid diglucoside | 484 | 483 > 331 | 40 / 20 | Gallic acid |
| *p*-coumaric acid | 164 | 163 > 119 | 35 / 10 | *p*-coumaric acid |
| Coumaric acid glucoside | 326 | 325 > 145 | 40 / 15 | *p*-coumaric acid |
| Coumaric acid derivate | 430 | 429 > 163 | 40 / 15 | *p*-coumaric acid |
| Caffeic acid | 180 | 179 > 135 | 35 / 15 | Caffeic acid |
| Caffeic acid glucoside | 342 | 341 > 179 | 60 / 20 | Caffeic acid |
| Vanillic acid | 168 | 167 > 123 | 30 / 10 | Vanillic acid |
| Vanillic acid hexoside | 330 | 329 > 167 | 40 / 15 | Vanillic acid |
| Vanillic acid derivate | 364 | 363 > 167 | 40 /15 | Vanillic acid |
| Ferulic acid | 194 | 193 > 134 | 30 / 15 | Ferulic acid |
| Ferulic acid hexoside | 356 | 355 > 193 | 40 / 25 | Ferulic acid |
| Ferulic acid derivate | 390 | 389 > 193 | 40 / 20 | Ferulic acid |
| Ferulic acid hexoside derivate | 450 | 449 > 355 | 40 / 20 | Ferulic acid |
| **Phenolic aldehydes** | | | | |
| Syringaldehyde | 182 | 181 > 166 | 35 / 10 | Gallic acid |
| **Flavan-3-ols** [2] | | | | |
| Catechin | 290 | 289 > 245 | 45 / 10 | Catechin |
| Epicatechin | 290 | 289 > 245 | 45 / 10 | Epicatechin |
| Dimer | 578 | 577 > 289 | 40 / 20 | Dimer B2 |
| Trimer | 866 | 865 > 287 | 60 / 30 | Catechin |
| **Flavonol** [2] | | | | |
| Kaempferol | 286 | 285 > 151 | 35 / 15 | Kaempferol |
| Kaempferol glucoside | 448 | 447 > 284 | 45 / 15 | Kaempferol |
| Kaempferol rutinoside | 594 | 593 > 285 | 50 / 25 | Kaempferol |
| Kaempferol coumaroyl glucoside | 594 | 593 > 285 | | Kaempferol |
| Mirycetin | 318 | 317 > 179 | 40 / 15 | Myricetin |
| Mirycetin rhamnoside | 464 | 463 > 316 | 50 / 25 | Myricetin |
| Mirycetin glucoside | 480 | 479 > 316 | 45 / 20 | Myricetin |
| Quercetin | 302 | 301 > 151 | 40 / 15 | Quercetin |
| Quercetin rutinoside (rutin) | 610 | 609 > 300 | 40 / 20 | Rutin |
| Quercetin coumaroyl glucoside | 610 | 609 > 463 | 40 / 20 | Quercetin |
| Syringetin | 346 | 345 > 315 | 60 / 25 | Quercetin |
| Syringetin glucoside | 508 | 507 > 327 | 60 / 30 | Quercetin |
| **Flavanone** [2] | | | | |
| Pinocembrin | 256 | 255 > 213 | 40 / 25 | Quercetin |

| | | | | |
|---|---|---|---|---|
| Naringenin | 272 | 271 > 151 | 40 / 15 | Quercetin |
| Eriodictyol glucoside | 450 | 449 > 287 | 45 / 10 | Quercetin |
| **Dihydrochalcone** [2] | | | | |
| Phloretin hexoside | 436 | 435 > 273 | 40 / 15 | Quercetin |
| **Lignans** [2] | | | | |
| Pinoresinol | 358 | 357 > 151 | 40 / 10 | Pinoresinol |
| Secoisolariciresinol | 362 | 361 > 346 | 50 / 15 | Secoisolariciresinol |
| Secoisolariciresinol glucoside | 524 | 523 > 361 | | Secoisolariciresinol |
| Cyclolariciresinol | 522 | 521 > 359 | 40 / 25 | Secoisolariciresinol |
| **Ellagic acid and ellagitannins** [2] | | | | |
| Ellagic acid | 302 | 301 > 245 | 45 / 25 | Ellagic acid |
| Ellagic acid pentoside | 434 | 433 > 300 | 40 / 30 | Ellagic acid |
| Ellagic acid deoxyhexoside | 448 | 447 > 301 | 40 / 25 | Ellagic acid |
| Ellagic acid hexoside | 464 | 463 > 301 | 40 / 25 | Ellagic acid |
| Dimethyl ellagic acid | 330 | 329 > 285 | 45 / 15 | Ellagic acid |
| Valoneic acid bilactone | 470 | 469 > 425 | 60 / 20 | Ellagic acid |
| Dehydrogalloyl HHDP hexoside | 616 | 615 > 301 | 60 / 25 | Ellagic acid |
| Galloyl HHDP glucoside | 634 | 633 > 301 | 60 / 30 | Ellagic acid |
| *Bis*-HHDP glucoside | 784 | 783 > 301 | 60 / 30 | Ellagic acid |
| Digalloyl HHDP glucoside | 786 | 785 > 301 | 70 / 35 | Ellagic acid |
| Ellagic acid derivative | 800 | 799 > 301 | 60 / 25 | Ellagic acid |
| Punigluconin | 802 | 801 > 301 | 50 / 30 | Ellagic acid |
| 2-*O*-galloylpunicalin | 934 | 933 > 721 | 70 / 35 | Ellagic acid |
| *Tris*-galloyl HHDP glucose isomer | 952 | 951 > 301 | 65 / 35 | Ellagic acid |
| Punicalagin A, B, isomer | 1084 | 1083 > 601 | 70 / 40 | Punicalagin |
| di(HHDP galloil glu) pentoside | 1416 | 1415 > 1397 | 70 / 35 | Ellagic acid |
| Digalloyl tri-HHDP diglucoside isomer | 1568 | 1567 > 765 | 70 / 35 | Ellagic acid |
| Brevifolin carboxylic acid | 292 | 291 > 247 | 45 / 20 | Ellagic acid |
| Galloyl HHDP gluconate isomer | 650 | 649 > 301 | 55 / 25 | Ellagic acid |
| Punicalin | 782 | 781 > 601 | 50 / 35 | Punicalagin |
| Galloyl *bis* HHDP hexoside | 936 | 935 > 633 | 60 / 25 | Ellagic acid |
| Castalagin derivate | 966 | 965 > 933 | 60 / 25 | Ellagic acid |
| **Urolithins** [1] | | | | |
| Hydroxy urolithin | 212 | 213 > 141 | 40 / 20 | Hydroxy urolithin |
| Di-hydroxy urolithin | 228 | 229 > 157 | 40 / 20 | Di-hydroxy urolithin |
| Tri-hydroxy urolithin | 244 | 245 > 155 | 40 / 30 | Tri-hydroxy urolithin |
| Tetra-hydroxy urolithin | 260 | 261 > 171 | 40 / 25 | Tri-hydroxy urolithin |
| Penta-hydroxy urolithin | 278 | 277 > 259 | 40 / 30 | Tri-hydroxy urolithin |
| **Other fermentation products** [2] | | | | |
| Catechol | 110 | 108.9 > 90.9 | 40 / 15 | Catechol |
| Phloroglucinol | 126 | 125 > 83 | 20 / 10 | Catechol |
| Tyrosol | 138 | 137 > 106 | 40 / 10 | Tyrosol |
| Hydroxytyrosol | 154 | 153 > 123 | 35/10 | Hydroxytyrosol |
| Hippuric acid | 179 | 178 > 134 | 40 / 10 | Hippuric acid |
| Hydroxyhippuric acid | 195 | 194 > 100 | 40 / 10 | Hippuric acid |
| Phenylacetic acid | 136 | 135 > 91 | 20 / 5 | Phenylacetic acid |
| *p*-Hydroxyphenylacetic acid | 152 | 151 > 107 | 20 / 10 | *p*-Hydroxyphenylacetic acid |
| 3,4-Dihydroxyphenylacetic acid | 168 | 167 > 123 | 20 / 10 | 3,4-Dihydroxyphenylacetic acid |
| Phenylpropionic acid | 150 | 149 > 105 | 20 / 5 | Phenylpropionic acid |
| 3-(4-Hydroxyphenyl)propionic acid | 166 | 165 > 121 | 20 / 10 | 3-(4-Hydroxyphenyl)propionic acid |
| 3-(2,4-Dihydroxyphenyl)propionic acid | 182 | 181 > 137 | 20 / 15 | 3-(2,4-Dihydroxyphenyl)propionic acid |
| Unkown | 334 | 333 > 259 | 40 / 20 | Hydroxytyrosol |

MW: Molecular weight. Ionisation mode: [1] negative mode, [2] positive mode. HHDP: hexahydroxydiphenic acid

**Table 2 Method.** Retention time (min), linearity, calibration curves, LOQs and LODs of the standard phenolic compounds used to quantity the pomegranate juice and the generated fermentation metabolites

| Phenolic compound | tr (min) | Linearity (mg/l) | Calibration curve | LOQ (µg/l) | LOD (µg/l) | Sample matrix |
|---|---|---|---|---|---|---|
| **Anthocyanins** | | | | | | |
| Cyanidin-3,5-O-diglucoside | 0.82 | 0.01-12.5 | y = 85913x – 131.25 | 10 | 3.5 | PJ |
| Cyanidin-3-O-glucoside | 1.85 | 0.001-1.25 | y = 716214x – 141.1 | 1 | 0.4 | PJ |
| **Rest of phenolic compounds** | | | | | | |
| Malic acid | 0.94 | 0.099-9.9 | y = 4536.8x – 642.5 | 99 | 35 | PJ |
| Gallic acid | 1.51 | 0.009-9 | y = 19793.3x – 103.6 | 9 | 3 | PJ, feces |
| Protocatechuic acid | 2.35 | 0.059-5.9 | y = 3708.8x – 246.9 | 59 | 20 | PJ, feces |
| Hydroxytyrosol | 2.72 | 0.01-10.00 | y = 3787.3x – 172.9 | 10 | 3 | Feces |
| 3,4-dihydroxyphenylacetic acid | 3.27 | 0.01-10.00 | y = 19717.0x – 241.9 | 10 | 3 | Feces |
| Tyrosol | 4.05 | 0.098-9.8 | y = 995.7x + 348.0 | 98 | 30 | Feces |
| *p*-hydroxybenzoic acid | 4.25 | 0.001-10.5 | y = 8142.7x – 23.4 | 1 | 0.3 | PJ, feces |
| Punicalagin | 4.75 | 30-1000 | y = 231.8x – 7255.3 | 30000 | 10000 | PJ |
| *p*-hydroxyphenylacetic acid | 4.95 | 0.017-1.7 | y = 13972.7x + 222.9 | 17 | 6 | Feces |
| Catechin | 5.01 | 0.098-10 | y = 648.5x – 81.9 | 98 | 32.5 | PJ |
| 3-(2,4-dihydroxyphenyl)propionic acid | 5.16 | 0.009-10 | y = 32136.3x – 99.9 | 9 | 2.5 | Feces |
| Vanillic acid | 5.49 | 0.0099-10 | y = 4565.3x + 1742.8 | 9.9 | 3.0 | PJ |
| Caffeic acid | 5.67 | 0.009-5.0 | y = 67897.3x – 792.5 | 9 | 3 | PJ |
| Dimer B2 | 6.35 | 0.1-10 | y = 1641.9x – 355.7 | 100 | 35 | PJ |
| Epicatechin | 7.21 | 0.098-10 | y = 814.7x – 184.6 | 98 | 30 | PJ |
| 3-(4-hydroxyphenyl)propionic acid | 7.64 | 0.018-20 | y = 7496.7x + 92.2 | 18 | 5 | Feces |
| *p*-coumaric acid | 8.57 | 0.009-10 | y = 80072.0x – 5.7 | 9 | 3 | PJ, feces |
| Ferulic acid | 10.60 | 0.0099-10 | y = 17006.7x – 33.9 | 9.9 | 3.0 | PJ |
| Ellagic acid | 12.02 | 0.99-10 | y =344.9x – 65.3 | 990 | 350 | PJ |
| Rutin | 12.25 | 0.0008-8 | y = 34110.0x + 642.1 | 0.8 | 2.5 | PJ |
| Phenylacetic acid | 12.47 | 0.002-17 | y = 28169.7x + 1006.0 | 2 | 0.6 | Feces |
| Urolithin C | 13.53 | 0.0007-2 | y = 100772x + 231.5 | 0.7 | 0.25 | Feces |
| Myricetin | 15.22 | 0.06-6 | y = 4473.3x – 625.5 | 60 | 22 | PJ |
| Seicolariciresinol | 15.85 | 0.099-10 | y = 1943.4 + 271.8 | 99 | 35 | PJ |
| Urolithin A | 16.64 | 0.008-10 | y = 24816.7x + 3098.9 | 8 | 2.5 | Feces |
| Phenylpropionic acid | 17.06 | 0.10-10 | y = 560.6x + 288.5 | 100 | 30 | Feces |
| Quercetin | 18.08 | 0.007-7 | y = 8978.7x + 2164.7 | 7 | 2.5 | PJ |
| Pinoresinol | 18.62 | 0.009-9 | y = 4544.7x + 87.4 | 9 | 3 | PJ |
| Urolithin B | 19.96 | 0.016-30 | y = 6878.7x + 769.1 | 16 | 5.0 | Feces |
| Kaempferol | 20.07 | 0.05-5.64 | y = 1499.7x + 291.5 | 50 | 15 | PJ |

tr: retention time. LOQ: quantification limit. LOD: detection limit
PJ: pomegranate juice

**Figure 1Method**. Extracted ion chromatograms of the anthocyanins determined in PJ by UPLC-MS/MS.

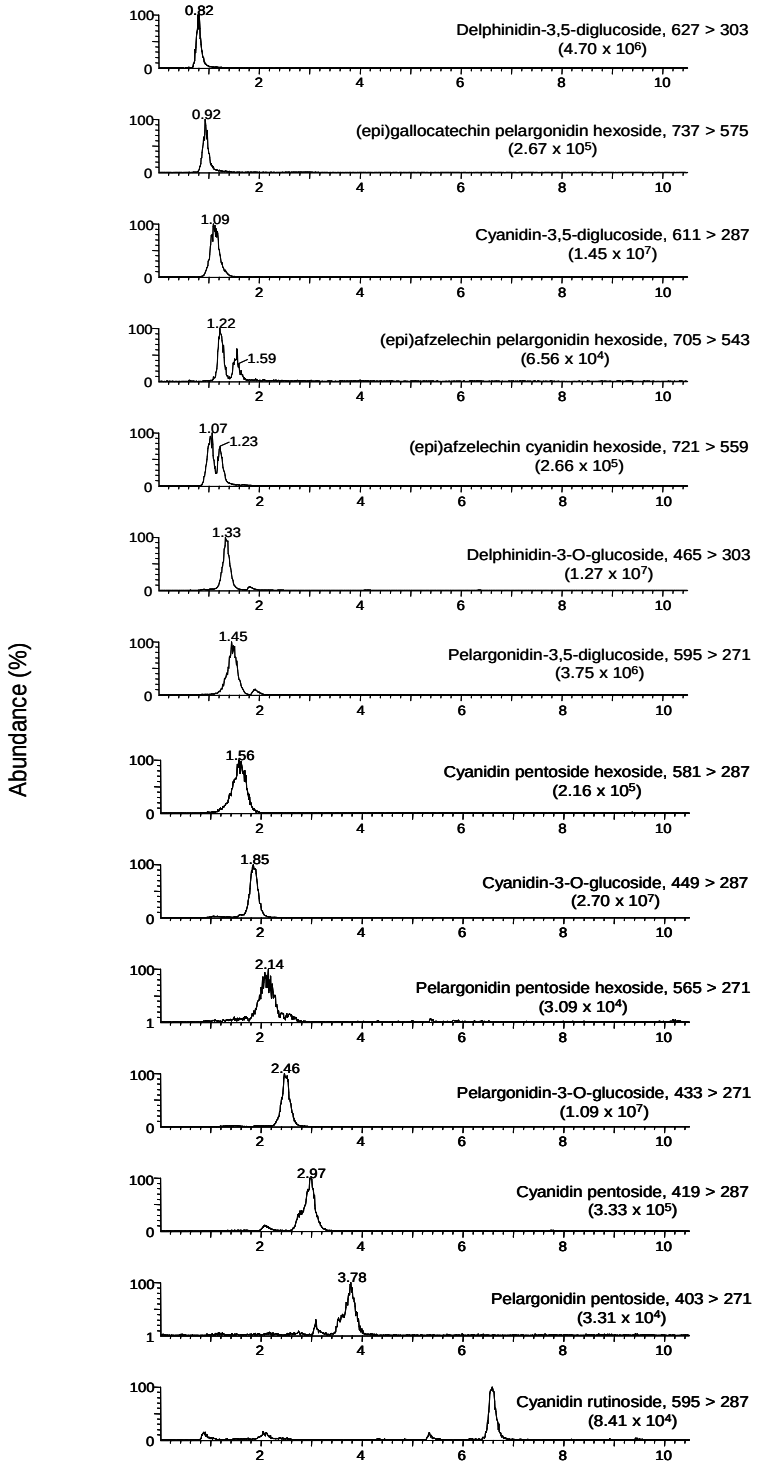


**Figure 2Method**. Extracted ion chromatograms of the main phenolic compounds (the rest of phenolic compounds) determined in PJ by UPLC-MS/MS.

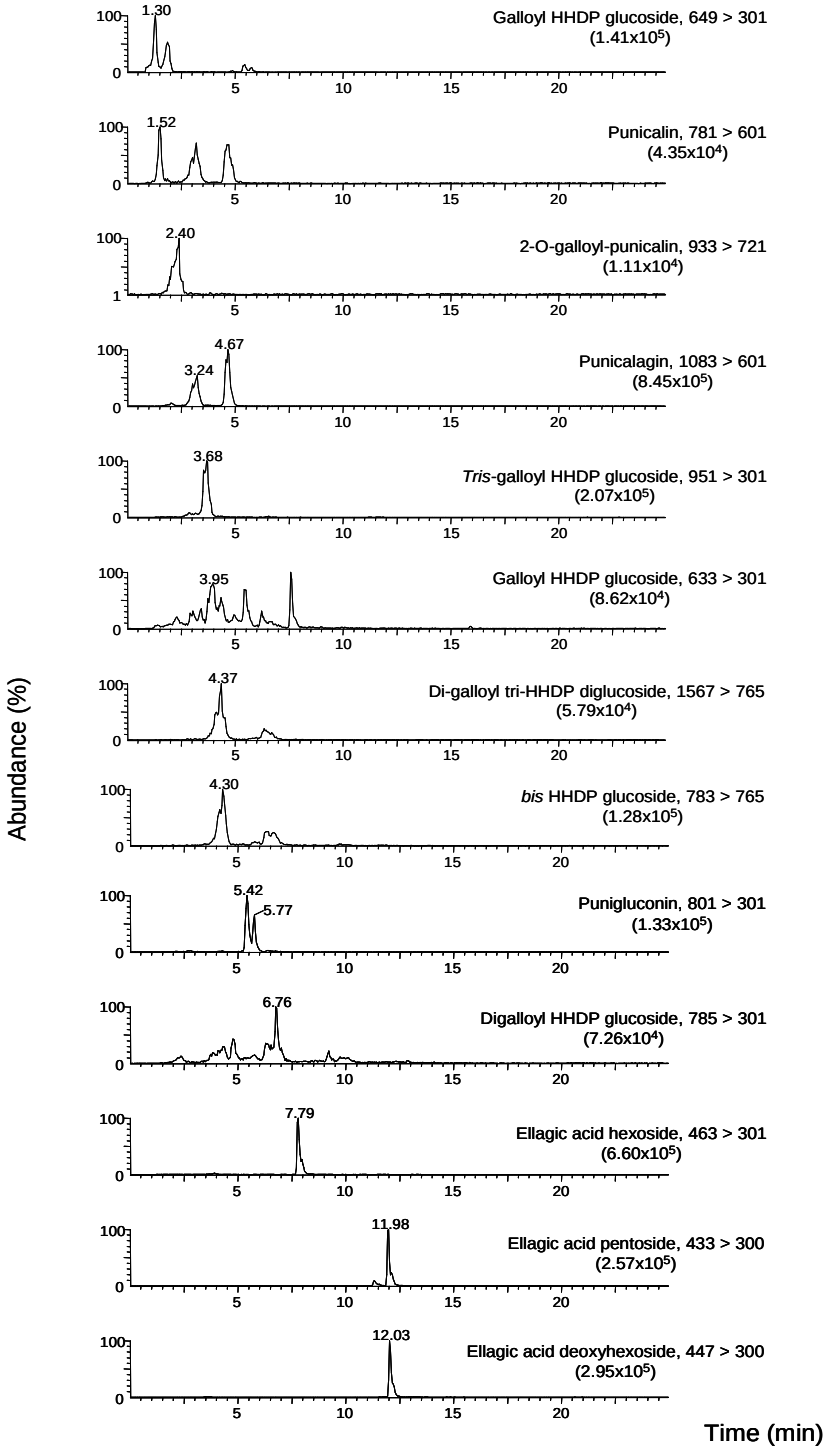


**Figure 1S:** GC/FID chromatogram of the SCFA (Acetic acid, propionic acid, butyric acid, isobutyric acid, isovaleric acid, valeric acid and 4-methylvaleric acid as internal standard, IS) quantified in feces.

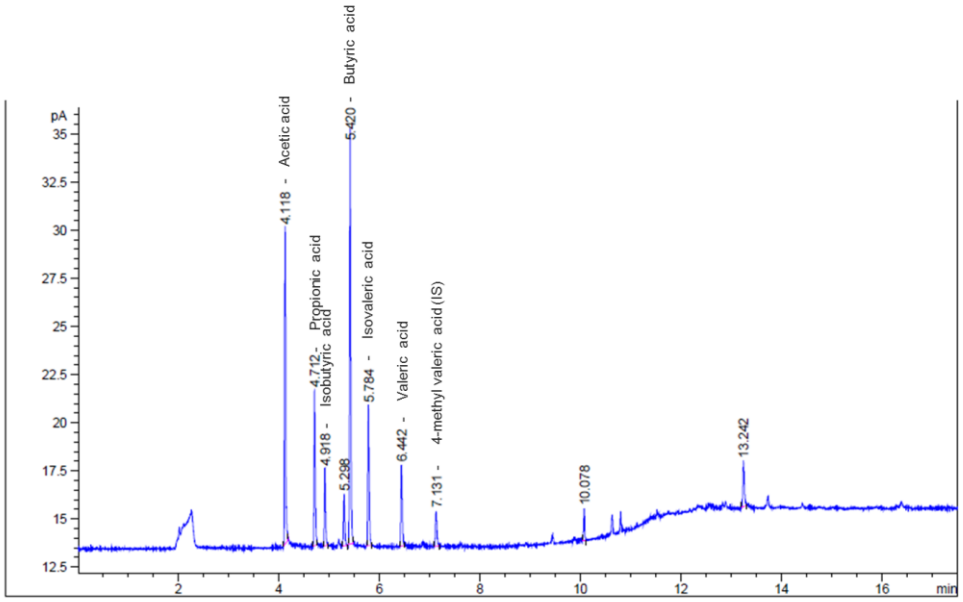
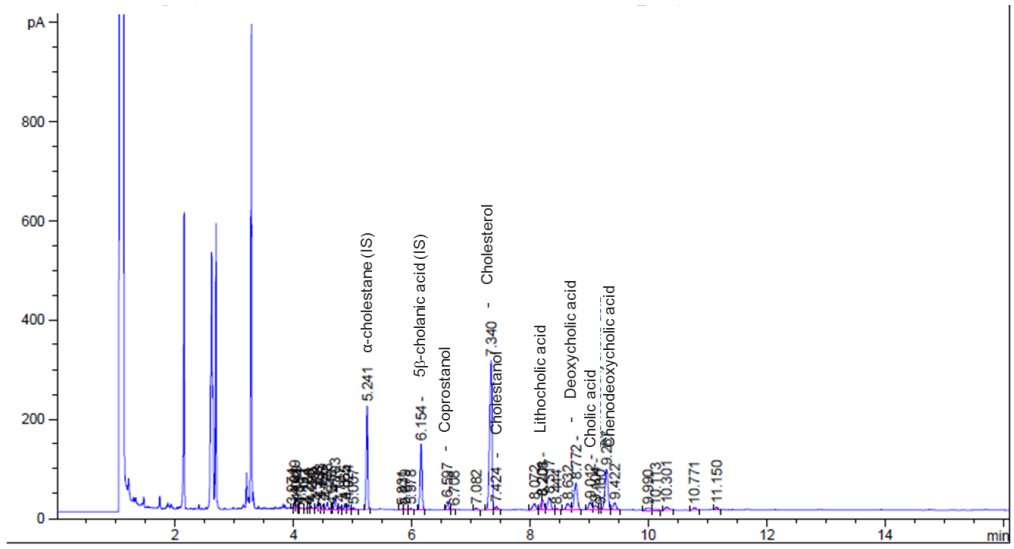
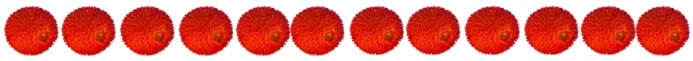



**Figure 2S**: GC/FID chromatogram of sterols (cholesterol, coprostanol, cholestenol, cholestanone and α-cholestane as internal standard, IS) and bile acids (cholic acid, deoxycholic acid, chenodeoxycholic acid, lithocholic acid and 5β-cholanic acid as internal standard, IS)



## **CHAPTER III:** ARBUTUS UNEDO



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# STABILITY AND METABOLISM OF *ARBUTUS UNEDO* BIOACTIVE COMPOUNDS (PHENOLICS AND ANTIOXIDANTS) UNDER *IN VITRO* DIGESTION AND COLONIC FERMENTATION

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**Abstract**

The natural antioxidants of *Arbutus unedo* highlight the importance of this fruit as natural source of bioactive compounds. In the present study, to evaluate the stability of phenolic compounds, ascorbic acid and fat-soluble antioxidants (α-tocopherol, β-carotene and lutein), *in vitro* gastrointestinal digestion was applied to *Arbutus unedo* fruit. After that, the non-absorbable fraction was anaerobically incubated with human faeces and the metabolic pathway for gallotannins, ellagitannins, flavan-3-ols and anthocyanins from *Arbutus unedo* fruit was proposed. The results showed that the presence of pectin from the fruit hampered the solubilisation of the phenolic compounds (with exception of gallic and ellagic acids) and fat-soluble vitamins during gastric digestion. Degradation of pectin-gel during the duodenal digestion favoured the release of the phenolic compounds and fat-soluble antioxidants to the media. The catabolic activity of human microbiota led to the generation of a wide range of simple phenols, such as *p*-hydroxybenzoic acid and catechol, derived from the catabolism of gallotannins, ellagitannins, flavan-3-ols and anthocyanins.

**Keywords**: antioxidants; *Arbutus unedo*; *in vitro* fermentation; *in vitro* gastrointestinal digestion; phenolic compounds

## 1. INTRODUCTION

Over recent years, a general scientific consensus has supported the association between the high intake of fruit and vegetables and the lower risk of chronic diseases; particularly, cardiovascular diseases (CVD), type-2 diabetes, and certain cancers (Mohamed, 2014). As a result, increasing fruit and vegetable consumption became a priority for international organisations as well as national governments, which have promoted different awareness campaigns. Fresh fruit constitute an important dietary source of such natural antioxidants as vitamins, fibre and certain minor compounds often known as phytochemicals (including phenolic compounds) or secondary plant metabolites, which are potentially associated with the prevention of chronic diseases (Del Rio et al., 2013). In response to unfavourable environmental conditions and to ensure survival, plants synthesize an important amount and variety of secondary metabolites, which participate in numerous mechanism of protection. Adaptation to the ecosystem may involve competition with other plants, soil nutrient availability, droughts or weather fluctuations, mould or bug infestation, amount of accessible sunlight and other factors

(Quideau, Deffieux, Douat-Casassus & Pouységu et al., 2011).

As result of these resilient and adaptive mechanisms, a wide range of protective compounds with antioxidant properties are accumulated in plants and fruits. So, the named "superfruits" are becoming increasingly popular among consumers for their accumulated phytochemicals that can be included in the diet. The promotion of their consumption could provide the food industry with possible new products and ingredients, while also stimulating local production. *A. unedo* L. (strawberry tree) is a Mediterranean shrub with a circum-Mediterranean Sea distribution, being found in western, central and southern Europe, north-eastern Africa (excluding Egypt and Libya), the Canary Islands and western Asia (Miguel, Faleiro, Guerreiro, & Antunes, 2014). The fruit of the *A. unedo* is generally used for preparing alcoholic beverages (wines, liqueurs and brandies), jams, jellies and marmalades, and less frequently eaten fresh (Santo, Galego, Gonçalves, & Quintas, 2012). It can also be added to yoghurts either in pieces or as flavours and be used, like other berries, in confectionery, such as for pie and pastry fillings and cereal products, among other applications (Miguel et al., 2014).

Several components belonging to diverse phenolic groups have been reported in the fruit of *A. unedo*. Gallotannins, such as mono-, di-, and tri-*O*-galloylshikimic acids and mono-, di-, and tri-*O*-galloylquinic acids have been described as being exclusive to this fruit, which gives it an extra added value. Other phenolic groups, such as flavan-3-ols, ellagitannins and anthocyanins, have also been described in *A. unedo* fruit in considerable amounts (Guimarães et al., 2013). This complex phenolic composition of *A. unedo* seems to be connected to antioxidant and anticarcinogenic effects (Del Rio et al., 2013). In addition, the particular composition in gallotannins could convert this singular fruit into an interesting source of antiviral agents against influenza (Ge et al., 2014; Saha et al., 2010) and HIV (Junior et al., 2013). Besides phenolic compounds, *A. unedo* fruit also contains considerable amounts of other antioxidants, such as ascorbic acid, and fat-soluble antioxidants like α-tocopherol, β-carotene and lutein (Oliveira et al., 2011; Pallauf, Rivas-Gonzalo, del Castillo, Cano, & de Pascual-Teresa, 2008), increasing its interest as a dietary source of multiple bioactivities related to the reduction of CVD risk.

From a nutritional and commercial point of view, the chemical composition of a fresh fruit is an important quality attribute. However, the total phenolic compounds and natural antioxidants present in food do not always reflect the total amount that can be absorbed and metabolized by the human body (bioavailability). During digestion, food components are constantly exposed to different physical (temperature), chemical (pH) and biochemical (enzymes) conditions. As a consequence of this, the bioavailability and bioactivity of the potential food bioactive compounds are affected (Hur, Lim, Decker, & McClements, 2011). Besides the stability, it is crucial to understand how the complex phenolic compounds found in *A. unedo* fruit, mainly gallotannins and ellagitannins, are released from the fruit matrix, solubilised in the digesta media and finally absorbed through the intestinal epithelium. These changes are a key point for elucidating the capability of food bioactive phytochemicals to be used by the human body, which is a key condition to achieve the levels at which health benefits have been observed. Moreover, part of the ingested phenolic compounds are not absorbed in the small intestine and pass to the large intestine, where the structural modifications promoted by gut microbiota result in the generation of specific active metabolites (González-Barrio, Edwards, & Crozier, 2011; Mosele, Macià, Romero, Motilva, Rubiò, 2015). Although *A. unedo* fruit has been proposed as a potential source of bioactive compounds, there is no information about their stability and changes in their molecular structure during the different steps of gastrointestinal digestion and subsequent colonic fermentation. In order to predict the stability of phenolic compounds, ascorbic acid and fat-soluble antioxidants (α-tocopherol, β-carotene and lutein), *in vitro* gastrointestinal digestion was carried out. As the large intestine is an active place where phytochemicals could be transformed and absorbed, it is also important to consider this

segment in *in vitro* assays. Therefore, the non-absorbable fraction obtained from the *in vitro* gastrointestinal digestion was anaerobically incubated with human faeces and the metabolic pathway for gallotannins, ellagitannins, flavan-3-ols and anthocyanins from *A. unedo* fruit was proposed.

## *2.* MATERIALS AND METHODS

### 2.1. Standards and reagents

Ultrapure water was obtained from a Milli-Q water purification system (Millipore Corp., Bedford, MA, USA). Alpha-amylase, pepsin, pancreatin, bile salts, $KH_2PO_4$ and $Na_2HPO_4{\cdot}2H_2O$ were obtained from Sigma Aldrich (St. Louis, MO, USA). Methanol, ethanol, hexane (all HPLC-grade), hydrochloric acid (37%), glacial acetic acid (99.8%) and methyl-terta-butyl-ether (MTBE) were from Scharlau S.L. (Barcelona, Spain). Trifluoroacetic acid (TFA), $NaHCO_3$, KCl, $CaCl_2$, $MgCl_2{\cdot}6H_2O$, $FeSO_4{\cdot}7H_2O$, $MnSO_4{\cdot}H_2O$, $ZnSO_4{\cdot}7H_2O$, $CoCl_2{\cdot}6H_2O$, $Mo(NH_4)_6O_{24}{\cdot}4H_2O$, and $CuSO_4{\cdot}5H_2O$, NaCl, $Na_2SO_4{\cdot}10H_2O$ were purchased from Pancreac Quimica S.A. (Barcelona, Spain). Acetonitrile (HPLC-grade) was purchased from Romil (Teknokroma, Barcenola, Spain)

For the identification and quantification of the phenolic compounds and antioxidants from *A. unedo* fruit, different commercial standards were used. Ellagic acid, 3-(2,4-dihydroxyphenyl) propionic acid and β-carotene were purchased from Fluka (Buchs, Switzerland); *p*-hydroxybenzoic acid, protocatechuic acid, catechin, epicatechin, α-tocopherol, lutein, 3',4'-(dihydroxyphenyl)acetic acid, *p*-(hydroxyphenyl) acetic acid, phenylacetic acid, 3-(4'-hydroxyphenyl)propionic acid, phenylpropionic acid, and catechol were from Sigma Aldrich (St. Louis, MO, USA); cyanidin-3-*O*-glucoside and dimer $B_2$ from Extrasynthese (Genay, France). Gallic acid was purchased from Panreac (Barcelona, Spain) and ascorbic acid from Sharlau (Barcelona, Spain). Standards of hydroxyl-6H-benzopyran-6-one derivatives (urolithins), such as urolithin A (dihydroxyurolithin), urolithin B (hydroxyurolithin) and urolithin C (trihydroxyurolithin) were kindly provided by Dr. Thasana from Chulabhorn Research Institute, Laksi, Bangkok (Nealmongkol, Tangdenpaisal, Sitthimonchai, Ruchirawat, & Thasana, 2013).

### 2.2. In vitro gastrointestinal digestion

Fruit from *A. unedo* was picked in December of 2013 in the vicinity of Lleida (Catalonia, Spain) **(Figure 1** of **Supplementary information)**. The fruit arrived in the laboratory on the same day it was picked and it was immediately washed by hand and cut into slices with a maximum thickness of 1.5 cm before being lyophilized (LyoBeta 15, Telstar, Netherlands). The lyophilisation cycle consisted in two phases, a first drying at 0.5 mbar (from -20 ºC to -5 ºC within 19 h) and a second drying performed at total vacuum (from 0 ºC to 15 ºC within 37 h). The freeze-dried samples were maintained at -80 ºC in amber containers until the day of the experiment. For the *in vitro* gastrointestinal digestion, the freeze-dried samples were grinded with a commercial coffee mill (Moulinex, France) until obtain a fine and visually homogenous powder (particle size < 2.5 mm). The *in vitro* gastrointestinal digestion was performed to imitate the physiological conditions during human oral, gastric and intestinal steps (Mosele et al., 2015). For this, 1.5 g of ground lyophilized *A. unedo* was incubated at 37ºC in a Gallenkamp IOI400.XX2.C orbital shaker (Lancashire, UK) for 5 min at 200 rpm in presence of artificial saliva (phosphate buffer 0.005 M at pH 6.9 with 0.04% of NaCl and 0.044% of $CaCl_2$) contained α-amylase (15 mg/1.5 g of lyophilized fruit). After the oral step, the sample was incubated under gastric conditions. For this, the pH of the digesta was adjusted to a value 2.0 by adding HCl 37% and incubated at 37ºC for 60 min under continuous shaking (200 rpm) in presence of pepsin (22.5 mg/1.5 g of lyophilized fruit). Then, to imitate intestinal digestion, the pH of the gastric digesta was corrected to 6.5 with $NaHCO_3$ (0.5 N) and pancreatin (30 mg/1.5 g of lyophilized fruit) and bile salts (200 mg/1.5 g of lyophilized fruit) were added. The resulted mixture was circulated (1 mL/min) in darkness for two hours thought a dialysis membrane (Sigma-Aldrich) immersed in phosphate buffer (pH 7.5) (Mosele et al., 2015).

