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CICLO XXXVI

***Multiple approaches to investigate the variability
in (poly)phenol bioavailability and metabolism***

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“Sapere aude”
Immanuel Kant

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Abbreviations

ADME	Absorption, distribution, metabolism, and excretion
ABC	ATP-binding cassette
ATP	Adenosine triphosphate
AUC	Area under the curve
BAs	Benzoic acids
BALs	Benzaldehydes
BCRP	Breast cancer resistance protein
BMI	Body mass index
BZs	Benzene derivatives
C-	Carbon atom
CAs	Cinnamic acids
CBG	Cytosolic β -glucosidase
C_{max}	Maximum concentration
COMT	Catechol- <i>O</i> -methyltransferase
COST-POSITIVE	European Cooperation in Science and Technology- Plant food bioactives interindividual variation
CMD	Cardiometabolic diseases
CVD	Cardiovascular diseases
CV%	Coefficient of variation
CYP	Cytochrome P450
DiH-	Dihydroxylated form on the B-ring
DPPOLs	1,3-Diphenylpropan-2-ols
ERC	European Research Council
ESI	Electronic supplementary information
GLUT	Glucose transporter
GWAS	Genome-wide association study
H-	Monohydroxylated form on the B-ring
HAs	Hippuric acids
IIV	Inter-individual variability
LC-MS	Liquid chromatography-mass spectrometry
LPH	Lactase phlorizin hydrolase
MRP	Multidrug resistance protein
OAT	Organic anion transporter
OATP	Organic anion-transporting polypeptide
O-DMA	<i>O</i> -Desmethylangolensin
OH-	Hydroxyl group on the lateral chain
PAAs	Phenylacetic acids
PCA	Principal Component Analysis
PPAs	Phenylpropanoic acids
PVLs	Phenyl- γ -valerolactones
PACs	Proanthocyanidins
PCs	Procyanidins
PD	Polymerization degree
P-gp	P-glycoprotein
RFMs	Ring-fission metabolites

SCFAs	Short chain fatty acids
SGLT	Sodium-dependent glucose transporter
SNP	Single nucleotide polymorphism
SRFMs	Structurally-related flavonoid metabolites
SULT	Sulfotransferase
T_{1/2}	Elimination half-life
TriH-	Trihydroxylated form on the B-ring
UGT	Uridine-5'-diphosphate glucuronosyltransferases
uHPLC-MS/MS	Ultra-high performance liquid chromatography coupled with tandem mass spectrometry
UM	Urolithin metabotype
Uro	Urolithin

Abstract

Increasing evidence suggests that modest, long-term intakes of (poly)phenol-rich foods can reduce the risk of chronic diseases. (Poly)phenols are the most abundant category of phytochemicals and constitute a heterogeneous family of more than 8000 molecules. Their cytoprotective activities derive from an indirect modulation of cellular signaling pathways controlling processes relevant to age-related disease progression. The main hindrance to exploiting their potential for the prevention of chronic diseases is the heterogeneity in the physiological response to their consumption, which mainly originates from inter-individual differences in their bioavailability and metabolism. An attempt to unravel metabotypes (aka metabolic phenotypes) has been made to manage this variability, based on qualitative, quantitative or quali-quantitative criteria. It is crucial to consider that the health effects attributed to (poly)phenol consumption may derive from a synergistic action exerted by the ingested (poly)phenols along the gastrointestinal tract, by their metabolites and catabolites acting systemically, and by the gut microbial ecology associated with their bioconversion. In this sense, the relationship between gut microbiota and (poly)phenols is bidirectional: gut microbiota is involved in (poly)phenol catabolism, while phenolic compounds can positively modulate the colonic microbial composition and function.

The aim of this Doctoral Thesis was to investigate the variability in (poly)phenol bioavailability and metabolism by applying multiple approaches. Two distinct factors contributing to the variation in (poly)phenol bioavailability and metabolism were analyzed: endogenous/host-related factors, including genetic background and gut microbiota, and chemical/structural properties, namely the native compound structure. Four different approaches were applied to achieve this objective: (1) an *in vitro* study using a human fermentation model, (2) an acute human intervention study, (3) a chronic human intervention study, (4) a systematic review of human studies. The first three approaches are based on targeted metabolomics analyses, while the fourth is based on a critical literature review.

In the first study, a protocol of *in vitro* colonic fermentation was optimized using pure flavan-3-ol compounds with a different structure ((-)-epicatechin and procyanidin dimers B2 and A2). The protocol optimization was achieved by extending the incubation time and monitoring fermentation conditions. The analysis of pH and faecal microbiota composition confirmed the stability and the colon-like features of the incubation environment, while the extension of the

fermentation time until 48 h and the monitoring of 5 different incubation times allowed to quantify additional low molecular weight catabolites. Results suggest that the flavan-3-ol structure influences microbial catabolism from a quantitative, qualitative and kinetic point of view: flavan-3-ol polymerization and the addition of an ether linkage in A-type procyanidins increase the steric hindrance and stiffness of the molecule, with a reduction in bioaccessibility resulting in delayed and reduced catabolism.

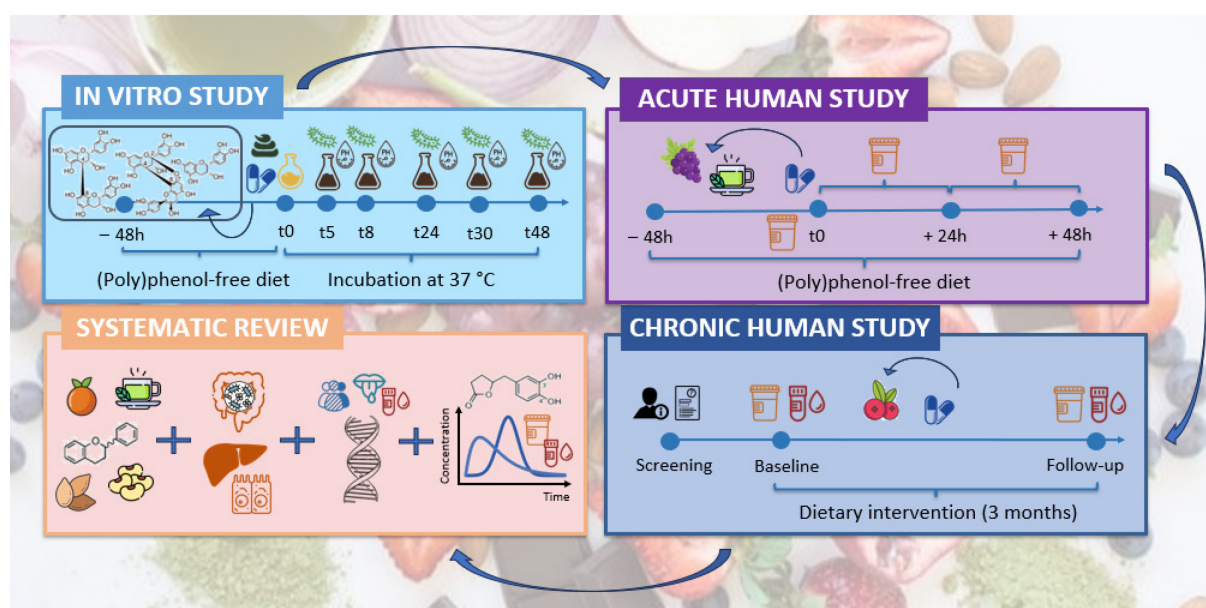
The impact of the flavan-3-ol structure on the production of phenolic metabolites was further investigated in an acute, cross-over intervention trial. Different plant extracts rich in flavan-3-ols were consumed by healthy subjects: a green tea extract, a grape seed extract with high monomeric content, and a grape seed extract with high procyanidin content. Three chemical/structural properties were considered: polymerization, B-ring hydroxylation extent, and presence of galloyl moieties. The contribution of endogenous metabolites (mainly low molecular weight phenolics) to the pool of urinary flavan-3-ol metabolites was evaluated to avoid biases in the bioavailability estimates. Differences between treatments in the 24 h and 48 h urinary excretion of phenolic metabolites were assessed, indicating that the native flavan-3-ol structure influences the bioavailability and metabolism of this (poly)phenol class. Results confirmed that procyanidins are resistant to microbial catabolism, while the peculiar composition of green tea flavan-3-ols results in the richest metabolic profile.

The third study consisted of the analysis of plasma and spot urine samples from a 12-week, placebo-controlled parallel intervention trial. The intervention involved the intake of freeze-dried cranberry powder in healthy older adults. Cranberry consumption led to changes in plasma metabolites but did not influence the urinary phenolic profile. A metabotyping approach was applied to handle inter-individual variability in (poly)phenol metabolism in free-living conditions. Three phenolic metabotypes characterized by quali-quantitative differences in the urinary excretion of some gut microbial metabolites were identified and further confirmed when applying a model focused only on flavan-3-ol colonic metabolites. Results suggest that colonic metabolism is the main driver for subject clustering, and flavan-3-ol metabolites may be key in determining (poly)phenol metabotypes, being flavan-3-ol metabotypes highly predictive of (poly)phenol metabotypes.

In the fourth study, a critical systematic review of human studies was performed to investigate the impact of genetic variations on (poly)phenol bioavailability and metabolism. In particular, the association between single nucleotide polymorphisms (SNPs) in genes involved in

(poly)phenol absorption, distribution, metabolism, and excretion (ADME) and the inter-individual variability in the plasma or urinary levels of (poly)phenol metabolites was explored. A total of twelve studies were selected, investigating different (poly)phenol sources and classes: green tea flavan-3-ols, orange flavanones, soy isoflavones, flaxseed lignans, and phenolic compounds from free diet. In total, 88 SNPs in 33 genes were analyzed, more than half of which were related to drug/xenobiotic metabolism. Phase II metabolism was the most investigated, confirming the crucial role of conjugation in (poly)phenol metabolism. The metabolites influenced by genetic variability were structurally-related flavonoid metabolites, mainly phase II conjugates, and gut microbiota metabolites. There was no consensus among studies allowing to associate a particular genetic variant with a change in the levels of a specific metabolite in urine or plasma, suggesting the need for more high-quality research in the field and new methodologies such as genome-wide association studies.

The findings of this Doctoral Thesis represent a starting point for improving the available knowledge on the variability in (poly)phenol bioavailability and metabolism, which is key to unveiling the health-promoting activity of phenolic metabolites associated with the consumption of (poly)phenol-rich foods. This information would help develop new concepts and methodologies and build a new scenario based on preventive, evidence-based, personalized nutrition strategies with these major dietary plant bioactives.



Graphical abstract of the Doctoral Thesis.

Chapter 1. Introduction

1.1. The role of dietary (poly)phenols in human health

Increasing evidence suggests that plant-based diets play an important role in the prevention of several chronic diseases, such as cardiovascular diseases (CVD), cardiometabolic diseases (CMD), some cancers, and neurodegenerative diseases (Liu, 2013). Besides providing low energy, dietary fiber, vitamins and minerals, plant-based foods are rich sources of a variety of biologically active phytochemicals with known health-promoting effects (Liu, 2013). (Poly)phenols or phenolic compounds are the most abundant category of phytochemicals and are widespread in fruits, vegetables, nuts, legumes, whole-grain cereals, oil seeds, and beverages like wine, coffee, and tea (Del Rio et al., 2013). However, many are present in only a limited number of species. (Poly)phenols constitute a heterogeneous family of more than 8000 molecules. *In planta*, they are produced as secondary metabolites, and are commonly involved in the defence against biotic and abiotic stress factors, and in the mutualistic or antagonistic interaction with other organisms (Del Rio et al., 2013). Moreover, they can be responsible for the color, taste, and astringency of some plant foods. Their biosynthetic pathway begins with the phenylpropanoid pathway, present in plants, fungi, and bacteria, which converts simple precursors into the amino acid phenylalanine (Sharma et al., 2019). Then, phenylalanine ammonia lyase catalyzes the conversion of phenylalanine to *trans*-cinnamic acid (Sharma et al., 2019). This enzyme is situated at the bifurcation between primary and secondary metabolism and is positively regulated by several environmental stressful stimuli.

Dietary intake of food (poly)phenols often exceeds 1 g/day (Zamora-Ros et al., 2013), and growing evidence from clinical trials and cohort studies suggests that modest, long-term intakes can reduce the risk of chronic diseases, especially CVD and type 2 diabetes (Del Rio et al., 2013; Rienks et al., 2018; Rodriguez-Mateos et al., 2014; Tresserra-Rimbau et al., 2014). The beneficial effects of (poly)phenol-rich foods on the promotion of cardiometabolic health have been widely demonstrated on key risk factors, including blood pressure, blood lipid profile, endothelial function, platelet activation, insulin sensitivity, glycemic response, and low-grade inflammation (Del Rio et al., 2013; Rodriguez-Mateos et al., 2014). Moreover, phenolic compounds have been associated with anticancer effects, mainly through the removal of carcinogenic agents, modulation of cancer cell signaling and cell cycle progression, and

promotion of apoptosis (Vauzour et al., 2010). Their regular consumption has been linked to neuroprotective effects, including a 50% reduction in the risk of dementia and age-related cognitive decline, a preservation of cognitive performance, a delay in the onset of Alzheimer's disease, and a reduction in the risk of Parkinson's disease (Vauzour, 2014) (Figure 1).