The temperature was maintained at 37 ºC during the digestion using a close circuit of distilled water (at 37 ºC), which surrounded the *in vitro* digestion system. After the dynamic duodenal digestion, two fractions were collected, the "IN" and the "OUT" fractions. The IN fraction (dialysis tube content) was considered the part of the digesta that reach the colon, whereas the OUT fraction (phosphate buffer), which contained the compounds capable of crossing the membrane, was considered as the bioavailable fraction of the *A. unedo*. Each *in vitro* digestion was performed in the absence of light and in triplicate. The samples obtained from the different digestion steps were lyophilized and stored at -80 °C in dark bottles until their chromatographic analysis for phenolic compounds and antioxidants (ascorbic acid, α-tocopherol, β-carotene and lutein). To evaluate their stability during digestion, a percentage of variation (%var) was calculated for each compound. This index provides the amount of each compound (ìmols) present in the complete digesta after gastric or duodenal (IN + OUT) digestions in relation to the amount (ìmols) quantified per 100 g of lyophilized *A. unedo* submitted to *in vitro* digestion. The percentage of absorption (%abs) defines the percentage of each compound (µmols) quantified in the OUT fraction in relation to the amount (µmols) quantified per 100 g of lyophilized *A. unedo* submitted to *in vitro* digestion.

### 2.3. *In vitro* colonic fermentation

The IN fraction obtained after the *in vitro* intestinal digestion was incubated *in vitro* in the presence of human gut microbiota, which was achieved with donations of faeces from three healthy females (27-35 years, BMI 18.5-24.9 kg/m$^2$). The protocol of the study was approved by the Ethical Committee of Clinical Research of Arnau Vilanova University Hospital, Lleida, Spain (Approval Number: CEIC-1326). Volunteers, who reported no intestinal alterations, no consumption of prebiotics and probiotics products and have declared no antibiotic treatment during the last three month previous to the donation day, transported the faecal samples in an airtight container provided with a gas generation sachet (BD GasPack™). In the laboratory, faecal samples were maintained in the containers at 4 ºC and used within the 2 h of defecation. For the fermentation procedure, a media (5% faeces) was prepared mixing the faeces with a pre-reduced carbonate-phosphate buffer prepared according to Mosele, et al., 2015. Ten millilitres of faecal inocula (5% faeces) were aliquoted in dispensable tubes (15 mL), mixed with 0.1 g of IN and incubated for different times (0, 2, 8, 24 and 48 h). These samples were prepared in parallel with two controls: Control 1, which was faecal medium without IN, and Control 2, which was carbonate-phosphate buffer with the IN fraction but without faeces. The incubation was carried out under anaerobic conditions in an orbital shaker (60 rpm) at 37 ºC and all incubations were performed in triplicate. Samples obtained from each time were freeze-dried and stored at -80 ºC until the chromatographic analysis of their phenolic compounds and antioxidants.

### 2.4. Chromatographic analysis of phenolic compounds

For the extraction of phenolic compounds, 0.25 g lyophilized *A. unedo* fruits, 0.25 g of the different fractions of the gastrointestinal digestion (oral, gastric, IN and OUT) and 0.1 g of the *in vitro* fermentation samples were diluted in 2.5 mL, 2.5 mL and 500 µL of methanol/$H_2O$/acetic acid (79.9:20:0.1, v/v/v), respectively. After 10 min of vigorous shacking, the samples were centrifuged for 10 min at 9000 rpm at room temperature. The supernatant was filtered with a 0.22 µm nylon syringe filter and injected into the column.

The analysis of the phenolic compounds in the *A. unedo* fruit, digestion and fermentation samples was performed by ultra-performance liquid chromatography (UPLC) coupled to tandem mass spectrometry (MS/MS) as the detector system (Waters, Milford, MA, USA). The chromatographic column was an Acquity BEH C18 (100 mm x 2.1 mm i.d.) with a 1.7 µm particle size (Waters). The UPLC™ system was equipped with a binary pump system, and a gradient elution was used. Two different mobile phases were used, one for the analysis of anthocyanins, and the second one for the other phenolic compounds. For the analysis of anthocyanins, eluent A was Milli-Q

water/acetic acid (90:10, v/v) and eluent B, acetonitrile. The gradient was performed as follows: 0-10 min, 3-25%B; 10-10.1 min, 25-80%B; 10.1-11 min, 80%B; 11-11.1 min, 80-3%B; 11.1-12.5 min, 3%B. On the other hand, for the analysis of the other phenolic compounds, eluent A was Milli-Q water/acetic acid (99.8:0.2, v/v) and eluent B, acetonitrile. The gradient was performed as follows: 0-5 min, 5-10%B; 5-10 min, 10-12.4%B; 10-18 min, 12.4-28 %B; 18-23 min, 28-100%B; 23-25.5 min, 100 %B; 25.5-27 min, 100-5%B; and 27-30 min, 5%B. For all the phenolic compounds, the flow-rate was 0.4 mL/min and the injection volume was 2.5 μL.

Tandem MS (MS/MS) analyses were carried out on a triple quadrupol detector (TQD) mass spectrometer (Waters) equipped with a Z-spray electrospray interface. The analyses were done in the positive ion mode for anthocyanins and urolithins and in the negative ion mode for the rest of the phenolic compounds. The data were acquired with the selected reaction monitoring mode (SRM). The MS/MS parameters were as follows: capillary voltage, 3 kV; source temperature, 150 ºC; cone gas flow-rate, 80 L/h and desolvatation gas flow-rate, 800 L/h; desolvation temperature, 400 ºC. Nitrogen (>99% purity) and argon (99% purity) were used as nebulizing and collision gases, respectively. The cone voltages and collision energies were optimized for each analyte by injecting each standard compound into a mixture of acetonitrile/water (50:50, v/v) at a concentration of 5 mg/L

Two transitions were studied for each compound, the most abundant was used for quantification, and the second one for confirmation purposes. The dwell time established for each transition was 30 ms. Data acquisition was carried out with the MassLynux v 4.1 Software. **Table 1** shows the SRM conditions used for quantification purposes, as well as the cone voltage and collision energy for each phenolic compound.

Gallic acid, ellagic acid, catechin, epicatechin, dimer, cyanidin glucoside, catechol, p-hydroxybenzoic acid, protocatechuic acid, phenylpropionic acid, hydroxyphenylpropionic acid, dihydroxyphenylpropionic acid, phenylacetic acid, p-hydroxyphenylacetic acid, dihydroxy phenylacetic acid, hydroxyurolithin, dihydroxy-urolithin, trihydroxyurolithin were quantified by their own calibration curves. Gallic acid hexoside, galloylshikimic acid, galloylquinic acid, di-*O*-galloylshikimic acid, di-*O*-galloylquinic acid, tri-*O*-galloylquinic acid and tetra-*O*-galloylquinic acid were tentatively quantified as gallic acid. Ellagic acid arabinoside and strictinin ellagitannin were tentatively quantified as ellagic acid. Epigallocatechin and gallocatechin + catechin were tentatively quantified as catechin while epicatechin gallate and epigallocatechin gallate were tentatively quantified as epicatechin. Hydroxyphenylvaleric acid was tentatively quantified as hydroxyphenylpropionic acid, and dihydroxyphenyl-γ-valerolactone and trihydroxy phenyl-γ-valerolactone were tentatively quantified as dihydroxyphenylpropionic acid. All the anthocyanins were tentatively quantified as cyanidin-3-*O*-glucoside. Tetrahydroxyurolithin and pentahydroxyurolithin were tentatively quantified as trihydroxyurolithin. **Table 1** also provides the information regarded the commercial standards used to quantify each phenolic compound.

### 2.5. Analysis of antioxidants: ascorbic acid, á-tocopherol, â-carotene and lutein

For the determination of the antioxidants in the *A. unedo* fruit, digestion and fermentation samples, high-performance liquid chromatography (HPLC) coupled to photodiode-array detector (DAD) (Waters, Milford, MA) was used. The HPLC-DAD system included a Waters 600 E pump, a Waters column heater (column temperature 22 ºC), a Waters 717 Plus autosampler and a Waters 996 DAD detector. Empower software was used. Before injection, all the samples were filtered with a 0.22 μm nylon syringe filter. All the compounds were quantified by their own calibration curve.

#### 2.5.1. Ascorbic acid analysis

To determine ascorbic acid from the *Arbutus unedo* fruit, oral and gastric digestions, 0.1 g of freeze dried samples was diluted in 10 mL of an aqueous solution of 0.1% TFA. The solutions obtained were vigorous shaken in a vortex for 1 min, and centrifuged for 5 min at 3500 rpm at room temperature. The supernatant was reserved

in dark bottles at 4 ºC and the process was repeated with the residue twice more.

**Table 1.** Optimized SRM conditions used for quantification purposes for the analysis of phenolic compounds by UPLC-MS/MS

| Phenolic compound | MW (g/mol) | SRM quantification | | | Standard used for quantification |
|---|---|---|---|---|---|
| | | Transition | Cone voltage (V) | Collision energy (eV) | |
| Anthocyanins * | | | | | |
| Cyanidin arabinoside | 418 | 419 > 287 | 40 | 20 | Cyanidin-3-O-glucoside |
| Cyanidin glucoside | 448 | 449 > 287 | 40 | 25 | Cyanidin-3-O-glucoside |
| Delphinidin arabinoside | 434 | 435 > 303 | 40 | 20 | Cyanidin-3-O-glucoside |
| Delphinidin glucoside | 464 | 465 > 303 | 40 | 25 | Cyanidin-3-O-glucoside |
| Pelargonidin arabinoside | 402 | 403 > 271 | 40 | 20 | Cyanidin-3-O-glucoside |
| Pelargonidin glucoside | 432 | 433 > 271 | 40 | 20 | |
| Peonidin arabinoside | 432 | 433 > 301 | 40 | 20 | Cyanidin-3-O-glucoside |
| Peonidin glucoside | 462 | 463 > 301 | 40 | 25 | Cyanidin-3-O-glucoside |
| | | | | | |
| Rest of the phenolic compounds | | | | | |
| Gallic acid | 170 | 169 > 125 | 35 | 10 | Gallic acid |
| Gallic acid hexoside | 332 | 331 > 169 | 40 | 15 | Gallic acid |
| Galloylshikimic acid | 326 | 325 > 169 | 40 | 20 | Gallic acid |
| Galloylquinic acid | 344 | 343 > 191 | 40 | 15 | Gallic acid |
| Di-O-galloylshikimic acid | 478 | 477 > 325 | 40 | 20 | Gallic acid |
| Di-O-galloylquinic acid | 496 | 495 > 191 | 40 | 25 | Gallic acid |
| Tri-O-galloylquinic acid | 648 | 647 > 495 | 40 | 15 | Gallic acid |
| Tetra-O-galloylquinic acid | 630 | 629 > 477 | 40 | 15 | Gallic acid |
| Ellagic acid | 302 | 301 > 245 | 45 | 25 | Ellagic acid |
| Ellagic acid arabinoside | 434 | 433 > 300 | 40 | 30 | Ellagic acid |
| Strictinin ellagitannin | 634 | 633 > 301 | 40 | 30 | Ellagic acid |
| Catechin | 290 | 289 > 245 | 45 | 15 | Catechin |
| Epicatechin | 290 | 289 > 245 | 45 | 15 | Epicatechin |
| Dimer | 578 | 577 > 289 | 40 | 20 | Dimer $B_2$ |
| Epigallocatechin | 306 | 305 > 125 | 40 | 15 | Catechin |
| Gallocatechin + catechin | 594 | 593 > 289 | 40 | 30 | Catechin |
| Epicatechin gallate | 442 | 441 > 169 | 40 | 20 | Epicatechin |
| Epigallocatechin gallate | 458 | 457 > 169 | 40 | 15 | Epicatechin |
| Catechol | 110 | 108.9 > 90.9 | 40 | 15 | Catechol |
| p-Hydroxybenzoic acid | 138 | 137 > 93 | 30 | 15 | p-Hydroxybenzoic acid |
| Protocatechuic acid | 154 | 153 > 109 | 45 | 15 | Protocatechuic acid |
| Phenylpropionic acid | 150 | 149 > 105 | 20 | 5 | Phenylpropionic acid |
| Hydroxyphenylpropionic acid | 166 | 165 > 121 | 20 | 10 | Hydroxyphenylpropionic acid |
| Dihydroxyphenylpropionic acid | 182 | 181 > 137 | 20 | 10 | Dihydroxyphenylpropionic acid |
| Phenylacetic acid | 136 | 135 > 91 | 20 | 15 | Phenylacetic acid |
| p-hydroxyphenylacetic acid | 152 | 151 > 107 | 20 | 10 | p-hydroxyphenylacetic acid |
| Dihydroxyphenylacetic acid | 168 | 167 > 123 | 15 | 5 | Dihydroxyphenylacetic acid |
| Hydroxyphenylvaleric acid | 194 | 193 > 175 | 40 | 15 | Hydroxyphenylpropionic acid |
| Dihydroxyphenyl-γ-valerolactone | 208 | 207 > 163 | 40 | 25 | Dihydroxyphenylpropionic acid |
| Trihydroxyphenyl-γ-valerolactone | 224 | 223 > 179 | 40 | 10 | Dihydroxyphenylpropionic acid |
| Hydroxy urolithin (urolithin B) * | 212 | 213 > 141 | 40 | 20 | Urolithin B |
| Dihydroxy urolithin (urolithin A) * | 228 | 229 > 157 | 40 | 20 | Urolithin A |
| Trihydroxy urolithin (urolithin C) * | 244 | 245 > 155 | 40 | 30 | Urolithin C |
| Tetrahydroxy urolithin * | 260 | 261 > 171 | 40 | 25 | Urolithin C |
| Pentahydroxy urolithin * | 278 | 277 > 187 | 40 | 30 | Urolithin C |

MW: Molecular weight
* Positive ionization mode

Then, the supernatants were combined and TFA solution was added until reaching 50 mL and analysed. In the case of the intestinal fractions (IN and OUT) and fermentation samples, 0.1 g was diluted in 1 mL and 500 µL of TFA solution, respectively, and the supernatant obtained after centrifugation was filtered and injected into the column. For the chromatographic determination of ascorbic acid, the analytical column was a BEH C18 column (100 x 4.6 mm, 2.5 ìm) (Waters), and the mobile phase was 0.1% TFA. An isocratic mode was used and the run time was 8 min. The chromatograms were obtained at 243 nm. The flow-rate was 0.4 mL/min, and the injection volume was 20 µL

**Table 2.** Content of phenolic compounds and antioxidants (µmol) in *Arbutus unedo* lyophilized fruit and in fractions of the gastric and duodenal (OUT + IN) steps of the *in vitro* gastrointestinal digestion of *Arbutus unedo*

| Compound (ìmols) | *Arbutus unedo* | Gastric | % var | Duodenal | % var | IN | OUT | % abs |
|---|---|---|---|---|---|---|---|---|
| **Gallic acid and gallotannins** | | | | | | | | |
| Tetra-O-galloylquinic acid | 21.9 | 19.5 | -11.8 | 0.43 | -98.0 | 0.40 | n.q. | 0.00 |
| Tri-O-galloylquinic acid | 2.44 | 2.05 | -16.0 | 0.14 | -94.3 | 0.10 | n.q. | 0.00 |
| Di-O-galloylquinic acid | 84.7 | 26.2 | -69.1 | 7.43 | -91.2 | 7.01 | 0.42 | 0.50 |
| Di-O-galloylshikimic acid | 201 | 122 | -39.1 | 10.6 | -94.7 | 10.2 | 0.38 | 0.19 |
| Galloylquinic acid | 1425 | 281 | -80.2 | 1275 | -10.5 | 943 | 332 | 23.3 |
| Galloyl shikimic acid | 104 | 42.9 | -59.1 | 272 | 159 | 216 | 55.3 | 52.8 |
| Gallic acid hexoside | 189 | 24.5 | -87.0 | 263 | 39.2 | 161 | 102 | 54.1 |
| Gallic acid | 17.2 | 150 | 772 | 156 | 807 | 91.0 | 65.5 | 379 |
| **Total gallotannins** | **2046** | **669** | **-67.3** | **1985** | **-3.00** | **1429** | **556** | **27.2** |
| **Flavan-3-ols** | | | | | | | | |
| Dimer | 26.8 | n.q. | -100 | 23.5 | -12.0 | 20.1 | 3.47 | 13.0 |
| Gallocatechin | 12.1 | n.q. | -100 | 6.07 | -50.1 | 5.54 | 0.53 | 4.37 |
| Epigallocatechin gallate | 2.06 | 0.23 | -88.8 | 0.1 | -31.9 | 0.99 | n.d. | n.d. |
| Catechin | 78.5 | 0.60 | -99.2 | 53.4 | 169 | 34.5 | 18.9 | 24.1 |
| Epicatechin | 0.72 | n.q. | -100 | 1.93 | 161 | 0.94 | 0.99 | 137 |
| Epigallocatechin | 0.93 | n.q. | -100 | 15.9 | -57.2 | 12.9 | 3.01 | 324 |
| Epicatechin gallate | 16.7 | n.q. | -100 | 7.16 | -51.5 | 6.12 | 1.05 | 6.25 |
| **Total flavan-3-ols** | **137** | **0.83** | **-99.4** | **109** | **-20.9** | **81.1** | **28.0** | **20.3** |
| **Ellagic acid and ellagitannins** | | | | | | | | |
| Strictinin ellagitannin | 184 | 29.1 | -84.2 | 104 | -43.3 | 99.2 | 4.80 | 2.61 |
| Ellagic acid arabinoside (xyloside) | 434 | 86.4 | -80.1 | 289 | -33.5 | 238 | 50.6 | 11.6 |
| Ellagic acid | 9.44 | 26.4 | 179 | 9.17 | -2.80 | 7.25 | 1.92 | 20.4 |
| **Total ellagitannins** | **628** | **142** | **-77.4** | **402** | **-35.9** | **345** | **57.3** | **9.13** |
| **Anthocyanins** | | | | | | | | |
| Pelargonidin arabinoside | 0.03 | 0.02 | -41.9 | 0.01 | -62.0 | 0.01 | 0.01 | 15.8 |
| Pelargonidin glucoside | 0.19 | 0.10 | -46.7 | 0.04 | -76.9 | 0.03 | 0.01 | 6.02 |
| Cyanidin arabinoside | 2.89 | 1.52 | -47.3 | 0.55 | -80.9 | 0.45 | 0.1 | 3.49 |
| Cyanidin glucoside | 15.5 | 13.6 | -12.7 | 2.53 | -83.7 | 2.08 | 0.45 | 2.89 |
| Peonidin arabinoside | 0.01 | 0.01 | 15.9 | 0.01 | -36.1 | 0.01 | n.q. | 0.00 |
| Peonidin glucoside | 0.03 | 0.10 | 261 | 0.04 | 28.5 | 0.03 | 0.01 | 22.5 |
| Delphinidin arabinoside | 0.38 | 0.14 | -64.5 | 0.16 | -58.5 | 0.07 | 0.09 | 24.0 |
| Delphinidin glucoside | 0.64 | 0.25 | -60.7 | 0.43 | -32.8 | 0.34 | 0.09 | 13.7 |
| **Total anthocyanins** | **19.7** | **15.7** | **-20.3** | **3.78** | **-80.8** | **3.02** | **0.75** | **3.82** |
| **Total phenolic compounds** | **2832** | **828** | **-70.8** | **2500** | **-11.7** | **1858** | **642** | **22.7** |
| **Other Antioxidants** | | | | | | | | |
| Ascorbic acid | 2496 | 2481 | -0.58 | 846 | -66.1 | 386 | 460 | 18.4 |
| α-tocopherol | 32.6 | 14.6 | -54.7 | 13.2 | -59.1 | 12.7 | 0.60 | 1.87 |
| β-carotene | 3.80 | 1.77 | -53.4 | 5.03 | -32.3 | 5.03 | n.q. | 0.00 |
| Lutein | 1.45 | n.q. | -100 | 1.84 | 10.3 | 1.84 | n.q. | 0.00 |

The results are expressed as umol of compounds in relation to the content in 100 g of lyophilized fruit submitted to digestion
n.d.: no quantified (lower than the detection limit (< LOD)). n.q.: its concentration between its LOD and LOQ
% var and % abs: see Materials and methods (section 2.2)

### *2.5.2.* α-tocopherol analysis

One gram of lyophilized *A. unedo* and digestion fractions (oral, gastric, IN and OUT) and 0.2 g of fermentation samples were vigorously mixed for 1 min with hexane and centrifuged at 9000 rpm, for 10 min at 4 ºC. The extraction procedure was repeated (n=3) until the α-tocopherol was not detected in the last extraction supernatant by chromatographic analysis. Then, the supernatants were combined and the mixture was rotavapored (Rotavapor® R-205, Büchi®, Switzerland) at 20 ºC (Heating bath B-490, Büchi, Switzerland) until fully dry. The *A. unedo* fruit and digestion samples were reconstituted in 1 mL of hexane whereas the fermentation samples were reconstituted in 200 μL.

For the chromatographic determination of α-tocopherol, the analytical column was Sulpelcosil LC-$NH_2$ (250 x 4.6 mm, 5μm) (Supelco, Bellefante, PA, USA), and the mobile phase was 70% hexane as eluent A, and 30% ethyl acetate as eluent B. An isocratic mode was used and the run time was 20 min. The chromatograms were obtained at 295 nm. The flow-rate was 1 mL/min, and the injection volume was 20 μL.

### *2.5.3.* β-carotene and lutein analysis

One g of the lyophilized *A. unedo* and digestions (oral, gastric, IN and OUT fractions) and 0.2 g fermentation samples were vigorous mixed for 1 min with a mixture of ethanol/hexane (60:40, v/v) and centrifuged at 9000 rpm, for 10 min at 4 ºC. The extraction procedure was repeated (n=3) until a colourless supernatant was obtained and the studied compounds were not detected in the last extraction supernatant by chromatographic analysis. The combined supernatants were rotavapored and the dry residue was redissolved in 2 mL of ethanol in the case of the fruit and digestions, and 200 μL for the fermentation residues.

For the chromatographic determination of β-carotene and lutein, the analytical column was an YMC C30 (150 × 4.6 mm, 3 μm) (Waters), and the mobile phase was methanol as eluent A, MTBE as eluent B, and Milli-Q water as eluent C. The gradient elution was of B into A with a constant 2%C, as follows: 0-27 min, 2-80 %B; 27-31 min, 80-2 %B; 31-35 min, 2 %B isocratic. The chromatograms were obtained at 450 nm. The flow-rate was 1 mL/min, and the injection volume was 20 μL.

### *2.6.* Total pectin content

Total pectin was determined in the lyophilized *A. unedo* fruit according to the method described by Maran, 2015, which is based on gravimetric determination. The results are expressed as g the pectin/100 g of lyophilized *A. unedo* fruit.

### *2.7.* Statistical analysis

All data are expressed as the average of the three replicates of the different digested fractions. To simplify the results shown in **Tables 1** and **2**, the standard deviations were omitted because they were lower than 10% of the values

## 3. RESULTS AND DISCUSSION

### 3.1. Phenolic and antioxidants content in *Arbutus unedo* fruit

The content of extractable polyphenols, ascorbic acid and fat-soluble antioxidants (α-tocopherol, β-carotene and lutein) in the *A. unedo* fruit is presented in **Table 2**. The data are expressed as μmols of compound per 100 g of lyophilized fruit submitted to *in vitro* digestion. Regarding the phenolic composition, the fruit of the *A. unedo* contains a wide variety of phenolic groups, including anthocyanins, flavan-3-ols, phenolic acids and hydrolysable tannins, such as ellagitannins and gallotannins. It is worth highlighting that the gallotannin tetra-*O*-galloylquinic acid is described for the first time in *A. unedo* fruit. As it is shown in **Table 2**, the gallotannins were the most abundant phenolic compounds in *A. unedo* fruit (2046 μmol/100 g lyophilized fruit) followed by ellagitannins (628 μmol/100 g lyophilized fruit). Nevertheless, other authors found flavan-3-ols to be the most abundant phenolic compounds in *A. unedo* (Guimarães et al., 2013; Pallauf, Rivas-Gonzalo, del Castillo, Cano, & de Pascual-Teresa, 2008). Considering the total phenolic content quantified

in the present study (2832 µmol/100 g lyophilized fruit), this was lower than the content determined in *A. unedo* fruit harvested in Portugal (Fortalezas et al., 2010).. This fact could be associated with the analytical technique used for determining the total phenols. These authors applied the Folin-Ciocalteau method (colorimetric), which is considered a non-specific analytical method that results in an overestimation of phenolic compounds due to the presence of ascorbic acid as a powerful reducing agent (Singleton, Orthofer, & Lamuela-Raventós, 1999). Regarding antioxidants, the *A. unedo* fruit could be considered an interesting dietary source of ascorbic acid (2496 µmol/100 g lyophilized fruit) and a lesser source of fat-soluble antioxidants such as α-tocopherol and carotenoids, β-carotene and lutein

The concentrations of these compounds detected in *A. unedo* fruit in the present study were also in agreement with previous data (Oliveira, Baptista, Malheiro, Casal, Bento, & Pereira, 2011), with the exception of lutein, which was found at higher concentration than that reported previously (Pallauf, Rivas-Gonzalo, del Castillo, Cano, & de Pascual-Teresa, 2008). It is important to highlight the high concentration of ascorbic acid in *A. unedo* fruit. Its concentration is higher than in other fruit commonly thought of as rich sources of vitamin C, such as oranges, strawberries and kiwis (Proteggente et al., 2002; Szeto, Tomlinson, & Benzie, 2002). In addition, when the concentrations of the fat-soluble antioxidants were compared to other fruits, such as apples, bananas, kiwis and oranges, *A. unedo* also presented higher levels of α-tocopherol (Chun, Lee, Ye, Exler, & Eitenmiller, 2006), β-carotene and lutein (USDA, 2015).

In order to test the stability of each individual phenolics and ascorbic acid and fat-soluble antioxidants during gastrointestinal digestion, an *in vitro* digestion model, mimicking the oral, gastric and duodenal conditions, was applied to *A. unedo* fruit samples.

### 3.2. Stability of Arbutus unedo fruit phenolic compounds and antioxidants during *in vitro* gastric step

After 5 min of oral digestion, minimal modifications were observed in the concentration of the phenolic compounds and vitamins (results not shown). No relevant differences were also observed in pomegranate juice, pulp and extract after oral digestion (Mosele et al., 2015)

The short exposure time and marginal effects of α-amylase could explain the lack of changes in the phytochemical profile of *A. unedo*, which would justify the omission of this step in other studies (Bouayed, Hoffmann, & Torsten 2011; Carnachan, Bootten, Mishra, Monro, & Sims, 2012; Green, Murphy, Schulz, Watkins & Ferruzzi, 2007; Vallejo et al., 2004). By contrast, simulated gastric digestion (pH 2.0 in presence of pepsin) caused an important decrease in the recovery of the phenolic compounds and fat-soluble vitamins (**Table 2**).

To perform a real balance of the effect of gastric digestion, the concentration of each individual compound was expressed as ìmols per 100 g of lyophilized *A. unedo* fruit, and as a percentage of variation (%var) between the initial µmols submitted to *in vitro* digestion and the µmols recovered in the total digesta after the incubationAs shown in **Table 2** and **Figure 1**, important differences were observed in the recovery (%var) of phenolic compounds and fat-soluble antioxidants after gastric digestion. The stability and potential absorption of low molecular weight compounds, such as gallic, ellagic and ascorbic acids, was slightly affected during gastric digestion. In fact, gallic and ellagic acids were recovered in larger amounts compared to the initial values (772 and 179 %var, respectively), and this could be due to the partial hydrolysis of the gallotannins and ellagitannins, respectively. By contrast, the most heavily-affected phenolic group was flavan-3-ols (-99.4 %var), followed by ellagitannins (-77.4 %var), gallotannins (-67.3 %var) and anthocyanins (-20.3 %var). High recovery of anthocyanins after gastric digestion was also observed in previous studies, this phenomena was attributed to their chemical characteristics that convert the anthocyanin in an acidic-tolerant molecule (Bouayed, Hoffmann, & Torsten 2011; Mosele et al., 2015). Regarding the fat-soluble antioxidants, the recovery after gastric digestion of α-tocopherol (-54.7 %var), β-carotene

(–53.4 %var), and lutein (-100 %var) was lower than the recovery of ascorbic acid, which showed a high stability under the pH (2.0) and temperature (37 ºC).

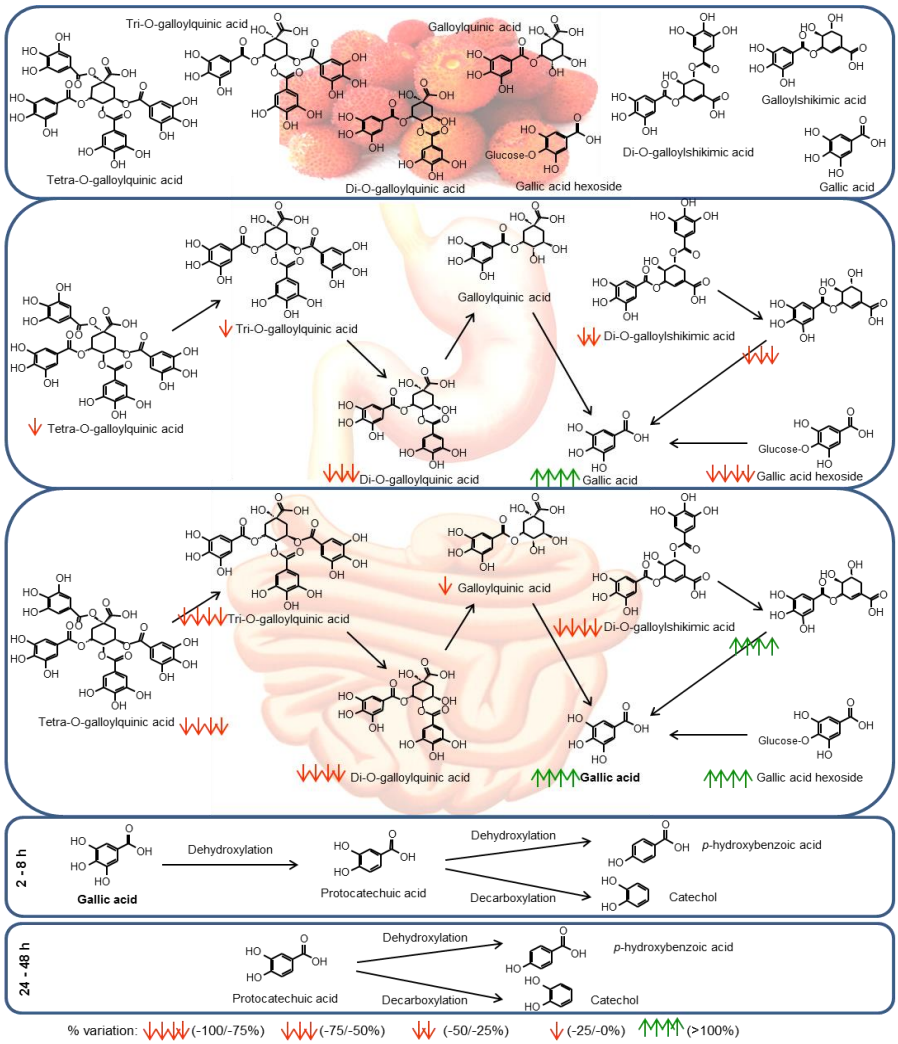


**Figure 1.** Schematic representation of the metabolic pathway of gallotannins from *Arbutus unedo* fruit after the simulated *in vitro* steps, stomach, small intestine and large intestine. The red down arrows represent a decrease in the recovery of compounds compared with the raw *A. unedo*. The green up arrows represent an increase in the recovery of compounds compared with the raw *A. unedo*.

To understand the low recovery of the some compounds studied in the digesta after gastric incubation, the composition of the food matrix submitted to digestion should be considered. Some studies have suggested that the presence of pectin could interact with bioactive compounds and affect their release into the digestive media due to their intragastric gelation properties (Padayache et al., 2012a; Padayache et al., 2012b). Thus, in order to connect the low recovery of bioactive compounds after gastric digestion, observed in the present study, with the

content of pectin in *A. unedo* fruit, this type of water-soluble fibre was measured and its average concentration was 4.1%. This amount was reported to be enough to promote the formation of gel under acidic conditions (Zhang, & Vardhanabhuti, 2014), which could explain the low recovery of gallotanins, ellagitannins and flavan-3-ols (**Table 2**). Similarly, a minor release of phenolic compounds was also observed after gastric incubation of whole apples (Bouayed, Hoffmann, & Torsten 2011), fruit with high amounts of pectin in its skin (Billy et al., 2008). Summarizing, the differences in the recovery of phenolic compounds and fat-soluble antioxidants could be attributed to the stability of the molecule under acidic conditions as well as their ease of entrapment in the pectin-gel.

### 3.3. Stability of *A. unedo* fruit phenolic compounds and antioxidants during *in vitro* intestinal digestion

After the intestinal digestion step, the two fractions were collected and analyzed separately. These were the outside dialysis solution (OUT), which was considered the dialyzable fraction, and the inside dialysis tub content (IN), referring to the non-dialyzable fraction. To study the effect of the intestinal conditions on the stability of the studied compounds, the µmols quantified in the IN and OUT fractions were summed (**Table 2**). Despite the low recovery of some of the compounds studied after gastric incubation, their recovery increased after simulated duodenal digestion. As can be seen in **Table 2**, the negative value (%var) of galloylquinic and galloyl shikimic acids, gallic acid hexoside, catechin and carotenoids observed after gastric digestion showed a positive value after duodenal digestion, the ìmols quantified in the total digesta (IN fraction plus OUT fraction) being similar to the ìmols quantified in 100 g of lyophilized *A. unedo* fruit prior to digestion. This behaviour could be explained by the link between some phenolic structures and the pectin-gel formed under the acidic conditions of the gastric digestion (pH 2.0), which could explain the low recovery of the main phenolic compounds. By contrast, the pH conditions of the duodenal phase (pH 6.5) could disrupt the gel structure due to depolymerisation by β-elimination (Carnachan, Bootten, Mishra, Monro, & Sims, 2012). The time and the speed at which the phenolics were released from the gel-structure could vary significantly according to the nature of the molecule, and this could influence their stability. Higher quantities of gallic acid, galloylshikimic acid, epicatechin and epigallocatechin were recovered compared with the initial amounts detected in the raw *A. unedo* fruit (prior to digestion) and the product obtained after gastric incubation. This fact could be explained by the partial hydrolysis (pH 6.5) of gallotannins and oligomeric flavan-3-ols, respectively. Based on the results observed after gastric and duodenal in vitro digestion of *A. unedo* fruit, we proposed the metabolic pathway of gallotannins based on the hydrolysis of tetra-*O*-galloylshikimic to tri-, di-, and mono-*O*-galloylshikimic until the simplest phenolic and gallic acids quantified in duodenal digesta (small intestine conditions) (**Figure 1**).

Besides gallotannins, anthocyanins also suffered an intense degradation after the duodenal step compared with the high recovery observed after gastric digestion (**Table 2**). This fact could suggest that anthocyanins were not embedded and hence protected by the pectin web, which could enhance the exposition of these flavonoids to the media. Previous studies where anthocyanins bioaccessibility was investigated under *in vitro* conditions have also observed important losses in the final amount recovered (Bouayed, Hoffmann, & Torsten 2011; Mosele et al., 2015). This was attribute to the sensitivity of anthocyanins to the medium-alkaline conditions of the intestinal environment (pH>6). Deglycosylation and subsequent liberation of the anthocyanins algycone to the media to explain the disappearance of anthocyanins should be discarded since β-deglycosylation activity has not been described for pancreatic enzymes. The presence of ascorbic acid has been described to confer protection to some phenolic classes such as flavan-3-ols during *in vitro* intestinal digestion (Green, Murphy, Schulz, Watkins & Ferruzzi, 2007). Probably, stability of anthocyanins was also negative affected by the parallel degradation of ascorbic acid during the duodenal step.

As far as antioxidants are concerned, ascorbic acid degradation through the gastric portion of the *in vitro* digestive model was not significant and degradation occurred primarily during the small intestinal phase (IN plus OUT fractions) (**Table 2**). The sensitivity of ascorbic acid to small intestinal conditions has been previously reported (Vallejo et al., 2004). By contrast, the α-tocopherol instability occurred primarily during the gastric phase. Interestingly, β-carotene and lutein were recovered in higher amounts after the duodenal step compared to their values in the *A. unedo* fruit prior to digestion (**Table 2**). These results suggest that fat-soluble vitamins could be retained by pectin-gel during gastric incubation and released when the media conditions changed, as is the case of the change from gastric (pH 2.0) to intestinal digestion (pH 6.5).

After the intestinal digestion step, the dialyzable fraction (OUT) was considered to be the fraction that could be available for absorption into the systematic circulation by passive diffusion. For each compound, the bioaccessibility (%abs) is defined as the percentage of compound that is quantified (µmols) in the soluble/dialyzable fraction (OUT) after the intestinal step (**Table 2**). The phenolic compounds recovered in major proportion in this fraction were gallic acid and gallotannins (27.2 %abs) and flavan-3-ols (20.3 %abs), followed by ellagic acid and ellagitannins (9.13 %abs) and anthocyanins (3.82 %abs). This tentative approach to predict the degree of intestinal epithelium absorption is in agreement with human studies where the bioavailability of phenolic compounds was studied after the intake of vegetable products (Clifford, Van Der Hooft, & Crozier, 2013; Stoner et al., 2005). Gallic acid and flavan-3-ols and their generated metabolites were detected in human plasma and urine after green tea intake (Clifford, Van Der Hooft, & Crozier, 2013). The poor efficiency of the ellagic acid and anthocyanins intestinal absorption was reported in human biological fluids after the intake of lyophilized black raspberries (Stoner et al., 2005).

**Table 3.** Phenolic precursors, phenolic microbial metabolites and antioxidants content (µmol compound / 100 g lyophilized *A. unedo* fruit) detected in the non dialyzable IN fraction and in the fecal media at different incubation times (2, 8, 24 and 48 h) for each fecal donor (Volunteers 1, 2 and 3)

| **Phenolic microbial metabolites (µmol/100 g *AU*)** | IN fraction | Fermentation time | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 2 h | | | 8 h | | | 24 h | | | 48 h | | |
| | | Vol 1 | Vol 2 | Vol 3 | Vol 1 | Vol 2 | Vol 3 | Vol 1 | Vol 2 | Vol 3 | Vol 1 | Vol 2 | Vol 3 |
| *Gallotannins* | | | | | | | | | | | | | |
| Galloylquinic acid | 943 | 133 | 177 | 177 | 22.1 | 4.16 | 16.9 | n.q. | n.q. | n.q. | n.q. | n.q. | n.q. |
| Galloylshikimic acid | 216 | 45.3 | 4.89 | 4.89 | n.q | n.q | n.q | n.q. | n.q. | n.q. | n.q. | n.q. | n.q. |
| *Ellagitannins* | | | | | | | | | | | | | |
| Ellagic acid | 7.25 | 1.61 | n.q. | n.q. | n.q | n.q | n.q | n.q. | n.q. | n.q. | n.q. | n.q. | n.q. |
| Pentahydroxy urolithin | n.d. | n.q. | n.q. | n.q. | n.q. | 0.01 | 0.05 | n.q. | n.q. | n.q. | n.q. | 0.01 | n.q. |
| Tetrahydroxy urolithin | n.d. | n.q. | 0.01 | 0.01 | n.q | n.q | n.q | n.q. | n.q. | n.q. | n.q. | n.q. | n.q. |
| Trihydroxy urolithin | n.d. | 0.02 | 0.01 | 0.01 | 0.17 | 0.82 | 0.19 | 0.57 | 0.14 | 0.03 | 0.14 | 0.02 | 0.01 |
| Dihydroxy urolithin | n.d. | n.q. | n.q. | n.q. | n.q. | n.q. | 6.1 | n.q. | 7.99 | 12.1 | 7.43 | 15.4 | 18.8 |
| Hydroxyurolithin | n.d. | n.q. | n.q. | n.q. | n.q. | n.q. | n.q. | n.q. | n.q. | 0.18 | n.q. | n.q. | 1.38 |
| *Hydroxyphenyl-γ-valerolactones* | | | | | | | | | | | | | |
| Dihydroxyphenyl-γ-valerolactone | n.d. | n.q. | n.q. | n.q. | 0.19 | n.q. | n.q. | 0.42 | 0.05 | 0.02 | 0.31 | n.q | n.q. |
| Trihydroxyphenyl-γ-valerolactone | n.d. | n.q. | n.q. | n.q. | 0.14 | n.q. | n.q. | 1.56 | 1.42 | 0.00 | 7.26 | n.q | 12.9 |
| *Valeric acids* | | | | | | | | | | | | | |
| Hydroxyphenylvaleric acid | n.d. | n.q. | n.q. | n.q. | n.q. | n.q. | n.q. | n.q. | n.q. | 79.2 | n.q. | 137 | 474 |
| *Hydroxyphenylpropionic acids* | | | | | | | | | | | | | |
| Dihydroxyphenylpropionic acid | n.d. | 0.18 | n.q. | n.q. | 11.8 | 15.6 | 10.6 | 22.2 | 37.7 | 13.2 | 21.9 | 13.7 | 4.71 |
| Hydroxyphenylpropionic acid | n.d. | 18.7 | n.q. | n.q. | 22.0 | 115 | 649 | 539 | 0.00 | 943 | 564 | n.q | 725 |
| Phenylpropionic acid | n.d. | n.q. | n.q. | n.q. | n.d | 118 | n.d | n.q. | 131 | n.q. | n.q. | 69.5 | 139 |
| *Hydroxyphenylacetic acids* | | | | | | | | | | | | | |
| Dihydroxyphenylacetic acid | n.d. | n.q. | n.q. | n.q. | 18.5 | 5.49 | 12.7 | 1.20 | 5.88 | 0.94 | n.q | 3.45 | 7.84 |
| *p*-hydroxyphenylacetic acid | n.d. | n.q. | n.q. | n.q. | 32.8 | n.q. | n.q. | 181 | 55.0 | 207 | 113 | 291 | 138 |
| Phenylacetic acid | n.d. | n.q. | n.q. | n.q. | n.q. | n.q. | n.q. | n.q. | n.q. | n.q. | 321 | n.q. | 550 |
| *Hydroxybenzoic acids* | | | | | | | | | | | | | |
| *p*-hydroxyenzoic acid | n.d. | 1.20 | n.q. | n.q. | 6.41 | 2.65 | 1.70 | 5.36 | 2.19 | 2.41 | 3.97 | 2.26 | 2.16 |
| Protocatechuic acid | n.d. | 8.85 | 15.2 | n.d. | 48.5 | 40.5 | 118 | n.q. | n.q. | n.q. | 0.00 | 0.00 | 2.79 |
| Gallic acid | 91.0 | 321 | 382 | 318 | 296 | 179 | 97.2 | n.q. | n.q. | n.q. | n.q. | n.q. | n.q. |
| *Benzenediols* | | | | | | | | | | | | | |
| Catechol | n.d. | 3.13 | 4.43 | n.q. | 76.7 | 85.6 | 167 | 43.1 | 70.1 | 259 | 24.6 | 279 | 238 |
| *Flavan-3-ols* | | | | | | | | | | | | | |
| Catechin | 34.5 | n.q. | n.q. | n.q. | n.q. | n.q. | n.q. | n.q. | n.q. | n.q. | n.q. | n.q. | n.q. |
| ***Antioxidants (µmol/100 g AU)*** | | | | | | | | | | | | | |
| α-tocopherol | 12.7 | 14.7 | 13.0 | 10.8 | 15.2 | 14.1 | 12.3 | 13.4 | 11.8 | 12.9 | 14.6 | 18.3 | 6.87 |
| β-carotene | 5.03 | 6.66 | 1.63 | 3.36 | 15.2 | 2.13 | 2.90 | 2.24 | 1.97 | 2.26 | 10.6 | 1.68 | 2.08 |

Vol.: volunteer
n.d.: no quantified (lower than the detection limit (< LOD))
n.q.: its concentration between its LOD and LOQ

Regarding the antioxidants in the OUT fraction, only ascorbic acid (18.4 %abs) and α-tocopherol, at a very low level (1.87 %abs), were quantified. Furthermore, β-carotene and lutein were not detected, indicating no diffusion through the dialysis membrane during the intestinal digestion phase. Although pectin could be considered a protector agent for some phenolic groups during the digestion process, its presence seemed to affect the bioavailability of β-carotene, lutein (Aschoff et al., 2015) and tocopherols (Richelle et al., 2004). In addition, the solubility of fat-soluble vitamins also depends on the presence of lipids (Read, Wright, & Abdel-Aal, 2015), but these are very limited in *A. unedo* fruit (Ruiz-Rodriguez et al., 2011), which could also explain the low recovery of fat-soluble vitamins in the OUT fraction. It could be concluded that most phenolic compounds present in the *A. unedo* fruit reach the large intestine. The non-absorbable fraction of phenolic compounds (non dialyzable IN fraction), which was susceptible to be catabolised by the gut microbiota, was composed mainly of gallic acid and gallotannins, ellagic acid and ellagitannins, and to a lesser extent, flavan-3-ols and anthocyanins (**Table 2**).

### 3.4. *In vitro* colonic fermentation of Arbutus unedo

After the gastric and intestinal *in vitro* digestion phases, the IN fraction (non dialyzable) was submitted to *in vitro* colonic fermentation using a human faeces inoculum from three healthy volunteers. The generation of phenolic catabolites was monitored at different incubation times from 0 to 48 h, considering the IN fraction as time-point 0 h. **Table 3** shows the microbial metabolites generated for each faecal donor at different fermentation times. The metabolites described in **Table 3** were those that appeared in major concentration in faecal media incubated with the IN fraction compared to control 1 and control 2; control 1 being faecal medium without IN, and control 2, carbonate-phosphate buffer with the IN fraction but without faeces. As was expected, different metabolites were generated at different fermentation times. Although an evident intervariability between the volunteers was observed, some behaviour patterns were repeated.

Since the IN fraction post-intestinal digestion of *A. unedo* contained gallic acid, gallotannins, flavan-3-ols, ellagic acid, ellagitannins and anthocyanins, a wide range of different microbial metabolites derived from their microbial catabolism were detected (**Table 3**). Based on the metabolites quantified at different incubation times, we proposed the colonic pathways for gallotannins, ellagitannins, flavan-3-ols and anthocyanins (**Figure 2**). Dihydroxyphenyl-γ-valerolactone and trihydroxyphenyl-γ-valerolactone were associated with the microbial catabolism of (epi)catechin and (epi)gallocatechin moieties, respectively (Jiménez-Girón, Muñóz-González, Martín-Álvarez, Moreno-Arribas, & Bartolomé, 2014; Serra et al., 2011). Then, dihydroxyphenylvaleric acid was generated from dihydroxyphenyl-γ-valerolactone (Jiménez-Girón, Muñóz-González, Martín-Álvarez, Moreno-Arribas, & Bartolomé, 2014). In the present study, we proposed that in the last phases of the colonic catabolism, the hydroxyphenylpropionic and hydroxyphenylacetic acids could be synthesised from dihydroxyphenylvaleric acid, followed by the simplest phenolic compounds, such as protocatechuic and *p*-hydroxybenzoic acids (**Figure 2**). Phenylpropionic and phenylacetic acids, in their hydroxylated forms, were determined at the final incubation times (24 and 48 h) (**Table 2**), and apart from being generated from flavan-3-ols, these have also been reported as coming from anthocyanins (González-Barrio, Edwards, & Crozier, 2011). In addition of these acids, protocatechuic and gallic acids have also been reported as coming from cyanidin-3-*O*-glucoside (Hanske et al., 2013; Vitaglione et al., 2007), this being the main anthocyanin detected in the IN fraction in the present study.

The other phenolic compounds detected in the faecal media were the urolithins. These compounds have been reported to be exclusive to ellagic acid microbial metabolism (García-Villalba et al., 2013). The kinetic generation of the different hydroxylated forms of urolithins detected in the present study (**Table 3**) was similar to that described after ellagic acid *in vitro* fermentation (García-Villalba et al., 2013). The more

hydroxylated urolithins were found at the initial fermentation times. Then, the bacterial metabolism was increased and led to the formation of the more lipophilic urolithins, such as dihydroxylated urolithins (urolithin A) and monohydroxylated urolithins (urolithin B). These two urolithin metabolites have been described as the main ellagic acid microbial metabolites (García-Villalba et al., 2013).

There is little information in the literature regarding the microbial colonic metabolism of flavan-3-ols, ellagitannins and anthocyanins. Nevertheless, no data is reported about the metabolic pathway of gallotannins. In order to fill this gap in the information, the gastrointestinal and colonic metabolic fate of gallotannins from the *A. unedo* fruit is proposed in **Figure 1**, while the colonic pathways of flavan-3-ols, ellagitannins and anthocyanins are represented in **Figure 2**.

Degradation of the gallotannins remaining in the IN fraction continued in the presence of human microbiota with an accumulation of gallic acid at early stages of incubation (2 h). At this same time (2 h), protocatechuic acid, *p*-hydroxybenzoic acid and catechol began to be detected in the faecal media. The maximum concentration of protocatechuic acid was detected at 8 h of incubation in the faecal media from the three volunteers. However, the maximum peak of the other microbial metabolites, *p*-hydroxybenzoic acid and catechol, depended on the faecal donor (Table 3). *p*-hydroxybenzoic acid was at 8 h for volunteers 1 and 2, and at 24 h for volunteer 3. Regarding catechol, its maximum concentration was at 8 h for volunteer 1, and at 48 h for volunteers 2 and 3. The microbial metabolites of gallotannins generated: gallic acid, protocatechuic acid and *p*-hydroxybenzoic acid, were also described for flavan-3-ols and anthocyanins (**Figure 2**) (Hanske et al, 2013; Serra et al., 2011). The gut microbiota composition, together with the quantity and quality profile of the phenolic compounds interacting with microorganisms (Cueva et al., 2013), are probably implicated in the types of microbial metabolites generated in the present study.

As far as antioxidants are concerned, a rapid degradation of ascorbic acid was observed in the faecal media, since this compound was no detected after any incubation time. On the other hand, α-tocopherol and β-carotene remained very stable, even after 48 h of incubation. Their resistance to microbial metabolism and media conditions was also observed in human faeces (Pierre et al., 2013).

## 4. CONCLUSIONS

The results obtained in the present study provide further evidence about the *in vitro* digestion stability and colonic metabolism of *A. unedo* antioxidants from the fruit to the colon. Depending on the phenolic groups and the antioxidant compound, different behaviour was observed under *in vitro* gastric and intestinal digestive conditions. The presence of pectin from the *A. unedo* fruit hampered the solubilisation of the phenolic compounds (with exception of gallic and ellagic acids) and fat-soluble vitamins during gastric digestion. The increase in the viscosity of bulk could lead to a slowdown in gastric emptying and this favour the gradual release of phenolic compounds from the pectin gel network into the small intestine. Therefore, a large proportion of the phenolic compounds and antioxidants from the *A. unedo* fruit remained in the non-absorbable fraction of the digesta (IN fraction), indicating that these compounds could reach the colon, where they are susceptible of further microbial transformations. The catabolic activity of human microbiota led to the generation of different phenolic microbial metabolites derived from the gallotannins, ellagitannins, flavan-3-ols and anthocyanins that remained stable after the in vitro intestinal digestion. These were urolithins, hydroxyphenylvalerolactones, hydroxyphenyl propionic acid, hydroxylphenylacetic acid, and the simplest phenols, such as gallic acid, *p*-hydroxybenzoic acid, protocatechuic acid and catechol.

Therefore, while these in vitro results cannot be fully extended directly to in vivo conditions, these data provide evidence that the fruit of the *A. unedo* may be interesting as a rich dietary source of a wide range of phenolic compounds, together with ascorbic acid and fat-soluble antioxidants.

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**ADDITIONAL INFORMATION**

**Figure 1**. *Arbutus unedo* fruit

**PUBLICATION VII** Study of human metabolism of Arbutus unedo phenols: Application of dried blood spot cards as a rapid sample pre-treatment method for determining phenolic metabolites in whole blood

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PAPER

# UNDERSTANDING OF HUMAN METABOLIC PATHWAYS OF DIFFERENT SUB-CLASSES OF PHENOLS FROM ARBUTUS UNEDO FRUIT AFTER AN ACUTE INTAKE

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**Abstract**

*Arbutus unedo is a small Mediterranean fruit, commonly named strawberry tree, which is a rich source of different sub-classes of phenolic compounds, the more representative being the gallic acid derivatives, including its mono and oligomeric forms esterified with quinic and shikimic acids. In addition, galloyl derivatives, particularly gallotannins, described in A. unedo, are part of a very selective phenolic group, present in a reduced number of plant-products. The aim of the present study is to provide a better understanding of human metabolic pathways of different sub-classes of phenols from the A. unedo fruit after an acute intake by healthy adults. Therefore, the A. unedo phenolic metabolites were studied in whole blood samples (0 to 24 h), urine (24 h) and feces (12 and 24 h). Special focus was placed on the application of dried blood spot (DBS) cards for the sample collection and for the analysis of phenolic metabolites in whole blood samples. The results of the blood analysis revealed two peaks for the maximum concentrations of the main phenolic metabolites. Furthermore, it is appropriate to highlight the application of DBS cards as an efficient and accurate way to collect blood samples in post-prandial bioavailability studies. The analysis of urine (24 h) gave a wide range of phenolic metabolites showing the extensive metabolism that A. unedo phenolic compounds underwent in the human body. The results of the study provide a relevant contribution to the understanding of the in vivo human bioavailability of phenolic compounds, especially galloyl derivatives, a singular phenolic sub-group present in the A. unedo fruit.*.

**Key words:** *Arbutus unedo*, human phenol bioavailability, dried spot cards, phenolic metabolism

## 1. INTRODUCTION

An eagerness to raise the value of local products rich in bioactive compounds has stimulated interest in the characterization of little-known plant products. *Arbutus unedo* (*A. unedo*) is a small Mediterranean fruit, commonly named strawberry tree that reaches it optimal ripeness at the end of autumn. *A. unedo* belongs to the Ericaceae family, native to the Mediterranean region and from the northeast of Europe to western France and Ireland. The macro- and micro- components of *A. unedo* have been previously characterized.[1-4] Between the minority fraction, phenolic compounds cover a broad range of different sub-classes, the more representative being the gallic acid derivatives, including its mono and oligomeric forms esterified with quinic and shikimic acids. In addition, galloyl derivatives, particularly gallotannins, described in *A. unedo*, are part of a very selective phenolic group, found in a limited number of plant products.[2,4-6] Other phenolic compounds, such as flavan-3-ols, ellagic acid derivatives, flavonols and anthocyanins, have also

been found in *A. unedo* in different concentrations.[1,2,4]

Diet phytochemicals, such as phenolic compounds, have been recognized as a natural defense against chronic disorders. This biological property has been related to an improvement in the antioxidant status of the body and modulation of the inflammatory response, which contribute to reducing the risks of suffering type-2 diabetes, cardiovascular diseases, cancer and degenerative diseases.[2,6-8] After intake, phenolic compounds undergo several transformations, which closely depend on their absorption and metabolism. In addition, over the last decade, many authors have highlighted the relevant role of diet phenols in the large intestine where these compounds are metabolized and can interact with local microbiota.[9-12]

The *in vitro* digestion models are useful resources for predicting chemical and structural changes, but do not fully cover the physiological conditions phenolic compounds are exposed to in the gastrointestinal tract of living organisms. *In vitro* digestion models should be considered as a preliminary evaluation in the bioavailability studies and it would be appropriate to combine these with *in vivo* studies. For *in vivo* studies, humans are preferred and the absorption and metabolism of bioactive compounds is normally studied through the analysis of such biological fluids as plasma and urine.[13-15] The identification of phenolic compounds and/or their biological metabolites in human biospecimens has been successfully used as markers of food intake and to associate their presence with disease preventive effects. Biological samples, such as urine and faeces, can be collected directly by the individuals involved, since no further training is required and the volunteers need not remain under clinical surveillance in special rooms for extended periods of time.

The analysis of food bioactive metabolites in blood is a good predictor of their absorption and metabolism and allows a reasonable estimate of the exposure of the target organs and/or tissues to these compounds. Nevertheless, qualified personnel and special supervision are required to obtain blood samples, and this could limit the objectives and increase the cost of the bioavailability studies. For this reason, it is necessary to find alternative solutions to simplify the blood sampling, especially in post-prandial bioavailability kinetic studies. In order to reduce these downsides, a simplified method using dried blood-spot (DBS) cards could be considered for the direct blood sampling and sample pre-treatment prior to the chromatographic analysis of phenolic metabolites. This sample pre-treatment strategy was previously applied by our group for the analysis of olive oil phenolic metabolites in human urine.[16] The successful results obtained and the simplified sampling technique encouraged us to apply this method to the analysis of blood samples.

The aim of the present study is to provide a better understanding of human metabolic pathways of different phenolic sub-classes from *A. unedo* fruit after an acute intake by healthy adults. Therefore, the purpose of this study was to determine *A. unedo* phenolic compounds and their generated metabolites in whole blood (0 to 24 h), urine (24 h) and faeces (12 and 24 h). Special focus was placed on the use of DBS cards for the direct analysis of phenolic metabolites in whole blood samples. To our knowledge, this is the first study in which DBS cards are applied to the analysis of blood phenolic metabolites.

## 2. MATERIALS AND METHODS

### 2.1. Chemicals and reagents

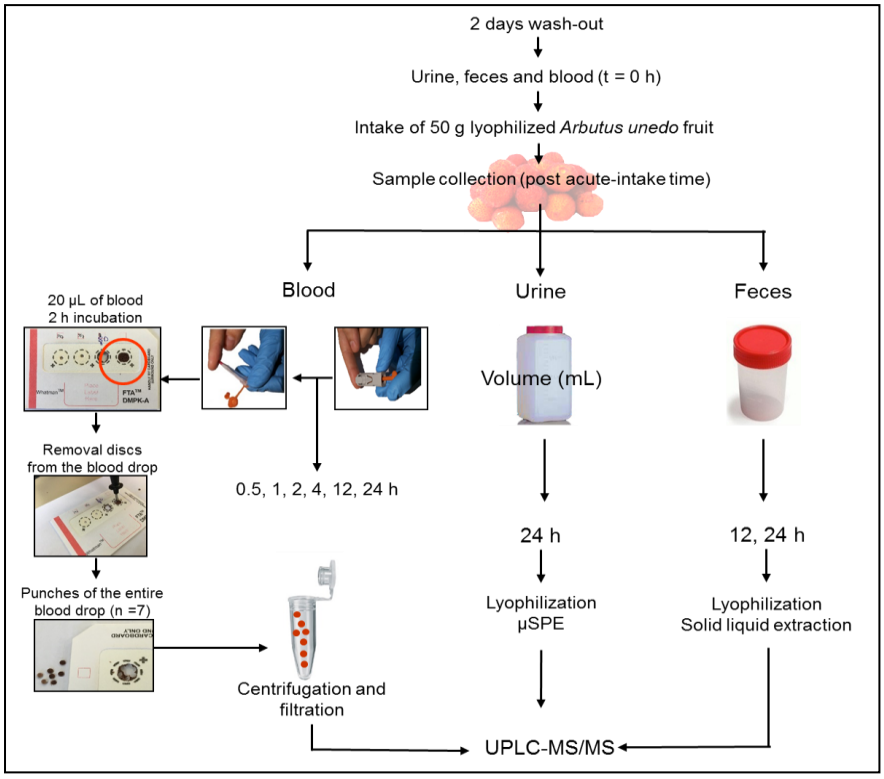
Ultrapure water was obtained from a Milli-Q water purification system (Millipore Corp., Bedford, MA, USA). Acetonitrile (HPLC-grade) was from Romil (Tecknokroma, Barcelona, Spain). Methanol and acetone (all HPLC-grade), hydrochloric acid (HCl) (37%), phosphoric acid (85%) and glacial acetic acid (99.8%) were from Scharlau S.L. (Barcelona, Spain).

Different commercial standards were used to identify and quantify the phenolic compounds from *A. unedo* fruit. Ellagic acid, syringic acid, 3-(4-hydroxyphenyl)propionic acid and 3-(2',4'-dihydroxyphenyl)propionic acid were purchased from Fluka (Buchs, Switzerland); *p*-hydroxybenzoic acid, protocatechuic acid, catechin, epicatechin, hippuric acid, myricetin and catechol were from Sigma Aldrich (St. Louis, MO,

USA); cyanidin-3-*O*-glucoside, quercetin, kaempferol, taxifolin (as internal standard (IS)), and dimer $B_2$ were from Extrasynthese (Genay, France). Gallic acid was purchased from Panreac (Barcelona, Spain). Standards of hydroxyl-6H-benzopyran-6-one derivatives (urolithins), such as urolithin A (dihydroxyurolithin), urolithin B (hydroxyurolithin) and urolithin C (trihydroxyurolithin) were kindly provided by Dr. Thasana from Chulabhorn Research Institute, Laksi, Bangkok.[17]

### 2.2. *Arbutus unedo* phenolic composition

*A. unedo* fruit were manually harvested in November 2014 in the vicinity of Lleida (Catalonia, Spain). The fruit were delivered to the laboratory on the same day they were picked. There, after being washed manually and cut radially (1 cm sized pieces), they were lyophilized (Telstar Lyobeta 15). The freeze-dried fruit was maintained in a desiccator protected for the light. The acute intake assay was run on the following week harvest. For the extraction of phenolic compounds from *A. unedo*, 0.1 g of ground lyophilized fruit was mixed with 5 mL of an acidified aqueous methanol solution (methanol/Milli-Q water/acetic acid, 79.9:20:0.1, v/v/v) and shaken for 10 min. After this time, the samples were centrifuged (9000 rpm for 10 min at room temperature), and the supernatant was filtered through a 0.22 µm nylon syringe filter (Tecknokroma, Barcelona, Spain) and transferred to the autosampler vial before the chromatographic analysis.




**Fgure 1.** Schematic representation of the study design of acute intake of *A. unedo* by healthy volunteer.

### 2.3. Study design

Three healthy humans with normal weight (IBM 18.5–24.9 kg $m^{-2}$) participated in the study. They were in good health, with no declared gastrointestinal disorders and not having received antibiotic treatment during the three months prior

to the study. The subjects gave written informed consent before starting the experiment protocol. The experiment was performed in compliance with the Helsinki Declaration and good clinical practice guidelines of the International Conference of Harmonization (ICH GCP). The protocol of the study was approved by the Ethical Committee of Human Clinical Research of Arnau Vilanova University Hospital, Lleida, Spain (approval number: CEIC-1326). **Fig. 1** shows a schematic depiction of the study design. After 2 days of wash-out and fasting overnight, the three volunteers consumed a single helping of 50 g of the lyophilized A. unedo fruit (equivalent to 163 g of fresh weight). Fasting samples of blood, urine and feces were collected before the acute intake. Following the A. unedo consumption, blood (0.5, 1, 2, 4, 12 and 24 h), urine (24 h) and feces (12 and 24 h) were collected. The intake of the single dose of A. unedo was preceded by a two-day wash-out period consisting of restriction on the consumption of fruit, vegetables, tea, red wine, chocolate, cocoa, whole grain foods and coffee. The same diet limitations were also respected on the day of the study, in which volunteers received low polyphenol meals for lunch and dinner, which consisted of refined carbohydrates, cheese, ham and plain yoghurt. Detailed information of food intake during the two days of the wash-out period and the day of the study was gathered from each volunteer in order to confirm dietary compliance.

**2.4. Biological samples pre-treatment**

The sample pre-treatment for the extraction of phenolic compounds and their generated metabolites in urine, blood and feces was carried out as follows.

*Blood analysis*. Blood samples at baseline (0 h) and different post-intake times (0.5, 1, 2, 4, and 24 h) were collected in the laboratory. The blood samples at 12 h were taken by each volunteer at home, stored at -20 ºC and transported to the laboratory the next day in refrigerated containers. The whole blood was taken by pricking the volunteers' fingers with disposable lancets (Unistik®, Owen Mumford Ltd, Woodstock, UK). Then, the blood drops were directly collected with micro-capillary blood collection tubes that contained lithium-heparin as anticoagulant (Microvette® CB 300 LH, sample volume 300 µL Sarstedt, Numbrecht, Germany). 20 µL of this whole blood was spotted onto a pre-marked circle on the FTA® DMPK cards (DBS filter paper) (GB Healthcare, Buckinghamshire, UK). The cards were then dried in the dark at room temperature for 2 h in a desiccator. After this time, 7 disks of 2 mm diameter were punched out from the card and were placed into an Eppendorf with 100 µL of methanol/water (50:50, v/v) (**Figure 1**). The Harris Uni-Core punch and a Cutting Mat were supplied by Whatman Inc. (Sanford, ME, USA). The Eppendorf was then vortexed for 5 min and centrifuged at 9000 rpm for 10 min at room temperature. The supernatant was filtered through a 0.22 µm nylon syringe filter and directly injected into the chromatographic system. Each blood sample was analyzed in triplicate.

*Urine analysis*. Urine samples were collected by the volunteers in appropriate containers at the baseline (0 h) and over 24 h after the *A. unedo* fruit intake. After measuring the total urine volume, aliquots of 6 mL were freeze-dried. Before the chromatographic analysis, the dry residue was reconstituted with 200 µL of an aqueous solution of 4% phosphoric acid. 100 µL of this solution was mixed in an Eppendorf containing 50 µL of taxifolin (IS) (at the concentration of 10 mg/L and prepared with 4% phosphoric acid) and 50 µL of 4% phosphoric acid. This solution was then centrifuged at 9000 rpm for 10 min at room temperature and the supernatant was cleaned up with µElution solid-phase extraction (µSPE). OASIS hydrophilic-lipophilic balance (HLB) µElution plates 30 µm (Waters, Milford, MA, USA) were used. These were activated with 250 µL of methanol and equilibrated with 250 µL of 0.2% acetic acid. 100 µL of urine supernatant with IS (50 µL) and 50 µL of 40% phosphoric acid were loaded into the plate. The retained phenolic compounds were then eluted with 2 x 50 µL of the solution acetone/Milli-Q water/acetic acid (70:29.5:0.5, v/v/v). 2.5 µL of the eluted solution were directly injected into the chromatographic system (UPLC-MS/MS). Each urine sample was analyzed in triplicate.

*Feces analysis*. Feces collected by volunteers before and after the acute intake of *A. unedo* were

freeze-dried in order to avoid possible variability due to the water content. Before the chromatographic analysis, freeze-dried feces (0.1 g) were mixed with 1 mL of an acidified aqueous methanol solution (methanol/Milli-Q water/HCl, 79.9:20:0.1, v/v/v) and shaken in vortex for 10 min. Then, the solution was centrifuged at 9000 rpm for 10 min at room temperature. The supernatants were collected and filtered with a syringe with a 0.2 um filter pore size and analyzed by UPLC-MS/MS. The fecal samples were analyzed in triplicate.

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**Table 1.** Phenolic content in the single dose of A. unedo lyophilized fruit consumed by the volunteers (mean value ± standard deviation).

| Phenolic compound | µmol/50 g lyophilized *A. unedo* fruit | Phenolic compound | µmol/50 g lyophilized *A. unedo* fruit |
|---|---|---|---|
| *Galloyl derivatives* | | *Flavonols* | |
| Gallic acid | 72.5 ± 9.41 | Myricetin | 0.18 ± 0.00 |
| Gallic acid hexoside | 327 ± 25.0 | Dihydroquercetin | 0.8 ± 0.17 |
| Galloyl shikimic acid | 340 ± 25.0 | Quercetin arabinoside | 8.32 ± 0.34 |
| Digalloyl shikimic acid | 181 ± 17.0 | Quercetin rhamnoside | 16.1 ± 1.33 |
| Galloyl quinic acid | 618 ± 80.1 | Quercetin glucoside | 5.73 ± 0.53 |
| Digalloyl quinic acid | 64.9 ± 7.16 | Quercetin-3-*O*-glucoside | 0.07 ± 0.04 |
| Trigalloyl quinic acid | 3.22 ± 0.15 | Quercetin galloyl hexoside | 4.03 ± 0.60 |
| Tetragalloyl quinic acid | 25.4 ± 1.69 | Dihydroxykaempferol | 9.01 ± 0.59 |
| Total | 1632 ± 144 | Kaempferol-3-*O*-rhamnoside | 1.17 ± 0.33 |
| *Ellagic acid derivatives* | | Kaempferol-3-*O*-glucoside | 0.11 ± 0.07 |
| Ellagic acid | 8.1 ± 0.59 | Kaempferol-3-*O*-rutinoside | 0.61 ± 0.27 |
| Ellagic acid arabinoside | 657 ± 76.9 | Dihydrokaempferol hexoside | 23.3 ± 3.30 |
| Strictinin ellagitannin | 371 ± 51.0 | Myricetin rhamnoside | 21.3 ± 0.59 |
| Total | 1036±122 | Myricetin glucoside | 7.25 ± 0.58 |
| *Flavan-3-ols* | | Total | 98 ± 5.34 |
| Catechin | 48.1 ± 3.11 | *Anthocyanins* | |
| Epicatechin gallate | 11.2 ± 1.04 | Cyanidin glucoside | 20.6 ± 2.76 |
| Epigallocatechin gallate | 3.97 ± 0.61 | Cyanidin arabinoside | 7.31 ± 1.16 |
| Gallocatechin-catechin | 12.4 ± 1.26 | Delphinidin arabinoside | 1.71 ± 0.29 |
| Dimer $B_2$ | 0.28 ± 0.06 | Delphinidin glucoside | 2.1 ± 0.33 |
| Other dimers | 30.4 ± 2.02 | Pelargonidin glucoside | 0.25 ± 0.04 |
| Trimer | 1.37 ± 0.16 | Pelargonidin arabinoside | 0.09 ± 0.01 |
| Total | 108 ± 7.94 | Peonidin glucoside | 0.12 ± 0.01 |
| | | Peonidin arabinoside | 0.07 ± 0.01 |
| | | Total | 32.2 ± 3.92 |
| | | *Total phenolic content* | *2905 ± 276* |

### 2.5. Liquid-chromatography coupled to tandem mass spectrometry (UPLC-MS/MS)

The analysis of phenolic compounds in samples of *A. unedo* fruit, and in the biological samples (blood, urine and faeces) was performed by ultra-performance liquid chromatography (UPLC) coupled to tandem mass spectrometry (MS/MS) as the detector system (Waters, Milford, MA, USA). The chromatographic column was an Acquity BEH C18 (100 mm x 2.1 mm i.d.) with a 1.7 µm particle size (Waters). The UPLC™ system was equipped with a binary pump system, and a gradient elution was used. Two different mobile phases were used, one for the analysis of anthocyanins, and the second one for the other phenolic compounds. For the analysis of anthocyanins, eluent A was Milli-Q water/acetic acid (90:10, v/v) and eluent B, acetonitrile. The gradient was performed as follows: 0-10 min, 3-25 %B; 10-10.1 min, 25-80 %B; 10.1-11 min, 80 %B; 11-11.1 min, 80-3 %B; 11.1-12.5 min, 3 %B. On the other hand, eluent A for the analysis of the rest of the phenolic compounds was Milli-Q water/acetic acid (99.8:0.2, v/v) and eluent B, acetonitrile. The gradient was performed as follows: 0-5 min, 5-10 %B; 5-10 min, 10-12.4 %B; 10-18 min, 12.4-28 %B; 18-23 min, 28-100 %B; 23-25.5 min, 100 %B; 25.5-27 min, 100-5 %B; and

27-30 min, 5%B. For all the phenolic compounds, the flow-rate was 0.4 mL/min and the injection volume was 2.5 μL. Prior to chromatographic analysis, the samples were filtered with a 0.22 μm nylon syringe filter.