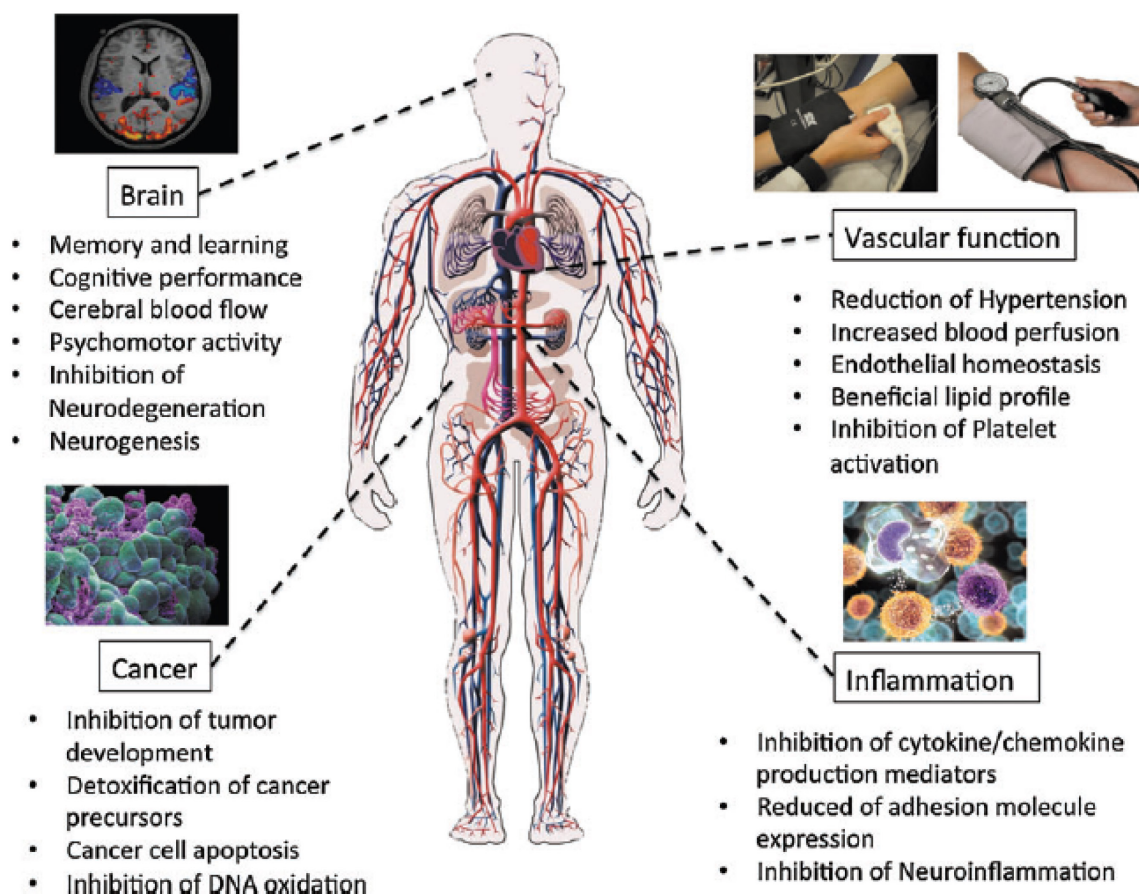


Figure 1. Potential protective effects exerted by (poly)phenols on human health (from Del Rio et al., 2013).

(Poly)phenols can act as antioxidant agents thanks to their electron-donor phenolic groups. However, their plasma concentration is far lower than that of antioxidant vitamins or pro-vitamins. So, it has been suggested and demonstrated in several *in vitro*, *in silico* and animal studies that their cytoprotective activities could derive from an indirect modulation of cellular signaling pathways and related machinery that mediate cell function under physiological and pathological conditions (Vauzour et al., 2010). Among these pathways, there are MAPK (ERK, JNK, p38), PI3K/Akt, NF- κ B, PPAR, SIRT1 and AMPK pathways (Monfoulet et al., 2021; Ruskovska et al., 2021). (Poly)phenols appear to regulate these pathways to control several pathogenic processes relevant to chronic, age-related disease progression. Of note, some (poly)phenols structurally resemble inhibitors of cell signaling cascades, such as PD98059, a MAPK inhibitor, and LY294002, a PI3K inhibitor (Vauzour et al., 2010).

1.2. Phenolic compounds: plant origin and human bioavailability and metabolism

1.2.1. Dietary sources, structure, and classification of (poly)phenols

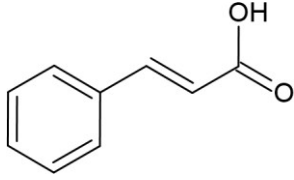
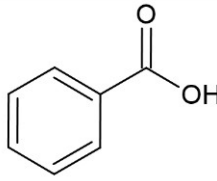
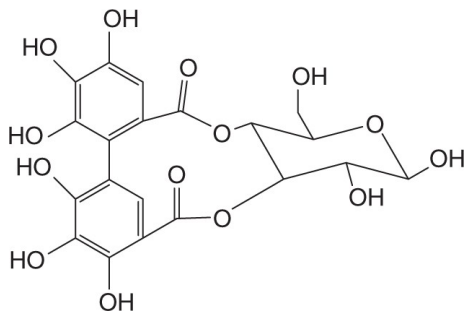
(Poly)phenols are characterized by a common structure with one or more hydroxyl groups on at least one aromatic ring (Del Rio et al., 2013). They can be classified into two main groups according to their structural properties: flavonoids and non-flavonoids. Non-flavonoids are a very heterogeneous group ranging from simple, low molecular weight phenolics, characterized by one aromatic ring, to complex, polymerized molecules of relatively high molecular weight (Del Rio et al., 2013). This group includes different subclasses (Table 1):

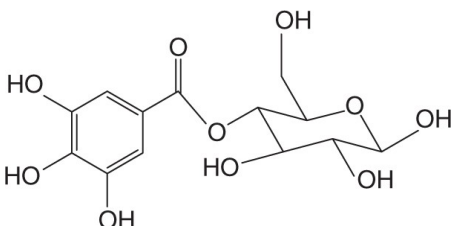
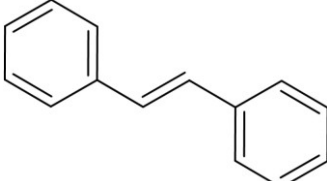
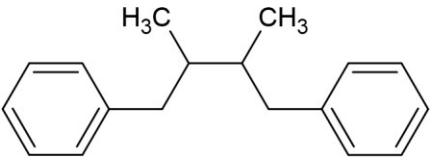
- **Phenolic acids**, the simplest molecules, are divided into benzoic acids, characterized by a carboxyl group linked to the benzene ring (C6-C1 structure), and cinnamic acids, with a C6-C3 structure and a double bond in the lateral chain. The concentration of benzoic acid derivatives (hydroxybenzoic acids) in edible plants is very low, except for red fruits, black radish, onions, and tea, which is a good source of gallic acid. On the contrary, hydroxycinnamic acids are more widespread and are abundant in coffee, cereals, and several fruits (blueberries, kiwis, plums, cherries, apples), where they can be found in a glycosylated form or as esters of quinic acid, shikimic acid and tartaric acid (Manach et al., 2004). The two main cinnamic acids, caffeic and ferulic acid, are found *in planta* mainly in the *trans* form; caffeic acid is abundant in coffee and in the outer parts of ripe fruits, and is often esterified with quinic acid, forming chlorogenic acids, while ferulic acid is abundant in the cereal bran, where it is esterified to arabinoxylans and hemicelluloses in the aleurone and pericarp (Manach et al., 2004).
- **Hydrolyzable tannins** are divided into gallotannins and ellagitannins, which are sugar esters of gallic acid and ellagic acid derivatives, respectively. While gallotannins are poorly present in foods, ellagitannins are abundant in berries (strawberry, raspberry, blackberry), pomegranate, nuts (walnut, chestnut, hazelnut), and green and black tea (Manach et al., 2004).
- **Stilbenes** are phytoalexins characterized by a structure composed of two aromatic rings linked by a 2-carbon bridge (C6-C2-C6) and are found in low quantities in the human diet (Manach et al., 2004). Their main representative is resveratrol, which is found mainly in

red wine and grapes, where it occurs both as *cis* and *trans* isomers and in a conjugated form (Del Rio et al., 2013).

- **Lignans** are formed by two phenylpropane units, and are present in high quantities in linseed, mainly as secoisolariciresinol and matairesinol, and in lower amounts in other oil seeds, whole-grain cereals (triticale, wheat), legumes (lentils), vegetables (garlic, asparagus, carrots), and fruits (pears, prunes).
- Other minor classes include: coumarins, oxygenated *O*-heterocyclic compounds present in the seeds, roots and leaves of plants (Küpel Akkol et al., 2020); xanthones, *O*-heterocyclic compounds found in mango fruits and mangosteen (Gutierrez-Orozco & Failla, 2013); curcuminoids, derivatives of curcumin, which is also known as diferulic methane and is found in turmeric (Priyadarsini, 2014); alkylresorcinols, consisting of a benzene ring linked to an alkyl chain, found in the outer layers of wheat and rye kernels (Zabolotneva et al., 2022); tyrosols, phenolic alcohols present in virgin olive oil (Karković Marković et al., 2019).

Table 1. Basic structures (on the right column) and main representative compounds (on the left column) of the principal classes of non-flavonoids.

Phenolic acids <u>Cinnamic acids</u>  <u>Benzoic acids</u> 	<i>p</i>-Coumaric acid Caffeic acid Ferulic acid Sinapic acid Gallic acid Protocatechuic acid Syringic acid Vanillic acid
Hydrolyzable Tannins <u>Ellagitannins</u> 	Sanguin H6 Punicalagin Pedunculagin

<u>Gallotannins</u> 	Galloyl-hexoside Digalloyl-hexoside
Stilbenes 	Resveratrol
Lignans 	Secoisolariciresinol Matairesinol Pinoresinol Lariciresinol

Flavonoids represent the broader group of (poly)phenols, with more than 4000 structures already identified. These compounds have a common C6-C3-C6 structure with two aromatic rings (A and B) connected by a 3-carbon bridge that forms a heterocyclic pyran ring (C-ring) (Del Rio et al., 2013). The basic flavonoid skeleton can have numerous substituents on A- and B rings, including hydroxyl, methoxy and alkyl groups. Flavonoids exist *in planta* mainly as O-glycosides and, to a lesser extent, as C-glycosides, with the aglycone moiety linked to a sugar, usually a monosaccharide such as glucose, galactose, arabinose, rhamnose and xylose, or a disaccharide such as rutinose (6-β-L-rhamnosyl-D-glucose) and neohesperidose (6-β-L-rhamnosyl-D-glucose). Based on the degree of oxidation, saturation, and hydroxylation of the central pyran ring, flavonoids can be divided into different subclasses (Table 2):

- **Flavonols** occur widely throughout the plant kingdom and are found as glycosides, with conjugation occurring at the 5, 7, 3', 4', and 5' positions (Del Rio et al., 2013). The richest sources are yellow and red onions, curly kale, leeks, broccoli, and blueberries. The principal

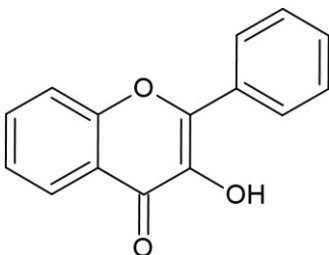
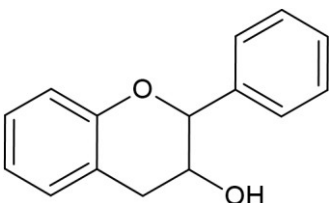
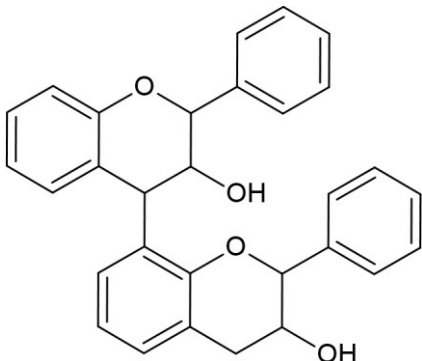
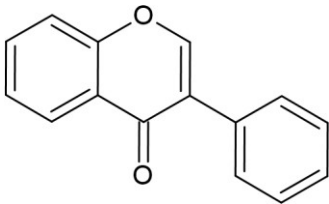
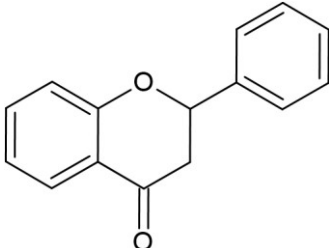
molecule is quercetin, present mainly in 3 different glycosylated forms: quercetin-4'-O-glucoside, quercetin-3,4'-O-diglucoside, and quercetin-3-O-rutinoside (rutin) (Del Rio et al., 2013). Flavonols accumulate in the outer and aerial tissues (skin and leaves) of the plant because their biosynthesis is stimulated by light (Manach et al., 2004).