Tandem MS (MS/MS) analyses were carried out on a triple quadrupol detector (TQD) mass spectrometer (Waters, Milford, MA, USA) equipped with a Z-spray electrospray interface. Ionization was achieved using electrospray (ESI) interface operating in the negative mode [M-H]- and the data was acquired through selected reaction monitoring (SRM). The ionization source parameters were capillary voltage 3 kV, source temperature 150 ºC and desolvation gas temperature 400 ºC, with a flow rate of 800 L/h. Nitrogen (99.99% purity, N2LCMS nitrogen generator, Claind, Lenno, Italy) and argon (≥ 99.99% purity, Aphagaz, Madrid, Spain) were used as cone and collision gases respectively. Two SRM transitions were studied, selecting the most sensitive transition for quantification and a second one for confirmation purposes. The SRM transition for quantification and the individual cone voltage and collision energy for each phenolic compound and metabolite are shown in **Table 1** of **Supplementary Information**. In this table is also shown in which standard compound is quantified or tentatively quantified each phenolics and metabolites. The dwell time established for each transition was 30 ms. Data acquisition was carried out with the MassLynx 4.1software.

The calibration curves were prepared by spiking blank blood, urine and faeces samples, obtained from volunteers under fasting conditions, with known concentrations of the standard compounds. Gallic acid, catechin, dimer $B_2$, ellagic acid, myricetin, cyanidin-3-*O*-glucoside, *p*-hydroxy benzoic acid, catechol, hippuric acid, protocatechuic acid, 3-(4-hydroxyphenyl) propionic acid, mono-, di-, and tri-hydroxy urolithins were quantified with their own calibration curves. On the other hand, gallic acid hexoside, mono-, di-, tri- and tetra-*O*-galloylquinic acid, mono- and di-*O*-galloylshikimic acid, gallic acid sulfate and gallic acid glucuronide were tentatively quantified by using the calibration curve of gallic acid. Pyrogallol sulfate, methyl pyrogallol sulphate, pyrogallol glucuronide, and pyrogallol glucuronide-sulfate, catechol sulfate, and catechol glucuronide were tentatively quantified as catechol. Ellagic acid arabinoside, ellagic acid glucuronide and strictinin ellagitannin were tentatively quantified as ellagic acid. Gallocatechin-catechin, catechin glucuronide, methyl catechin glucuronide and catechin sulfate were tentatively quantified as catechin. Epigallocatechin, epicatechin gallate, epigallocatechin gallate, epicatehin glucuronide, methyl epicatechin glucuronide and methyl epicatechin sulfate were tentatively quantified as epicatechin. Dimer and trimer were tentatively quantified as dimer $B_2$. Dihydroquercetin, quercetin arabinoside, quercetin rhamnoside, quercetin glucoside, quercetin-3-*O*-glucoside and quercetin galloyl hexoside were tentatively quantified as quercetin. Dihydroxykaempferol, kaempferol-3-*O*-rhamnoside, kaempferol-3-*O*-glucoside, kaempferol-3-*O*-rutinoside and dihydro kaempferol hexoside were tentatively quantified as kaempferol. Myricetin rhamnoside and myricetin glucoside were tentatively quantified as myricetin. All the anthocyanins were tentatively quantified as cyanidin-3-*O*-glucoside. Hydroxy phenylpropionic acid was tentatively quantified as 4-hydroxyphenylpropionic. Hydroxyurolithin glucuronide was tentatively quantified as hydroxyurolithin; and dihydroxyurolithin glucuronide as dihydroxyurolithin. Tetra hydroxyurolithin, pentahydroxyurolithin, and tetra hydroxyurolithin sulfate were tentatively quantified as trihydroxyurolithin.

### 2.6. Method validation

The instrumental quality parameters of the method, such as linearity, repeatability, accuracy, detection limits (LODs), and quantification limits, as well as the extraction recovery (%R) and matrix effect (%ME) for the determination of phenolic compounds in biological samples, were determined by spiking blank biological samples (blood, urine and faeces obtained prior to *A. unedo* intake) with known concentrations of standard compounds. These instrumental quality parameters, as well as the %R and %ME, were determined as reported in our previous study.[16] The results obtained are shown in **Table 1** of the

**Supplementary Information**. The %R for all the phenolic compounds studied was higher than 75% and the %ME was lower than 15%.

## 3. RESULTS

**Table 1** shows the dose of *A. unedo* phenols ingested thought 50 g of lyophilized fruit (expressed as µmols of phenolic compound). As can be seen, the main phenolic compounds are galloyl and ellagic acid derivatives whereas minor fractions correspond to flavan-3-ols, flavonols and anthocyanins. After the intake of the single dose of 50 g of lyophilized *A. unedo* fruit (equivalent to approximately 2900 µmols phenolic compounds), the phenolic metabolism was studied through the analysis of blood, urine and faeces

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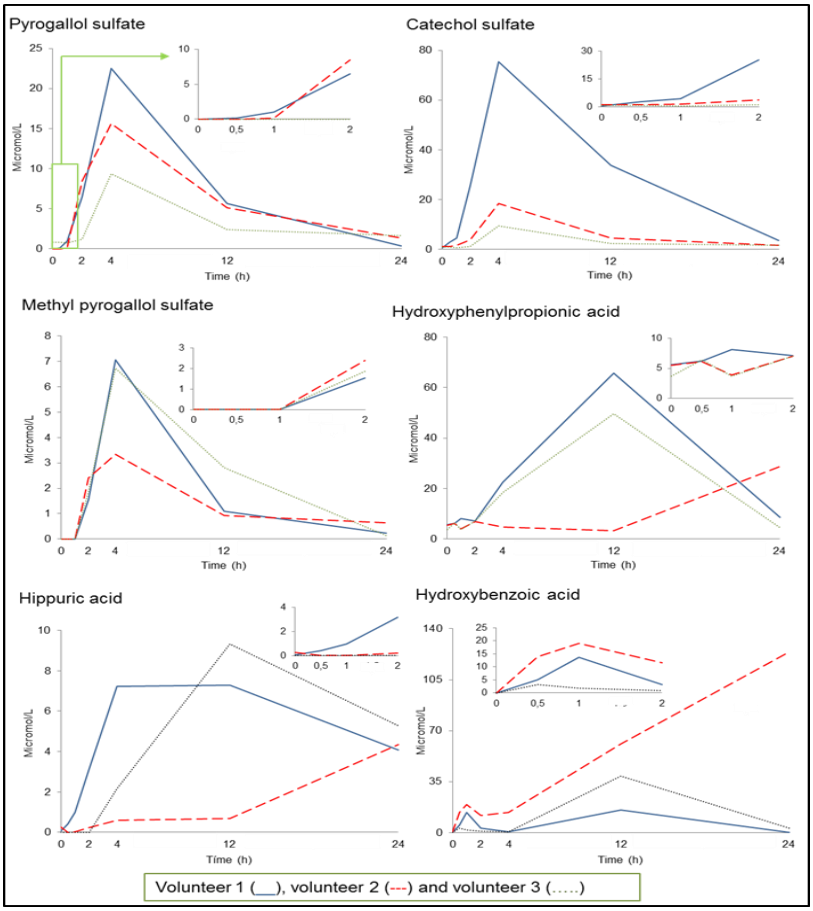
**Figure 2.** Pharmacokinetic representation of the main phenolic metabolites detected in blood samples at different times (0, 0.5, 1, 2, 4, 12 and 24 h) of healthy adults after the intake of a single dose of 50 g of lyophilized *A. unedo.*




### 3.1. Phenolic metabolites in blood samples

**Figure 2** shows the individual kinetics of the main phenolic metabolites detected in the blood samples of the three volunteers after the acute intake of 50 g of lyophilized *A. unedo* (numerical data is available in **Table 2** of the **Supplementary Information**). In general, a progressive increase with time (with small oscillations) and two maximum peaks were observed for the main of the phenolic metabolites generated, one at 4 h, and a second one between 12-24 h (**Figure 2**).

The blood concentration of catechol, pyrogallol and methyl pyrogallol, all in their sulfate forms, reached a maximum at 4 h post-intake of *A. unedo* in the three volunteers. On the other hand, the concentration of the hydroxybenzoic acid, hydroxyphenylpropionic acid and hippuric acid showed a maximum at 12 h post-intake, with except in volunteer 2 who showed the maximum concentration at 24 h. No native or conjugated forms of gallotannins, flavan-3-ols, ellagic acid, flavonols and anthocyanins were detected in the whole blood. One aspect to be noted is the person-to-person variability, which was particular obvious in terms of the concentrations and the time when the different phenolic metabolites reach their maximum peak, especially between 12 and 24 h after the intake of the *A. unedo* fruit (**Figure 2** and **Table 2** of the **Supplementary Information**).

**Table 2**. Phenolic compounds and their metabolites (μmol/day) detected in the volunteer's 24 h urine after the acute intake of 50 g of lyophilized *Arbutus unedo*

| Phenolic metabolites | Concentration (μmol/day) ± SD | | |
|---|---|---|---|
| | Vol. 1 | Vol. 2 | Vol. 3 |
| *Gallic acid and its derivatives* | | | |
| Gallic acid | 0.66 ± 0.06 | 1.66 ± 0.76 | n.d. |
| Gallic acid sulfate | 4.37 ± 1.73 | 12.7 ± 5.04 | 0.78 ±0.12 |
| Gallic acid glucuronide | 0.17 ± 0.09 | 0.86 ± 0.37 | 0.14 ±0.07 |
| Galloylshikimic acid | 0.94 ± 0.40 | 2.29 ± 0.30 | 0.49 ± 0.07 |
| Galloylquinic acid | n.d. | 0.26 ± 0.13 | 0.01 ± 0.01 |
| *Phenolic acids* | | | |
| Protocatechuic acid | 0.54 ± 0.09 | 0.82 ± 0.25 | n.d. |
| Syringic acid | 0.14 ± 0.01 | 0.12 ± 0.03 | 0.07 ± 0.02 |
| Syringic acid sulfate | 0.13 ± 0.04 | 0.49 ± 0.21 | 0.04 ± 0.03 |
| Hydroxyphenylpropionic acid | 3.18 ± 0.30 | 1.57 ± 0.22 | 0.54 ± 0.06 |
| Hippuric acid | 31.3 ± 3.58 | 48.6 ± 5.25 | 18.3 ± 9.67 |
| *Flavan-3-ols and their metabolites* | | | |
| Catechin | 0.18 ± 0.00 | 0.45 ± 0.14 | 0.08 ± 0.05 |
| Catechin sulfate | 3.92 ± 0.58 | 10.5 ± 1.40 | 1.69 ± 0.57 |
| Catechin glucuronide | 0.05 ± 0.01 | 0.16 ± 0.06 | 0.03 ± 0.00 |
| Epicatechin glucuronide | 0.11 ± 0.01 | 0.35 ± 0.23 | 0.03 ± 0.00 |
| Methyl catechin sulfate | 0.66 ± 0.09 | 1.49 ± 0.24 | 0.26 ± 0.02 |
| Methyl catechin glucuronide | 0.08 ± 0.02 | 0.30 ± 0.06 | 0.07 ± 0.01 |
| Methyl epicatechin sulfate | 0.22 ± 0.05 | 0.57 ± 0.06 | 0.08 ± 0.02 |
| Methyl epicatechin glucuronide | 0.43 ± 0.07 | 0.94 ± 0.17 | 0.23 ± 0.03 |
| *Catechol and pyrogallol and their metabolites* | | | |
| Catechol sulfate | 89.1 ± 14.7 | 65.7 ± 22.0 | 12.3 ± 3.89 |
| Catechol glucuronide | 6.72 ± 1.02 | 6.88 ± 2.79 | 1.05 ± 0.14 |
| Pyrogallol sulfate | 56.4 ± 14.5 | 169 ± 43.5 | 27.1 ± 17.2 |
| Methyl pyrogallol sulfate | 8.78 ± 1.79 | 28.2 ± 7.19 | 18.0 ± 23.1 |
| Pyrogallol glucuronide | 12.6 ± 2.75 | 29.0 ± 11.2 | 6.45 ± 1.86 |
| Pyrogallol glucuronide sulfate | 0.30 ± 0.04 | 0.50 ± 0.16 | 0.31 ± 0.04 |
| *Ellagic acid and its metabolites* | | | |
| Ellagic acid glucuronide | 17.0 ± 0.25 | 19.9 ± 3.96 | 10.2 ± 1.63 |
| Hydroxyurolithin glucuronide | 0.01 ± 0.01 | 0.03 ± 0.01 | 0.04 ± 0.00 |
| Dihydroxyurolithin glucuronide | 0.14 ± 0.01 | n.d. | 0.55 ± 0.05 |
| Tetrahydroxyurolithin sulfate | 0.01 ± 0.00 | 0.02 ± 0.00 | n.d. |

Results are expressed as mean (*n*=3) SD: standard deviation n.d.: no detected (Its concentration is below its detection limit (LOD)). Vol.: Volunteer

### 3.4. Urinary excretion of phenolic compounds

**Table 2** shows the mean urinary concentrations of phenolic metabolites detected in the three volunteers followed the intake of the single dose of lyophilized *A. unedo* fruit (μmols excreted/24 h). The high number of phenolic compounds and their metabolites detected in urine show the extensive metabolism that the *A. unedo* phenols underwent in the human body. A total of 28 phenolic

metabolites were selected and quantified after comparing their concentrations (μmol/L) between the baseline (0 h) and the cumulative urinary excretion (24 h). Among these, gallic acid, galloyl-derivatives, protocatechuic acid, syringic acid (including its sulfate form), flavan-3-ols (including sulfate and glucuronide metabolites) and urolithin metabolites, could be characterized as exclusive to the *A. unedo* fruit intake, since they were not detected in the urine at 0 h (**Table 2**).

**Table 3.** Concentration of the microbial phenolic metabolites (nmol/g lyophilized feces) after 12 h (volunteer 1) and 24 h (volunteers 1, 2 and 3) the intake of 50 g of lyophilized *A. unedo* (results are expressed as mean ± standard deviation

| Phenolic metabolites | Concentration (nmol/g lyophilized feces) | | | |
|---|---|---|---|---|
| | 12 h | 24 h | 24 h | 24 h |
| | Vol 1 | Vol 1 | Vol 2 | Vol 3 |
| Gallic acid | 8.85 ± 1.75 | n.d. | n.d. | n.d. |
| Hippuric acid | n.d. | 5.35 ± 0.71 | 27.4 ± 2.11 | n.d. |
| *p*-hydroxybenzoic acid | 12.3 ± 0.62 | 15.2 ± 4.73 | 133 ± 7.52 | 7.91 ± 1.45 |
| Hydroxybenzoic acid | 0.06 ± 0.07 | 0.21 ± 0.36 | n.d. | n.d. |
| Protocatechuic acid | 1.37 ± 1.26 | n.d. | n.d. | n.d. |
| Catechol | 397 ± 19.9 | 206 ± 19.2 | 71.5 ± 3.06 | 16.3 ± 1.15 |
| Phenylpropionic acid | n.d. | n.d. | n.d. | 2.42 ± 1.39 |
| 3-(4'-hydroxyphenyl)propionic acid | n.d. | n.d. | 92.6 ± 11.9 | n.d. |
| Hydroxyphenylpropionic acid | 1069 ± 23.7 | 297 ± 6.35 | 15158 ± 1042 | 547 ± 173 |
| 3-(2',4'-dihydroxyphenyl)propionic acid | n.d. | n.d. | 2.58 ± 2.86 | n.d. |
| Dihydroxyphenylpropionic acid | n.d. | n.d. | 529 ± 34.6 | 42.9 ± 7.50 |
| Hydroxyurolithin | n.d. | n.d. | n.d. | n.d. |
| Dihydroxyurolithin | n.d. | 33.81 ± 12.5 | 46.0 ± 22.7 | 84.6 ± 1.91 |
| Trihydroxyurolithin | 0.07 ± 0.06 | n.d. | 5.94 ± 5.14 | n.d. |
| Tetrahydroxyurolithin | 0.17 ± 0.04 | n.d. | 0.26 ± 0.22 | n.d. |
| Pentahydroxyurolithin | 0.34 ± 0.02 | 1.61 ± 0.24 | 0.16 ± 0.10 | n.d. |

n.d.: no detected (its concentration is below its LOD)
Vol.: volunteer

Of these compounds, galloyl shikimic acid (2.29-0.49 μmols excreted/24 h) and gallic acid sulfate (12.7-4.37 μmols excreted/24 h) were the most predominantly excreted. Other phenolic metabolites were detected in urine at the baseline (0 h), but a large increase in their concentration was observed after the intake of *A. unedo* fruit. The most abundant were catechol sulfate (89.1-12.3 μmols excreted/24 h), pyrogallol sulfate (169-27.1 μmols excreted/24 h) and hippuric acid (48.6-18.3 μmols excreted/24 h). Other metabolites that contributed considerably to the urinary phenolic pool were pyrogallol glucuronide (29.0-6.54 μmols excreted/24 h), ellagic acid glucuronide (19.9-10.2 μmols excreted/24 h) and methyl pyrogallol sulfate (28.2-8.78 μmols excreted/24 h).

### 3.3. Phenolic metabolites in fecal samples

The three volunteers provided the fecal samples 24 h after the *A. unedo* fruit intake, but only one of them (Vol. 1) was able to provide an extra sample after 12 h. The post-intake concentration of fecal metabolites, expressed in nmol/g lyophilized feces, is shown in Table 3. Similarly to what was observed in the blood and urine samples, important inter-individual variability was observed in the feces obtained 24 h post-intake. The results showed an intense colonic metabolism of *A. unedo* phenolic compounds since no native structures detected in the fruit (**Table 1**) were found in the feces taken 24 h after ingestion (**Table 3**). Hydroxyphenylpropionic acid (297 to 15158 nmols/g lyophilized feces) was the main microbial metabolite quantified in the feces of the three volunteers. Despite notable differences between individuals, other metabolites, such as *p*-hydroxybenzoic acid, hippuric acid, catechol, dihydroxyphenylpropionic acid and urolithins (especially urolithin A), were also detected at higher concentration levels (nmols/g lyophilized faeces) compared with the baseline (t = 0 h)

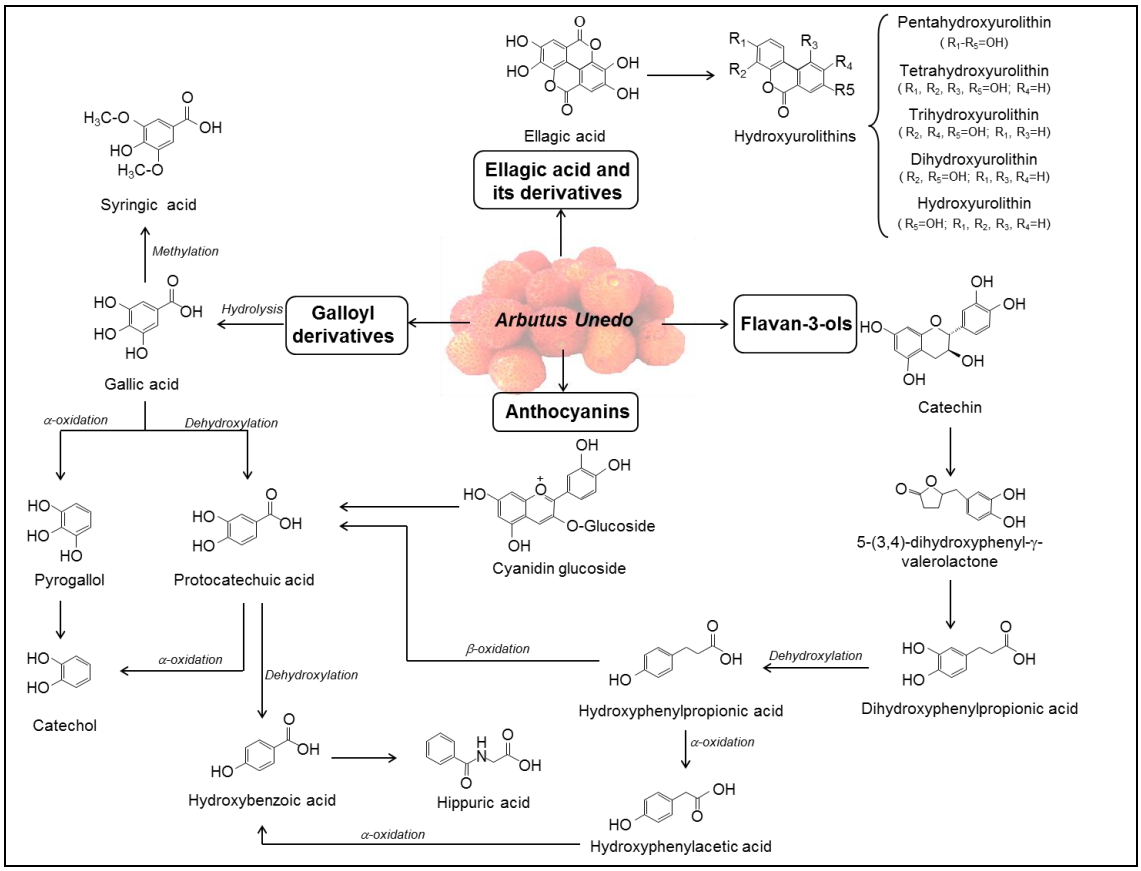



**Figure 3.** Possible phenol metabolic pathways for the generation of the phenolic metabolites (only the aglycones) detected in blood, urine and feces after the acute intake of *A. unedo* fruit

## 4. DISCUSSION

Human studies regarded the evaluation of the absorption, metabolism and excretion of phenolic compounds are highly appreciated because the results obtained are useful for tentatively determining their effective bioavailability. The bioavailability studies are based on the collection of biological samples, normally blood and urine, after the acute intake of a specific food or pure compound. Although the urine samples can be collected by volunteers themselves, the collection of blood/plasma has serious limitations as it requires qualified staff and special infrastructures where volunteers must spend a long time. During this period, the subjects have numerous blood samples taken and where it is sometimes necessary to use a cannula for hours to facilitate the procedure. Beyond the discomfort this means, some people have a particular aversion to needles, which could be a reason to refuse to participate in a study like this and consequently making it more difficult to find enough volunteers. The satisfactory results obtained previously by our group with the application of DBS cards for the sample-pretreatment to determine olive oil phenolic metabolites in urine samples[16] gave us the motivation to extend the use of this easy strategy for the volunteers to collect their own samples for bioavailability studies using whole blood.

It is highly desirable to promote phytochemical-rich products because this would be an effective way to increase opportunities to enrich the variety

and nutritional value of the diet. Moreover, bioavailability studies, especially those with human subjects, are required to determine the way the body uses the phenolic compounds. In an effort to investigate whether the phenolic compounds from *A. unedo* fruit are bioavailable in the human body, for the first time, we conducted an acute study to determine the possible potential of this fruit as a rich source of phenolic compounds for a healthy population. A special feature of the *A. unedo* fruit is that it ripens in the autumn-winter period of generally low availability of fruit with high phenolic antioxidant contents. The analysis of the phenolic composition of the blood, urine and faeces revealed an increase in the concentrations of a wide variety of phenolic metabolites (including phase-II metabolites and microbial catabolism), which suggests the active metabolism that *A. unedo* phytochemicals undergo in the human body. The results of the present study also demonstrate, for the first time, that the phenolic compounds from *A. unedo* are bioavailable in healthy adults and, in addition, the main phenolic compounds absorbed can be quantified in blood samples by using DBS cards for the self-management of blood collection and easy sample-pretreatment before the chromatographic analysis.

Over the last few years, the use of DBS cards has gained ground in studies concerning drug pharmacokinetics and other applications, such as the detection of prohibited substances (doping),[18,19] therapeutic drug monitoring, assessing adherence to medications and preventing toxicity.[20] In contrast, to the best of our knowledge, this is the first time that DBS cards have been used for the determination of phenolic metabolites in whole blood. Easy sample collection, the possibility of self-management of blood collection, easy sample pre-treatment and the reduction of invasive techniques could convert the use of this method into a promising way to increase the number of human studies in the area of phytochemical bioavailability. One downside of DBS cards could be the low sensitivity when trace metabolites are determined. In our study, in order to enhance the sensitivity of this sample pre-treatment, the entire blood circle was punched out from the card and analyzed, which was equivalent to 7 disk extractions, instead of only one extraction (disk).

Two transient maximum concentrations of phenolic metabolites were noticed in blood samples after the intake of *A. unedo* fruit. The maximum concentrations of catechol sulfate, pyrogallol sulfate and methyl pyrogallol sulfate were found in the blood of the three volunteers 4 h after the *A. unedo* fruit intake. By contrast, the maximum concentration of hydroxyphenyl propionic acid, hippuric acid and hydroxybenzoic acid was detected at 12 or 24 h after the *A. unedo* fruit intake, depending on the volunteer. Discrepancies between volunteers in the concentration and time when each metabolite reached its maximum blood concentration could be related to individual differences in the dynamics of gastrointestinal transit time, diverse absorption efficiency and particular microbiota composition. An increase in the plasma concentration of the sulfated forms of catechol and pyrogallol have also been described by Pimpao et al. (2015)[15] after an acute intake of 500 mL of a mixture of fruit (including *A. unedo*) by healthy adults. Considering the delay appearance (4 h) of sulfated pyrogallol in blood, this metabolite probably originated from the hydrolysis of galloyl phenol derivates (galloylshikimic acid, galloyl quinic acid, gallotannins, galloyl flavan-3-ols) in the proximal large intestine through the action of microbial esterases and carboxilases.[21] Microbial dehydroxilation, previous described in *in vitro* incubations,[9,12] could explain the appearance of catechol from pyrogallol. Based on the compounds detected in the blood, we proposed the possible human metabolic pathways of *A. unedo* phenolic compounds (**Figure 3**). This includes the generation of phase-II metabolites (sulfo-conjugates), probably originated in the liver after intestinal absorption. The microbial catabolism in the distal colon could explain the second peak observed in blood after 12-24 h of *A. unedo* intake, in which hydroxyphenylpropionic, hippuric and hydroxybenzoic acids were detected as the main metabolites. Gonthier et al. (2003)[22] assumed that quinic acid could be the precursor of hippuric acid, probably *via* the gut microbial shikimate pathway in the gastrointestinal tract.[23] Hippuric acid has also been described as a

hepatic and renal metabolite resulting from the conjugation of benzoic acid and glycine.[24] The presence of hydroxyphenylpropionic and hydroxybenzoic acids may be due to the microbial metabolism of flavan-3-ols[10,12,14,25] and/or flavonols[11,12,26] (**Figure 3**).

In the present study, a greater diversity of the phenolic spectrum was noticed in urine than in the blood. This ties well with the results reported by other authors, where the phenolic profile of human or animal urine reaches a greater complexity than that of the plasma.[22,27] Nevertheless, catechol and pyrogallol, the larger compounds excreted in urine after the intake of *A. unedo* fruit, were also the predominant phenolic compounds detected in whole blood in our study. Free galloyl shikimic and quinic acids were found in the urine, which indicates that they could be absorbed in their intact form by the intestinal epithelium. Therefore, in view of the fact that these galloyl derivatives are the most representative phenolic compounds of *A. unedo*, their presence in biological fluids make them particularly suitable markers for monitor the dietary exposure. Further consideration should be given to galloylshikimic acid since it appeared in the urine of all the volunteers.

The chromatographic analysis of the urine revealed the presence of other phenolic compounds and their phase-II and microbial metabolites. Most of these compounds have been described before in human bioespeciments and their origin could be from a combination of phenolic metabolic pathways (**Figure 3**). Protocatechuic acid could be formed by the microbial degradation of cyanidin glucoside,[13] since this is the most abundant anthocyanin in the *A. unedo* fruit. In the case of syringic acid and its sulfated form, these could be generated by the methylation of gallic acid (phase-II metabolism). Epicatechin and catechin metabolites could originate from flavan-3-ols (**Table 1**) through the hepatic metabolism (in the case of conjugates) and microbial catabolism (hydrolysis of galloyl derivatives).[25] Several studies have described urolithins as exclusively microbial products of ellagic acid colonic metabolism.[12]

In the present study, we detected a relationship between the phenolic metabolites detected in the blood, urine and feces. Simpler compounds, derived from microbial metabolism, were the most abundant phenolic metabolites found in the human fluids. This highlights the importance of the full screening of bioespeciments to establish the bioavailability of dietary compounds, since these could sometimes be underestimated, especially because the analyses mainly focused on the detection of the native phenolic compounds of the food.[28] In accordance with our results, Pimpao et al. (2015)[15] and Margalef et al. (2015)[25] observed that microbial phenolic metabolites contribute an important percentage of the absorbed and circulated phenolic structures in humans and animals.

## 5. CONCLUSIONS

The results of the present study, based on the analysis of biological fluids, like whole blood and urine, together with the analysis of faeces, have allowed us to establish the metabolic pathways of different phenolic groups of *A. unedo* fruit in humans for the first time. Unsupervised sampling of blood by volunteers at home was found to be suitable for the analysis of blood phenolic metabolites using the DBS card methodology. The analysis of whole blood revealed two peaks in the maximum concentrations of the main phenolic metabolites. In parallel, the analysis of urine (24 h) displayed a wide range of phenolic metabolites showing the extensive metabolism that *A. unedo* phenolic compounds underwent in the human body. Galloylshikimic and galloylquinic acids could be selected as exclusive to the *A. unedo* intake since they were not detected in the analysis of urine at 0 h. We believe our results provide a relevant contribution to the understanding of the *in vivo* human bioavailability of phenolic compounds, especially galloyl derivatives, a singular phenolic sub-group present in *A. unedo* fruit.

Additionally, the use of DBS cards offers the advantage of easy sample pre-treatment before the chromatographic analysis, where a low volume of blood (up to 20 µL), obtained from minimally invasive techniques, is needed. The proposed use of DBS cards might be considered in future studies, with the possibility of extending the analysis with the inclusion of other micronutrients for simultaneous determination.

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**ADDITIONAL INFORMATION**

**Table 1 Supplementary Information.** Optimized SRM conditions used for quantification for the analysis of phenolic compounds by UPLC-MS/MS.

| Phenolic compound | MW | Quantification | | | Standard used for quantification |
|---|---|---|---|---|---|
| | | SRM | Cone voltage (v) | Collision energy (eV) | |
| Catechol | 110 | 108.9 > 90.9 | 40 | 15 | Catechol |
| Catechol sulfate | 190 | 189 > 109 | 20 | 15 | Catechol |
| Catechol glucuronide | 286 | 285 > 123 | 40 | 15 | Catechol |
| Pyrogallol sulfate | 206 | 205 > 125 | 20 | 15 | Catechol |
| Methyl pyrogallol sulfate | 220 | 219 > 124 | 20 | 25 | Catechol |
| Pyrogallol glucuronide | 302 | 301 > 125 | 20 | 10 | Catechol |
| Pyrogallol glucuronide-sulfate | 382 | 381 > 125 | 20 | 10 | Catechol |
| *p*-Hydroxybenzoic acid | 138 | 137 > 93 | 30 | 15 | *p*-Hydroxybenzoic acid |
| Hydroxybenzoic acid | 138 | 137 > 93 | 30 | 15 | *p*-Hydroxybenzoic acid |
| Protocatechuic acid | 154 | 153 > 109 | 40 | 15 | Protocatechuic acid |
| Gallic acid | 170 | 169 > 125 | 35 | 10 | Gallic acid |
| Gallic acid hexoside | 332 | 331 > 169 | 40 | 15 | Gallic acid |
| Mono-*O*-galloylquinic acid | 344 | 343 > 191 | 40 | 15 | Gallic acid |
| Di-*O*-galloylquinic acid | 496 | 495 > 191 | 40 | 25 | Gallic acid |
| Tri-*O*-galloylquinic acid | 648 | 647 > 495 | 40 | 15 | Gallic acid |
| Tetra-*O*-galloylquinic acid | 630 | 629 > 477 | 40 | 15 | Gallic acid |
| Mono-*O*-galloylshikimic acid | 326 | 325 > 169 | 40 | 20 | Gallic acid |
| Di-*O*-galloylshikimic acid | 478 | 477 > 325 | 40 | 20 | Gallic acid |
| Gallic acid sulphate | 250 | 249 > 169 | 35 | 15 | Gallic acid |
| Gallic acid glucuronide | 346 | 345 > 169 | 35 | 15 | Gallic acid |
| Syringic acid | 198 | 197 > 182 | 30 | 10 | Syringic acid |
| Ellagic acid arabinoside | 434 | 433 > 300 | 40 | 30 | Ellagic acid |
| Ellagic acid glucuronide | 478 | 477 > 301 | 40 | 20 | Ellagic acid |
| Strictinin ellagitannin | 634 | 633 > 301 | 40 | 30 | Ellagic acid |
| *p*-Hydroxyphenylacetic acid | 152 | 151 > 107 | 20 | 10 | *p*-Hydroxyphenylacetic acid |
| Dihydroxyphenylacetic acid | 168 | 167 > 123 | 20 | 10 | 3,4-Dihydroxyphenylacetic acid |
| 3-(4-Hydroxyphenyl)propionic acid | 166 | 165 > 121 | 20 | 10 | 3-(4-Hydroxyphenyl)propionic acid |
| Hydroxyphenylpropionic acid | 166 | 165 > 121 | 20 | 10 | 3-(4-Hydroxyphenyl)propionic acid |
| 3-(2',4'-Dihydroxyphenyl)propionic acid | 181 | 181 > 137 | 20 | 15 | 3-(2',4'-Dihydroxyphenyl)propionic acid |
| Dihydroxyphenylpropionic acid | 181 | 181 > 137 | 20 | 15 | 3-(2',4'-Dihydroxyphenyl)propionic acid |
| Hippuric acid | 179 | 178 > 134 | 40 | 10 | Hippuric acid |
| Catechin | 290 | 289 > 245 | 45 | 15 | Catechin |
| Gallocatechin – catechin | 594 | 593 > 289 | 40 | 30 | Catechin |
| Catechin sulfate | 370 | 369 > 289 | 40 | 20 | Catechin |
| Catechin glucuronide | 466 | 465 > 289 | 40 | 20 | Catechin |
| Methyl catechin glucuronide | 480 | 479 > 303 | 40 | 25 | Catechin |
| Epicatechin | 290 | 289 > 245 | 45 | 15 | Epicatechin |
| Epigallocatechin | 306 | 305 > 125 | 40 | 15 | Epicatechin |
| Epicatechin gallate | 442 | 441 > 169 | 40 | 20 | Epicatechin |
| Epigallocatechin gallate | 458 | 457 > 169 | 40 | 15 | Epicatechin |
| Methyl epicatechin sulfate | 384 | 383 > 303 | 45 | 15 | Epicatechin |
| Methyl epicatechin glucuronide | 480 | 479 > 303 | 40 | 25 | Epicatechin |
| Dimer | 578 | 577 > 289 | 45 | 20 | Dimer $B_2$ |
| Trimer | 865 | 865 > 287 | 60 | 30 | Dimer $B_2$ |
| Quercetin-3-*O*-glucoside | 464 | 463 > 301 | 45 | 25 | Quercetin |
| Quercetin glucoside | 464 | 463 > 301 | 45 | 25 | Quercetin |
| Quercetin arabinoside | 434 | 433 > 300 | 45 | 20 | Quercetin |
| Quercetin rhamnoside | 448 | 447 > 301 | 40 | 15 | Quercetin |
| Dihydroquercetin | 304 | 303 > 285 | 45 | 15 | Quercetin |
| Quercetin galloyl hexoside | 616 | 615 > 463 | 40 | 30 | Quercetin |
| Kaempferol-3-*O*-glucoside | 448 | 447 > 285 | 45 | 15 | Kaempferol |
| Kaempferol-3-*O*-rhamnoside | 432 | 431 > 285 | 45 | 20 | Kaempferol |
| Kaempferol-3-*O*-rutinoside | 594 | 593 > 285 | 50 | 25 | Kaempferol |
| Dihydrokaempferol | 288 | 287 > 259 | 45 | 10 | Kaempferol |
| Dihydrokaempferol hexoside | 450 | 449 > 269 | 40 | 15 | Kaempferol |
| Myricetin glucoside | 480 | 479 > 317 | 45 | 20 | Myricetin |
| Myricetin rhamnoside | 464 | 463 > 317 | 50 | 25 | Myricetin |
| Cyanidin-3-*O*-glucoside * | 450 | 449 > 287 | 40 | 20 | Cyanidin-3-*O*-Glucoside |
| Cyanidin arabinoside * | 420 | 419 > 287 | 40 | 20 | Cyanidin-3-*O*-Glucoside |
| Delphinidin arabinoside * | 436 | 435 > 303 | 40 | 20 | Cyanidin-3-*O*-Glucoside |

| | | | | | |
|---|---|---|---|---|---|
| Delphinidin glucoside * | 466 | 465 > 303 | 40 | 20 | Cyanidin-3-*O*-Glucoside |
| Pelargonidin arabinoside * | 404 | 403 > 271 | 40 | 20 | Cyanidin-3-*O*-Glucoside |
| Pelargonidin glucoside * | 434 | 433 > 271 | 40 | 20 | Cyanidin-3-*O*-Glucoside |
| Peonidin arabinoside * | 434 | 433 > 301 | 40 | 20 | Cyanidin-3-*O*-Glucoside |
| Peonidin glucoside * | 464 | 463 > 301 | 40 | 20 | Cyanidin-3-*O*-Glucoside |
| Hydroxy urolithin (urolithin B) * | 212 | 213 > 141 | 40 | 20 | Hydroxy urolithin (urolithin B) |
| Dihydroxy urolithin (urolithin A) * | 230 | 229 > 157 | 40 | 20 | Dihydroxy urolithin (urolithin A) |
| Trihydroxy urolithin (urolithin C) * | 246 | 245 > 155 | 40 | 30 | Trihydroxy urolithin (urolithin C) |
| Hydroxy urolithin glucuronide | 388 | 387 > 211 | 40 | 20 | Hydroxy urolithin (urolithin B) |
| Dihydroxy urolithin glucuronide | 404 | 403 > 227 | 40 | 30 | Dihydroxy urolithin (urolithin A) |
| Tetrahydroxy urolithin * | 262 | 261 > 171 | 40 | 25 | Trihydroxy urolithin (urolithin C) |
| Tetrahydroxy urolithin sulfate | 340 | 339 > 259 | 40 | 20 | Trihydroxy urolithin (urolithin C) |
| Pentahydroxy urolithin * | 278 | 277 > 187 | 40 | 30 | Trihydroxy urolithin (urolithin C) |

MW: molecular weight (g/mol)
* ESI positive

**Table 2 of Supplementary Information.** Instrumental quality parameters for the determination of phenolic compounds in biological samples after the *Arbutus unedo* intake.

| Phenolic compound | Linearity range (μM) | Calibration curve | RSD (%) (25 μM), *n*=3 | Accuracy (%) (25 μM), *n*=3 | LOD (μM) | LOQ (μM) |
|---|---|---|---|---|---|---|
| **Blood samples** | | | | | | |
| Catechol | 0.10-110 | y = 239.47 x – 17.75 | 104 | 104 | 0.04 | 0.10 |
| Hippuric acid | 0.10-70 | y = 96.07 x + 202.89 | 99 | 98 | 0.03 | 0.10 |
| *p*-Hydroxybenzoic acid | 0.10-90 | y = 174.15 x + 424.29 | 98 | 102 | 0.03 | 0.10 |
| 3-(4-hydroxyphenyl)propionic acid | 0.10-80 | y = 170.21 x + 127.05 | 103 | 99 | 0.03 | 0.10 |
| **Urine samples** | | | | | | |
| Catechol | 0.9-50 | y = 473.25 x – 30.27 | 102 | 103 | 0.3 | 0.9 |
| 3-(4-hydroxyphenyl)propionic acid | 0.06-35 | y = 346.51 x + 253.21 | 100 | 99 | 0.02 | 0.06 |
| Protocatechuic acid | 0.06-30 | y = 891.56 x – 15.21 | 105 | 98 | 0.02 | 0.06 |
| Gallic acid | 0.06-30 | y = 1247.39 x + 34.30 | 101 | 96 | 0.02 | 0.06 |
| Hippuric acid | 0.05-50 | y = 949.4 x + 795.26 | 101 | 97 | 0.03 | 0.08 |
| Syringic acid | 0.05-25 | y = 1189.2 x + 710.42 | 95 | 104 | 0.03 | 0.05 |
| Catechin | 0.03-25 | y = 625.25 x – 203.77 | 95 | 105 | 0.01 | 0.03 |
| Epicatechin | 0.03-25 | y = 413.75 x – 181.54 | 98 | 101 | 0.01 | 0.03 |
| Hydroxy urolithin (urolithin B) | 0.07-100 | y = 4373 x + 230.70 | 99 | 102 | 0.03 | 0.07 |
| Dihydroxy urolithin (urolithin A) | 0.03-50 | y = 15862 x + 730.52 | 102 | 104 | 0.01 | 0.03 |
| Trihydroxy urolithin (urolithin C) | 0.003-30 | y = 64963 x + 5420.5 | 103 | 99 | 0.001 | 0.003 |
| **Faeces samples** | | | | | | |
| Catechol | 14-710 (1) | y = 12.46 x + 318.28 (2) | 102 (1) | 103 (1) | 4.5 (2) | 14 (2) |
| 3-(2',4'-dihydroxyphenyl)propionic acid | 0.9-500 (1) | y = 112.31 x + 2638.5 (2) | 101 (1) | 98 (1) | 0.3 (2) | 0.9 (2) |
| 3,4-dihydroxyphenylacetic acid | 1.0-500 (1) | y = 85.67 x + 448.35 (2) | 98 (1) | 97 (1) | 0.3(2) | 1.0 (2) |
| 3-(4-hydroxyphenyl)propionic acid | 1.0-500 (1) | y = 375.56 x + 409.94 (2) | 97 (1) | 102 (1) | 0.3 (2) | 1.0 (2) |
| Dihydroxy urolithin (urolithin A) | 0.3-500 (1) | y = 1697.5 x + 929.66 (2) | 102 (1) | 96 (1) | 0.1 (2) | 0.3 (2) |
| Gallic acid | 0.7-400 (1 | y = 108.46 x + 13.63 (2) | 96 (1) | 95 (1) | 0.2 (2) | 0.7 (2) |
| Hippuric acid | 0.7-400 (1) | y = 111.32 x + 92.88 (2) | 99 (1) | 98 (1) | 0.2 (2) | 0.7 (2) |
| *p*-Hydroxyphenylacetic acid | 1.0-560 (1) | y = 54.56 x – 6516.4 (2) | 97 (1) | 104 (1) | 0.3 (2) | 1.0 (2) |
| *p*-Hydroxybenzoic acid | 1.0-500 (1) | y = 303.59 x + 2205.1 (2) | 100 (1) | 99 (1) | 0.3 (2) | 1.0 (2) |
| Protocatechuic acid | 0.9-500 (1 | y = 74.02 x + 27.07 (2) | 104 (1) | 105 (1) | 0.3 (2) | 0.9 (2) |
| Trihydroxy urolithin | 0.03-300 (1) | y = 5988.2 x + 678.26 | 103 (1) | 102 (1) | 0.01 (2) | 0.03 (2) |

(1): Linearity range, calibration curve, LODs and LOQs in feces are nmols/g lyophilized feces.
(2): %RSD and %accuracy in feces are 100 nmols/g lyophilized feces

**Table 2.** Concentration of phenolic compounds and their generated metabolites and its standard deviation (μmol/L ± SD) detected at different times in volunteer's (1, 2 and 3) blood after the acute ingestion of 50 g of lyophilized *A. unedo*

| Phenolic compound (μmol/L blood) | Time 0 h | | | Time 0.5 h | | | Time 1 h | | | Time 2 h | | | Time 4 h | | | Time 12 h | | | Time 24 h | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| *p*-Hydroxybenzoic acid | n.d. | n.d. | n.d. | n.d. | n.d. | 7.52 ± 1.20 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 0.11 ± 0.15 | n.d. | n.d. | 0.18 ± 0.06 | 0.85 ± 0.28 | 0.10 ± 0.05 |
| Hydroxybenzoic acid | n.d. | n.d. | n.d. | 5.13 ± 3.83 | 13.9 ± 1.90 | 8.39 ± 5.35 | 13.7 ± 4.91 | 19.1 ± 3.21 | 1.78 ± 2.52 | 3.26 ± 0.37 | 11.7 ± 3.44 | 0.79 ± 0.12 | 0.85 ± 0.15 | 13.8 ± 3.74 | 0.73 ± 0.11 | 15.5 ± 1.53 | 60.9 ± 8.91 | 38.9 ± 3.45 | 0.33 ± 0.13 | 124 ± 8.38 | 2.97 ± 0.80 |
| Hydroxybenzoic acid sulfate | n.d. | n.d. | n.d. | n.d. | n.d. | 0.83 ± 0.46 | 4.85 ± 1.33 | 1.81 ± 0.13 | 0.73 ± 0.73 | 25.4 ± 8.73 | 3.73 ± 1.17 | 1.21 ± 0.14 | 75.5 ± 10.4 | 18.5 ± 0.78 | 9.37 ± 1.23 | 33.7 ± 7.48 | 4.62 ± 0.98 | 2.41 ± 0.22 | 3.62 ± 0.35 | 1.75 ± 0.20 | 1.87 ± 0.40 |
| Catechol sulfate | 0.71 ± 0.13 | 1.16 ± 0.12 | 0.88 ± 0.09 | 2.83 ± 0.85 | 1.16 ± 0.75 | n.d. | 1.00 ± 0.44 | 0.17 ± 0.11 | n.d. | 6.46 ± 2.67 | 8.44 ± 0.68 | n.d. | 22.5 ± 1.86 | 15.8 ± 1.17 | 7.88 ± 1.05 | 5.88 ± 0.38 | 5.15 ± 1.14 | 1.39 ± 0.32 | 0.38 ± 0.11 | 1.35 ± 0.40 | 0.32 ± 0.07 |
| Catechol glucuronide | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 0.16 ± 0.00 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Pyrogallol sulfate | n.d. | n.d. | n.d. | 0.13 ± 0.06 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 0.05 ± 0.00 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Pyrogallol glucuronide | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 7.08 ± 0.74 | 3.35 ± 0.30 | 8.73 ± 0.99 | 1.09 ± 0.08 | 0.92 ± 0.06 | 2.82 ± 0.76 | 0.24 ± 0.12 | 0.85 ± 0.15 | 0.15 ± 0.02 |
| Methyl pyrogallol sulfate | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 1.55 ± 0.43 | 2.40 ± 0.29 | 1.85 ± 0.31 | 22.5 ± 1.86 | 15.8 ± 1.17 | 7.88 ± 1.05 | 5.88 ± 0.38 | 5.15 ± 1.14 | 1.39 ± 0.32 | 0.38 ± 0.11 | 1.35 ± 0.40 | 0.32 ± 0.07 |
| Hippuric acid | 0.08 ± 0.01 | 0.27 ± 0.07 | n.d. | 0.43 ± 0.17 | n.d. | n.d. | 0.88 ± 0.41 | 0.02 ± 0.03 | n.d. | 3.16 ± 1.57 | 0.24 ± 0.05 | n.d. | 7.24 ± 0.70 | 0.60 ± 0.08 | 2.17 ± 0.54 | 7.28 ± 1.00 | 0.68 ± 0.04 | 9.34 ± 1.13 | 4.07 ± 0.45 | 4.38 ± 1.35 | 5.29 ± 1.44 |
| Hydroxyphenylpropionic acid | 5.83 ± 2.83 | 5.46 ± 1.05 | 3.74 ± 0.85 | 8.23 ± 1.94 | 6.13 ± 2.95 | 8.39 ± 5.35 | 8.09 ± 4.82 | 3.95 ± 2.34 | 3.71 ± 1.96 | 7.12 ± 0.25 | 6.98 ± 2.50 | 7.03 ± 1.23 | 22.4 ± 0.38 | 4.70 ± 1.13 | 19.4 ± 2.23 | 65.8 ± 8.54 | 3.37 ± 0.43 | 49.7 ± 3.63 | 8.57 ± 1.71 | 28.7 ± 9.10 | 4.57 ± 1.79 |

Concentrations are expressed as mean ± SD ($n$=2)
n.d.: no detected (its concentration is below its detection limit (LOD))

# GENERAL DISCUSSION

The inclusion of plant-based products, especially fruit and vegetables, as part of everyday diet, has been linked to a reduced risk of chronic disease (Del Rio et al., 2013; Johnson et al., 2004; Liu et al., 2003). This association appears to be related to their composition, based on moderate amounts of carbohydrates, fibre, vitamins, minerals and other minor compounds commonly known as phytochemicals (Liu et al., 2003). Regarding phytochemicals, phenolic compounds have attracted much attention in the last few decades in view of their antioxidant, anti-inflammatory and anticarcinogenic properties (Del Rio et al., 2013).

The present Doctoral Thesis proposes a series of works concerning *in-vitro* and *in-vivo* studies in order to evaluate the colonic fate of different groups of phenolic compounds as well as their possible repercussion on the local ecosystem (microbial and metabolic implications). Three different plant-based products were selected for this purpose: enriched olive oil with olive and olive/thyme phenolic extracts, pomegranate and *Arbutus unedo* (*A. unedo*) fruit (**Figure 14**). Each of these products has its distinctive phenolic spectrum whose behaviour during digestion does not necessarily have to be equal; therefore, they deserve individual evaluation.

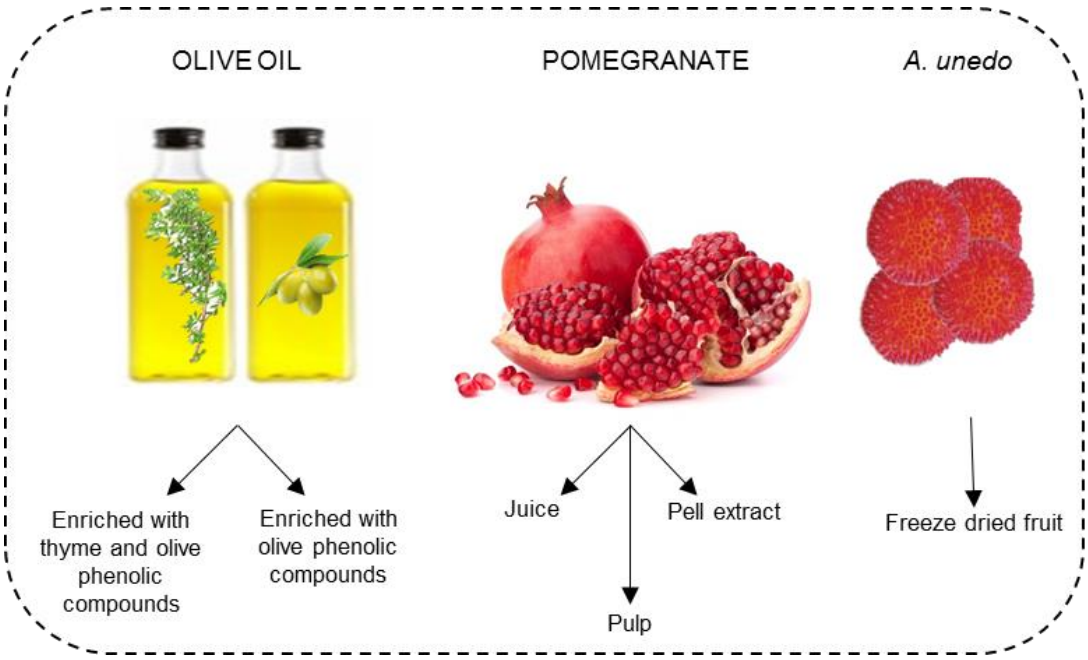


**Figure 14**. Rich-phenolic sources selected to study the colonic fate of their phenolic compounds

The results derived from each study comprising this Doctoral Thesis could be analyzed from different perspectives (**Figure 15**). On the one hand, by comparing the data obtained from *in-vitro* and *in-vivo* models to establish possible correlations. On the other hand, the discussion could also be formulated from a cross-sectional approach, based on the comparison of the colonic metabolism between the different types of phenolic compounds tested. In addition, a short review associated with the possible implication of phenolic compounds in the gut ecosystem should be mentioned.

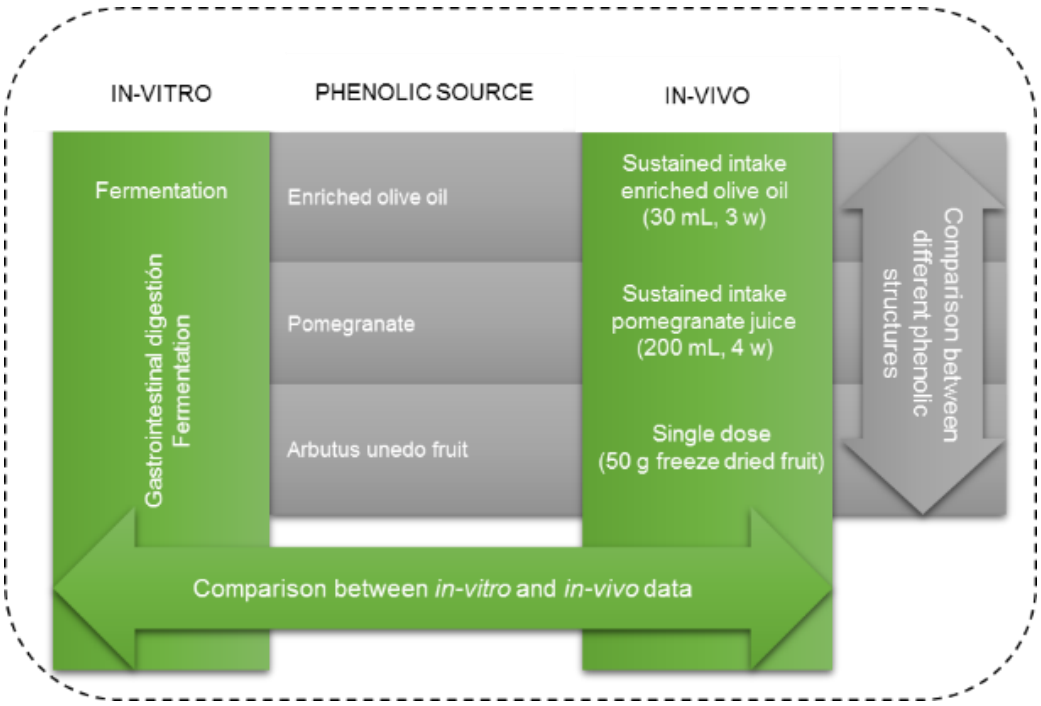


**Figure 15**. Different perspectives to face the discussion of the results obtained from the individual works that comprises the core of the present Doctoral Thesis.

## Phenol colonic metabolism: from *in vitro* to *in vivo* approaches

*In-vitro* digestion models are valuable sources of preliminary results to screen and predict the metabolic behaviour of several food components that, in many cases, represent the first entry point for further investigations (Guergoletto et al., 2016; Hur et al., 2011; Bode et al., 2013). For example, the identification of a specific metabolite generated during colonic

fermentation by *in-vitro* model can be subjected to further tests to confirm its *in-vivo* generation and its possible physiological relevance. It is rather enigmatic how the observations from *in-vitro* digestion models can be extended to humans. However, comparing results might help to discriminate which considerations are more relevant in *in-vitro* experiments if is intended to extrapolate to *in-vivo* systems (Bode et al., 2013).

The most common elements that make up artificial digestion are *in-vitro* gastrointestinal digestion combined with *in-vitro* colonic fermentation. According to the desired end points of the study, there are considerable differences between the parameters measured. These may include structural changes in the native molecule, stability, release from the food matrix (bioaccessibility), prediction of the potential bioavailability, and interference with other food components (competitive process) (Coates et al., 2007; Hur et al., 2011; Rubió et al., 2014a; Saura-Calixto et al., 2007). The pursued objective of this Doctoral Thesis was the qualitative and quantitative characterization of the phenolic fraction that presumably reaches the colon (Mosele et al., 2015a; Mosele et al., 2016a) after the gastro-intestinal digestion process.

On the other hand, *in-vitro* fermentations were implemented to study the time-based biotransformation of the most representative phenolic compounds of the three phenolic sources selected: phenol-rich olive oils, pomegranate and *A. unedo* fruit, in order to propose their colonic metabolic pathways (Mosele et al., 2014a; Mosele et al., 2014b; Mosele et al., 2015a; Mosele et al., 2016a). Using the information obtained from *in-vitro* and *in-vivo* studies, an overall picture of the colonic pathways of various phenolic compounds has been proposed. Unfortunately, due to ethical issues, the *in-situ* study of the digestive process in humans was not considered. Therefore, the phenolic composition of faeces collected after the sustained intake of phenol-rich olive oils and pomegranate juice, and obtained following the acute intake of *A. unedo* fruit, was explored in order to understand the colonic metabolism of their respective phenolic components. Interestingly, the global results of this Doctoral Thesis revealed a suggestive relationship between those colonic metabolites detected after *in-vitro* fermentation and those increased in the faeces after the phenolic human diet intervention (**Table 5**).

**Table 5**. Common observations between *in vitro* fermentations and the phenolic profile of human faeces after dietary supplementation.

| Phenolic source | *In vitro* | *In vivo* | Reference |
|---|---|---|---|
| Olive oil enriched with their own phenolic compounds | Generation of HT as main metabolite of HT acetate and OLE | Significant increase of HT in faeces after sustained intake of olive oil during 3 w | **Mosele et al., 2014 Martín-Peláez et al., 2015** |
| | Good stability of HT during fermentation | | |
| | Marginal microbial catabolism of HT | No microbial metabolites were considerable increased | |
| Pomegranate juice | Generation of different hydroxylated forms of utolithins | Significant increase of urolithin A | **Mosele et al., 2015a Mosele et al., 2015b** |
| | Generation of catechol | Significant increase of catechol | |
| | Different profile of metabolizers | Different profile of metabolizers | |
| *A. unedo juice* | Generation of urolithins, phenylpropionic and phenylacetic acids, hydroxybenzoic acid, catechol and gallic acid | Increase in the concentration of most metabolites described *in vitro* | **Mosele et al., 2016a Mosele et al., 2016b** |
| | Generation of gallic acid and protocatechuic acid at initial times of fermentation | Detection of gallic acid and protocatechuic acid only in early faeces | |

HY: hydroxytyrosol; OLE: oleuropein; w: weeks

It is also worth mentioning the similarity existing between the time of defecation and the *in-vitro* fermentation kinetic. Those metabolites detected only at initial times of *in-vitro* fermentation of *A.unedo* (gallic and protocatechuic acids) were detected in the faeces excreted after more than 12 h after *A. unedo* fruit intake, but not in those excreted after more than 24 h (Mosele et al., 2016a; Mosele et al., 2016b). This fact is in accordance with

the previous conclusion of Stephen et al. (1987), who associated a rapid transit time with increased amounts of unmetabolized dietary products in faeces.

Diverse categories of human metabolizers (inter-individual variability) were distinguished in both *in-vitro* and *in-vivo* models. Differences were not only in the amount of metabolites produced but also in their nature (Mosele et al., 2015a; Mosele et al., 2015b; Mosele et al., 2016a; Mosele et al., 2016b). Based on the different profile of urolithins detected in human biological fluids, Tomás-Barberán et al., 2014, differentiated between three phenotypes of urolithin converters. These include phenotype A (produces only urolithin A), phenotype B (produces urolithin A and/or isourolithin A plus urolithin B) and phenotype 0 (non-urolithin producer). Something similar occurs with the isoflavones, since there is a percentage of individuals who do not have the capacity to convert daidzein into equol (Decroos et al., 2005; Guadamuro et al., 2015).

Despite the commonalities, some discordance between *in-vitro* and *in-vivo* outcomes was also noted (**Table 6**). The metabolic profile of faeces after phenolic treatment did not completely reproduce the same spectra as *in-vitro* samples. These contradictions, which may seem trivial, can lead to over or underestimations of *in-vivo* metabolism. Over-estimation can occur when certain metabolites increase considerably after *in-vitro* fermentation, but their presence in faeces after phenolic treatment lacks significance. This is the case of the olive oil enriched with a combination of olive and thyme phenolic extracts. After diet supplementation with olive oil enriched with olive and thyme phenols (500 mg total phenols/kg oil) during 3 weeks at a dose of 25 mL/day), the analysis of human faeces did not show changes in the concentration of hydroxyphenylpropionic acid (Martín-Peláez et al., 2015), despite the fact that this compound was the main metabolite formed from rosmarinic acid and eriodictyol after the *in-vitro* colonic fermentation (Mosele et al., 2014b). In the same way, *p*-coumaric and protocatechuic acids were not significantly increased in human faeces after the diet supplementation with pomegranate juice (200 mL/day during 4 weeks) (Mosele et al., 2015b), although results from *in-vitro* fermentation of pomegranate-based products showed an intense colonic metabolism (Mosele et al., 2015a). In the case of *A. unedo* fruit, valerolactones and phenylacetic acids do not make an outstanding contribution to the phenolic content of faeces after acute ingestion (Mosele et al., 2016a; Mosele et al., 2016b).

**Table 6**. Discrepances between results from *in vitro* fermentation of phenolic compounds and the phenolic profile of human faeces after dietary supplementation.

| Phenolic source | *In vitro* | *In vivo* | Reference |
|---|---|---|---|
| Olive oil enriched with their own phenolic compounds | Not increased | Significant increase in the concentration of protocatechuic acid | **Mosele et al., 2014** **Martín-Peláez et al., 2015** |
| Olive oil enriched with thyme phenolic compounds | No microbial degradation and high stability of phenolic monoterpenes | Not detected | **Mosele et al., 2014** **Martín-Peláez et al., 2015** |
| | Generation of hydrohydroxyphenylpropionic acid as main metabolite of rosmarinic acid and eriodictyol | Not significant increased | |
| Pomegranate juice | Not increased | Significant increase of hydroxytyrosol | **Mosele et al., 2015** **Mosele et al., 2015** |
| | Not increased | Significant increase of phenylpropionic acid | |
| | Not detected | Significative increase of an unidentified metabolite | |
| *A. unedo* | Valerolactones generation | Not detected | **Mosele et al., 2016** **Mosele et al., 2016** |
| | Penylacetic acids increment | Not increased | |

Contrary to the latter perspective, the presence of non-described *in-vitro* phenolic compounds in biological specimens after phenolic treatment can lead to omissions of *in-vivo* metabolism. This has been noted after the daily dietary supplementation with olive oil enriched with olive/thyme phenols (Martín-Peláez et al., 2015), resulting in an increase in the concentration of protocatechuic acid in faeces, while this phenolic metabolite was not detected as a consequence of the *in-vitro* colonic fermentation of olive or thyme phenolic extracts (Mosele et al., 2014a; Mosele et al., 2014b). In this respect, phenylpropionic acid and hydroxytyrosol were detected in considerable amounts in human faeces after a daily

diet supplementation with pomegranate juice (200 mL/day, 4 weeks), but these compounds were not detected after *in-vitro* fermentation of pomegranate products (Mosele et al., 2015a; Mosele et al., 2015b). Similar to the latter was the increase of hippuric acid in faeces of some volunteers after the acute intake of *A. unedo* fruit, which was not observed after the *in-vitro* fermentation of *A. unedo* fruit (Mosele et al., 2016a; Mosele et al., 2016b).

Certain reasons explain differences in the phenolic metabolism when *in-vitro* models are compared with the metabolism in living beings. Firstly, the difficulty to reproduce the microbiota associated with the colon mucosa and other mammalian contributions, such as active intestinal absorption, effect of brush border enzymes, cellular efflux and the contribution of enterohepatic recirculation, and other compounds present in the diet.

Collectively, the results of the Doctoral Thesis maintain the previous opinions set out by other authors who insist on the necessity to contrast *in-vitro* data with animal or human monitoring (Hur et al., 2011). *In-vitro* data is obtained mainly from chemical analyses, and the lack of many factors present in *in-vivo* systems could be crucial in some physiological aspects. This does not mean that *in-vitro* assessments should be discarded to study the behaviour of phenolic compounds during digestion. Further, *in-vitro* gastrointestinal digestion and fermentation could be considered as a valuable approach to (1) determine the metabolites generated from the action of gut microbiota to elucidate the metabolic pathways involved in colon metabolism, (2) distinguish between different phenotypes of metabolizers, (3) establish the potential stability of parent compounds present in food and their microbial derivatives during the digestion process, (4) identify potential biomarkers of consumption, and (5) promote the identification and subsequent study of phenolic derivatives based on their chemical and physiological properties. In synthesis, information derived from *in-vitro* experiments can be used as the basis for the design of proper human studies, in which the definition of biomarkers and therapeutic targets may be necessary.

## Colonic metabolism according to the type of phenolic structure and the food matrix composition

To associate the improvement of specific physiological responses with the intake of phenolic compounds, it is crucial to determine the chemical structure and the concentration that reach the target tissues. For this purpose, researchers are making increasing use of

data derived from urine and plasma analysis, paying particular attention to the native structure of phenols present in the food (Bergmann et al., 2010; Rubió et al., 2014b; Serra et al., 2010). Apart from a limited number of exceptions, the balance between the amount of phenolic compounds ingested and that detected in biological fluids (urine and plasma) is far from satisfactory, suggesting limited bioavailability (Bergmann et al., 2010; Rubió et al., 2014b; Serra et al., 2010). In this context, the beneficial effects associated with phenolic ingestion are perhaps related to structures derived from diverse metabolic pathways, with special regard to the colon metabolism (Bergmann et al., 2010; Del Rio et al., 2013; Guadamuro et al., 2015; Johnson et al., 2004; Martín-Peláez et al., 2015; Tomás-Barberán et al., 2014; Verbeke et al., 2015).

The three different phenolic sources studied in the Doctoral Thesis, phenol-rich olive oils, pomegranate and *A. unedo* fruit, are particularly rich in one or two phenolic subclasses and have modest concentrations of others. The addition of pomace phenolic extract (waste product of virgin olive oil extraction) to olive oil increases and standardizes the concentration of the main virgin olive oil phenols, such as phenolic alcohols (hydroxytyrosol and tyrosol) and secoiridoids (oleuropein derivatives) with minor sensorial modifications (Rubió et al., 2012; Rubió et al., 2014b). Additionally, the incorporation of the thyme phenolic extract provides supplementary phenols, such as monoterpenes (thymol and carvacrol), rosmarinic acid and flavonoids (Rubió et al., 2012; Rubió et al., 2014b). The analysis of the pomegranate products reported high concentration of ellagitannins, ellagic acid related compounds and anthocyanins (in juice and pulp), besides other minor phenols (Mosele et al., 2015a; Mosele et al., 2015b). *A. unedo* fruit possesses a singular phenolic composition characterized by high amounts of gallotannins (Mosele et al., 2016a; Mosele et al., 2016b).

In accordance with previous studies by other authors, our results have shown that, during *in-vitro* digestion, the stability of phenolic compounds depends on their chemical characteristics (Bergmann et al., 2010; Coates et al., 2007; Kahle et al., 2005; Rubió et al., 2014a; Serra et al., 2010; Tagliazucchi et al., 2010). Anthocyanins and polymeric phenols (ellagitannins, gallotannins and proanthocyanins) are especially sensitive to small intestine conditions. The native phenols present in the food are degraded to unknown compounds (probably chalcones), while the latter are recovered as monomers or minor polymerized forms (Bergmann et al., 2010; Coates et al., 2007; Kahle et al., 2005; Mosele et al., 2015a;

Mosele et al., 2016a; Tagliazucchi et al., 2010; Serra et al., 2010). The food matrix characteristics also have an impact on the phenolic stability during digestion, particularly delaying the rate of release to the digesta, which confers some resistance to degradation (Mosele et al., 2015a; Mosele et al., 2016a). The characteristics of the alimentary bolus that reaches the colon will depend on how much the phenolic fraction is affected during gastrointestinal digestion (Coates et al., 2007; Kahle et al., 2005; Mosele et al., 2015a; Mosele et al., 2016a; Rubió et al., 2014a).

Once phenolic species are in contact with the gut microbiota, many of them suffer further degradation with the concomitant generation of microbial derivatives in parallel with the decrease of the parent compounds (Mosele et al., 2014a; Mosele et al., 2014b; Mosele et al., 2015a; Mosele et al., 2016a). The degree of microbial degradation also seems to be influenced by the characteristics of the molecule. For example, low molecular weight phenols are less affected. This is the case of hydroxytyrosol, tyrosol and phenolic monoterpenes (Mosele et al., 2014a; Mosele et al., 2014b) detected in human faeces. Moreover, it could be expected that hydroxytyrosol was transformed to tyrosol by dehydroxylation. Nevertheless, this reaction was not observed under *in-vitro* conditions (Mosele et al., 2014a), corresponding to the absence of tyrosol in faeces after olive oil intake (Martín-Peláez et al., 2015). Nevertheless, other minor compounds, such as phenolic acids (e.g. gallic acid), that can initially be present in the food (pomegranate and *A. unedo* fruit) or else generated by the catabolism of more complex precursors, are easily degraded (Mosele et al., 2015a; Mosele et al., 2016b). This fact could be explained by the characteristics of the side chain of the phenolic molecule that may govern the affinity as microbial substrate.

The colonic fermentation of phenolic compounds is a gradual process of degradation in which intermediate metabolites are generated early. In turn, these intermediate or initial metabolites are subsequently catabolized, promoting the accumulation of simpler end products of fermentation in the gut lumen. Some of these intermediate or end microbial derivatives can be exclusive or common to several precursors (**Table 7**). Ellagitannins (or ellagic acid derivatives) and other phenolic compounds, such as isoflavones, lignans and resveratrol, have been described as producing exclusive microbial metabolites (Bode et al., 2013; Decroos et al., 2005; Guadamuro et al., 2015; Heinonen et al., 2001; Mosele et al., 2015a; Tomás-Barberán et al., 2014). On the other hand, there is a wide range of phenolic

compounds that produce common metabolites. This was reported for eriodictyol and rosmarinic acid, which produced hydroxyphenylpropionic acid as the main microbial metabolite (Mosele et al., 2014b). Hydroxyphenylpropionic acid was also generated after the *in-vitro* fermentation of coffee (rich in chlorogenic acid) (Ludwig et al., 2013).

The dominance of colonic metabolism intermediate products over end-products in faeces is influenced by host-intrinsic features, such as transit time, rate of absorption, and composition and activity of microbiota. Different phenolic compounds sharing common microbial metabolites certainly do not guarantee a specific biomarker of consumption.