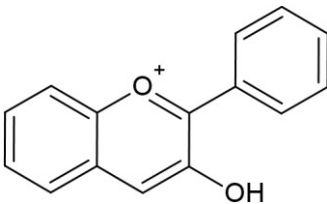
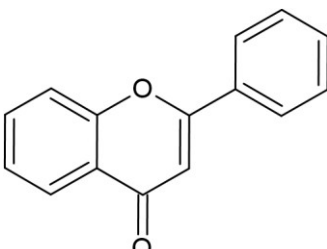
- **Flavan-3-ols** are the most ubiquitous and the principal class of flavonoids in the Western diet. The main sources are cocoa and dark chocolate, green tea, red wine, several fruits (apple, pear, peach, cherry, prune, black grape, berries), legumes, and nuts. They are the only class that occurs *in planta* in an aglyconic form and constitute the most structurally complex group, ranging from monomeric forms (commonly named catechins) to oligomeric (2-10 units) and polymeric (>10 units) structures of up to 190 units, named proanthocyanidins (PACs) or condensed tannins (Del Rio et al., 2013). The basic structure can be esterified at position 3 with a gallic acid moiety. The two chiral centers at C2 and C3 of the monomeric flavan-3-ol produce four stereoisomers for each level of B-ring hydroxylation. The hydroxyl groups in the B-ring can range from one to three, yielding different structures: (epi)afzelechins, with one hydroxyl group at C4'; (epi)catechins, with two hydroxyl groups at C3' and C4'; (epi)gallocatechins, with three hydroxyl groups at C3', C4', and C5'. (+)-Catechin and (–)-epicatechin are the most widespread flavan-3-ol monomers in nature, while (epi)gallocatechins and their galloylated forms are characteristic compounds of green tea (Manach et al., 2004). According to the number of linkages between monomeric units, two types of PACs are distinguished: type B and type A. Type B PACs are formed from flavan-3-ol monomers by oxidative coupling between the C4 of the upper unit and the C6 or C8 of the adjacent lower or extension unit. Type A PACs have an additional ether bond between C2 and C7 and are less widespread in the plant kingdom than type B PACs, characteristic of cranberry (Rodriguez-Mateos et al., 2014). Oligomeric and polymeric structures have an additional chiral center at C4 of every additional flavan-3-ol unit. Condensed tannins made up exclusively of (epi)catechin units are called procyanidins (PCs), whereas propelargonidins and prodelphinidins are the oligomeric and polymeric structures consisting of at least one subunit of (epi)afzelechin or (epi)gallocatechin, respectively (Del Rio et al., 2013; Rodriguez-Mateos et al., 2014). Procyanidins are the most abundant type of PACs in plants, while propelargonidins and prodelphinidins are rare.

- **Isoflavones** are biologically active compounds with estrogenic properties and are often referred to as phytoestrogens, as it happens for lignans. They have hydroxyl groups in positions 7 and 4' in a configuration analogous to the hydroxyls in the estradiol molecule. The richest sources are leguminous plants, especially soybean, which contains three main molecules: genistein, daidzein and glycitein (Manach et al., 2004).
- **Flavanones** are present in exceptionally high amounts in citrus fruits. The most common flavanone glycoside is hesperetin-7-*O*-rutinoside (hesperidin), while the main aglycones are naringenin in grapefruit, hesperetin in oranges, and eriodictyol in lemons. Flavanones are generally glycosylated by a disaccharide at position 7: either a neohesperidose, which is responsible for the bitter taste, or a rutinose, which is flavorless.
- **Anthocyanins** are water-soluble pigments present in the vacuolar sap of the epidermal tissues of flowers and fruits, which they give a pink, red, blue, or purple color (Manach et al., 2004). They represent the conjugated forms of anthocyanidin aglycones, with sugars (mainly glucose at position 3) or organic acids (citric and malic acids) as conjugates (Del Rio et al., 2013). In the human diet, anthocyanins are found in red wine, certain leafy and root vegetables, and different fruits, such as red grapes, pomegranates, black plums, cherries, and berries. In fruits, they are found mainly in the skin, except for cherries and strawberries, in which they also occur in the flesh. Food contents are generally proportional to color intensity (Manach et al., 2004).
- **Flavones** consist chiefly of glycosides of luteolin and apigenin and are present in parsley, celery, and some herbs (Del Rio et al., 2013; Manach et al., 2004). Cereals such as millet and wheat contain C-glycosides of flavones, while the skin of citrus fruit contains large quantities of polymethoxylated flavones (Manach et al., 2004).
- Other minor classes are flavanonols, dihydroflavonols, flavan-3,4-diols, chalcones, dihydrochalcones, auronones and prenylflavonoids (Braune & Blaut, 2016).

Certain polyphenols such as quercetin are found in all plant products (fruit, vegetables, cereals, legumes, tea, wine), whereas others are specific to particular foods (gallo catechins in green tea, flavanones in citrus fruit, isoflavones in soybean, phloridzin in apples, type A PACs in cranberry) (Manach et al., 2004). In most cases, foods contain complex mixtures of (poly)phenols, which may synergistically affect health outcomes.

Table 2. Basic structures (on the right column) and main representative compounds (on the left column) of the principal classes of flavonoids.

Flavonols 	Quercetin Kaempferol Myricetin Isorhamnetin
Flavan-3-ols <u>Monomers</u>  <u>Proanthocyanidins</u> 	(+)-Catechin (-)-Epicatechin (-)-Epicatechin-gallate (-)-Epigallocatechin-gallate (+)-Gallocatechin Procyanidin B1 Procyanidin B2 Procyanidin A2
Isoflavones 	Daidzein Genistein Glycitein
Flavanones 	Naringenin Hesperetin Eriodictyol Isosakuranetin

Anthocyanidins 	Cyanidin Delphinidin Pelargonidin Peonidin Malvidin Petunidin
Flavones 	Apigenin Luteolin

1.2.2. Absorption, distribution, metabolism, and excretion of (poly)phenols

Despite the large body of evidence about the health effects of (poly)phenols and (poly)phenol-rich foods, their protective role, especially towards cardiometabolic outcomes, has not been demonstrated consistently yet (Cassidy & Minihane, 2017; Gibney et al., 2019; González-Sarrías et al., 2017). The main hindrance to unravelling and exploiting the potential of dietary (poly)phenols for the prevention of chronic diseases is the heterogeneity in the individual response to their consumption, which conditions the efficacy of these compounds in promoting health (Manach et al., 2017; Milenkovic et al., 2017). The inter-individual variability (IIV) in the physiological response mainly originates from differences in the bioavailability and metabolism of (poly)phenols (Gibney et al., 2019; Landberg et al., 2019) and is influenced by several factors including personal characteristics (sex, age, ethnicity), lifestyle (diet, smoking, physical activity), (patho)physiological status, genetic background and gut microbiota composition and activity (Cassidy & Minihane, 2017; Gibney et al., 2019). These factors condition the nutrkinetic process of absorption, distribution, metabolism and excretion (ADME) of (poly)phenols, determining the amount and type of phenolic metabolites in circulation. Phenolic metabolites are the molecules potentially available to target tissues and can act as mediators of diet-induced effects on health (Roager & Dragsted, 2019).

Following ingestion, dietary (poly)phenols remain relatively stable during gastric transit and reach the small intestine, where only a small proportion is absorbed. The absorption of

glycosylated (poly)phenols is associated with a preliminary reaction of hydrolysis that releases the aglycones (Del Rio et al., 2013). This reaction can be catalyzed by lactase phlorizin hydrolase (LPH), located in the brush border of the small intestine epithelial cells, which exhibits broad substrate specificity for flavonoid-*O*- β -D-glucosides. The released aglycone may then enter the epithelial cells by passive diffusion because of its increased lipophilicity and proximity to the cellular membrane. An alternative hydrolytic step is mediated by a cytosolic β -glucosidase (CBG), after the active transport of the (poly)phenol glycosides into the epithelial cells through the sodium-dependent glucose transporter 1 (SGLT1). Regarding oligomeric and polymeric (poly)phenols, small amounts of some PC dimers have been detected in plasma in their native form, indicating that intact PCs with a low polymerization degree (PD) can be partially absorbed. However, their plasma concentrations represent <1% of the circulating flavan-3-ol metabolites and are subjected to a high inter-individual variability (Mena et al., 2019). Once absorbed, phenolic compounds follow the common metabolic pathway of xenobiotics, undergoing phase II enzymatic metabolism, to increase their hydrophilicity and be excreted through urine. They can be conjugated with one or moieties of glucuronic acid, sulfate and methyl groups (usually added to sulfated/glucuronidated molecules) in reactions catalysed by uridine-5'-diphosphate glucuronosyltransferases (UGTs), sulfotransferases (SULTs) and catechol-*O*-methyltransferases (COMT), respectively (Rodríguez-Mateos et al., 2014). Of less importance, the liver metabolizing enzymes of the family of cytochrome P450 (CYP) are involved in phase I metabolism, in particular hydroxylation and *O*-demethylation of fully methylated flavonoids with no hydroxyl groups available, before their conjugation (Liu et al., 2017).

While the phase I metabolic pathway is a minor contributor to flavonoid disposition, phase II metabolism is a key player. These reactions first occur in enterocytes, after which metabolites pass through the portal vein to the liver, where they may undergo further conjugation before entering the systemic circulation and eventually undergoing renal excretion. The conjugation mechanisms are highly efficient: aglycones are either absent in blood or present in low concentrations after nutritional doses, and circulating polyphenols are conjugated derivatives generally bound to albumin (Manach et al., 2004). Before renal excretion, polyphenols can penetrate tissues, particularly those in which they are metabolized, but their ability to accumulate within specific target cells needs to be further investigated. Some recycling of phase II metabolites from the liver back to the small intestine may occur via enterohepatic

recirculation in the bile, and efflux of at least some of the metabolites back into the lumen of the small intestine could happen with the involvement of members of the adenosine triphosphate (ATP)-binding cassette (ABC) family of transporters, including multidrug resistance protein 2 (MRP2), P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP) (Crozier et al., 2010; Liu et al., 2017). Conjugated metabolites reaching the duodenum can be then subjected to the action of bacterial enzymes, especially β -glucuronidase, in the distal segments of the intestine, after which they may be reabsorbed (Manach et al., 2004). MRP-3 and the glucose transporter GLUT2 are implicated in the efflux of metabolites from the basolateral membrane of the enterocytes into the circulation (Del Rio et al., 2013). Several organic anion transporters (OATs), organic anion transporting polypeptides (OATPs) and multidrug resistance proteins (MRPs) are essential for excretion: OAT(P)s are involved in the uptake of (poly)phenol metabolites by the liver and the kidney proximal tubes, while MRPs are implicated in the efflux from hepatocytes into the bloodstream and in the efflux from renal proximal tubular cells into urine (Liu et al., 2017) (Figure 2).

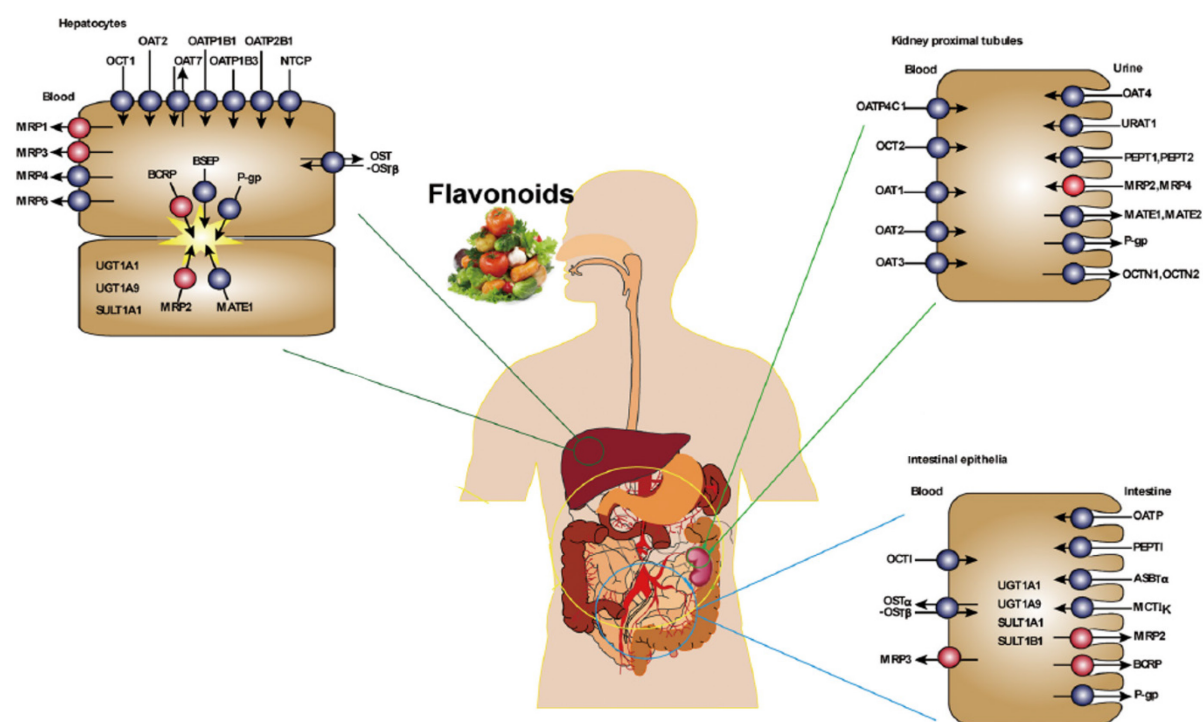


Figure 2. Disposition mechanisms of flavonoids in humans. Boxes represent hepatocytes, kidney proximal tubular cells and enterocytes with their cytoplasmic phase II metabolizing enzymes and plasma membrane transporters. Transporters coloured in red are of importance in the transport process of flavonoids, while those coloured in blue are not relevant (modified from Liu et al., 2017).

(Poly)phenol conjugates with sugar moieties that are resistant to the action of LPH/CBG (all C-glycosides and O-glycosides with sugar moieties other than glucose), together with most

esterified molecules and oligo/polymeric (poly)phenols (>90%), are not absorbed in the small intestine and reach the colon, where they undergo modifications by the gut microbiota (Del Rio et al., 2013). After the hydrolytic release of aglycones from esterified and glycosylated flavonoids and the release of monomers from tannins, flavonoids undergo C-ring fission (also, A-ring fission for flavan-3-ols) and following reactions of demethylation, dehydroxylation, α and β -oxidation of the aliphatic lateral carbon chain, reduction of carbon-carbon double bonds, isomerization, and decarboxylation, being converted to low molecular weight catabolites (phenolic acids and derivatives) that are efficiently absorbed *in situ* through passive diffusion, with some undergoing further phase II metabolism locally and in the liver before entering the circulation (Braune & Blaut, 2016). These microbial catabolites are named ring fission metabolites (RFMs) and are excreted in urine in substantial quantities that, in most instances, exceed the excretion of (poly)phenol metabolites absorbed in the upper gastrointestinal tract, which will be referred to as structurally related flavonoid metabolites (SRFMs) (Crozier et al., 2010). In addition, more than half the carbon of glycosides and the flavonoid A ring may be metabolized to short-chain fatty acids (SCFAs) and, therefore, be available for energy metabolism by the host, being oxidized and ultimately exhaled as carbon dioxide (Rodriguez-Mateos et al., 2014). In this way, obligate or facultative anaerobic bacteria may utilize glycosylated flavonoids entering the intestine as additional growth substrates: flavonoids can serve as the sole carbon and energy source, with the attached sugar moieties being preferentially fermented. However, complete degradation of flavonoids by gut bacteria, including cleavage of the aromatic A- and B-ring metabolites is usually not observed (Braune & Blaut, 2016).