**Table 7.** Microbial metabolites generated from different phenolic sources.

| Phenolic precursors | Intermediates metabolites | Final metabolites | Reference |
|---|---|---|---|
| Ellagitannins (ellagic acid) | Pentahydroxyurolithins<br>Tetrahydroxyurolithins<br>Trihydroxyurolithins (urolithin C) | Dihydroxyurolithins (urolithin A)<br>Hydroxyurolithins (urolithin B) | **Mosele et al., 2015** |
| Isoflavones (Daidzein) | Dihydrodaidzein | *O*-desmethyllangolesin<br>Equol | **Decross et al., 2005** |
| Lignans | | Enterodiol<br>Enterolactone | **Heinonen et al., 2001** |
| Resveratrol | | Dihydroresveratrol<br>3,4′-dihydroxy-*trans*-stilbene<br>3,4′-dihydroxybibenzyl (lunularin) | **Bode et al., 2014** |
| Eriodictyol | Dihydroxyphenylpropionic acid | Hydroxyphenylpropionic acid | **Mosele et al., 2015** |
| Rosmarinic acid | Dihydroxyphenylpropionic acid | Hydroxyphenylpropionic acid | **Mosele et al., 2015** |
| Chlorogenic acid | Caffeic acid | Hydroxyphenylpropionic acid | **Ludwig et al., 2013** |

A more complex picture emerges when the food matrix composition and food structure is considered. The behaviour in the phenolic metabolism resulting from the *in-vitro*

gastrointestinal digestion of pomegranate juice and pulp was quite similar, and some differences were observed with respect to the pomegranate peel extract (Mosele et al., 2015a). The metabolites formed during *in-vitro* digestion did not differ in nature, but some aspects relative to their amount were evident. Although major quantities of urolithins were collected during *in-vitro* fermentation of the extract, the relative rate of urolithin generation respect to the initial precursors (ellagitannins) was greater for juice and pulp (Mosele et al., 2015a). Seeram et al., 2008, observed that the administration of liquid, semi-liquid and solid pomegranate extracts with the same phenolic concentration did not promote significant differences in the concentration of plasmatic urolithin A. Only the time of maximum concentration was delayed for the solid pomegranate extract compared to liquid and semiliquid preparations. Based on the knowledge of matrix effects, food and nutraceuticals could be designed to be focused on a particular mission. The delivery of parent compounds could be manipulated to fit the needs of each specific individual. For example, promoting the generation of microbial metabolites in different segments of the colon may be possible with a food material that controls the release of parent compounds.

## Methods of phenolic analysis in *in vitro* models and human biological samples

Prior to the qualitative and quantitative characterization of *in-vitro* digestion samples and human faeces, phenolic compounds and their microbial metabolites need to be extracted from the sample. Several pre-treatment sample methods, including water or chemical solvents (e.g. methanol), alone or in combination, have been used (Martín-Peláez et al., 2015; Mena et al., 2015; Mosele et al., 2014a; Mosele et al., 2014b; Mosele et al., 2015a; Mosele et al., 2016a; Tagliazucchi et al., 2010). Marked contrast with the reality is the main justification put forward for rejecting the use of chemical solvents, since extractions performed with water (or aqueous buffers) adjust better to the physiological conditions (Pierre et al., 2013). However, the use of chemical solvents is the most favourable approach since, if chosen correctly, they are best suited to the characteristics of the matrix and selectively retain the compounds of interest (Mena et al., 2015; Saura-Calixto et al., 2007). The use of chemical solvents for the analysis of samples obtained from *in-vitro*

and/or *in-vivo* digestion is not considered to be physiological, since the major effects of extraction are achieved due to the digestion process itself. In addition, the isolation of those phenolic compounds that remain closely associated with the food matrix require far more aggressive treatments that include high temperatures in the presence of strong acids for long periods of time (Saura-Calixto et al., 2007).

*In-vitro* gastrointestinal digestion and colonic fermentation have been widely used to study stability, bioaccessibility and transformation of several individual phenolic compounds (Mena et al., 2015; Mosele et al., 2014a; Mosele et al., 2014b) and whole food (Saura-Calixto et al., 2007; Mosele et al., 2015a; Mosele et al., 2016a). Study-to-study comparisons may become risky due to the lack of harmonized protocols between the digestion models, the experimental conditions and the modality used for phenolic detection and quantification. In the current situation, it is unrealistic to expect all the laboratories to use the same digestion model, since each one should be adapted to the available resources. Certain parameters, such as the experimental conditions, may be easy to apply in an attempt to harmonize inter-laboratory procedures. Some of them could be: (1) the time-course of incubation in each individual digestion step, (2) the pH values of each compartment, (3) the ratio between the amount of buffer and food (or compound), (4) the amount and type of enzymes and bile acids, (5) the extraction procedures, (6) the proportion of faeces in faecal slurries, (7) the composition of buffers and (8) the expression of results.

The analysis of faeces before and after a dietary intervention based on the ingestion of phenolic-rich products is not a common practice (Rubió et al., 2014b; Seeram et al., 2008; Serra et al., 2010; Tomás-Barberán et al., 2014). The composition of faeces is a reflection of the intra-colonic environment and their characterization before and after phenolic treatment includes information regarding the impact of the local residence of phenolic compounds. Collection of faeces is easy and does not pose any particular risk, since it does not require any invasive technique, but it can be somewhat embarrassing for volunteers. Handling of fresh faeces for analysis may be impracticable and unpleasant; therefore, lyophilization was conducted in most of the *in-vitro* and human biological fluids analyzed in the present work. A free water material permits better homogenization and the reduction of sample-to-sample variability. In addition, the results can be expressed based on dry weight or also on fresh weight, if required.

In parallel, the analysis of bioactive food metabolites in plasma is a good predictor of their absorption and metabolism, and allows a reasonable estimation of their body distribution and lifespan (Rubió et al., 2014b). Nevertheless, qualified personnel and special supervision are required for blood extraction, which could limit the objectives and increase the cost of the bioavailability studies. For this reason, with the aim of developing an alternative solution to simplify blood sampling, we proposed the use of dry blood spot (DBS) cards as a simple sample-pre-treatment for the analysis of phenolic compounds in whole blood. This original approach was sufficient to detect and quantify the most abundant phenolic species in blood after the acute intake of *A. unedo* fruit (Mosele et al., 2016b). We observed that the main circulating metabolites detected were of colonic origin, which is in accordance with previous findings that also noticed the important contribution of colonic metabolites to total oral bioavailability of dietary phenolic compounds (Rubió et al., 2014b; Tomás-Barberán et al., 2014).

## The role of dietary phenolic compounds in the large intestine

### Phenolic compounds - microbiota interactions-faeces metabolites

Modulation of gut microbiota is a concept associated with positive modifications due to the influence of external factors (diet and antibiotics). These changes range from the increasing abundance of beneficial microorganisms to the lower number of harmful inhabitants (Cammarota et al., 2014). With regard to foodstuff, the term “prebiotic” has emerged to define those compounds that possess the capacity to increase health-promoting probiotic bacteria (especially *Bifidobacterium* and *Lactobacillus*). Gibson et al., 1994 defined a prebiotic as “*a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health*”. Considering this, phenolic compounds, if they increase the concentration of beneficial bacteria, would automatically be included in this group. However, the prebiotic concept has mutated over recent years and currently there is not an agreed definition. In general, only alimentary fibre and certain non-digestible carbohydrates are considered as potential candidates (Cammarota et al., 2014; Hutkins et al., 2004).

From a global point of view, data obtained from *in-vitro* fermentations and animal studies have shown positive responses to phenolic treatments (Appendix; Guergoletto et al., 2016).

Nevertheless, the latter expectations are not really encouraging in human trials due to the low amount of studies and the incongruence between results (Appendix). Another objective of this Doctoral Thesis was to study the ability of different phenolic dietary treatments (enriched olive oil and pomegranate juice) to reshape the human gut microbiota. This is intended to provide extra data obtained from human studies in order to stimulate the debate about the possibility of including phenolic compounds in dietary therapies to promote gut microbiota homeostasis.

Analyzing the results of the present work together, we observed two different scenarios. First, an increase in the abundance of *Bifidobacterium* after 3 weeks of sustained intake of 25 mL of enriched olive oil with thyme and olive extracts (Martín-Peláez et al., 2015). On the other hand, substantially higher amounts of phenolic compounds supplied by 200 mL/day of pomegranate juice during 4 weeks did not produce any change in the gut community (Mosele et al., 2015b). The incongruence in the responses to dietary treatments between enriched olive oil and pomegranate juice may be related to the health status of volunteers. The participants in the study of dietary supplementation with phenol-enriched olive oil were hypercholesterolaemic subjects, while participants in the study of dietary supplementation with pomegranate juice were healthy and younger subjects. Healthy gut microbiota promotes colon homeostasis, and different strategies could be employed by microorganisms to maintain this stable state for short or medium-term periods (Lozupone et al., 2012). Hypercholesterolaemia has been associated with an altered microbiota, which may be more susceptible to dietary changes (Martínez et al., 2009). Whether a phenolic treatment is capable of promoting changes in the gut microbiota may depend on the resilience of the microorganism to the type and amount of phenolic compounds and the length of treatment (Lozupone et al., 2012).

In any case, differences between studies in the techniques used to assess the gut microbiota unfortunately make any comparison incompatible. Pyrosequencing is a large-scale high-throughput screening method used for the characterization of the general composition of the gut community (Sekirov et al., 2010; Mosele et al., 2015). On the other hand, the fluorescence in situ hybridization (FISH) technique utilizes specific probes to target distinct groups of bacteria, and hence identified certain bacterial groups previously selected with the researcher's criteria (Martín-Peláez et al., 2015).

The characterization of faecal metabolites can subsequently be analyzed to establish possible connections with certain gut inhabitants. The relationship between phenolic microbial metabolites, faecal sterols and short chain fatty acids (SCFAs) and intestinal microorganisms was studied. Metabolic-microbial associations displayed both positive and negative correlations and were only detected for catechol and phenylpropionic acid, two microbial metabolites that increased significantly after the intake of pomegranate juice (Mosele et al., 2015b). All this confirms the existence of a relationship between phenolic compounds and certain bacteria groups at colon level. Metatranscriptomic as well as the introduction of proteomic studies in phenolic interventions could be an interesting approach to understand how the microbiota functionality responds to dietary phenolic interventions (Lozupone et al., 2012).

It is very difficult to select a single connecting factor that explains the association between phenolic compounds from the diet and microbial modulation, due to the large heterogeneity between individuals' responses. Different factors, such as dose administered, treatment time, form of administration, and number and characteristics of the volunteers (healthy or presenting certain risk of disease), add even more complexity.

Regarding the faecal metabolites other than phenolic metabolites, the gut epithelium is continuously exposed to a varied amount and type of substances. The overall balance of the lumen composition is determined by non-absorbed food and internal secretions (enzymes, bile acids, mucus and shedding of cells). Due to the high density of microorganisms in the large intestine, most of these products are successfully biotransformed (Appendix; Verbeke et al., 2015). The result is the generation of a wide range of metabolites. Some microbial metabolites, such as those derived from proteins and sterols (cholesterol and bile acids), are likely to play a role in many different intestinal diseases, whereas SCFAs maintain the gut homeostasis (Appendix; Verbeke et al., 2015). In spite of some shortcomings, the analysis of faecal samples is a suitable alternative to estimate, by the regulation of the abundance of toxic/beneficial compounds, the potential impact that dietary phenolic compounds may have on the intra-colonic environment (Pierre et al., 2013). Verbeke et al. (2015) propose that "*analyzing the activity of the microbiota rather than its composition and structure may be more relevant to assess the impact of prebiotic interventions*".

The present Doctoral Thesis includes innovative aspects of dietary phenolic compounds in the regulation of SCFAs and faecal sterols (Martín-Peláez et al., 2015; Mosele et al., 2015b), since these parameters have been poorly explored in previous human dietary interventions with phenols. We observed that the concentration of SCFAs and primary and secondary bile acids did not undergo modification after the intervention with phenol-rich olive oils and with pomegranate juice, respectively. Therefore, the microbial metabolism of luminal cholesteroll was, in both cases, modified due to the dietary intervention (Martín-Peláez et al., 2015; Mosele et al., 2015b).

It is worth mentioning that the existence of statistical significance in the faecal metabolic profile does not necessarily indicate clinical significance and *vice versa*. Normally, in clinical medicine, clinical significance is estimated based on a cutoff value (on occasions represented by a range including low and high values) (Colodner et al., 2006). Regarding the parameters studied in the present work, there is no consensus about the range of faecal SCFAs and sterols in order to discriminate between “healthy” and “unhealthy” state. Once the healthy composition of faeces has been defined, the impact of dietary interventions on intestinal homeostasis will be more realistic and precise. However, the complexity of the gut ecosystem and the intra and inter-individual variability complicate the definition of cutoff values of faecal metabolites (Verbeke et al., 2015). To make this possible, the inclusion of a larger number of individuals is necessary in order to better adjust the range of plausible values.

The third volume of the Word Cancer Report prepared in 2014 by the International Agency for Research on Cancer (which forms part of the World Health Organization –WHO-) includes a controversial issue in which processed meat and meat products are categorized as group 1 (carcinogenic to humans) and group 2A (probably carcinogenic) carcinogenics, respectively (www.who.int). The decision of the committee was made based on the results reported by numerous epidemiological studies that associate consumption of these products with an increased risk of cancer development, especially colorectal cancer.

Several components present in meat and processed meat foods may explain the association between their consumption and the risk of colorectal cancer. Among them, positive association of risk was described for N-nitroso compounds (Pierre et al., 2013), fat (Van Hecke et al., 2014), proteins (Appendix; Belobrajdic et al., 2003), heme iron (Bastide

et al., 2015; Pierre et al., 2013), heterocyclic amines and polycyclic aromatic hydrocarbons (Helmus et al., 2013). Their mechanisms of action include increase of oxidation, genotoxicity and cytotoxicity that promote the appearance of pre-carcinogenic lesions (Bastide et al., 2015; Pierre et al., 2013; Van Hecke et al., 2014). Some compounds, such as heme iron, heterocyclic amines and polycyclic aromatic hydrocarbons, are originally present in the food, whereas N-nitroso compounds and other toxic products derived from fat and proteins are formed endogenously through the action of microbiota (Van Hecke et al., 2014). In addition, the presence of fat in meat and processed meat products promotes the secretion of bile acids, which increase the pool of potential carcinogens in the gut (Ajouz et al., 2014; Appendix).

Reduction of certain colorectal cancer biomarkers has been observed after the simultaneous consumption of processed meat products, and $\alpha$-tocopherol may reveal an important role of dietary antioxidants as protector agents (Pierre et al., 2013). In addition, Arts et al. (2002) observed that a diet rich in proteins and iron reduced the absorption of flavonoids, which would increase the presence of protective molecules in the lumen. The contribution of dietary phenolic compounds should be studied experimentally to evaluate the possible reduction of cancer risk in meat eaters. For this, the implementation of studies concerning the effectiveness of phenolic compounds to reduce the formation of harmful substances by gut microbiota and/or to block the pro-carcinogenic mechanism activated by toxic compounds is needed.

**Development of phenolic-rich products to improve colonic health status**

The natural occurrence of phenolic compounds in the diet and their healthy properties have converted these phytochemicals into an attractive ingredient for the design, development and production of high value-added foods and nutraceuticals (Rubió et al., 2014b). Phenolic compounds are part of the minor fraction of plant-based foods but, despite their low concentration, it has been noticed that they confer protection against colonic injuries (Appendix; Coates et al., 2007; Johnson et al., 2004; Martín et al., 2015). The lumen enrichment with phenolic species provides a wide assortment of compounds associated with antioxidant, anti-inflammatory and antiproliferative

purposes (Appendix; Coates et al., 2007). This phenolic cocktail is expected to protect the gut from several toxic substances formed during gastro-intestinal digestion and to promote the development of a healthy gut microbiota (Appendix; Guergoletto et al., 2016 Johnson et al., 2004)

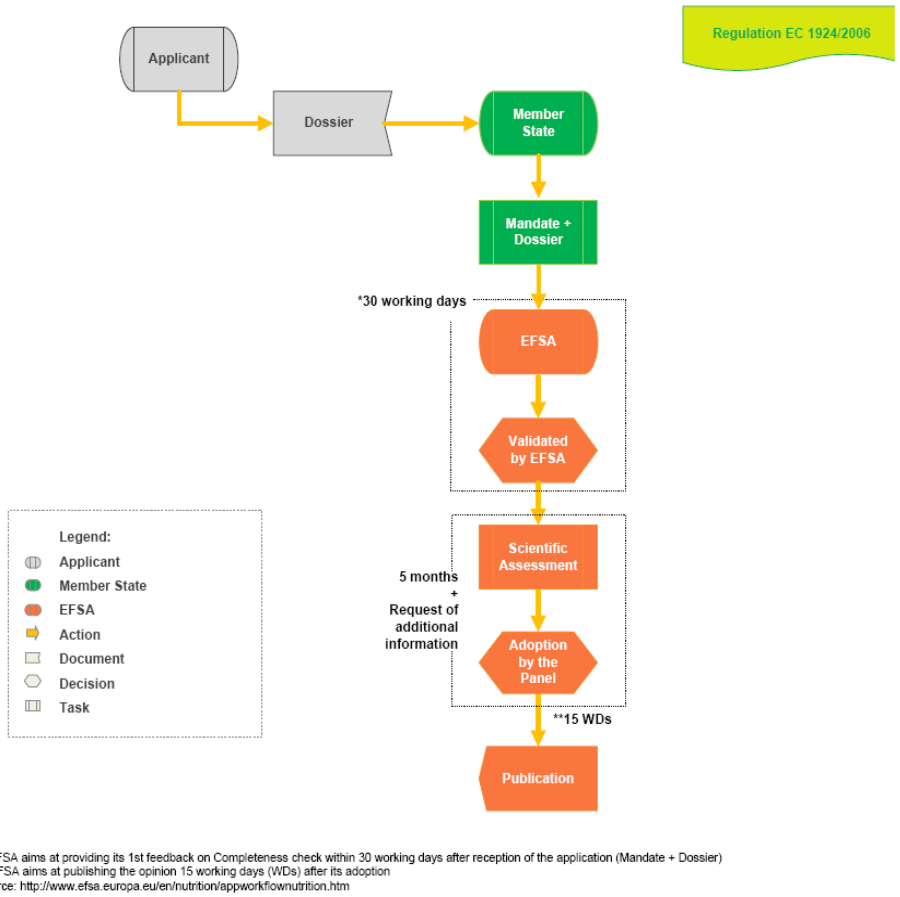


**Figure 16**. Applicants who wish submit an application for authorizations of a health claim under Articles 13.5 or 14 or Regulation EC 1924/2006 or for modification of an existing authorizations should consult the guidance documents and complete the relevant application forms. Applications should be submitted to the national competent authority of a Member State. The competent authority passes the application and any supplementary information supplied by the applicant to EFSA, which carries out the scientific evaluation. From www.efsa.europa.eu

Considering the potential and growing applications of phenol-rich foods for the maintenance of an optimal colonic metabolism, there are regulatory policies that hinder their commercialization, including *claims* associated with health. The European Food Safety Agency (EFSA) is the authority responsible for conducting and verifying compliance with the strict rules that foods must fulfil to be placed on the European market as health

claim products (**Figure 16**). The idea is to protect consumers from unscrupulous traders or industries. However, at the same time, the high standards may not stimulate the scientific and industrial advancement in the study of phenolic compounds and their repercussion on human health, which may have a negative impact on the development of functional foods (Katan et al., 2012).

Substantial information from *in-vitro* and *in-vivo* animal studies is available in the literature to explain the positive impact of phenolic compounds on the colon metabolism (Appendix; Del Rio et al., 2013; Guergoletto et al., 2016; Martin et al., 2015). However, the lack of consistent results obtained from human clinical trials is usually the main reason that conditioned the recognition of *health claims* for phenol-rich foods (Katan et al., 2012). Much remains to be done to demonstrate the importance of phenolic compounds in human health. In the case of colonic metabolism, there are a number of outstanding issues that need to be resolved. For this, the establishment of multidisciplinary groups of experts could be optimal in order to establish:

i. The amount of phenolic compounds that needs to reach the colon intact to produce the expected effects.

ii. The amount of phenolic compounds that should be ingested to reach the effective concentration in the colon.

iii. The active form of the molecule (parent compound or microbial metabolites).

iv. The possible interactions with the food matrix or other dietary components that may be ingested simultaneously.

v. The different profile of metabolizers to design specific dietary strategies (for example the oral administration of the microbial metabolite).

vi. The identification of specific biomarkers of colonic disease progression and their control by diet phenolic compounds.

In synthesis, the potential of the phenol-rich products and the biological activity of the metabolites formed as a consequence of digestion and colonic catabolism must be confirmed through more studies (especially involving humans) in which specific biomarkers of colonic disease progression are identified.

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# CONCLUSIONS

The main conclusions drawn from the results obtained from this Doctoral Thesis are presented below. The structure of the Conclusion section has been organized based on the proposed objectives, from phenolic characterization (phenol-enriched olive oil, pomegranate and *A. unedo* fruit) to the colonic metabolism of phenolic compounds.

**Objective 1. Phenolic characterization of enriched olive oils, pomegranate products (juice, pulp and peel extract) and *A. unedo* fruit.**

**Conclusion 1**. The present Doctoral Thesis offers relevant information that contributes to expanding the existing data regarding the phenolic composition of plant-based products, including some indigenous fruits. The phenolic sources studied present a distinctive phenolic spectrum normally dominated by a specific group of phenolic compounds.

Olive oil enriched with its own phenolic compounds possesses large amounts of hydroxytyrosol derivatives, mainly oleuropein aglycone derivatives (secoiridoids).

The incorporation of thyme phenolic extract into olive oil enriched the product with complementary phenols, mainly phenolic monoterpenes (thymol and carvacrol), flavonoids and rosmarinic acid.

The main phenolic sub-classes of the pomegranate products (juice, pulp and phenolic extract) are ellagitannins and ellagic acid derivatives.

The *Arbutus unedo* fruit is a rich source of gallotannins.

**Objective 2. Application of the *in-vitro* colonic fermentation model to define the behaviour of phenolic compounds in the large intestine.**

**Conclusion II**. The *in-vitro* colon fermentation of the most representative phenols of virgin olive oil (tyrosol, hydroxytyrosol, hydroxytyrosol acetate and oleuropein) revealed that (i) phenolic acids are the main catabolites formed after the fermentation of tyrosol and hydroxytyrosol, (ii) hydroxytyrosol and tyrosol are very stable during colonic fermentation, and (iii), by contrast, hydroxytyrosol acetate and oleuropein are completely degraded during colonic fermentation, generating hydroxytyrosol as the main microbial metabolite.

**Conclusion III**: The thyme phenolic monoterpenes thymol and carvacrol are not degraded by human microbiota after *in-vitro* colonic fermentation. Other phenols from thyme extract, such as rosmarinic acid and the flavonoid eridictyol are completely degraded and generate hydroxyphenylpropionic acid as the main microbial metabolite.

**Conclusion IV**. The application of *in-vitro* gastrointestinal digestion to pomegranate products (juice, pulp and peel extract) and to *A. unedo* fruit allows the stability of phenolic compounds to be predicted according to their particular chemical structures and the influence of the food matrix during digestion. The *in-vitro* colonic fermentation model is a useful tool to probe the affinity of phenolic compounds as microbial substrates (rate of metabolism) and the main metabolites generated as a consequence of the faecal microbiota metabolism.

The absence of native phenolic compounds (ellagitannins and ellagic acid derivatives) present in the pomegranate products at final times of incubation indicates good microbial affinity. The *in-vitro* fermentation of the three pomegranate products (juice, pulp and peel extract) results in the same metabolic products, urolithins being the main microbial metabolites.

The *in-vitro* fermentation of *A. unedo* fruit promotes the total disappearance of native phenolic compounds (mainly gallotannins) phenylacetic and phenylpropionic acids being the main catabolites formed.

**Objective 3. Colonic metabolic pathways of phenolic compounds**

**Conclusion V.** *In-vitro* fermentation models offer the possibility of assessing the degradation rate of phenolic compounds according to the time of exposure to faecal microorganisms. This allows pictures to be captured that represent the different states of phenolic compound degradation. Putting all the pictures together allows the metabolic pathways of phenolic compounds in the colon to be defined. Following this premise, the present Doctoral Thesis proposes the colonic pathways of the main phenolic compounds present in enriched olive oils, pomegranate products and *A. unedo* fruit. The human colonic pathways of oleuropein, hydroxytyrosol, tyrosol,

hydroxytyrosol acetate, thymol, carvacrol and gallotannins constitute a valuable source of information, since they had not previously been described.

**Objective 4. *In-vivo* colonic metabolism of phenolic compounds: human studies**.

**Conclusion VI.** Daily dietary supplementation with phenol-enriched olive oils and pomegranate juice during three and four weeks, respectively, as well as the acute intake of *A. unedo* fruit, increases the amount of phenolic compounds and their microbial metabolites in faeces.

Three weeks of sustained intake of olive oil enriched with its own phenolic compounds increases the concentration of hydroxytyrosol and dihydroxyphenylacetic acid in human faeces. Similarly, the sustained intake of olive oil enriched with a mixture of olive and thyme phenols increases the concentration of protocatechuic acid in human faeces.

Daily supplementation of pomegranate juice during four weeks increases the amount of urolithins, hydroxytyrosol, phenylpropionic acid, catechol and an unknown metabolite in the faeces of healthy volunteers.

The acute intake of *A.unedo* fruit increases most but not all the phenolic metabolites generated during *in-vitro* fermentation.

**Objective 5. Changes in the gut ecosystem (microbial and metabolic modifications) promoted by the intake of enriched olive oil and pomegranate juice**

**Conclusion VII**. Daily moderate consumption of olive oil enriched with phenolic compounds from olive and thyme during three weeks increases the amount of gut beneficial bacteria (bifidobacteria) in hypercholesterolaemic human volunteers. By contrast, no changes in the microbiota composition of healthy volunteers are observed after daily diet supplementation with pomegranate juice.

**Conclusion VIII**. Regarding faecal metabolites, diet supplementation with olive oil enriched with its own phenolic compounds interferes with the gut microbial metabolism of cholesterol, increasing the amount of coprostanone in human faeces. In a similar way, the sustained intake of pomegranate juice affects the colonic

metabolism of cholesterol, decreasing the generation rate of coprostanol and increasing the presence of unmetabolized cholesterol in faeces. Neither the sustained intake of enriched olive oils nor that of pomegranate juice modifies the concentration of short chain fatty acids and bile acids in human faeces.

**Objective 6. Comparison between *in-vitro* and *in-vivo* results**

**Conclusion IX**. The phenolic profile of human faeces after daily dietary supplementation during a period of three-four weeks with phenol-enriched olive oils and pomegranate juice, after the acute intake of *A. unedo* fruit, is partly aligned with the results obtained from the *in-vitro* fermentations. However, the use of *in-vitro* colonic fermentation models could be proposed as a useful tool to elucidate the colonic pathways of dietary phenols; therefore, they must necessarily be combined with dietary intervention studies in humans.

# APPENDIX

*Review*

**METABOLIC AND MICROBIAL MODULATION OF THE LARGE INTESTINE ECOSYSTEM BY NON-ABSORBED DIET PHENOLIC COMPOUNDS: A REVIEW**

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**Abstract:** Phenolic compounds represent a diverse group of phytochemicals whose intake is associated with a wide spectrum of health benefits. As consequence of their low bioavailability, most of them reach the large intestine where, mediated by the action of local microbiota, a series of related microbial metabolites are accumulated. In the present review, gut microbial transformations of non-absorbed phenolic compounds are summarized. Several studies have reached a general consensus that unbalanced diets are associated with undesirable changes in gut metabolism that could be detrimental to intestinal health. In terms of explaining the possible effects of non-absorbed phenolic compounds, we have also gathered information regarded their influence on the local metabolism. For this purpose, a number of issues are discussed. Firstly, we consider the possible implications of phenolic compounds in the metabolism of colonic products, such as short chain fatty acids (SCFA), sterols (cholesterol and bile acids), and microbial products of non-absorbed proteins. Due to their being recognized as affective antioxidant and anti-inflammatory agents, the ability of phenolic compounds to counteract or suppress pro-oxidant and/or pro-inflammatory responses, triggered by bowel diseases, is also presented. The modulation of gut microbiota through dietetic maneuvers including phenolic compounds is also commented on. Although the available data seems to assume positive effects in terms of gut health protection, it is still insufficient for solid conclusions to be extracted, basically due to the lack of human trials to confirm the results obtained by the *in vitro* and animal studies. We consider that more emphasis should be focused on the study of phenolic compounds, particularly in their microbial metabolites, and their power to influence different aspects of gut health.

**Keywords:** colon metabolites; gut fermentation; microbiota; phenolic compounds

## 1. INTRODUCTION

Most of the beneficial health properties of fruit, vegetables, and whole grains have been attributed to bioactive non-nutritional chemical compounds commonly named phytochemicals, which include phenolic compounds. Plant phenols embrace a wide range of secondary metabolites that are synthesized from carbohydrates via the shikimate pathway, occurring as soluble conjugated (glycosides) and insoluble or bound forms [1]. Based on the extensive intake of these kinds of phytochemicals through the diet, a complex mixture of hundreds of phenolic compounds enters the gastrointestinal tract where they can be partially released and absorbed, or survive stomach and intestinal digestion and reach the colon until excretion via feces. Several studies have reported that an important part of the ingested phenolic compounds reaches the large intestine where it undergoes a series of microbial transformations that leads to the generation of related metabolites [2–4].

On occasion, gut microbiota has been defined as a biological reactor since it possesses powerful metabolic functions which include the transformation of many compounds that reach the colon. This activity is possible through the capacity of microorganisms to produce a huge and varied range of enzymes. In the particular case of phenolic compounds, their intestinal transformations include several steps. First, aglycones must be released to the media. For this, different classes of enzymes are needed to

deconjugate the specific moiety associated to the molecule or, in the case of polymeric forms, to break phenolic polymers into individual monomers. Phenolic compounds are also strongly linked to some components of the food matrix, and this interaction is also disrupted by microbiota [1]. The released aglycones undergo subsequent microbial transformations which may include ring fission, α or β-oxidation, dehydrogenation, dehydroxylation, and demethylation, and these result in the generation of simpler related compounds.

An overview of the bioactivity that is probably carried out by phenolic compounds in the large intestine is represented in Figure 1. The passage of digesta through the small intestine is estimated to take around 2–4 h, but the transit time increases considerably in the large intestine, extending to as long as 24 h or more [4,5]. This time is probably long enough to accumulate substantial amounts of phenolic compounds and induce metabolic and microbial changes in the gut lumen. Phenolic compounds are recognized antioxidants and anti-inflammatory agents which can protect intestinal cells from pro-oxidant and inflammatory injuries [6–8]. The presence of luminal phenolic compounds could also impact the metabolic profile, enhancing or inhibiting the generation of fermentation products derived from endogenous and dietary compounds. In turn, changes in the metabolic profile of the gut are sometimes associated with the modification of the shape of the intestinal inhabitants. In fact, some authors have associated the phenolic compounds–gut microbiota interaction with a presumable modulation effect, which could prevent or, indeed, restore microbiota alterations observed in disease [9,10]

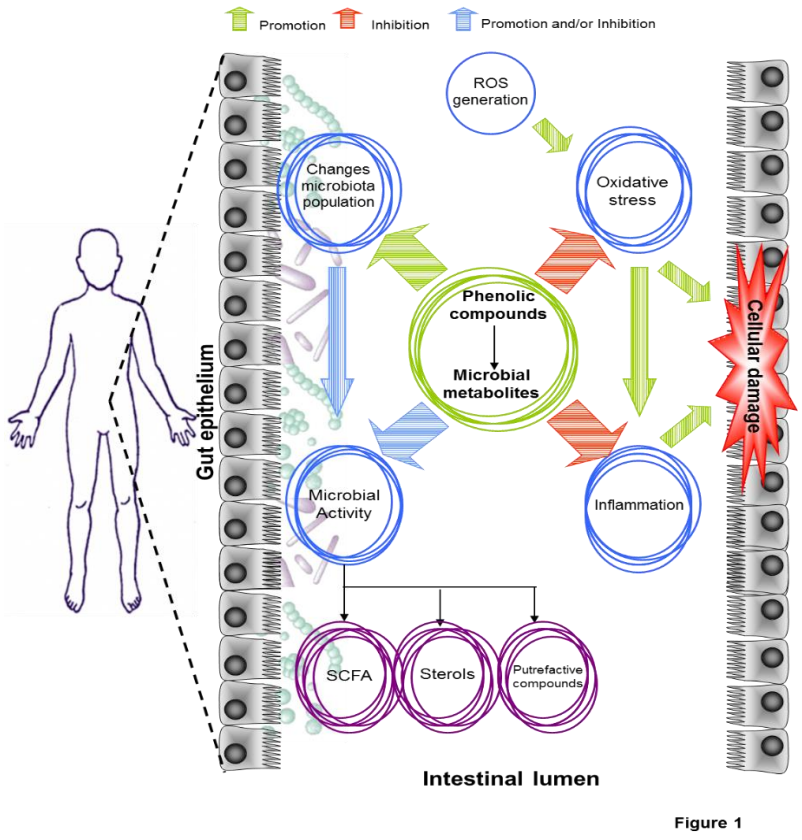



**Figure 1.** Overview of the possible implications of phenolic compounds at the gut level.

The aim of this manuscript was to consult the information available from *in vitro* and *in vivo* studies to review the transformations that the non-digested phenolic compounds undergo when they reach the colon and the possible influence they have on the transformation of other intestinal metabolites, such as short chain fatty acids (SCFAs), sterols, and microbial products of non-absorbed proteins. In addition, their possible implications in metabolic and microbial changes as well as in antioxidant and anti-inflammatory effects at the gut level were also considered.

## 2. Microbial Phenolic Metabolites

Phenolic compounds are commonly classified into two main groups, flavonoids and non-flavonoids. In the next section we will describe the catabolism pathways and the phenolic metabolites formed related with the chemical structures of flavonoids and non-flavonoids.

### *2.1. Flavonoids*

Flavonoids possess an aglycone skeleton of two flavonoid rings (A- and B-rings) connected by a heterocyclic C-ring. In foods, except for flavan-3-ols, flavonoids are normally presented as glycosides or organic acid conjugates.

The sub-class of flavan-3-ols includes the diastereomers catechin and epicatechin, and their corresponding gallate esters, epigallocatechin and epigallocatechin gallate. In foods, flavan-3-ols are present as monomers (catechin and epicatechin) or proanthocyanidins (variable attached monomers). To study the microbial modifications of flavan-3-ols, pure standards as well as grapes, tea, cocoa, berries, red wine, and their extracts have been used. The colonic catabolism of proanthocyanins undergoes a first degradation step resulting in the release of monomer structures with the subsequent hydrolysis of gallic acid from the galloylated forms [11–14]. Based on the literature, we propose the colonic pathways of monomeric flavan-3-ols and their corresponding gallate esters (**Figure 2**). The early appearance of simple phenolics (like catechol) suggests further degradation of gallic acid generated by hydrolysis of epigallocatechin gallate in the first steps of the colonic catabolism [11,12,14,15]. Similarly, the initial C-ring fission of flavan-3-ols (**Figure 2A**) produces the corresponding diphenylpropan-2-ol. Then, this is further converted into 5-(3′,4′,5′)-tri- and 5-(3′,4′)-dihydroxyphenyl-γ-valerolactone in the cases of gallate esters and monomers, respectively [11,14,16]. The subsequent catabolism of the tri- and dihydroxyphenyl-γ-valerolactones originates different hydroxylated forms of phenylvaleric acid [11–15,17]. Neither trihydroxyphenylvaleric acid nor trihydroxyphenylpropionic acid have been described as microbial metabolites of gallate esters in *in vitro* fermentations. However, trihydroxyphenylvaleric acid was identified in human plasma [16]. Phenyl-γ-valerolactones and phenylvaleric acids have been described as exclusive microbial metabolites of flavan-3-ols. Their progressive microbial transformation releases different hydroxylated forms of phenyl (**Figure 2B**) and benzoic acids (**Figure 2C**), whose profile and abundance seem to depend on the particular metabolic activity of each individual microbiota and the composition of flavan-3-ols in the substrate [11–15,17]. Furthermore, other minor catabolites, such as hippuric acid, *p*-coumaric acid, and vanillic acid, homovanillic acid, homovanillyl alcohol, and 3-*O*-methylgallic acid have been associated with the *in vivo* colon metabolism of flavan-3-ols [11,13,16,18].