1.2.3. Colonic degradation of flavonoids by human gut microbiota

Several bacterial species and strains are involved in flavonoid degradation in the colon. The ability to cleave the glucose moiety is widely distributed among Bifidobacteriaceae and prevalent among Lactobacillaceae, Lachnospiraceae and Enterococcaceae. Several bacteria with β -glucosidase activity are dominant members of the human gut microbiota, namely *Bifidobacterium adolescentis*, *Bifidobacterium longum*, *Enterococcus faecalis*, *Bacteroides ovatus*, *Bacteroides uniformis*, *Parabacteroides distasonis* and *Escherichia coli* (Braune & Blaut, 2016). Bacteria acting on flavonoids conjugated with disaccharides (rutinose and neohesperidoside) possess α -rhamnosidases, which have been characterized from strains of

Lactobacillus acidophilus, *Lactobacillus plantarum* and *Bifidobacterium dentium*. Some members of the Lachnospiraceae, Enterococcaceae and Streptococcaceae were reported to cleave C-glucosylated flavones and isoflavones. However, C-deglycosylation is less prevalent and has so far been scarcely investigated. After O-deglycosylation, anthocyanidins undergo spontaneous cleavage of the C-ring, resulting in the production of benzoic acids (BAs) and, to a lesser extent, 3-phenylpropanoic acids (PPAs), which can be oxidized to 2-phenylacetic acids (PAAs) and mandelic acids (2-hydroxy-2-phenylacetic acids) (Del Rio et al., 2013). Moreover, after the preliminary deglycosylation step, A- and B-ring of all flavonoid aglycones can be O-demethylated and dehydroxylated. In particular, *Eubacterium limosum* and *Blautia* sp. MRG-PMF1 can O-demethylate flavones, isoflavones or prenylated flavanones.

The microbial catabolism of flavonol and flavone aglycones starts with the reduction of the C2-C3 double bond, yielding the corresponding flavanonols and flavanones, respectively. These flavonoids undergo C-ring fission, catalyzed by *Eggerthella* sp. SDG-2, *Flavonifractor plautii*, *Eubacterium ramulus* and *Clostridium butyricum*. The B-ring of flavonols is thus transformed to PAAs, while the B-ring of flavones and flavanones is converted into phenylhydracrylic acids (3-hydroxy-3-phenylpropanoic acids) and PPAs, which can be oxidized to PAAs and BAs (Braune & Blaut, 2016; Crozier et al., 2010). The A-ring is instead converted into phloroglucinol (benzene-1,3,5-triol) and resorcinol (benzene-1,3-diol) and can be finally fermented to produce SCFAs (Figure 3).

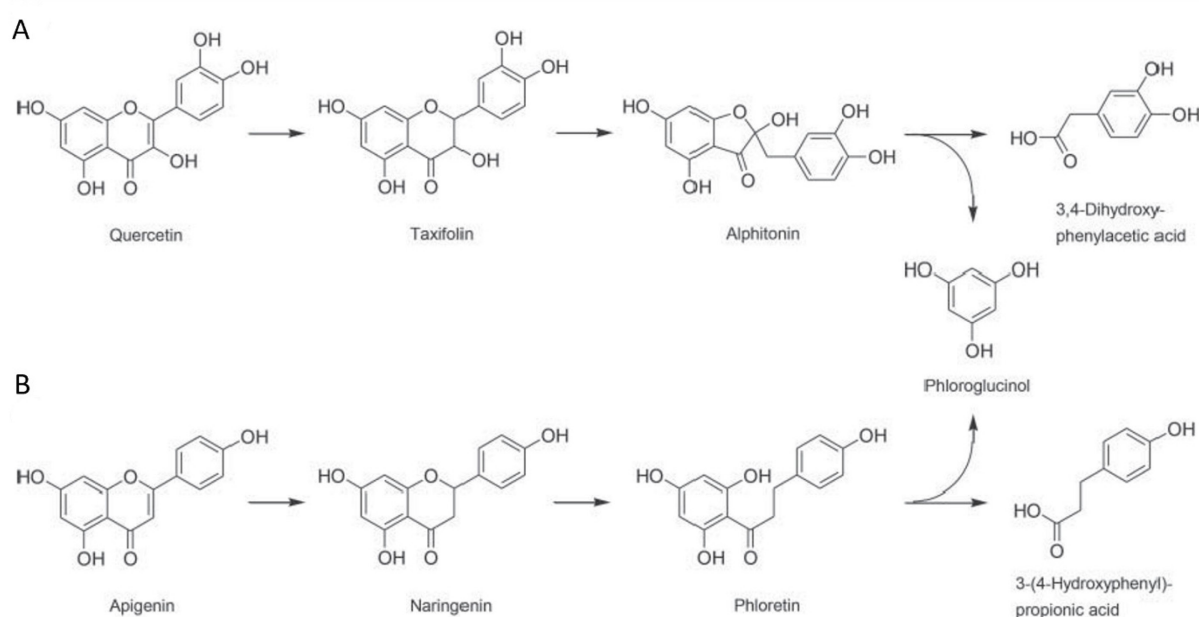


Figure 3. Pathways of bacterial conversion of (A) flavonols (using quercetin as example) and (B) flavones and the corresponding flavanones (using apigenin and naringenin as examples) (from Braune & Blaut, 2016).

Ring fission of isoflavone aglycones results in the production of *O*-desmethylangolensins (*O*-DMA), which may be further degraded, producing 2-phenylpropanoic acids from B-ring and phloroglucinol and resorcinol from A-ring. Bacterial catabolism of isoflavone aglycones can also involve a stepwise reduction to isoflavan structures producing dihydroisoflavones, tetrahydroisoflavones and equol consequently (Braune & Blaut, 2016) (Figure 4). Fission of the C-ring of isoflavones is catalyzed by two strains of *Eubacterium ramulus* and by strain SY8519, all of which are members of the Lachnospiraceae. Members of the Coriobacteriaceae, including *Slakia isoflavoniconvertens* and *Slakia equolifaciens*, are responsible for the complete reduction of isoflavones to equol and hydroxy-equol (Braune & Blaut, 2016). Unlike phenolic acids, which are common catabolites of all flavonoid classes, equol and *O*-DMA are metabolites specific to isoflavones.

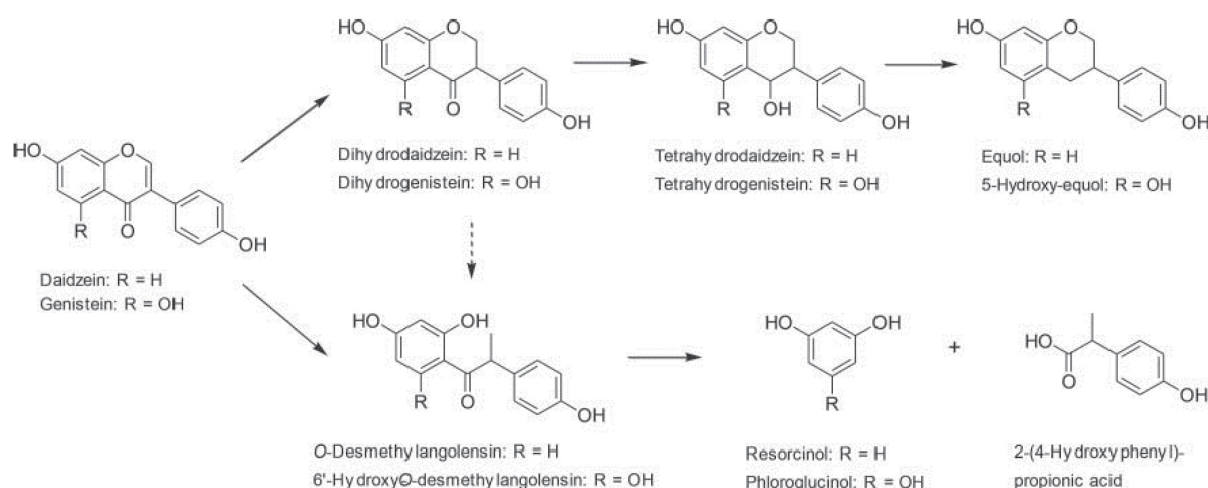


Figure 4. Pathways of bacterial conversion of isoflavones using the examples of daidzein and genistein (from Braune & Blaut, 2016).

Bacterial conversion of monomeric flavan-3-ols begins with the hydrolysis of ester bonds (for galloylated flavan-3-ols) by microbial esterases, followed by the reductive cleavage of the C-ring, yielding 1,3-diphenylpropan-2-ol derivatives (DPPOLs). C-ring fission is catalyzed by strains of *Slakia equolifaciens*, *Eggerthella lenta* and *Adlercreutzia equolifaciens*. The last two bacteria can dehydroxylate flavan-3-ols and their C-ring cleavage products at the B-ring (Braune & Blaut, 2016). Further degradation includes A-ring fission catalyzed by *Flavonifractor plautii*, with the production of 5-phenyl- γ -valerolactones (PVLs), which can be hydrolyzed forming 4-hydroxy-5-phenylvaleric acids (4-OH-PVAs) and, through dehydroxylation, 5-phenylvaleric acids (PVAs). Formation of PVLs is favoured with respect to 4-OH-PVAs, and 4-OH-PVA concentration exceeds the concentration of PVAs. Of note, PVLs, 4-OH-PVAs and PVAs,

collectively referred to as 5-carbon chain RFMs (5C-RFMs), are metabolites specific to flavan-3-ols and can be used as biomarkers of intake of flavan-3-ol-rich foods (Mena et al., 2019). Flavan-3-ol catabolism can continue converting PVAs into low molecular weight phenolic acids: 3-carbon chain RFMs (3C-RFMs), namely PPAs and cinnamic acids (CAs), produced through β -oxidation of the lateral chain of PVAs; 2-carbon chain RFMs (2C-RFMs) or PAAs, arising from α -oxidation of PPAs; 1-carbon chain RFMs or BAs, generated by β -oxidation of PPAs or α -oxidation of PAAs (Mena et al., 2019). Finally, BAs deriving from the metabolism of flavan-3-ols and other flavonoids can be further metabolized in two different ways: (1) reduction to benzaldehydes (BALs) and benzene derivatives (pyrogallol and catechol from trihydroxylated and dihydroxylated B-rings, respectively) by the gut microbiota, or (2) conjugation with glycine by hepatic enzymes, forming hippuric acid derivatives (HAs) (Crozier et al., 2010). All the microbial catabolites generated from parent flavonoids can be easily absorbed by passive diffusion and further conjugated at the colonocyte/hepatocyte level by phase II enzymes to produce conjugated derivatives that are subsequently eliminated in urine, although such conjugations do not necessarily account for 100% of the substrate (Mena et al., 2019).

Regarding B-type PAC colonic degradation, four different pathways have been postulated: (1) a minor route (<10%) involving the interflavan cleavage and yielding (epi)(gallo)catechin monomers; (2) the degradation of the lower unit, involving C-ring fission with formation of fission PACs and the following A-ring cleavage that yields PVLs; (3) the degradation of the upper unit, yielding 5-(3',4'-dihydroxyphenyl)acetic acid; the simultaneous degradation of the upper and lower units, with the production of 5-(2',4'-dihydroxyphenyl)valer-2-enic acid, a metabolite specific of PACs (Figure 5). Microbial breakdown of A-type PACs has been so far less investigated. It is known that they are more resistant to microbial catabolism than B-type because of their more rigid and complex interflavan ether bonds. Their degradation starts with the cleavage of monomeric unit C-rings, yielding fission PACs that can be further degraded.

The final PVL metabolites arising from flavan-3-ol degradation depend on the type of the parent compound in terms of stereogenic configuration and extent of B-ring hydroxylation. The presence of a stereogenic center at position C4 of PVLs and 4-OH-PVAs can help distinguish metabolites deriving from parent (+)-(gallo)catechin, having an *S*-configuration, from metabolites of (-)-(gallo)epicatechin, with an *R*-configuration. When (epi)gallocatechin or (epi)gallocatechin-gallate is the colonic precursor, the 3',4',5'-trihydroxyphenylpropan-2-ol derivative is converted into 5-(3',4',5'-trihydroxyphenyl)- γ -valerolactone and the

corresponding PVA. These trihydroxyphenyl catabolites can be dehydroxylated to yield the analogous dehydroxylated derivatives (both 3',4' and 3',5'), which can be subsequently dehydroxylated to generate 3'-hydroxyphenyl derivatives and, to a lesser extent, 4'-hydroxyphenyl derivatives (Mena et al., 2019). When (epi)catechin or (epi)catechin gallate is the colonic precursor, the 3',4'-dihydroxyphenylpropan-2-ol is converted into 5-(3',4'-dihydroxyphenyl)- γ -valerolactone, which can be dehydroxylated to yield a 3'-hydroxyphenyl derivative and, less frequently, a 4'-hydroxyphenyl derivative (Figure 6). The concentration of dihydroxyphenyl derivatives exceeds the concentration of hydroxyphenyl ones.

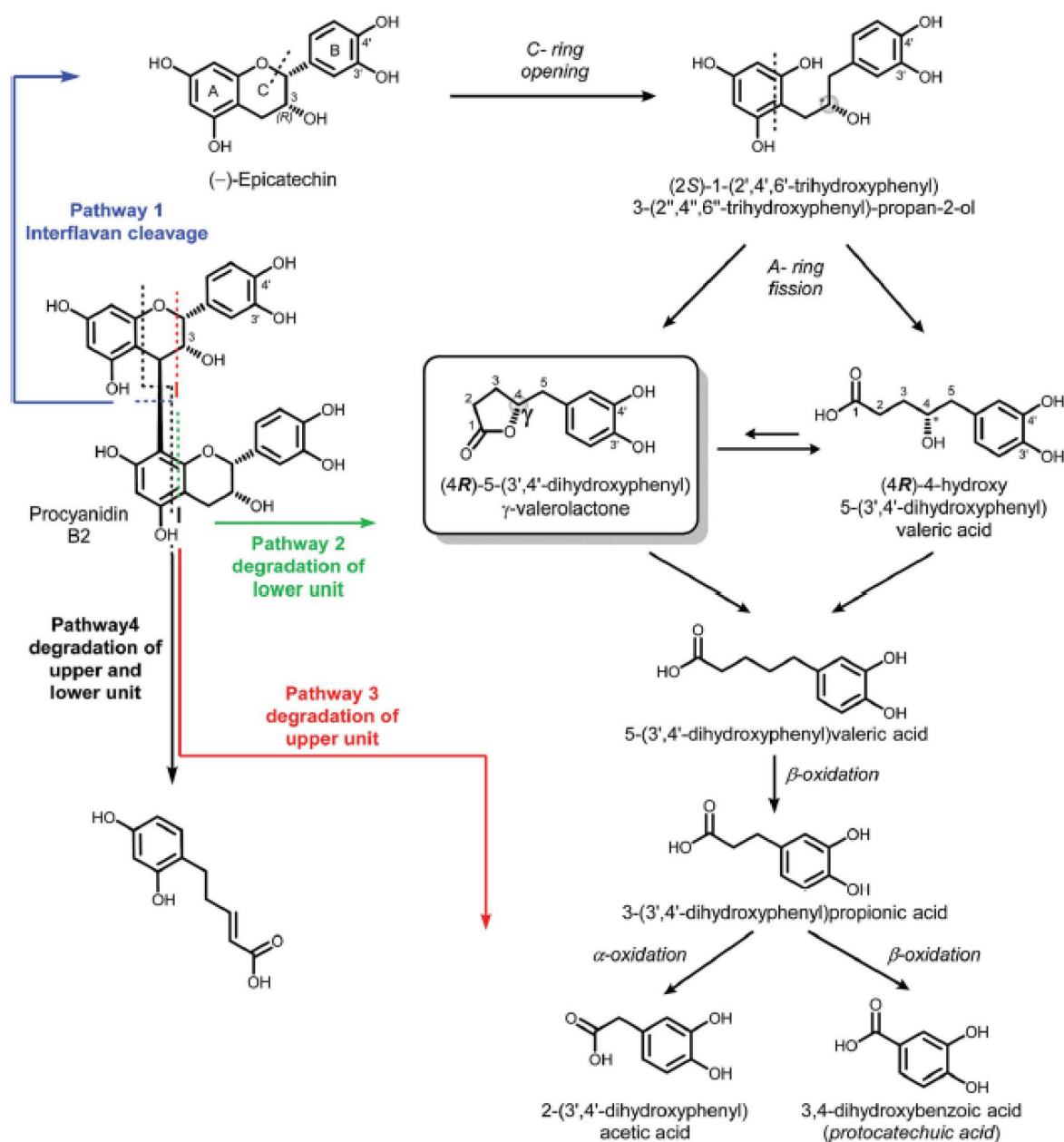


Figure 5. Gut microbiota degradation of procyanidin B2 and epicatechin in the colon (from Mena et al., 2019).

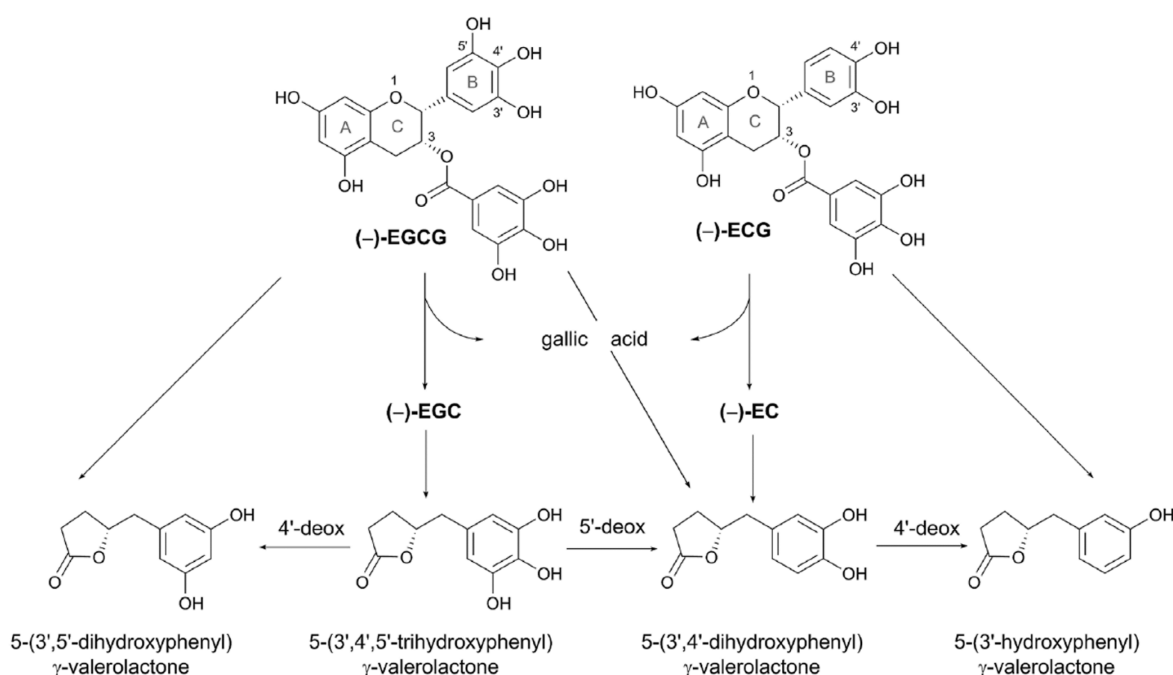


Figure 6. Phenyl- γ -valerolactone metabolites derived from (-)-epigallocatechin-gallate (EGCG) and (-)-epicatechin-gallate (ECG). “deox” indicates dehydroxylation (from Mena et al., 2019).

Flavan-3-ols circulate principally as phase II conjugates of RFMs, as up to 97 host-gut microbiota metabolites were found (Di Pede et al., 2023). Moreover, their half-life is far higher than that of SRFMs, indicating that they remain in systemic circulation for a relatively long time and can be relevant for human health, acting as mediators of the cross-talk between host and gut microbiota induced by diet (Roager & Dragsted, 2019). The appearance of flavan-3-ol metabolites in circulation reflects their absorption and microbial catabolism: SRFMs appear rapidly, while 5C-RFMs and especially 3/2/1C-RFMs are late-occurring and remain in plasma for 24 and 48 hours, respectively (Ottaviani et al., 2016). Phase II conjugates of PVLs are the main flavan-3-ol metabolites and are excreted in urine prevalently as sulfate conjugates and, to a lesser extent, as glucuronide forms (Di Pede et al., 2023). For this reason, they have been evaluated as biomarkers of flavan-3-ol intake in large epidemiological studies (Ottaviani et al., 2018). In particular, 5-(4'-hydroxyphenyl)- γ -valerolactone-3'-sulfate is the most representative metabolite, excreted in urine in amounts higher than 15% of the ingested dose. While PVLs are specific metabolites of flavan-3-ols, low molecular weight phenolics are common microbial degradation metabolites obtained from the different subclasses of flavonoids through confluence microbial metabolic pathways (Figure 7). Moreover, BAs are common late metabolites of both flavonoid and non-flavonoid phenolics (Selma et al., 2009).

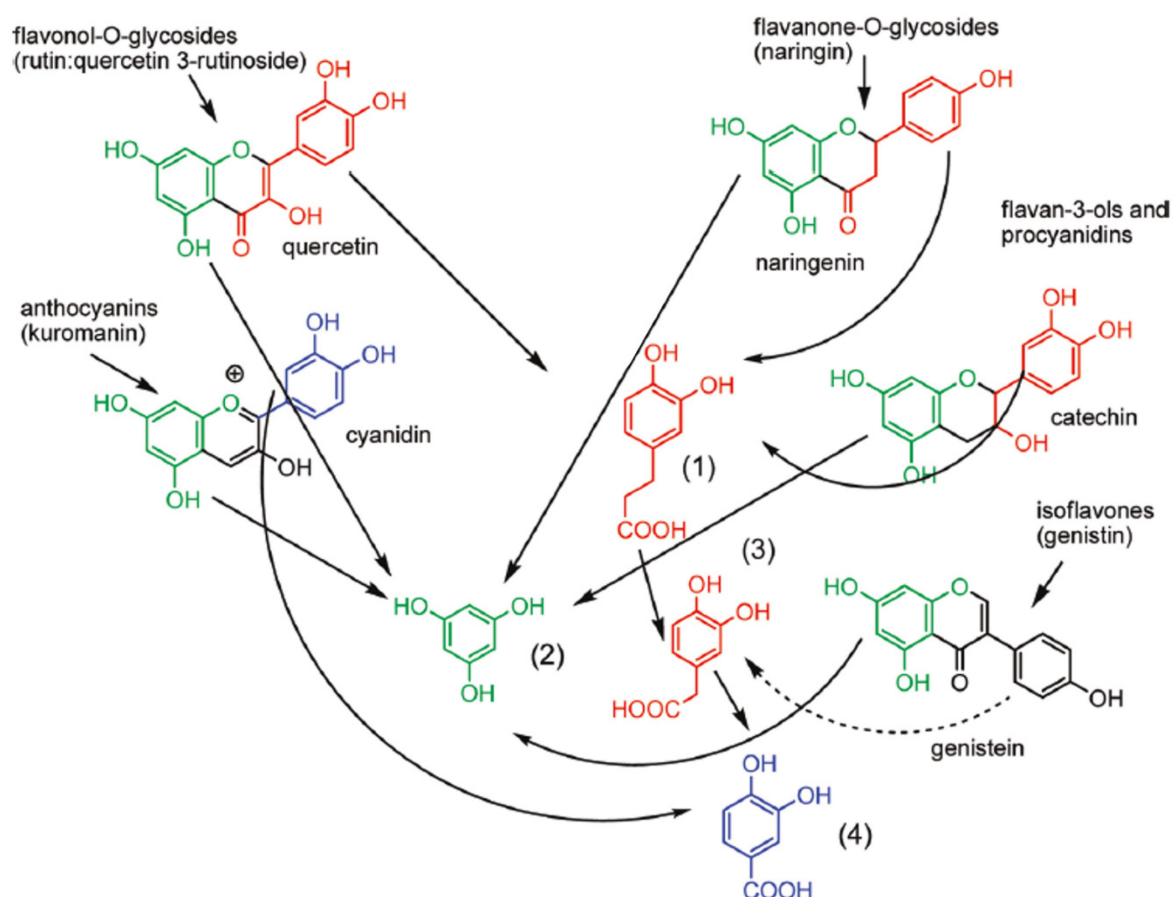


Figure 7. Confluence metabolic pathways and common metabolites of dietary flavonoids. (→) indicates demonstrated pathways and (- - ->) indicates hypothetical pathways (modified from Selma et al., 2009).

1.3. Inter-individual variability in (poly)phenol bioavailability and metabolism

In recent years, the divergent responses obtained in epidemiological studies about the protective health effects of dietary (poly)phenols (Bondonno et al., 2019; Tresserra-Rimbau et al., 2019) have led to controversial results in clinical trials, implying that a “one-size-fits-all” approach is not adequate to explore the potential beneficial effects attributed to phenolic compounds (Marino et al., 2020; Morand & Tomás-Barberán, 2019). In addition, it is not clear yet whether the health effects attributed to (poly)phenol consumption are influenced by the ingested (poly)phenols, which may exert their effects along the gastrointestinal tract by their derived bioavailable metabolites and catabolites, exerting effects systemically, and/or by the gut microbial ecology associated with (poly)phenol metabolism (Iglesias-Aguirre et al., 2021). There is evidence of extensive variability in the bioavailability of flavonoids: the amount

measured in biological samples ranges between 15-99% of the original flavonoid dose and is recovered as a wide range of metabolites (Manach et al., 2005). The existing IIV in (poly)phenol metabolism and response may hide the potential of (poly)phenol consumption on human health. An effort by researchers involved in the COST Action POSITIVE was made to define IIV in (poly)phenol ADME and to assess its impact on cardiovascular health outcomes (Almeida et al., 2018; Bayram et al., 2018; Bento-Silva et al., 2020; Eker Özkacar et al., 2019; García-Conesa et al., 2018; Gibney et al., 2019; González-Sarrías, Combet, et al., 2017; Hålldin et al., 2019; Landberg et al., 2019; Manach et al., 2017; Martini et al., 2019; Menezes et al., 2017; Milenkovic et al., 2017; Morand & Tomás-Barberán, 2019). Determinants such as age, sex, genotype, gut microbiota, and (patho)physiological status could lead to differences among subjects in (poly)phenol metabolism and bioavailability, generating a heterogeneous response upon their consumption and highlighting how IIV represents a key aspect to be undeniably considered (Bento-Silva et al., 2020; Manach et al., 2017; Morand & Tomás-Barberán, 2019). Nevertheless, the actual contribution and influence of each factor in determining IIV is still unknown for most of the (poly)phenol classes.