Flavonols are the most common phenolic compounds in foods such as red wine, apples, onions, beer, spices, herbs, berries, and cocoa, with quercetin, kaempferol, and myricetin being the most studied. During the first steps of the colonic catabolism of the aglycone forms of quercetin and kaempferol, the initial metabolites formed are dihydroquercetin (taxifolin) and dihydrokaempferol, respectively, which are further metabolized to di- and hydroxyphenylpropionic acid, respectively (**Figure 3A**) [19–23]. These metabolites then enter the catabolic route of phenyl acids (**Figure 2B**) and benzoic acids (**Figure 2C**) to generate minor related compounds [19,22,23]. Following the intake of radiolabeled quercetin-4′-*O*-glucoside in rats, high concentrations of hippuric acid were found in the jejunum/ileum, urine, and plasma [22]. Furthermore, 3′,4′,5′-trihydroxyphenylacetic acid and 3′,5′-dihydroxyphenylacetic acid were the main microbial

metabolites detected in rat feces after the administration of myricetin [24], probably derived from the intermediate dyhydromyricetin. However, the generation of quercetin from myricetin should not be discarded [25].

The flavanones hesperetin and naringenin are found in larger concentration in oranges, whereas eriodictyol is a flavanone commonly presented in aromatic herbs and nuts. Hydroxyphenylpropionic and phenylpropionic acids have been described as major fermentation products of eriodictyol and naringenin [23,26,27]. In addition, phloroglucynol was also detected in the fermentation vessels of naringenin [23] and eriodyctiol [28] which could be formed from the A-ring. In the case of hesperidin, 3-(3-hydroxy-4-methoxyphenyl)propionic acid (dihydroisoferulic acid) and different hydroxylated forms of phenylpropionic acid have been reported as products of it colonic catabolism [27] (**Figure 3B**).

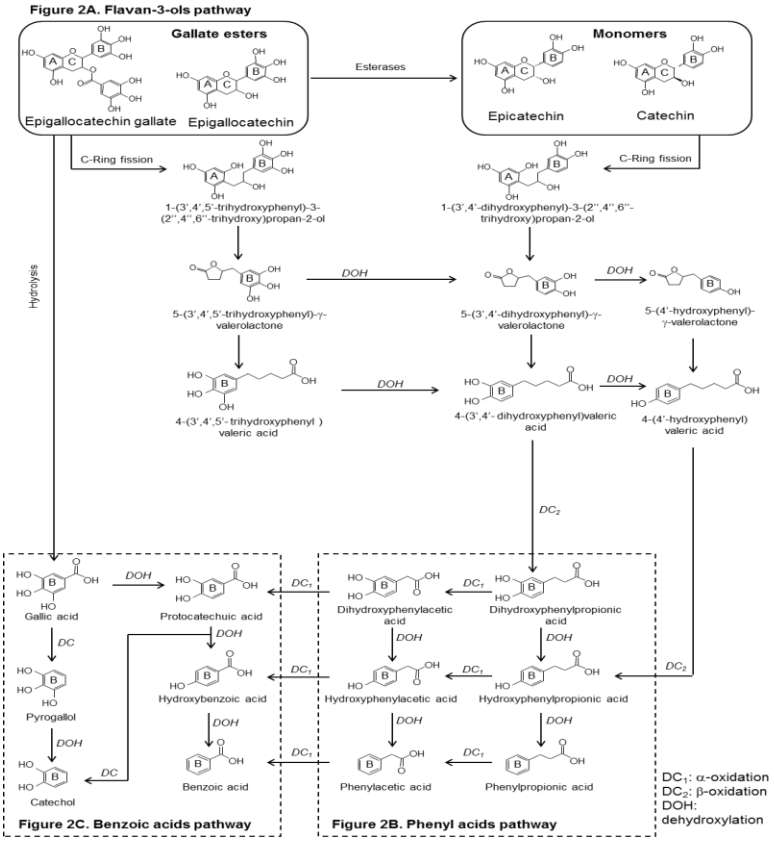



**Figure 2.** Proposed colonic pathways of monomeric flavan-3-ols and their corresponding gallate esters

Flavones, like apigenin and luteolin and their glycosides, are normally found in beer, olive oil, aromatic herbs, and nuts. In the same way as with the other flavonoid sub-classes, deglycosylation is the first microbial action. Apigenin aglycone is transformed into naringenin, whose microbial catabolism produces phenylpropionic acids [21,29] (**Figure 3C**). Luteolin undergoes an initial isomerization to form eriodictyol as an intermediate metabolite which is transformed to 3-(3′,4′-dihydroxypheyl)propionic acid [21,30] (**Figure**

**3C**). In coincidence with *in vitro* data, 3-(4′-hydroxyphenyl)propionic acid was the main metabolite detected in urine after the administration of apigenin-7-*O*-glucoside to human microbiota-associated rats [29].

Genistein and daidzein are the most representative isoflavones and are normally present in their glycoside forms in soy products. The results of *in vitro* incubations of genistein, together with experiments where gnotobiotic rats were inoculated with isolated human equol-forming bacteria, have shown that, during the first steps of colonic catabolism, genistein, aglycone generated dihydrogenistein (**Figure 4**) which is partly converted into 5-hydroxy-equol [31,32]. After *in vitro* fermentation of genistein, Coldham *et al.* [33] observed that dihydrogenistein was metabolized into 6′-hydroxy-*O*-demethylangolensin which, in turn, was transformed into 2-(4-hydroxyphenyl)propionic acid and phloroglucinol. On the other hand, daidzein aglycone can be reduced to dihydrodaidzein and then converted into equol or *O*-desmethylangolensin [31,32,34] (**Figure 4**). Although more than one bacteria has been described as an isoflavone-converter [31,32], not all humans have the capacity to convert genistein and daidzein into their respective microbial metabolites [35], which could indicate that isoflavone transformer bacteria are not common among human intestinal flora

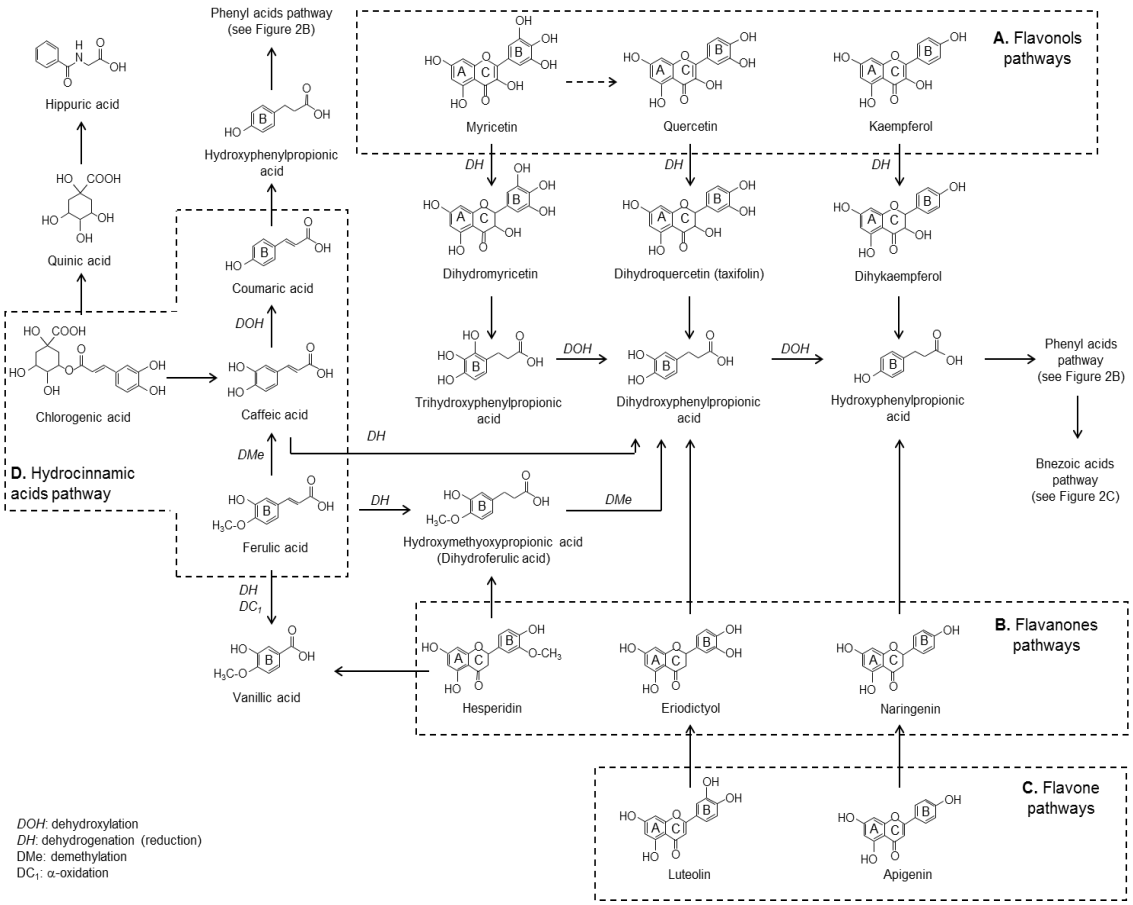



**Figure 3.** Proposed colonic pathways of flavonols, flavanones, flavones, and hydrocinnamic acids

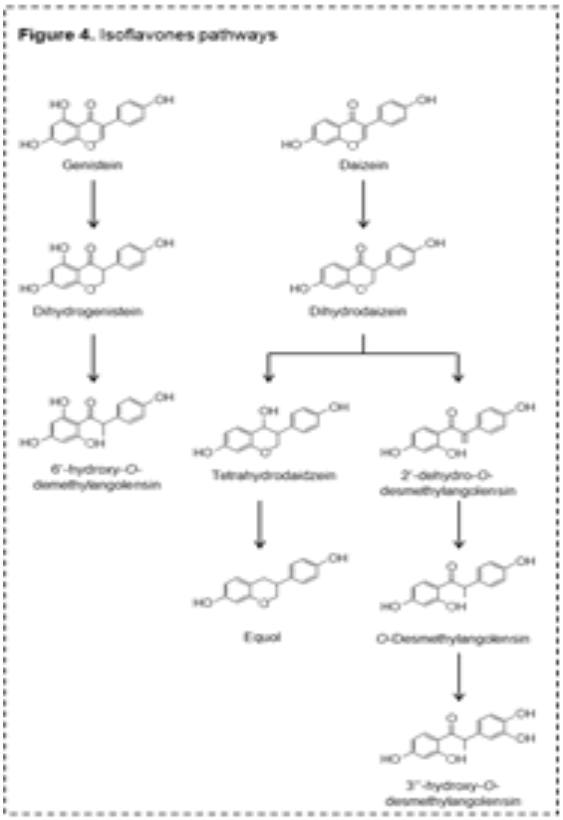



**Figure 4.** Proposed colonic pathways of isoflavones

Anthocyanins are widely dispersed throughout the plant kingdom, being responsible for red, blue, and purple colors. Red wine, grapes, berries, and pomegranate are examples of anthocyanin-ri**ch** products. After microbial deglycosylation, ring fission of the aglycone releases two parts, one from the A-ring and the second one from the B-ring, which then undergo simultaneous catabolism [36,37]. Regarding the fission of the B-ring, both phenyl acids and benzoic acids have been reported as the microbial metabolites of anthocyanins (**Figure 5**). Gonzalez-Barrio *et al.* [37] proposed a complete colonic pathway for cyanidin including different alternative conversion pathways for the B-ring, and protocatechuic acid (benzoic acid) and 3-(3′,4′)-dihydroxyphenylpropionic acid (phenyl acid) were proposed as initial metabolites. The subsequent microbial metabolism of these initial products generates simpler compounds. Regarding benzoic acids, hydroxybenzoic acid has been reported to be a microbial metabolite of pelargonidin glucoside, protocatechuic acid of cyanidin glucoside, vanillic acid of peonidin glucoside, syringic acid of malvidin glucoside, methyl gallic acid of petunidin glucoside, and gallic acid of delphinidin glucoside [36–40]. Considering the results obtained from previous studies, the generation of benzoic acids prevails over that of phenyl acids (**Figures 2B,C and 5**)

Apart from B-ring fission, other microbial metabolites could also be generated from A-ring fission. The fission of ring A of anthocyanins could generate trihydroxybenzaldehyde [39,40], which could further be converted to phloroglucinol [37]. Due to the lack of complete information regarding the description of the colonic fate of some anthocyanins, we tentatively propose alternative pathways in **Figure 5**. Obviously, the acceptance or rejection of these must be confirmed by future studies. Along with some of the metabolites

described in *in vitro* studies, hippuric acid was also detected in human urine after the ingestion of raspberries [37].

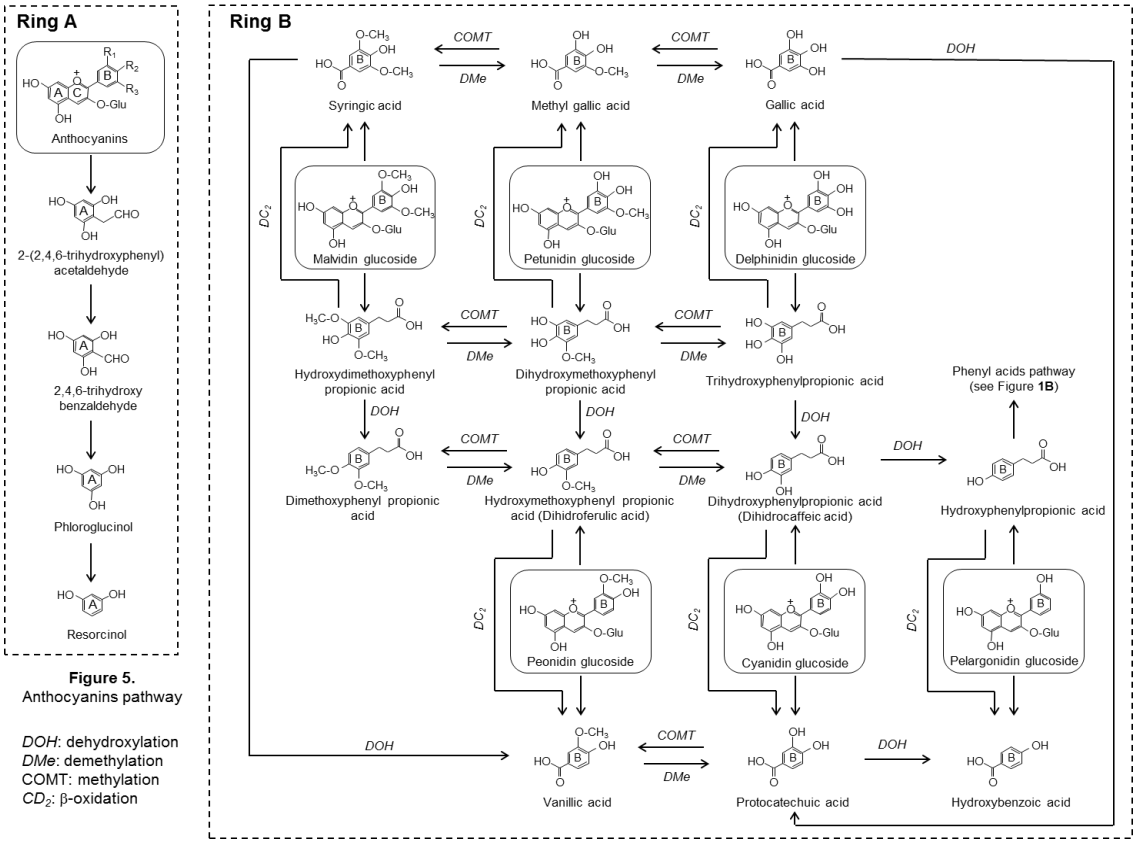



**Figure 5.** Proposed colonic pathways of anthocyanins.

*2.2. No Flavonoids*

Phenolic acids, such as hydroxycinnamic and hydroxybenzoic acids, are present in numerous plant products. In plant tissues, phenolic acids form ether linkages with lignin through their hydroxyl groups in the aromatic ring and ester linkages with structural carbohydrates and proteins through their carboxylic group [1]. These bound phenolics survive stomach and intestinal digestion and reach the colon since, being the substrate to colon microbiota, cell wall fibrous materials are difficult to digest.

Hydroxycinnamic acids are widely distributed in nature, with coffee, whole cereals, dried drupes, wine, berries, spices, and aromatic herbs being the richest sources. Caffeic, ferulic, and *p*-coumaric acids, as well as their tartaric and quinate esters, such as chlorogenic acid (quinate ester of caffeic acid), are included in this group, and their microbial degradation steps are proposed in **Figure 3D**. Chlorogenic acid underwent dihydroxylation, dehydrogenation, or ester hydrolysis as a first microbial transformation in simulated conditions [41]. *In vitro* fermentation of freeze-dried coffee confirmed the initial hydrolysis of chlorogenic acid into caffeic acid which later suffers degradation, with di- and mono-hydroxylated phenylpropionic acids the main metabolites detected [23,42]. Other metabolites, such as *m*-coumaric and hippuric acids, were detected in the urine of rats after the administration of chlorogenic acid [43]. As an

intermediate product of chlorogenic acid, caffeic acid degradation was expected to produce a similar metabolic profile [43] (**Figure 3D**). Dihydroferulic acid (3-(3-methoxy-4-hydroxyphenyl)propionic acid) [27,39] and 4-vinylguaiacol [44] together with minor degradation compounds have been described after *in vitro* fermentation of ferulic acid.

On the other hand, hydroxybenzoic acids are made up of gallic acid and ellagic acid. Gallic acid is also part of the hydrolysable tannins' and flavan-3-ols' molecular structure. Its microbial transformation is proposed in **Figure 2B**, whereas the colonic fate of ellagic acid is explained in the following section.

Ellagitannins are the main group of hydrolysable tannins. Particularly high concentrations of ellagitannins are found in muscardine grapes, pomegranates, and some berries and nuts. Intestinal breakdown of ellagitannins into ellagic acid was observed *in vitro* [2,37,45] and *in vivo* [46]. Ellagic acid is further metabolized by local bacteria, giving pentahydroxy-urolithins as the first metabolite which is successively dehydroxylated to tetra-, tri-, di-, and mono-hydroxy-urolithins, [2,37,45–47], as proposed in **Figure 6A**. An important person-to-person variability in the profile and amounts of urolithins has been observed with major urolithin A (dihydroxy-urolithin) or urolithin B (hydroxy-urolithin) producers, and others are incapable of producing any class of urolithins [2,3,37,45,47]. *Gordonibacter urolithinfaciens* and *Gordonibacter pamelaeae* were described as responsible for the transformation of ellagic acid into penta-, tetra-, and trihydroxyurolithin [48] and its absence could be associated with the inability of some individuals to produce urolithins

Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) is the most common stilbene, present in grapes and wine, and it has been extensively studied. Regarding the *in vitro* experiments, dihydroresveratrol, 3,4′-dihydoxy-*tran*s-stilbene, and lunularin (3,4′-dihydroxy-bibenzyl) were described as microbial derivatives [49,50] (**Figure 6B**). Bode *et al.* [49] concluded that three colonic pathways of resveratrol can be distinguished in terms of the quantity of end products as a lunularin producer, dihydroresveratrol/lunularin producer, or dihydroresveratrol producer. Whereas dihydroresveratrol and lunularin were considered the main end products, the low amounts of 3,4′-dihydroxy-*trans*-stilbene detected seem to indicate that this product is intermediate or marginally produced by gut microbiota. The urine metabolic profile after an acute dose of *trans*-resveratrol confirmed the results found under *in vitro* fermentation [49,50].

Syringaresinol, pinoresinol, lariciresinol, secoisolariciresinol, and matairesinol are the most common lignans found in plant products. Besides being present in foods, secoisolariciresinol is a microbial degradation product of syringaresinol and also of lariciresinol, which, in turn, is derived from the microbial fermentation of pinoresinol [51,52] (**Figure 6C**). The final microbial products of secoisolariciresinol, with several intermediates, are enterodiol and its oxidized product enterolactone [53,54]. Secoisolariciresinol can also be converted to matairesinol, from which only enterolactone is obtained from microbial catabolism [52].

The phenolic alcohols, tyrosol, hydroxytyrosol and its precursors, oleuropein, and hydroxytyrosol acetate are the most representative compounds typically found in olives and virgin olive oil. Few studies have focused on the colonic pathway of these phenolic compounds. Oleuropein was transformed *in vitro* into its aglycone, elenolic acid, hydroxytyrosol, and hydroxytyrosol acetate [55,56] (**Figure 7**). Individual fermentation of tyrosol and hydroxytyrosol confirmed the low microbial metabolism of these compounds and their relative stability under *in vitro* colonic conditions [55]. An *in vivo* experiment following the administration of oleuropein to rats detected other related metabolites such as homovanillic acid [57].

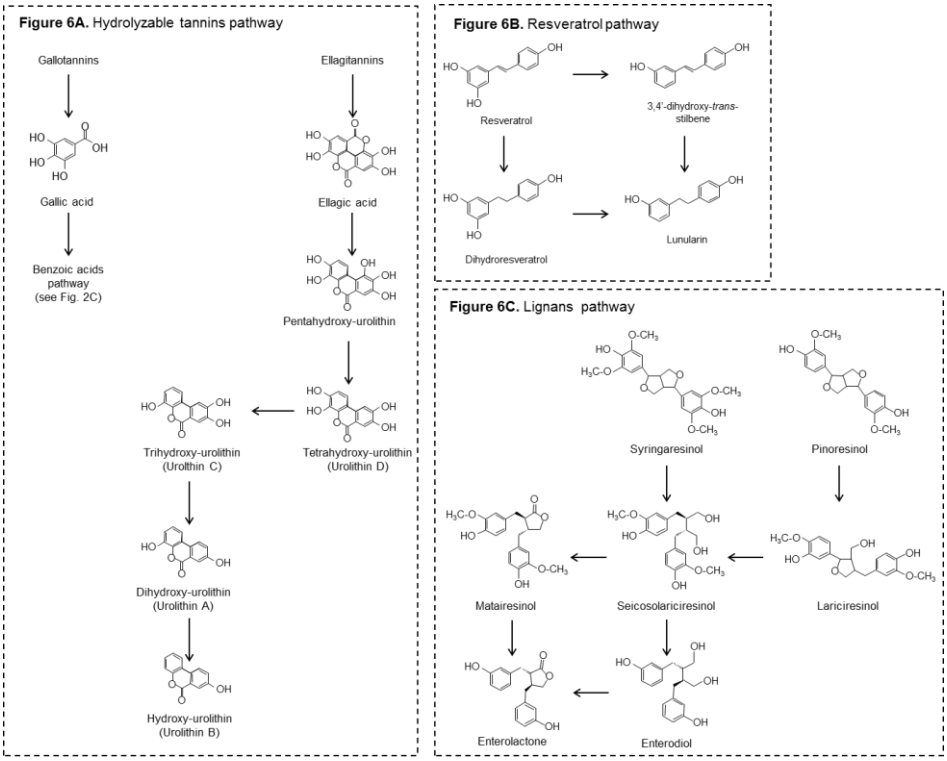



**Figure 6.** Proposed colonic pathways of hydrolysable tannins, resveratrol, and lignans.

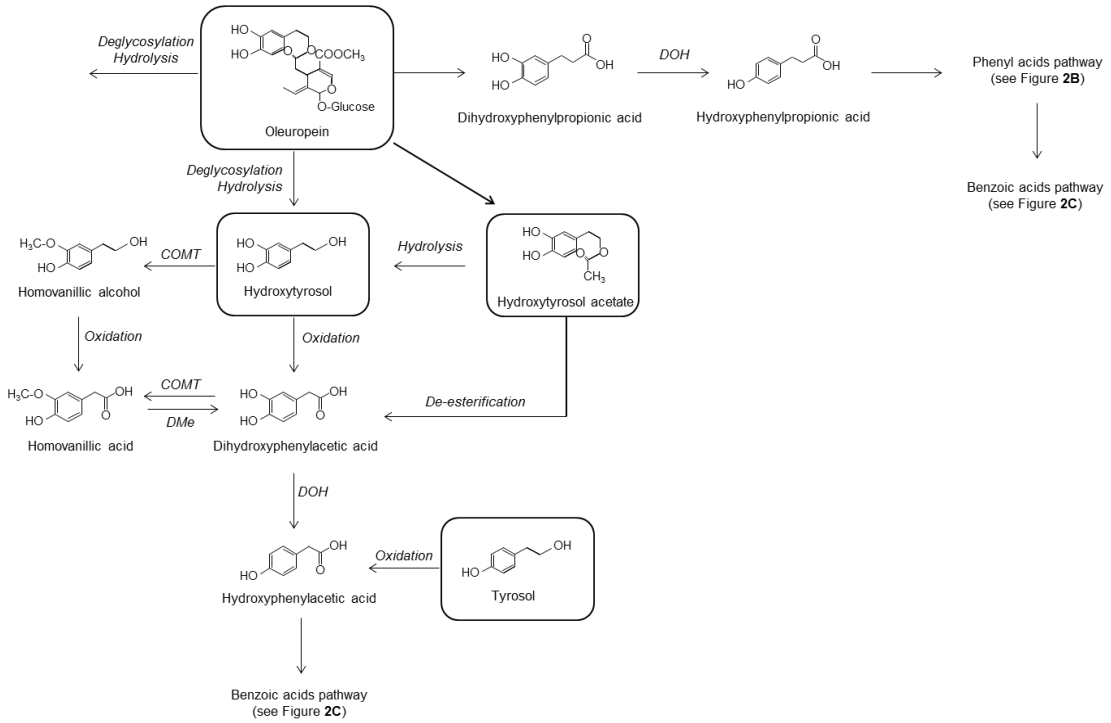
Considering the complex colonic pathways, it is possible to conclude that the first microbial transformation of phenolic compounds leads to the accumulation of initial metabolites that share characteristics from the original compound (particularly conserving their functional groups) and are considered markers of an early fermentation stage. The continuous exposure of the initial metabolites to microbiota leads to the accumulation of final catabolites, which can be common to different phenolic sub-groups. An elevated accumulation of some microbial metabolites would be indicative of the main microbial pathways probably common to several individuals. On the contrary, thos etabolites present in minor concentrations or in a smaller proportion of individuals could be related to a specific microbiota composition capable of activating secondary metabolic pathways

Different profiles of metabolites observed after *in vivo* interventions suggest hepatic and renal metabolism prior to excretion via urine. Hippuric acid was the common phenolic metabolite detected in the largest amounts in plasma and urine after the intake of varied phenolic sources. The origin of hippuric acid can be through the microbial transformation of the quinic acid moiety or the hepatic metabolism of benzoic acids [58,59]. Nevertheless, the concentration of hippuric acid in urine decreased considerably after the antibiotic treatment of rats [60] and in the urine of ileostomy [11], suggesting an important contribution by the gut microbiota.

*In vitro* and *in vivo* studies have contributed enough so far to confirm the capacity of gut microbiota to metabolize phenolic compounds (**Box 1**). The degradation pathway routes involve several reactions in which the side chain groups and the heterocyclic C-ring of flavonoids are more likely to be used by microorganisms, while the benzoic ring remains intact. Depletion of phenolic compounds along with the increase in bacteria growth observed in *in vitro* fermentations could be considered good evidence to

believe that these phytochemicals are used as carbon and energy sources. However, there are other reasons that can explain the utilization of phenolic compounds by gut microbiota. As xenobiotics, they may be degraded to reduce the toxicity that these compounds may have on resident bacteria

**Figure 7.** Phenolic alcohols pathway




*DOH*: dehydroxylaton
*COMT*: methylation
DMe: demethylation

**Figure 7.** Proposed colonic pathways of oleuropein.

## 3. Impact of Diet Phenolic Compounds on Gut Microbial Fermentation Metabolites

### *3.1. Short Chain Fatty Acids (SCFAs)*

SCFAs are saturated aliphatic organic acids consisting of one to six carbons, of which acetic (C2), propionic (C3), and butyric (C4) acids are the most abundant. SCFAs production mainly occurs in the proximal part of the colon, where the availability of substrates is most abundant. The majority of SCFAs (up to 95%) are rapidly absorbed by the colonocytes, leading to decreasing concentrations from the proximal to distal colon. Only a minor fraction of the SCFAs (about 5%) is excreted in the feces [63]. Isobutyric, isovaleric, and valeric acids, also called branched chain fatty acids (BCFAs), constitute the remaining 5% and are derived from the microbial fermentation of branched chain amino acids [64,65]. A disruption in the concentration of SCFAs, especially due to an increase in the proportion of BCFAs, could be a possible signal of loss of intestinal homeostasis [66,67].

***Box 1.*** *Models Concerning the Study of Phenolic Microbial Metabolites*

*In vitro* and *in vivo* studies have been essential for building the colonic pathways of phenolic compounds. Basically, *in vitro* studies consist of anaerobic incubations where phenolic compounds represent the microbial substrate and bacteria cultures, human or animal feces, the microbial inoculum. The phenolic substrate used in these studies may include pure standards [11,19,20,26,30,38,49,55], isolated compounds [36,38,45], phenolic extracts [12,15,38,39,45], and food or pre-digested food [2,37,42,57]. *In vitro* fermentation models have some questionable points, mainly their limited representativeness of *in vivo* conditions. For example, *in vitro* incubations do not include enterohepatic recirculation, colonocyte absorption, mucosa-associated microbiota, and changes in physiological conditions during the transit time. The latter point has been partly overcome with the development of modern controlled batch cultures called "Simulator of Human Intestinal Microbial Ecosystem" (SHIME). The sample circulates through different reactors that represent the ascending, transversal, and descending colon in which pH and temperature are continuously controlled and the growth of microbiota is in line with the environmental conditions and substrate availability [12,17,61]. Despite the mentioned limitations, *in vitro* studies provide valuable information regarding the phenolic metabolites generated by gut microbiota. Fermentations are usually performed for 24–48 h during which sub-samples are collected at different time-points. Time-course metabolite generation allows colon metabolic pathways that probably take place under *in vivo* conditions to be defined. As microbial metabolites can be absorbed by colonocytes, their *in vitro* identification provides a useful tool for identifying these phenolic metabolites in plasma and/or urine in the human bioavailability or dietary intervention studies.

*In vivo* studies are ideal complements to the *in vitro* ones. Nevertheless, complex ethical issues, concerning the study of the phenomena occurring during human digestion, limit *in situ* observations and sample collection. In general, phenolic compounds are usually analyzed in fractionated blood (plasma, serum…) and urine to determine which compounds are absorbed and their absorption and excretion kinetics. In general, phenolic compounds are quickly absorbed, reaching the Cmax in plasma between 0.5 and four hours after the intake, corresponding to the stomach and/or small intestine absorption [16,18,37,62], whereas the colonic metabolites appear in plasma later, indicating their gradual colonic biotransformation and, in some cases, showing a second increase in the plasma concentration after 4 h [16,18]

Acetic acid, propionic acid, and particularly butyric acid have been extensively studied due to their involvement in the maintenance of correct body functions [68,69]. Butyric acid is considered essential for maintaining the colon cellular function since it is the main energy source for colonocytes [70]. Chemopreventive properties have also been described for butyric acid due to its capacity to prevent the formation of malignant cells and to induce apoptosis in colonic cancer cells [71]. In fact, lower amounts of SCFAs [72] and butyric acid-producing bacteria [73] were found in the feces of colorectal patients than in those of healthy subjects. Contrarily, elevated amounts of BCFAs are presumed to be prejudicial to colonic health [74,75] and high concentrations have been observed in inflammatory bowel diseases (IBDs) [66] and obesity [67].

*In vitro* fermentation of pure phenolic compounds enables the extent to which phenolic compounds and gut microbiota are exclusively involved in SCFAs production to be seen. An improvement in the total production of SCFAs was observed after the *in vitro* incubation of chlorogenic acid, rutin, caffeic acid, quercetin [76], and olive oil polyphenols [77]. To the contrary, isolated proanthocyanidins [78] and punicalagins [45] suppressed the *in vitro* generation of SCFAs. The increments observed leave interpretations open to the thought that phenolic compounds could be transformed into SCFAs. However, this alternative is practically discarded since the ring cleavage of the aromatic ring of phenolic compounds was not observed under anaerobic conditions [79]. Rather than direct production, the increase in SCFAs observed after *in vitro* fermentation of pure phenolic standards is probably related to a higher fermentation rate of the released glycoside moieties and/or of the remaining carbohydrates in the culture media or the fece

Phenolic-rich extracts have increased in interest since they can provide added value as food ingredients and can also be used in nutraceutical applications. Their involvement in SCFAs generation has shown mixed results. In two experiments, black tea extract, red wine-grape juice extract [17], and soy germ

powder [61] were studied in a SHIME *in vitro* system. The tea extract and soy germ powder increased the concentration of acetic and propionic acids, whereas the soy germ and the mix extract only increased the concentration of propionic acid. In parallel, a slight decrease in the amount of butyric acid was observed after continuous feeding of black tea and red wine-grape juice extracts. A rise in the production of the three main SCFAs was observed after incubation of pomegranate extract [45]. Higher recoveries of urolithins in the batch cultures of pomegranate extract compared with that of punicalagins suggest major microbial activity and a possible involvement of phenolic microbial metabolites. Nevertheless, rats fed with raspberry seed extract produced more SCFAs and minor urolithins than rats that received strawberry seed extract [80]. Thus, the results of different studies are contradictory and phenolic compounds with similar chemical characteristics showed different behavior.

Because the later data was obtained under *in vitro* conditions, it risky to extrapolate the same effects to *in vivo* conditions when extracts or phenolic compounds are ingested through the diet. Heterogeneous data have also been obtained from animal and human studies after exposure to a phenolic-rich diet. The concentration of acetic acid increased in the feces of pigs after two weeks of receiving a diet enriched with 0.2% of green tea phenolic compounds [81]. In healthy humans, the inclusion of a red wine-grape juice extract, but not grape juice extract, over four weeks, reduced the concentration of isobutyric acid [82]. Furthermore, a decrease in the production of SCFAs in rats was noticed after the administration of an aqueous passion fruit leaf extract [83]. No changes in the production of SCFAs were observed after the inclusion of trans-resveratrol or quercetin (alone or in combination) in rats fed with a high-fat sucrose diet for six weeks [84] or in healthy humans after the intake of pomegranate juice for four weeks [85].

The role of non-digestible carbohydrates (dietary fiber) in the production of SCFAs is unquestionable. Based on its chemical and physical properties, dietary fiber is normally classified into soluble and insoluble. Pectin, oligosaccharides (fructooligosaccharides, FOS), and inulin are considered soluble fiber. The fermentability of soluble fiber is high due to its capacity for water solubilization, which allows the action of microbial enzymes. Insoluble fiber corresponds basically to cellulose, some hemicelluloses, lignin, and arabinoxylans, which are also fermented, but at a much lower rate than soluble fiber [86]. Many authors have postulated that the production of SCFAs may be enhanced when dietary fiber is administered in combination with phenolic compounds. However, this claim remains inconclusive.

Replacement of part of the cellulose (maize starch) by strawberry and raspberry defatted seed extracts rich in ellagitannins enhanced the total SCFAs production and the proportion of butyric acid in rats after four weeks [80]. However, the results of the latter study were not only attributed to the phenolic compounds since their effectiveness was more marked in the raspberry extracts, coinciding with a major content of soluble fiber. In a similar experimental design, equivalent concentrations of dietary fiber were included in the diets of rats through soluble and insoluble fractions of bilberries, blackcurrants, and raspberries [87]. Rats fed with the soluble fraction of bilberries showed the highest caecal formation of SCFAs and fiber fermentability in coincidence with higher anthocyanin intake.

Moreover, the addition of apple-pomace extract, in combination with FOS and cellulose, for four weeks [88], or lyophilized apple together with apple pectin [89], increased the proportion of butyric acid in rats compared with when fiber was consumed alone, suggesting an additional benefit that could be attributed to the presence of phenolic compounds in the extract.

In some cases, the combination of fiber with phenolic compounds seems not to alter the gut metabolism of SCFAs. For example, apple fiber rich in phenolic compounds did not produce any change in the generation of SCFAs, either in combination with apple pectin under *in vitro* conditions [71] or together with boysenberry juice after four weeks of human consumption [90]. In line with the previous studies, rats fed with apple-pomace extract rich in proanthocyanins showed no difference in the production of SCFAs compared with rats fed with apple-pomace extract deprived of phenolic compounds [91], and similar

results were observed after combination of grape extract and inulin in the diet of rats [92]. Inhibitory effects in the production of SCFAs were observed when isolated apple polysaccharides were fermented *in vitro* together with purified apple phenolic extract rich in proanthocyanins [79]. Similarly, the suppression of SCFAs was also noticed in rats after four weeks of a combination of wheat and oat fiber with blackcurrant and chokeberry extracts (both rich in proanthocyanidins) [93] and low or high ellagitannin-rich strawberry extract in combination with FOS [94]. The conversion of SCFAs from starch was also suppressed by the addition of grape-seed extract to pig fecal incubations [95].