Among the factors driving IIV, gut microbiota undoubtedly plays a crucial role in (poly)phenol metabolism, and it is believed to underpin much of the IIV observed (Morand et al., 2020). Well-established examples of microbiota-dependent variation are the formation of urolithins from ellagic acid and ellagitannins, and equol and *O*-DMA from the isoflavone daidzein. In these cases, metabolic phenotypes (aka metabotypes) have been described and characterized by the selective production or non-production of these catabolites, due to a specific gut microbiota ecology, defining producers/non-producers in the population (Setchell et al., 2013; Tomás-Barberán et al., 2014). For other (poly)phenol classes, an attempt to unravel metabotypes has been made based on quantitative (catabolite production gradient) or qualitative (different production proportions among specific catabolite classes) criteria without firmly establishing the causes responsible for the observed differences. Quantitative differences can define high- and low-producers/excretors in the population, as demonstrated for flavanones, hop prenylflavonoids (isoxanthohumol), and lignans (Aschoff et al., 2016; Bolca et al., 2007; Fraga et al., 2021; Rowland et al., 2000). Quali-quantitative differences are instead related to different metabolite excretion patterns deriving from the production of different proportions of the same metabolites, as observed for flavan-3-ols (Mena et al., 2018, 2022). Similarly, differences in the ratio between phase II sulfates and glucuronides have been

proposed as putative indicators of inter-person variation in the metabolism of (poly)phenols, attributable to variable enzymatic activities and perhaps also to genotypic diversity (Anesi et al., 2019; Cortés-Martín et al., 2019; Favari et al., 2020). Since phenolic metabolites may be mediators of the health effects attributed to (poly)phenols, it is essential to assess the IIV in their production, as well as understand the main factors affecting this phenomenon.

1.3.1. Flavonoids

Flavan-3-ols

Flavan-3-ol metabolites for which IIV has been reported are both native ones, as PACs and (epi)(gallo)catechins, and gut microbiota-derived catabolites, such as tri/di/mono-hydroxylated PVLs and PVAs, either described as aglycones or as conjugates. In general, high and low producers of flavan-3-ol metabolites have been observed (*continuous production*), as reported by authors or highlighted by high coefficient of variation (CV%) and fold-variations (Actis-Goretta et al., 2013; Andreu-Fernández et al., 2020; Castello et al., 2018; Favari et al., 2020; Feliciano et al., 2017; Gee et al., 2017; Hakeem Said et al., 2021; Jiménez-Girón et al., 2013; McKay et al., 2015; Mena et al., 2018; Ottaviani et al., 2016; Romain et al., 2022; Scholl et al., 2018; van Duynhoven et al., 2014; Velzen et al., 2014). However, high producers of native compounds are not necessarily high or low producers of gut microbiota-derived catabolites, as the inter-relationship between these two metabolite classes (SRFMs and RFM) has rarely been assessed (van Duynhoven et al., 2014).

Regarding native metabolites, IIV in their plasma concentrations or urinary excretion has been assessed without determining specific factors responsible for the observed IIV. Actis-Goretta et al. (2013) found large IIV in the absorption of (–)-epicatechin after perfusion of purified (–)-epicatechin into an isolated jejunal segment of 6 volunteers, as determined by inter-subject different recovery of (–)-epicatechin in intestinal perfusates. Similarly, the amount of (–)-epicatechin phase II metabolites detected in bile and urine varied considerably among individuals, with one subject excreting higher amounts of (–)-epicatechin metabolites in bile than the rest of the volunteers and, consequently, presenting lower amounts of these metabolites in urine. Despite the small number of volunteers prevented from reaching any firm conclusion, authors assumed that different rates of metabolism could be one of the most

important variables determining the overall absorption of (–)-epicatechin (Actis-Goretta et al., 2013).

In another study, age was hypothesized as a potential cause for IIV in the ADME of native flavan-3-ol metabolites (Rodriguez-Mateos et al., 2015). Authors only found slight differences between young and elderly when the highest dose of cocoa flavan-3-ols foreseen in the study was administered, while no significant difference was observed between age groups when the lowest cocoa flavan-3-ol dose was consumed. In detail, differences were observed in plasma levels of sulfated and glucuronidated (epi)catechin metabolites and were explained by differences in the elimination rate, suggesting that the expected decline of renal function with age may influence the plasma levels of phase II (epi)catechin metabolites in elderly (Rodriguez-Mateos et al., 2015).

When a green tea extract was administered to 84 healthy participants (Scholl et al., 2018), IIV in the pharmacokinetics of (–)-epigallocatechin, (–)-epigallocatechin-gallate, and (–)-epicatechin-gallate was not associated with sex, age, weight, height, body mass index (BMI), lifestyle parameters (such as smoking, alcohol consumption or habitual consumption of green tea), nor with polymorphisms in the genes coding P-gp and sulfotransferase 1A1 (SULT1A1). However, inter-person differences were associated with the use of contraceptives and inherent genetic variations in genes coding for MRP2 and organic anion transporting polypeptide 1B1 (OATP1B1) for (–)-epigallocatechin and (–)-epigallocatechin-gallate (Scholl et al., 2018).

Finally, Fernández et al. (2020) reported greater IIV differences in AUC (Area Under the Curve) values for epigallocatechin-gallate in males than females, suggesting a potential influence of sex. Further studies that corroborate all these findings are necessary.

Concerning gut microbiota catabolites, attempts to define flavan-3-ol metabotypes based on the differential quali-quantitative urinary excretion of PVLs and 3'-hydroxylated PPAs (3'-HPPAs) (*cluster production*) have been performed in recent years. Firstly, three putative metabotypes were proposed after consumption of an (epi)gallocatechin-rich green tea extract: metabotype 1 was characterized by high excretion of trihydroxylated PVLs (triHPVLs) and dihydroxylated PVLs (diHPVLs) and reduced excretion of 3'-HPPAs; metabotype 2 was distinguished by a medium excretion of diHPVLs and a limited excretion of triHPVLs and 3'-HPPAs; and metabotype 3 was characterized by a limited production of PVLs and high amounts of 3'-HPPAs (Mena et al., 2018) (Figure 8). Later works reported similar findings, although flavan-3-ol precursors differed from those of green tea and did not include trihydroxylated

compounds, namely (epi)gallocatechin derivatives (Cortés-Martín et al., 2019; Mena et al., 2022). The resulting picture was more straightforward but consistent, as authors reported at least two urinary flavan-3-ol metabolotypes after consumption of cranberry and nuts: one cluster characterized by a high excretion of 3'-HPPAs and monohydroxylated PVLs (HPVLs), and a reduced excretion of diHPVLs, whereas the other cluster by a high excretion of diHPVLs and low excretions of HPVLs and smaller catabolites (3'-HPPAs) (Cortés-Martín et al., 2019; Mena et al., 2022). At the same time, depending on the clustering technique used, a third cluster, characterized by a low excretion of all the metabolites, was also observed (Cortés-Martín et al., 2019; Mena et al., 2022). Indeed, a particular influence of the normalization method, as well as the clustering algorithm used (i.e., distance- or density-based), has emerged when defining metabolotypes originating from quali-quantitative differences in metabolite production (Mena et al., 2022).

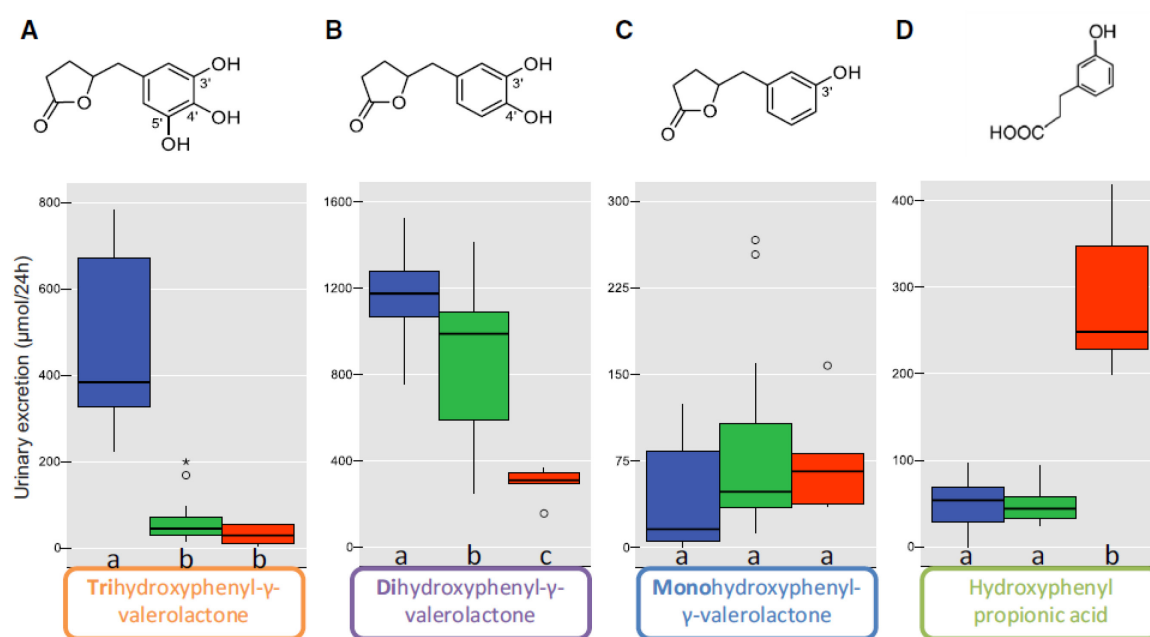


Figure 8. Urinary excretion of trihydroxyphenyl-γ-valerolactones (a), dihydroxyphenyl-γ-valerolactones (b), monohydroxyphenyl-γ-valerolactones (c), and 3-(3'-hydroxyphenyl)propionic acid (d) by groups of subjects. Boxplots are built using the mean of the urinary excretion of the sum of all the derivatives belonging to the same aglycone moiety. The blue group represents metabolotype 1, the green one is metabolotype 2, and the red stands for metabolotype 3. Different letters indicate statistically significant differences ($p < 0.05$) among groups. Circles and asterisks indicate outliers and extreme outliers (more than three times the interquartile range), respectively (from Mena et al., 2018).

Despite a certain robustness of the evidence for colonic metabolotypes of flavan-3-ols, further *ad hoc* confirmatory research is required to consolidate this observation and fully understand the factors behind these differences, as well as their possible health-related consequences. Among the factors to be considered, gut microbiota composition and bioconversion capacity

are likely the main variables determining the observed IIV, but efforts are needed to identify the microbial species responsible for the different catabolism of flavan-3-ols. Information on specific bacterial strains responsible for the bioconversion of flavan-3-ols into PVLs and low molecular weight phenolics is currently scarce. Van Velzen et al. (2014) positively correlated diHPVL production with members of Clostridia and Actinobacteria, including *Clostridium leptum*, *Flavonifractor plautii*, *Ruminococcus bromii*, *Sporobacter termitidis*, and *Eubacterium ramulus*, and the genus *Propionibacterium*. *Lactobacillus plantarum* IFPL935, *Eggerthella lenta*, *Adlercreutzia equolifaciens*, and *Flavonifractor plautii* are the only bacteria identified being able to convert flavan-3-ols into 5-(3',4'-dihydroxyphenyl)- γ -valerolactone and 5-(3'-hydroxyphenyl)- γ -valerolactone, but no microorganisms responsible for further catabolism into PVAs and 3-HPPAs have yet been identified (Kutschera et al., 2011; Sánchez-Patán et al., 2012). On the other hand, transit time should also be considered as it may drive the metabolotypes elucidated so far.

Lastly, a certain IIV in phase II metabolism of gut-microbiota catabolites has been reported, mainly related to diHPVLs, with subjects excreting higher amounts of glucuronide than sulfate derivatives, or vice versa (Anesi et al., 2019; Cortés-Martín et al., 2019; Mena et al., 2022). This fact has also been observed for (epi)catechin derivatives. This variability could be related to genetic polymorphisms in phase II enzymes (Haldar et al., 2014; Inoue-Choi et al., 2010; Miners et al., 2001), but also to the dose of flavan-3-ols consumed. It has been reported that the sulfonation pathway has higher affinity but lower efficiency than the glucuronidation one, and when the ingested amount of flavan-3-ols increases, a shift from sulfation toward glucuronidation might occur (Koster et al., 1981). Further studies will elucidate this and its potential relevance for human health.

Flavonols

Data on IIV in the ADME of flavonol metabolites have been mainly reported for kaempferol, quercetin, and myricetin, either as aglycones or phase II conjugates, and for two methylated derivatives of quercetin, namely isorhamnetin and tamarixetin. For flavonol native metabolites, IIV in their plasma concentration and urinary excretion has been observed, as expressed by the high CV% (*continuous production*), but no specific factor has been associated to the observed inter-individual differences (DuPont et al., 2004; Egert et al., 2008; Feliciano et al., 2017; Guo et al., 2014; McKay et al., 2015; Mecha et al., 2020; Romain et al., 2022; Y.

Wang et al., 2016). One study performed on just 7 healthy volunteers reported a correlation between the percentage of kaempferol excreted upon bean consumption and the BMI of volunteers, with higher kaempferol excretion observed for lower BMI (Bonetti et al., 2007). Sex and gut microbiota were also investigated as potentially responsible for the IIV observed in quercetin AUC values after consumption of quercetin and rutin (quercetin-rutinoside) by 16 healthy subjects (Erlund et al., 2000). While a small level of IIV was observed after quercetin aglycone intake, rutin consumption by females led to higher quercetin AUC and C_{max} (maximum concentration) values, with subjects using oral contraceptives having the highest values. It was hypothesized that women's microbiota was able to hydrolyze sugar moieties more efficiently than men's, and this might be linked to the use of oral contraceptives (Erlund et al., 2000). Almeida et al. (2018) recently reviewed the IIV in quercetin bioavailability in humans and did not manage to establish the causes behind the reported IIV, although suggesting a potential implication of genetic polymorphisms, dietary adaptation, composition of gut microbiota, drug exposure, and other subject characteristics such as BMI and health

Flavones

Scarce IIV data are available for flavones, mainly related to luteolin and apigenin phase II metabolites, which are characterized by high CV% in urinary excretion. A recent study reported different levels of urinary excretion of phase II derivatives of hydroxy-polymethoxyflavones, demethylated metabolites of specific flavones found in orange juice (Tomás-Navarro et al., 2021). Upon definition of excretion levels as high (if above 10% of the intake), medium (between 5 and 10% of the intake), and low (if below 5% of the intake), the possible existence of three quantitative groups of high, medium and low excretors of these polymethoxyflavone metabolites was suggested (*continuous production*) (Tomás-Navarro et al., 2021). The involvement of gut microbiota and CYP1A1 polymorphisms in the demethylation of polymethoxyflavones was hypothesized (Tomás-Navarro et al., 2021).

Flavanones

Regarding flavanones, IIV has been observed for unchanged compounds and their phase II metabolites and for gut microbiota-derived phenolic acids. For unchanged and phase II metabolites, a continuous production was reported, defining the existence of two/three groups of excretors, depending on the amount of flavanone metabolites excreted and

thresholds considered, thus classifying subjects into high, medium and low/no producers (Bourian et al., 1999; Nishioka et al., 2021; Tomás-Navarro et al., 2014, 2021) or high and low/no producers (Ávila-Gálvez et al., 2021; Erlund et al., 2002; Fraga et al., 2022). In some instances, polymorphisms in phase II enzymes and transporters have been associated with the observed quantitative metabolotypes. For example, Fraga et al. (2022) associated high excretion of phase II flavanone metabolites with GGCC and GCTG haplotypes for the ABCC2_rs8187710, SULT1A1_rs3760091, SULT1A1_rs4788068, and SULT1C4_rs1402467 enzymes involved in the efflux of flavanone metabolites back to the intestinal lumen (which may limit the bioavailability of these compounds) and their conjugation with a sulfate moiety (which favours their elimination in urine). In some other cases, the influence of polymorphisms in phase I enzymes (in particular CYP1A1 and CYP2A6) has been suggested as a potential explanation of the observed IIV (Bourian et al., 1999; Tomás-Navarro et al., 2021). Nishioka et al. (2021) observed two different flavanone metabolotypes, one characterized by a lower excretion, a slower excretion rate and a lower glucuronidation rate (profile A), and the other one conversely showing higher excretion, a faster excretion rate, and higher glucuronidation rate (profile B). These differences were attributed to a possible involvement of polymorphic variants of the phase II enzyme UGT. Subjects classified as profile B showed a greater abundance of *Bacteroides uniformis* and *Bifidobacterium bifidum*, species responsible for the O-deglycosylation of flavanones, while profile A-individuals exclusively presented *Clostridium* sp., related to the C-ring fission, but the meaning of these observations still needs to be clarified (Nishioka et al., 2021).

The urinary excretion of phase II flavanones and the ratio of some phenolic acids (mainly hippuric acid, 4'-hydroxyphenylacetic acid, and 3-(4'-hydroxyphenyl)propanoic acid) was used to classify individuals consuming orange juice according to three different metabolic patterns (Fraga et al., 2021). Of note, subjects with a low or intermediate ratio (i.e., higher production of phase II metabolites) saw a significant reduction in body fat percentage and blood pressure after 60 days of orange juice consumption (Fraga et al., 2021). In other studies, authors did not define quantitative metabolotypes for flavanone metabolites, but similarly observed high CV% in their plasma concentrations or amounts in urine, suggesting that individuals are producing more and others producing fewer metabolites (Castello et al., 2020; Erlund et al., 2001; Hernández-Prieto, Fernández, et al., 2023; Hernández-Prieto, Garre, et al., 2023; Pereira-Caro et al., 2017; Romain et al., 2022). Pereira-Caro et al. (2017) reported CV% up to 300% in the

urinary excretion of phase II flavanone metabolites in a study where the effect of training on flavanone metabolism after supplementation of the diet with orange juice was evaluated. Exercise did not significantly modify the amount of phase II flavanones in circulation. On the other hand, machine learning has been used to better understand the contribution of sex and sweetener presence to the pool of flavanones and phenolic acids in circulation, both in plasma and urine, after chronic consumption of a maqui-lemon beverage (Hernández-Prieto, Fernández, et al., 2023; Hernández-Prieto, Garre, et al., 2023). Results accounted for an effect of sex on some metabolites and indicated that sweeteners like stevia and sucrose may enhance the bioavailability of some compounds.

Anthocyanins

Data on IIV in the ADME of anthocyanins have been mainly reported for glycosylated forms of cyanidin, delphinidin, malvidin, pelargonidin, and petunidin. Regarding native anthocyanidin metabolites, a substantial variation in their plasma concentration and urinary excretion among individuals has been observed, as expressed by the high CV% (*continuous production*), but no specific cause responsible for the reported IIV has been identified (Kalt et al., 2017; McKay et al., 2015; Milbury et al., 2010; Teixeira et al., 2017; Xie et al., 2016). An attempt to review the factors that affect anthocyanidin bioavailability in humans has been recently done, without reaching any firm conclusion due to a lack of literature addressing this topic (Eker Özkacar et al., 2019). Authors assumed that differences in enzymes involved in anthocyanidin metabolism and transport and in gut microbiota composition and activity, which in turn can be affected by further factors such as genetics, age, and diet, may be the main responsible for IIV in anthocyanidin ADME (Eker Özkacar et al., 2019), but studies are needed to demonstrate these assumptions.

Isoflavones

IIV reported for isoflavones has been suggested for both unchanged compounds, mainly daidzein, genistein, glycitein, formononetin, and biochanin A, and for gut microbiota-derived catabolites, namely dihydrodaidzein, dihydrogenistein, equol, *O*-DMA, and 6'-hydroxy-*O*-DMA. Differences among subjects have been described mainly in the production or non-production of equol and *O*-DMA (*cluster production*), the two main microbial-derived catabolites of daidzein. In fact, for both metabolites, two distinct metabotypes have generally been

identified, clustering subjects between producers and non-producers (Frankenfeld et al., 2004). These observed metabolotypes are determined by a specific gut microbiota composition and activity. It was reported that the gut microbes involved in equol production are more than ten, including *Slackia equolifaciens*, *Slakia isoflavoniconvertens* and *Adlercreutzia equolifaciens*, while, unfortunately, there are fewer reports on the bacterial species specifically linked to *O*-DMA production (Iglesias-Aguirre et al., 2021). According to Zheng et al. (2019), the equol-producing metabolotype shows a higher abundance of the equol-producing bacteria *Adlercreutzia equolifaciens* and *Bifidobacterium bifidum*, and a different microbial composition enriched in the genera *Prevotella*, *Megamonas*, *Allistipes*, *Desulfovibrio*, *Collinsella*, and *Eubacterium*. This was in agreement with the findings of Nakatsu et al. (2014), who reported that post-menopausal women equol producers presented an enrichment in *Bifidobacterium* and *Eubacterium* compared to non-producers after consumption of soy bars for one week. Interestingly, Yoshikata et al. (2019) identified equol-producing bacteria in 97% of women participating in their study, although only 22% of women characterized by that microbial composition were equol producers, suggesting that equol production might not depend on the quantity, but on the quality of equol-producing bacteria consortiums. In addition, equol producers presented higher microbiota diversity than non-producers, which might favour equol production.

The equol-producing and *O*-DMA-producing metabolotypes are unrelated: the capacity to harbour equol-producing bacteria is not associated with the ability to harbour *O*-DMA-producing bacteria. The studied populations have displayed a distinct distribution of these metabolotypes, but, in most cases, equol-producers were below 50%, and *O*-DMA producers were more than 50% of total individuals (de Oliveira Silva et al., 2020; Frankenfeld et al., 2006; Frankenfeld et al., 2004; Iino et al., 2019; Kleinloog et al., 2021; Mitchell & Collins, 1999; Miura et al., 2016; Sakane et al., 2014; Setchell & Cole, 2006; Usui et al., 2013; Van der Velpen et al., 2014; Wiseman et al., 2004; Yoshikata et al., 2016, 2021a). Ethnicity may also play an important role in the distribution of equol- and *O*-DMA-producing metabolotypes, as observed by Song et al. (2006) in a population of more than 300 Korean American and Caucasian-American female subjects. Equol-producer phenotype was significantly more prevalent in Korean American than in Caucasian American women and girls and, conversely, the *O*-DMA-producer phenotype was significantly less prevalent in Korean American than in Caucasian American females (Song et

al., 2006). However, further investigation is needed to evaluate the role of ethnicity and dietary habits in the observed differences between sub-groups of individuals.

Some studies looked for associations between the incidence of the equol- or *O*-DMA-producing metabotypes and sociodemographic characteristics of the population other than ethnicity, including age and education level, anthropometric values (height, weight and BMI, among others) and lifestyle factors (as dietary habits) (Bolca et al., 2009; Frankenfeld et al., 2006; Frankenfeld et al., 2004; Iino et al., 2019; Kawabata et al., 2019; Liu et al., 2014; Rowland et al., 2000; Sakane et al., 2014; Setchell & Cole, 2006). No clear associations with any factor were consistently reported across studies.

An increasing number of studies have associated equol- or *O*-DMA-producing metabotypes with potential effects on health, mainly related to protection against menopausal symptoms (Frankenfeld et al., 2006; Wu et al., 2007; Yoshikata et al., 2021) and cardiovascular risk, as comprehensively reviewed by Frankenfeld (2017). Equol- and/or *O*-DMA-producers seemed to have better cardiovascular health profiles than non-producers (Z.-M. Liu et al., 2014; Reverri et al., 2017; Yoshikata et al., 2016; Zheng et al., 2019). In addition, acute vascular benefits of equol in men have been reported only in equol producers upon soy consumption, while the same benefits were not observed in equol non-producers after intake of synthetic equol, suggesting that the microbial ecology associated with the equol-producing capacity is crucial for determining health benefits (Hazim et al., 2016). These results were promising, even though not fully supported by other scientific evidence: the study by Usui et al. (2013) described a significant improvement of cardiometabolic risk biomarkers after chronic daily oral administration of *S*-equol, particularly in equol non-producer females. Clarifying whether the health effects exerted by equol and/or *O*-DMA are due to the presence of a specific microbial ecology associated with metabolite production is needed, also to understand whether the health status has an impact on the production of these metabolites or are the metabolites to condition the health status.

Regarding other isoflavone metabolites, they are produced by all individuals but in different amounts (*continuous production*), as evidenced by high CV% values (Bolca et al., 2009; Kleinloog et al., 2021; Nakatsu et al., 2014; Nayeem et al., 2019; Rowland et al., 2000; Tsangalis et al., 2005; Tsunoda et al., 2002; Wiseman et al., 2004). The factors responsible for the observed IIV have not been defined yet, and further studies are needed to elucidate them. Interestingly, it can be noted that unchanged isoflavone metabolites (specifically daidzein,

genistein, and glycitein) are excreted in higher amounts in equol non-producers (Liu et al., 2014; Miura et al., 2016). On the other hand, recent work has reported that AUC_{0-24h} and C_{max} values for 5-hydroxyequol but not for other isoflavone metabolites changed according to the enterotype, with subjects belonging to *Prevotella*-rich enterotype presenting higher values than in the *Bacteroides* and Ruminococcaceae enterotypes (Song et al., 2023). Recently, Soukup et al. (2023) assessed the metabolic profile of isoflavones considering both daidzein and genistein, in 59 postmenopausal women consuming a soy supplement for 12 weeks. The study population was classified into 5 isoflavone metabotypes (*cluster production*), characterized by different proportions of daidzein, genistein, equol, dihydrodaidzein, dihydrogenistein, and 4-ethylphenol. Clusters were associated with estrogenic potencies. This study opened the door to more comprehensive metabotyping approaches with soy products, avoiding reductionist approaches focused just on equol or *O*-DMA.

1.3.2. Non-flavonoids

Phenolic acids

Phenolic acid metabolites can originate from human metabolism or from resident microbiota catabolism of phenolic acids occurring in foods, namely hydroxycinnamic and benzoic acid derivatives, as well as from gut microbial catabolism of dietary flavonoids (Bento-Silva et al., 2020). They can also undergo subsequent phase II metabolism. Phenolic acid metabolites, for which data on IIV has been reported, mainly include CAs, PPAs, PAAs, BAs, and HAs, either as aglycones or phase II conjugates. Their plasma concentration and urinary levels are affected by a substantial IIV, suggesting that high and low producers of these metabolites would exist (*continuous production*) (Alkhaldy et al., 2019; Castello et al., 2018, 2020; Chandra et al., 2019; Cortés-Martín, Selma, et al., 2018; Domínguez-Fernández et al., 2022; Esteban-Fernández et al., 2020; Feliciano et al., 2016, 2017; Gill et al., 2010; Gutierrez Diaz et al., 2016, 2018; Hernández-Prieto, Fernández, et al., 2023; Hernández-Prieto, Garre, et al., 2023; Hidalgo-Liberona et al., 2020; Jiménez-Girón et al., 2013; Kerimi et al., 2020; McKay et al., 2015; Mecha et al., 2020; Monteiro et al., 2007; Muñoz-González et al., 2013; Ottaviani et al., 2016; Rodríguez-Mateos et al., 2016; Romain et al., 2022; Sánchez et al., 2018; Velzen et al., 2014; Xie et al., 2016). Indeed, some studies focused on flavonoids and reporting on phenolic acids have discussed the role of phenolic acids while classifying metabolic patterns (*cluster*

production) (Fraga et al., 2021; Mena et al., 2018, 2022). PPAs, in particular 3'-HPPAs, have proved to be fundamental for the determination of colonic metabolotypes of flavan-3-ols, showing different production patterns about PVL production (Cortés-Martín et al., 2019; Mena et al., 2018, 2022). The excretion of a few phenolic acids served to classify orange juice consumers into three groups (low, medium, and high excretors) and, together with the excretion ratio of phase II flavanones, explore the benefits of orange juice on subjects presenting different metabolization patterns (Fraga et al., 2021). This is interesting as phenolic acids usually contribute broadly to the amount of (poly)phenols in circulation. So, investigating different phenolic acid production patterns may help better understand the role of (poly)phenols on human health.