Since many factors such as the abundance and activity of specialized carbohydrate-fermented bacteria and enzyme expression and activity, as well as substrate availability, are involved in SCFAs synthesis, phenolic compounds could interact with one or more of these factors. Depending on the type and amount of the phenolic compounds ingested, they reach the colon in different proportions and degrees of polymerization [4,62]. In accordance with this, they could promote or inhibit the growth of carbohydrate-fermented bacteria [76,96]. Bacterial enzymatic expression and activity is crucial for the release of monomeric compounds from polysaccharides to form SCFAs. In addition, different works suggest that polyphenols might modulate the activity of glyosidic enzymes differently [80,92,93].

In plant foods, phenolic compounds are naturally associated with dietary fiber. Thus, the physiological effects of fiber may depend not only on its chemical and physical properties, but also on its particular linkage and the composition of its phenolic compounds [44,97]. The effects observed to be hampered, particularly by proanthocyanins, may be associated with tight bonds among fiber and phenolic compounds which result in a delay in degradation and, thus, less fermentation. Nevertheless, Snelders *et al.* [44] observed that fiber fermentability was more related to the amounts of phenolic compounds present in the fiber matrix than to their form (free or bonded to fiber). Thus, performing studies to define the intrinsic composition of dietary phenolics and food fiber could be of great interest for understanding the role of both food components when these are alone or together.

However, it is still questionable whether it is really desirable to stimulate the production of SCFAs. As SCFAs also represent energy extraction of non-absorbed macronutrients, they can provide approximately 10% of the total energy intake [98]. This aspect is considered positive in populations with a diet rich in plant-derived polysaccharide food. Nevertheless, this may be detrimental in Western populations, because a greater capacity to harvest energy from the diet could contribute to the positive calorie intake balance typical of obesity [99]. In fact, the gut microbiota of obese individuals are especially rich in bacteria specialized in carbohydrate fermentation [99,100]. Hindering of SCFA production observed for some classes of phenolic compounds may be effective in reducing the energy harvest in overweight and obesity, which might partially explain the weight loss observed after the intake of food rich in phenolic compounds [101]. In synthesis, the versatility of phenolic compounds and their association with fiber, free or bound, could stimulate the design of different food products or recommendations depending on the effects expected to be attained.

### *3.2. Bile Acids and Sterols*

Bile acids (BAs) are classified into primary and secondary. In humans, the primary BAs cholic and chenodeoxycholic acids are synthesized from cholesterol by hepatic enzymes and stored in the gallbladder (conjugated with the amino acids glycine and taurine) until being excreted into the duodenum to facilitate the digestion and absorption of lipophilic compounds. Most of the conjugated primary BAs are reabsorbed in the ileum and the remaining fraction reaches the colon, where cholic and chenodeoxycholic acids are metabolized into deoxycholic and lithocholic acids, respectively, by gut microbiota [102]. The main microbial catabolism steps include the deconjugation of amino acids and dihydroxylation by microbial 7-α-dehydroxylase [102,103].

Fecal cholesterol represents the non-absorbed dietary cholesterol in addition to that provided by intestinal desquamated cells. The microbial reduction of cholesterol releases coprostanol as the main metabolite, generating cholestanone and coprostanone as intermediates [104]. High excretion of sterols and their microbial metabolites through feces has been associated with hipocholesterolemic effects [105], but is also a common feature of high fat diets [106]. A continuous exposure to elevated luminal levels of secondary BAs and cholesterol microbial metabolites has been suggested to increase the susceptibility to intestinal inflammation and colorectal cancer [74,107–109]. In addition to the toxic effects of secondary sterols *per se*, the concentration of putrefactive compounds (see Section 3.3 below) could also increase the lumen toxicity due to the release of amino acids (glycine and taurine), these being products of the microbial hydrolysis of BAs.

There is scarce data regarding the impact of phenolic compounds on the gut microbial transformation of BAs and cholesterol. Different studies have demonstrated that phenolic compounds have suppressing effects on the microbial conversion of sterols. For example, the amount of secondary BAs was reduced in rats fed with a diet containing tea polyphenols and gallotannins [110] or apple, grape, and red beet juices [111]. In another study with rats, the supplementation of a high fat diet with curcumin and caffeic acid reduced the concentration of deoxycholic acid in feces, whereas the administration of caffeic acid, catechin, rutin, and ellagic acid reduced the amount of lithocholic acid [112]. However, there was no evidence of lower conversion of primary to secondary BAs when red wine tannins were added to rat diets [113], or when healthy humans received pomegranate juice over a period of four weeks [85]. Regarding sterols, it seems that dietary phenolic compounds could also influence the conversion of cholesterol into their respective microbial derivatives, especially coprostanol. Compared with a control diet, rats that received tea polyphenols and gallotannins increased their excretion of fecal cholesterol in detriment to the excretion of coprostanol [110]. Similarly, pomegranate juice intake reduces the conversion rate of cholesterol to coprostanol in healthy adults [85]. On the other hand, a large proportion of coprostanol was observed in rats after the intake of apple, grape, and red beet juices [111]. These results suggest that the conversion of primary BAs to secondary BAs and cholesterol to coprostanol is hampered by phenolic-rich diets, but more data regarding the role of phenolic compounds on colonic sterol gut conversion in humans is needed.

The negative implications of the secondary metabolites of sterols in the colon may be mitigated by phenolic luminal compounds. The inclusion of chlorogenic acid in the diet of mice reduced the deoxycholic acid's tumor-promoting effects [109]. In this line, protection against the cytotoxicity effects of deoxycholic acid in Caco-2 cells was observed when cells were incubated with proanthocyanidins [114]. In another *in vitro* study in which deoxycholic acid was incubated with HCT-116 cells, antigenotoxic and aticytotoxic activity was observed for quercetin, resveratrol, and rottlerin [115]. In the same study, genistein, curcumin, and epicallocatechin gallate showed no effect.

High fat diets have also been associated with the increase of the activity of β-glucuronidase [106]. Many toxic metabolites are neutralized by glucuronide conjugation and it is the form that promotes their excretion from the body. Thus, deglucuronidation not only promotes the retention of toxic metabolites in the body but also increases gut environment toxicity. It has been also proposed that phenolic compounds could interfere with the activity of β-glucuronidase, but the results are inconsistent. A decrease in the activity of β-glucuronidase has been observed in rats after diet supplementation with grape extract [92] and ellagitannins in combination with cellulose [94]. On the contrary, higher activity of β-glucuronidase was noticed in rats after the addition of blackcurrant polyphenols to the diet [93].

To date, the effects of phenolic compounds on the microbial transformation of colonic sterols have not been explored in depth and there is no information about the role of phenolic microbial metabolites. Regarding the changes in fecal sterol composition through the effect of phenolic-rich products, the data available in the literature is too scarce to enable solid conclusions to be drawn. Sterols are a non-invasive

and easy parameter to be analyzed in fecal samples with a relatively high sensitivity to change when the characteristics of the diet also change [106]. This converts them into good candidates for reflecting alterations in the gut lumen after dietary interventions, particularly those containing high fat.

### *3.3. Products of Non-Absorbed Protein Metabolism*

Every day, variable amounts of nitrogenous compounds of dietary and endogenous origins reach the colon. Protein-rich diets, particularly those containing red meat, increase gut toxicity as a consequence of an excessive production of microbial products derived from protein fermentation [64,65,116,117]. These substances, also called putrefactive compounds, include ammonia, fecal phenolic (phenol and *p*-cresol) and indolic compounds (indol and skatole), sulphur compounds (hydrogen sulphide, methyl mercaptan, and dimethyl sulphide), branched chain fatty acids, and polyamides (putrescine, agmatine, cadaverine, tyramine, and histamine). Intestinal ammonia is generated through the microbial deamination of urea and amino acids [65,118]. Phenolic and indolic compounds are mainly derived from the microbial catabolism of aromatic amino acids (phenylalanine, tyrosine, and tryptophan) [65]. Sulphate-reducing bacteria produce sulphur metabolites as a product of anaerobic respiration during the catabolism of cysteine and methionine [119]. Polyamides, besides being present in food, are also released in the gut by the bacterial metabolism [120].

Elevated undigested luminal proteins could alter the intestinal ecology, stimulating the activity of nitrogen-degrading bacteria, which are considered to have detrimental effects in gut homeostasis [121,122]. In parallel, microbial enzymatic expression and activity increase proportionally with the amount of substrate [122], thus promoting the accumulation of putrefactive compounds which are associated with gut integrity and function loss due to their potential cytotoxicity, genotoxicity, and carcinogenic activity, increasing the risk of inflammatory bowel disease (IBD) and the development of colorectal cancer [66,74,116,123].

The modification of dietary patterns, such as including dietary carbohydrates [89,116,117], and restricting energy intake [124], has shown an inverse relationship with the concentration of these putrefactive fecal compounds. However, there is insufficient information regarding the effects of phenolic compounds in this aspect. Among the limited number of studies focused on researching the relation between protein fermentation and phenolic compounds, the most numerous are those on polymeric and monomeric flavan-3-ols. In the case of animal studies, a tea phenol-enriched diet reduced the pH and concentration of ammonia, phenol, *p*-cresol, and skatole in pig feces after two weeks of treatment [81]. Satisfactory results were also observed in humans after supplementation with grape seed extract [125] and flavan-3-ols [126]. Both products reduced fecal pH and hampered the production of sulphur compounds. A trend toward a decrease in phenol, *p*-cresol, 4-ethylphenol, indole, and skatole in feces was observed after consumption of grape seed extract, but these compounds were not studied after flavan-3-ol supplementation. In the case of ammonia, its fecal amounts were significantly reduced in the flavan-3-ol group and tended to decrease after grape seed extract intake. More consistent results, observed after the intervention with flavan-3-ols, may be related to the duration of the study, this being two and six weeks for grape seed extract and flavan-3-ols treatments, respectively. On the contrary, no changes in the production of ammonia and sulphides were noticed after continuous feeding of soygerm powder in a SHIME system [61].

A reduction of the pH of the feces observed after phenolic intervention could partly explain the decrease in the concentration of putrefactive substances, since microbial proteases are more active at neutral or slightly alkaline pH [127]. However, this is not a common effect. In some cases, phenolic compounds increase [92] or have no effects [80,83] on lumen pH. In addition, a reduction in fecal putrefactive compounds does not always respond to a reduction in the pH [80,92]. The differences may depend on the predominant class of bacteria and the availability preference of the substrate type. For example, *Escherichia coli* and *Proteus mirabilis* produced ammonia from amino acids even at low pH [118].

Phenolic compounds and their microbial metabolites possibly collaborate with additional factors to reduce the accumulation of protein fermentation products. A reduction of protein conversion, beyond the decrease in pH in the gut lumen, may also be associated with the capacity of phenolic compounds to inhibit proteolytic bacteria and interfere with their enzymatic activity. Gram-negative Proteobacteria (especially *E. coli*), *Bacillus* spp., and Gram-positive Clostridia [118,128] have been implicated in gut proteolysis and their abundance in IBDs is probably associated with the typical morphological and physiological alterations [129,130]. Beside the capacity of phenolic compounds to inhibit the growth of several members of the proteolytic bacteria, it has been observed that phenols also possess the capacity to attenuate the expression of genes involved in the secretion of proteases, inhibiting their synthesis and their activity [125,131–133]. The degree to which the enzymatic activity is blocked depends on the phenolic concentration, its molecular structure, and its functional groups [133].

Abnormal concentrations of proteinases and putrefactive compounds in the lumen alter the permeability of the gut epithelium, promoting the movement of toxic substances across the intestinal barrier, which leads to a tendency to activate inflammatory mediators [129,134]. One of the reported mechanisms by which phenolic compounds improve barrier integrity is by increasing the expression of tight junction proteins [84,134–137]. This may mean that although some phenolic compounds are unable to reduce protein fermentation, they offer an alternative way of protecting colon integrity, enhancing the host tolerance to susceptible molecules present in the internal environment.

Large intestine digestion is a dynamic system in which entrapped compounds in the digesta pass through the ascending, transversal, and descending colon before being excreted by feces. Local microbiota is active throughout the lumen, metabolizing non-absorbed products in the function of substrate preference and availability, and is also conditioned by the physiological conditions of the gut (pH, redox potential, transit time) [138]. The pattern of the native phenolic compounds present in food change between the colonic segments due to the microbial fermentation, resulting in the accumulation of different metabolites with different activity than their precursors. As major proteolytic activity is found in the terminal colon [64,123], it would be interesting to study the interaction of microbial proteolysis-phenolic metabolites in this part of the gut. In addition, we have found only one paper related to the study of the modification of fecal nitrogen compounds after the addition of phenolic-rich products in a high red meat diet in humans [139]. However, to have a better understanding of the role of dietary phenolic compounds in protein fermentation at gut level, more human and animal studies are needed.

## 4. Impact of Phenolic Compounds on Intestinal Function

### *4.1. Intestinal Redox Homeostasis*

Oxidative stress is defined as the damage promoted by an imbalance between pro-oxidant agents (free radicals) and endogenous (superoxide dismutases, SOD, catalase glutathione peroxidase, GPx, and glutathione, GSH) and exogenous mechanisms to neutralize their effects. Free radicals are highly reactive molecules capable of reacting with the biological components of cellular structures, such as the structural lipids of cell membranes, proteins, and nucleic acids (DNA damage), compromising good cell function and integrity. Moreover, the alteration of cellular components caused by oxidative stress in many cases precedes the over-expression of pro-inflammatory agents that are responsible for the activation of mechanisms that trigger cell damage [140]. Repeated bouts of inflammation can lead to the occurrence of IBDs, such as ulcerative colitis and Crohn's disease, which are, in turn, risk factors of colon cancer [141].

Pro-oxidant substances are products of normal aerobic metabolism, but their levels increase as a consequence of toxic exposure (smoke, excessive alcohol intake, irradiation, some drugs, food components, toxins) and other particular situations (age, infection, stress). The gastrointestinal tract produces, receives, and retains these types of substances, which implies an elevated risk of alterations in cells and tissue in situations of over-production of free radicals and/or depletion of antioxidant reparative

mechanisms [142]. Dietary phenolic compounds could act as an antioxidant system in the gastrointestinal lumen and epithelium, reducing the oxidative damage in different ways: safeguarding the activity of endogenous antioxidants, decreasing free radical production, or neutralizing the latter by radical scavenging [6,7,9,140,143–147].

*In vitro* studies using cell-based methods are considered a good tool for deciphering the mechanisms by which phenolic compounds attenuate oxidative responses. Treatment of intestinal Caco-2 cells with different fractions of cranberry and apple peel phenolic extracts [140,143], as well as red wine extracts [144], effectively mitigated the membrane structure disruption caused by the pro-oxidants Fe/ascorbate and tertra-butyl hydroperoxide, respectively. Direct scavenging activity is the proposal mechanism by which urolithins suppress the generation of free radicals from activated neutrophils [145]. On some occasions, foods also collaborate with the pro-oxidant potential of the gut environment. For example, an overproduction of free radicals and a reduction of GSH activity were evident in Caco-2 cells when they were incubated with acrylamide, but the latter effects were reduced by cocoa phenolic extract, procyanidin dimer B2, and epicatechin [146]. In a similar way, protection against oxidative damage induced by the micotoxin deoxynivaleol in HT-29 cells was also observed when the culture media was enriched with epigallocatechin gallate [147].

The main entrance route for phenolic compounds is the gastrointestinal tract, where they reach a greater lumen concentration and remain longer compared with those observed in blood circulation [22,62]. Also, the extensive phase II metabolism (glucuronidation, sulphatation, and methylation) that phenolic compounds undergo during absorption and body distribution created doubts concerning their effectiveness as antioxidant agents at the systemic level [13,145]. In the case of colon lumen, the non-absorbed phenolic compounds and their microbial metabolites prevail in native-unconjugated forms [148]. During digestion, the antioxidant capacity of the luminal content can indeed increase compared with the original food, probably due to the release of phenolic compounds from the food matrix promoted by the physiological conditions and action of digestive enzymes [149]. In the large intestine, microorganisms have the ability to release those phenolic compounds intimately entrapped in the food matrix (particularly dietary fiber), which also contribute to the total antioxidant capacity at the gut lumen level [150,151]. Moreover, the phenolic compounds released from the digestive bolus can be distributed into intestinal fluids and favor direct contact with the colonic epithelium, mitigating the detrimental effects of free radicals at the cell level.

The antioxidant status in *in vivo* studies (animals or humans) could be estimated by analyzing the antioxidant capacity of the intestinal content or fecal water by different methods, such as the oxygen radical absorbing capacity (ORAC), the ferric reducing antioxidant power (FRAP), the diphenyl-1-picrylhydrazyl (DPPH), the free radical scavenger (TBARS), and the 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonate) radical cation (ABTS) [6,13,14,19,20,149–151]. The total antioxidant capacity of the intestinal content increased after the ingestion of grape seed extract in rats [151] and limited the production of free radicals in human fecal water after polyphenol-rich chocolate intake [152]. In contrast, some studies have noted no changes in the antioxidant capacity in colon tissue after the intake of phenolic-rich products [10]. The difference in the bioactivity between microbial phenolic metabolites and their parent compounds together with the progressive decrease of the lumen phenolic concentration due to the active colonic absorption could be an explanation for the unobserved antioxidant effects. However, it should be stressed that lack of effect in the total antioxidant capacity does not necessarily encompass the loss of other biological properties [10] (see next Section 4.2.).

### *4.2. Intestinal Inflammation*

Inflammation is a biological phenomenon associated with the innate body immune response in the first line of defense to combat threats which could compromise human health. Episodes of controlled inflammation

are indispensable for suppressing chemical, biological, and physical injuries. However, in some cases, due to diverse genetic and environmental causes, uncontrolled inflammation responses predispose individuals to the development of chronic diseases. In the case of the gastrointestinal tract, IBDs are characterized by an unknown etymology of recurrent episodes of inflammation causing a progressive loss of cellular function and tissue degeneration that could develop into colon cancer [141,153].

The chronification of inflammation involves complex and interconnected mechanisms of molecular mediators, whose continued feedback promotes the perpetuation of inflammation. The over-production of free radicals (reactive oxygen and nitrogen species) and cytokines (TNF-α, IL-6, IL-8, PGE2, among the most studied), the up-regulation of nuclear factor kappa-B (Nf-κB) and monophosphate-activated protein kinase (MAPK), and the recruitment, activation, and adhesion of leukocytes have been identified as occurring in IBDs [7,8]. Phenolic compounds seem to inhibit or attenuate the intensity of the inflammatory response by modulating the cellular inflammatory mediators and/or neutralizing free radicals (see Section 4.1 above).

*In vitro* cultures of intestinal cells are particularly valuable models for identifying the target molecules involved in chronic inflammation which may be susceptible to regulation by phenolic compounds. The results obtained from these studies could be interesting for the design of new anti-inflammatory drugs capable of avoiding the adverse side effects observed for some drugs used to treat IBDs [154]. The possible mechanisms by which phenolic compounds could mitigate or suppress inflammatory responses *in vivo* can be predicted by cells activated by different types of inflammation promoters. The down-regulation of NF-κB is considered a therapeutic target in IBDs, since it prevents the activation of pro-inflammatory mediators, such as COX-2 and iNOS, which, in turn, are responsible for the production of cytokines and free radicals. Individual compounds, such as epigallocatechin gallate [147] and anthocyanins [155], and also phenolic mixtures obtained from apple peels [143], cocoa [8], cranberries [140], and red wine [156], have shown an *in vitro* ability to inhibit the activation of NF-κB pathways. In the latter studies, the inhibition of NF-κB activity was also associated with the down-expression of COX-2 [8,143,147,156] and iNOS [8,140,156]. Resveratrol [157] and cocoa phenolic compounds [146] were also effective as regulators of other signaling pathways such as JAK-STAT and MAPK, respectively. On the contrary, chlorogenic-rich fractions obtained from lyophilized blueberries did not show clear evidence as inhibitor agent of NF-κB [155].

Although less common compared with the data available regarding the anti-inflammatory potential of phenolic compounds, it seems that their microbial metabolites also possess similar properties. The down-regulation of COX-2 in human adenoma cells was observed after the incubation of (3′,4′-dihydroxyphenyl)acetic and 3-(3′,4′-dihydroxyphenyl)propionic acids [158], common phenolic catabolites detected in feces. Reclusion and adhesion of leukocytes play an important role in the development of inflammation since they also secrete pro-inflammatory cytokines. The secretion of cytokines TNF-α and IL-6 was effectively inhibited by urolithins after activation of THP-1 human monocyte cells [159]. Urolithins were also effective as anti-inflammatory mediators in activated neutrophils, inhibiting cytokines and the secretion of the proteinases necessary for adhesion [145]. Fibroblasts are a type of cell that synthesizes the extracellular matrix and collagen, responsible for tissue cicatrization. Their activation, mediated by cytokines, monocytes, and free radicals, also involves the secretion of pro-inflammatory agents. In addition, the exacerbated extracellular production of collagen could lead to intestinal stenosis (bowel obstruction) [160]. Urolithins, especially urolithin A, were also effective at inhibiting the activation, migration, and adhesion of colon fibroblasts with a concomitant reduction of several secretory pro-inflammatory mediators. These effects were even more evident than those shown by their precursor, ellagic acid [161].

Chemically induced IBD in animals is a revolutionary approach to studying the effect of phenolic compounds on inflammation under *in vivo* conditions [153]. The improvement in physical indicators (less

body weight loss) and hematological and histological probes, together with a reduction in the expression of pro-inflammatory mediators in combination with phenolic treatment, constitute evidence of the protection given by phenolic compounds against intestinal epithelium damage and the attenuation of the inflammatory response. Individual phenolic compounds [7,10,134,136,162], and phenolic extracts [7,8,163,164] have shown positive effects in the control of IBDs in animals, even to the same extent as sulphosalizine, a common medicine used in IBDs, but with fewer collateral effects [7]. Contrary to the later results, the administration of resveratrol to rats for six weeks induces the expression of inflammatory parameters [84]. Data from animal studies support the anti-inflammatory effect of phenolic compounds observed in *in vitro* experiments, but the role of phenolic metabolites in this aspect is still unclear, due to the lack of information about the phenolic disposition in tissues and feces in most of the later studies.

Dietary intervention in human studies including IBD patients is basically conducted to observe the remission of clinical symptoms or the diminution in the number or intensity of relapses [165–167]. Despite phenolic compounds have been extensively studied in animal models of IBDs, data regarding human trials is currently limited to one study in which the administration of epigallocatechin gallate to patients with ulcerative colitis over 56 days showed a satisfactory degree of remission of symptoms with minimal side effects [166]. Due to the ethical issues mentioned above, human studies to investigate the *in situ* effects promoted by phenolic compounds are not always possible. Nevertheless, there are promising fecal inflammatory markers that could potentially be used in human studies with minimal invasiveness.

Calprotectin and lactoferrin are binding proteins found in the cytoplasm of neutrophils, monocytes, and macrophages. Fecal calprotectin could be a good tool for the molecular screening of active intestinal inflammation, because its levels are substantially elevated in IBDs and colorectal cancer [167–169]. Furthermore, the amounts of calprotectin in feces have been shown to correlate well with the histological lesions caused by continuous inflammation in IBDs [167,168,170] and the tumor stage in colorectal cancer [169], indeed with better results than other common plasma biomarkers, such as the case of C-reactive protein [167,170]. A reduction in the fecal excretion of calprotectin was observed after surgical intervention [169] and oral probiotic administration [171]. Rats fed with a high fat diet in combination with grape seed extract showed lower levels of fecal calprotectin than the control animals [172].

Although collecting feces is easy and non-invasive, human studies concerning fecal inflammation biomarkers are scarce. To our knowledge, there is only one study involving IBDs patients and phenolic treatment. In this work, anthocyanin-rich bilberries were administrated to ulcerative colitis patients for nine weeks and the results at the end of the study showed a decrease in the fecal levels of calprotectin, which increased again when the bilberry treatment was stopped [173]. These results not only show the palliative effects of some phenolic compounds in IBDs, but also the inclusion of calprotectin as an index of inflammation response after dietary treatment. Exploring the association between phenolic compounds and intestinal inflammation through dietary treatment is a promising future approach to be considered.

**5. Phenolic Compounds and Gut Microbiota Modulation**

The gastrointestinal tract, especially the large intestine, houses the most abundant and complex microbiota in humans. The gut population, composed of approximately $10^{10}$–$10^{12}$ cells per gram of intestinal content, participates in several metabolic functions that the host cannot fulfill by itself [174–176]. These metabolic functions and their derivative end products depend on the quantitative and qualitative features of the gut inhabitants. A harmonious balance in the composition of the gut microbiota has been associated with maintaining health and a higher life expectancy accompanied by a satisfactory quality of life. An imbalance in the microbial population is known as dysbiosis. Dysbiosis has been associated with intestinal and non-intestinal metabolic disorders, which confirms the association between the microbiota and non-digestive functions [176].

Although full characterization of the microbiota in homeostasis and disease still remains to be completed, some trends in its profile could be helpful for discriminating between the two states. A lower diversity of bacteria, change in bacteria functionality, and a decrease in the beneficial inhabitants together with a major abundance of detrimental opportunistic bacteria seem to be involved in metabolic alterations such as obesity and type-2 diabetes, and diseases of the gut such as IBDs and colorectal cancer [9,10,73,100,177,178]. An alteration of the microbial equilibrium undermines bowel functionality, alters host immunity, and increases susceptibility to pathogen colonization.

The mechanisms by which the phenolic compounds modulate the gut microbiota still remain to be deciphered, but may involve direct and indirect interactions. Phenolic compounds could directly stimulate or inhibit bacterial growth. Inhibition is closely related to previously reported antimicrobial properties of phenolic compounds and stimulation presumably associated with the capacity of the bacteria to metabolize them [131,179]. The antimicrobial activity of phytochemicals has been extensively studied because is thought that it is the main cause by which phenolic compounds can modify the characteristics of the gut structure population. On the basis of several works [131], it could be said that phenolic compounds possess a selectively bacteriostatic or bactericide effect, inhibiting the growth of a wide range of potential pathogenic bacteria slightly affecting or even promoting the beneficial microbial population. However, it is important to consider the concentration and characteristics of the molecule (type of phenolic compound and if it is presented in conjugated or free form) because these factors seem to govern the modulation of the desired effects. Indirect implications include complex issues where numerous microbial-host and microbial-microbial interrelationships take place. Phenolic metabolites could affect the growth of other bacterial groups and, in turn, the over-growth of some bacterial groups could condition the development of others [76,179,180].

The prevalence of determinate microbiota members is preferred to others due to the efficacy they have shown in ameliorating the gut ecosystem with positive effects at the local and systemic levels. For this reason, most studies have focused on the effects of polyphenols on *Bifidobacterium* and *Lactobacillus*, but there is increasing interest in other emergent bacteria that could be relevant for the health of the host [9,101]. Under *in vitro* conditions, anthocyanins [38], phenolic compounds bound to the insoluble cocoa fraction [150], pomegranate extract [45], and soygerm powder [61] have stimulated the growth of *Lactobacillus* and *Bifidobacterium*. Pomegranate extract and apple pomace juice were also capable of increasing *Bifidobacterium* levels in rodents [111,163]. The *Lactobacillus* count was increased after the administration of apple and red beet pomace juice instead of water in rats [110]. Grape seed extract rich in proanthocyanins was able to increase the population of *Bifidobacterium* in healthy adults [125]. Although the phenolic compounds contained in a cocoa powder did not prevent the age-induced reduction of *Lactobacillus* and *Bifidobacterium* in rats after six weeks, they reduced the growth of Bacteroidetes, *Staphylococcu*s, and *Clostridium* [181]. The latter prebiotic effects could also have been related to the possible presence of fiber in the phenolic sources. Dietary fiber is known to influence the microbiota profile, especially through the increase in probiotic microorganisms [182]. However, other sources deprived of dietary fiber, as in the case of incubation of individual compounds [76], green tea phenolic compounds in pigs [81], fiber-free juice [101], resveratrol administrated to rats [10], isoflavone supplementation in women [183], and cocoa-isolated phenolic compounds in healthy adults [184], have also shown positive effects to favor the increase in the number of *Bifidobacterium* [10,76,183,184], *Lactobacillus* [10,101,184], and butyrate-producing bacteria in parallel with the modulation of growth of bacteria associated with gut dysbiosis [10,76,184].

Diet is probably the most easily manipulable factor to modulate the composition of the gut microbiota, and the latter results show that phenolic compounds *per se* are presumably able to interact with gut inhabitants and produce changes, most of them toward a healthier profile. However, in a recent study by our group [85], no changes were observed in the microbiota composition of healthy adults after four weeks of

sustained intake of pomegranate juice with a high phenolic content. Therefore, the production of the microbial phenolic metabolites catechol and phenylpropionic acid was correlated with the presence of a higher percentage of some bacterial groups, supporting the existence of phenolic compound-bacteria interrelationships. Despite this fact, the moderate consumption of red wine by healthy volunteers over a period of 20 days increased the number of Firmicutes, Bacteroidetes, *Bifidubacterium*, and *Prevotella* [185]. The *Bifidobacterium* population also increased in the feces of healthy humans after six weeks of sustained intake of blueberry drink [186], basically promoted by the higher amounts of some species [187].

Recent studies have proposed the use of phenolic-rich sources as a therapeutic strategy to prevent and, in some cases, reverse the dysbiosis associated with different pathologies. Obese rodents or animals fed with obesogenic diets are common tools for studying the role of the microbiota in the metabolic syndrome. The metabolic syndrome includes a series of alterations, such as obesity, dyslipidaemia, glycosemia, and loss of insulin sensitivity. Thus, the attenuation of one of the latter factors could be considered as a protective factor of type 2 diabetes and cardiovascular diseases

A high Firmicutes/Bacteroidetes ratio has been considered an index of obesity and its depletion a marker of successful treatment. However, the results in this context are ambiguous. The reduction in the incidence of obesity reflected by lesser weight gain and fat accumulation was observed after treatment with quercetin plus resveratrol [84], coffee [188], fiber-free plum [101] and cranberry extract [9], but no significant differences were observed after pomegranate peel extract intake by obese mice [163]. The majority of the latter phenolic treatments showed a modification of the microbial population with respect to their control counterparts, but it was not always encompassed by a decrease in the Firmicutes/Bacteroidetes ratio, suggesting the involvement of other bacteria or non-microbiota mediated effects. Nevertheless, a positive correlation between body mass index and the Firmicutes/Bacteroidetes ratio is still questionable because this parameter has not always been described in obesity [99,101,189]. The prevention of obesity is a target factor to reduce the incidence of type 2 diabetes, which could be reinforced by the additional effects provided by dietary phenolic compounds. For example, the administration of cranberry extract to mice for nine weeks increased the relative abundance of *Akkermansia* ssp., which has been linked with the enhancement of diabetic protective effects such as an increase in insulin sensitivity and better inflammatory parameters [9].

IBDs have also been associated with a microbial status in which *Enterobacteriacea*, especially *E. coli*, are more abundant than in the control subjects. The characterization of *E. coli* has revealed that the virulent types were more abundant in biopsy specimens in IBDs patients compared with healthy individuals, basically due to the expression of adhesines which facilitate the adherence of the bacteria to the epithelium surface [190]. The administration of resveratrol to IBD-induced rats for 25 days prevented the over-growth of *Enterobacteriaceae*, particularly *E. coli* [10]. Some studies confer the phenolic compounds with the *in vitro* properties of inhibiting the cellular adhesion of harmful bacteria with a minimal repercussion on the beneficial members of the gut microbiota [191].

All seem to indicate that the gut community responds positively to dietary phenolic compounds where the abundance of determinate bacteria could contribute to host health maintenance. After phenolic intervention, microbiota changes may by expressed on different taxonomic levels. Lack of difference at the phylum level does not necessarily indicate a lack of probiotic effects; sometimes the changes are evident at class, family, genus, or species levels [84].

Microbiota profile and, thus, microbiota modulation after diet intervention can be studied by different techniques, such as fluorescent *in situ* hybridization (FISH), quantitative PCR-based methods (qPCR) [76,101], and high-throughput sequencing; these are among the most widely used. The analyses by FISH and qPCR are based on identifying and quantifying specific bacterial groups, defined as oligonucleotide probes. For this, in most of the assays, the selected probes depend on the specific bacteria of interest in

the study and probably some important microbial changes may be omitted if the probe is not considered in the study. Other, more sophisticated techniques are based on the amplification and pyrosequencing of bacterial 16S ribosomal RNA and allow overall population bacterial genoma identification (metagenome) [101,177]. A more in-depth and detailed characterization of the whole microbiome provides information regarding changes not only in the phylum, class, family, and genus but also specific alterations observed at the species level. In summary, the phenolic compounds that may hamper the microbial imbalance in situations of risk of disease or those that may prevent dysbiosis by reinforcing beneficial bacteria could be included in therapeutic strategies to restore or maintain a healthy profile of the gut microbiota. It remains to be elucidated whether dysbiosis is a cause or consequence of several human pathologies associated with the microbiota.

**6. Conclusions and Future Perspectives**

The large intestine is a sophisticated and complex ecosystem where interrelationships among the host, microbiota, and its metabolic products (dietary and endogenous products) play a key role in local and systemic health. The inter-dependence between the latter factors is so tight that the alteration of one of them can govern the behavior of the others, altering the gut homeostasis and, thus, making the appearance of disease more likely.

Dietary phenolic compounds reach the colon in variable amounts, enriching the gut lumen of the related metabolites derived from the local microbiota metabolism. Lumen phenolics (parent compounds and their metabolites) can actively take part in the innumerable interactions that occur in the large intestine. Depending on their nature, diet phenolic compounds apparently enhance the generation of beneficial metabolic products and/or hamper the production and effects of detrimental luminal compounds, protecting and prolonging gut homeostasis. The mechanisms by which phenolic compounds can interact with the gut metabolism may involve interference with enzymatic expression and activity, changes in the characteristics of the gut environment, changes in the modulation of the signaling pathways responsible for pro-oxidant, inflammatory, and carcinogenic effects, enhancement in the protection of the intestinal epithelium from the negative effects of toxic substances, and/or the modulation of the microbial population.

There are still several aspects that require emphasizing. Phenolic compounds undergo active colonic microbial transformations, generating intermediate and final related metabolites that could be present in the digesta in higher concentrations than their precursors, without necessarily sharing the same bioactivity. In this context, studies focused on analyzing the parent compounds in food probably fail to estimate the phenomena occurring in the colon correctly. It would be important to identify whether the health benefits are associated with the parent compounds or their respective microbial derivatives in order to design the correct dietetic or technological strategies to obtain major and better results from phenolic dietary therapies.

Phenolic compounds represent a wide group including several sub-groups that, although they have similar characteristics, are not completely equal. Differences in chemical structure, number of functional groups, and the combination of different moieties probably lead to these molecules having different functions. Despite the information obtained and published over recent years, the effects and mechanisms by which phenolic compounds promote changes in the human gut ecosystem are not yet completely elucidated. A solution could be partly promoted by the combination of metabolomic (characterization of metabolite changes in the gut environment after dietary intervention with phenolic compounds or phenol-rich foods), microbiome (gut bacteria collective genome), and metagenomic (for the identification of down- or over-expressed proteins involved in microbial activities, whose classification thus depends on the function) disciplines to describe a more complete scenario promoted by phenolic compounds.

Given all of the above, the design and performance of more research, especially focused on human trials, is encouraged to confirm the efficacy of phenolic compounds at the gut level. The results provided by previous and future studies could be useful to the design of dietary recommendations not only to suppress or reduce symptoms in disease but also to provide the healthy population with simple tools to promote the maintenance of health.

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