Specific factors influencing IIV related to phenolic acid metabolites have not been well defined yet, although a certain role of gut microbiota composition and microbial activity has been suggested (Gill et al., 2010; Gutierrez Diaz et al., 2016, 2018; Sánchez et al., 2018; Velzen et al., 2014). The impact of age, BMI, and lifestyle factors, including dietary habits, alcohol consumption and physical activity, are still not robustly characterized by available studies (Alkhaldy et al., 2019; Cortés-Martín et al., 2019; Gutierrez Diaz et al., 2018; Hernández-Prieto, Fernández, et al., 2023; Hernández-Prieto, Garre, et al., 2023; Vázquez-Fresno et al., 2015). Bento-Silva et al. (2020) recently reviewed the variables affecting IIV associated to phenolic acids, suggesting a major contribution of gut microbiota and genetic polymorphisms, without reaching any firm conclusion.

Hydrolysable tannins

No literature dealing with IIV in the ADME of gallotannins is available, probably due to their rare occurrence in plant-based foods. Conversely, there is much information about ellagitannins, and this is likely related to the fact that metabolotypes involved in the metabolism of ellagitannins/ellagic acid, characterized by the production or not (*cluster production*) of specific gut microbiota-derived metabolites, namely urolithins (Uros), were described for the first time years ago and extensively studied later (Cerdá et al., 2005; Cortés-Martín et al., 2018; González-Sarriás et al., 2015, 2017; Romo Vaquero et al., 2015; Selma et al., 2015, 2017; Truchado et al., 2012). Three urolithin metabolotypes (UMs) have been described: UM-A includes producers of only Uro-A (3,8-dihydroxy-urolithin), UM-B includes producers of IsoUro-A (3,9-dihydroxy-urolithin) and Uro-B (3-hydroxy-urolithin) in addition to Uro-A, whereas UM-

0 identifies non-producers (García-Villalba et al., 2022; Tomás-Barberán et al., 2017) (Figure 9).

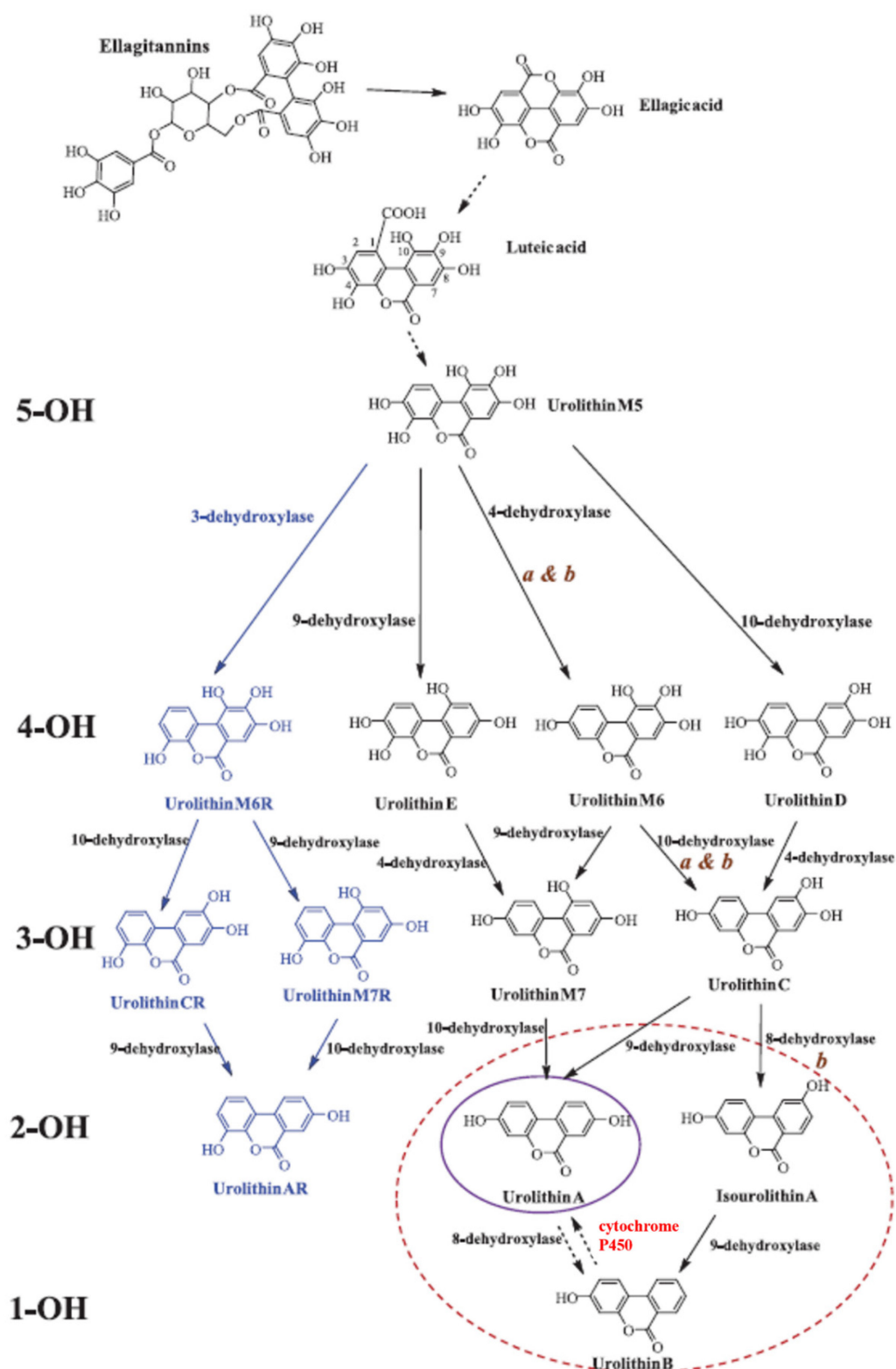


Figure 9. Catabolic pathway of ellagitannins to urolithins, showing the gut microbial enzymatic activities involved (in red, the only human enzyme putatively involved). 5-OH, 4-OH, 3-OH, 2-OH, and 1-OH refer to the number of hydroxyl groups for each urolithin group (penta-, tetra-, tri-, di- and monohydroxy urolithins, respectively). (a) *Gordonibacter urolithinifaciens*, *G. pamelaee*; (b) *Ellagibacter isourolithinifaciens*. The purple and red circles designate the final urolithins produced in UM-A and UM-B, respectively. Uro-AR, produced through the catabolic pathway coloured in blue, can be found in both metabolotypes (modified from García-Villalba et al., 2022).

Urolithins occur naturally in the gut by the action of the gut microbiota on dietary ellagitannins, but ellagic acid needs to be produced first. Ellagic acid can be obtained from ellagic acid glycosides or ellagitannins by enzymatic hydrolyses, catalyzed by microbial β -glucosidases or ellagitannases (ellagitannin acyl hydrolases), respectively (García-Villalba et al., 2022). Lactic acid bacteria such as *Lactobacillus plantarum*, *L. paraplantarum* and *L. pentosus* have shown a tannase activity. Ellagic acid can be then hydrolyzed, decarboxylated and subsequently dehydroxylated by the gut microbiota producing urolithins. Two species of the genus *Gordonibacter* (*G. urolithinifaciens* and *G. pamelaee*) can metabolize ellagic acid into Uro-M5, Uro-M6, and Uro-C. *Ellagibacter isourolithinifaciens* can also convert ellagic acid into Uro-M5, Uro-M6, Uro-C, and isoUro-A. *E. isourolithinifaciens* is more efficient than *G. urolithinifaciens* in the catechol-dehydroxylase activity, while the reductase activity is more relevant in *G. urolithinifaciens* (García-Villalba et al., 2022).

The distribution of UM-A and UM-B is affected by aging: UM-A is more prevalent at an early age, decreasing from 85% up to 55% in adulthood, parallel to an increase of UM-B from 15% up to 45%; from 35 to 40 years of age, the proportion of UM-A and UM-B (55%, and 45%, respectively) remains approximately unaltered (Cortés-Martín et al., 2018). UMs have been associated with human gut microbiota: lower richness and diversity was found in UM-0 individuals compared to UM-A and UM-B, and UM-A has been associated with a protective gut microbial ecology, while UM-B individuals may be prone to dysbiosis and cardiometabolic risk (Iglesias-Aguirre et al., 2021; Romo-Vaquero et al., 2019). Moreover, an improvement of cardiometabolic risk biomarkers was observed by González-Sarrías et al. (2017) in UM-B individuals after consuming an ellagitannin-rich pomegranate extract. In contrast, no statistically significant effects were observed in UM-A individuals or the whole group before clustering individuals according to their UMs (González-Sarrías et al., 2017).

Lignans

Enterolactone and enterodiol, and in a few cases the native compound secoisolariciresinol, are the main lignan catabolites for which IIV in their production has been observed. Enterolactone is the most frequently assessed catabolite. Two quantitative phenotypes have been mostly described, namely high and low enterolactone producers (*continuous production*) (Frankenfeld, 2013; Mullens et al., 2022; Navarro et al., 2020; Vanharanta et al., 2002). The most critical determinants associated with IIV in the human plasma concentration of

enterolactone are the composition and activity of the gut microbiota, followed by the use of antibiotics (Hålldin et al., 2019).

Enterolactone is produced by the gut microbiota, starting from plant lignans, through a series of deglycosylation, demethylation, dehydroxylation, and dehydrogenation reactions (Figure 10). Some bacterial genera have been identified and associated with each conversion step of lignans to enterolactone: *Bacteroides* and *Clostridium* with deglycosylation, *Eubacterium*, *Butyribacterium* and *Blautia* for demethylation, *Clostridium* and *Eggerthella* for dehydroxylation, and the species *Lactonifactor longoviformis* for dehydrogenation (Hålldin et al., 2019). Consequently, the lack of certain bacteria, their interaction, and/or an inappropriate intestinal environment could decrease enterolactone production, determining the different resulting metabotypes (Hålldin et al., 2019). Lignan-rich food intake, constipation, BMI, sex and other lifestyle factors, such as smoking, could also influence the total variability among subjects (Hålldin et al., 2019). Regarding the health implications, high enterolactone production has been related to better anthropometric parameters (Frankenfeld, 2013), while gene expression of fecal exfoliated cells has recently revealed that high enterolactone production is associated with a suppressed inflammatory status (Mullens et al., 2022).

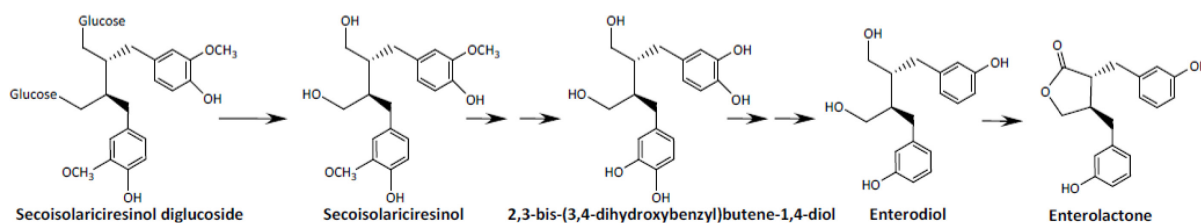


Figure 10. The main bacterial conversion steps of secoisolariciresinol-diglucoside to enterolactone (modified from Iglesias-Aguirre et al., 2021).

Besides enterolactone, enterodiol is a peculiar microbial catabolite of lignans. Nowadays, it is not clear if high and low producer phenotypes can be clearly discriminated also for enterodiol, but undoubtedly its production, as well as secoisolariciresinol metabolism, are affected by a high IIV, as shown by high reported CV% (*continuous production*) (Bolca et al., 2009; Frankenfeld, 2013; Mullens et al., 2022; Navarro et al., 2020). Whether high enterolactone producers are also high enterodiol and secoisolariciresinol producers is not clear yet.

Stilbenes

Regarding stilbenes, IIV has been reported for unchanged compounds, namely *trans*-resveratrol (3,5,4'-trihydroxy-*trans*-stilbene) and piceid (resveratrol-3-glucoside), and for

glucuronide derivatives of *trans*-resveratrol and gut microbiota-derived catabolites. *Trans*-resveratrol is produced by all subjects, but in different amounts, as indicated by high CV% (*continuous production*) (Almeida et al., 2009; Vaz-da-Silva et al., 2008; Velzen et al., 2014). At the same time, its glucuronide derivatives seem to be selectively produced only by specific subjects (Vitaglione et al., 2005). Regarding gut microbiota-derived catabolites, different routes of microbial *trans*-resveratrol conversion have been described: one leading to the production and excretion of dihydroresveratrol, another one producing lunularin, and a third one leading to a similar production of both catabolites (Bode et al., 2013). In detail, lunularin (3,4'-dihydroxybibenzyl) is a *trans*-resveratrol catabolite obtained by its reduction to dihydroresveratrol and subsequent dehydroxylation at the 5-position (Figure 11). Almost all lunularin producers also excrete 3,4'-dihydroxy-*trans*-stilbene.

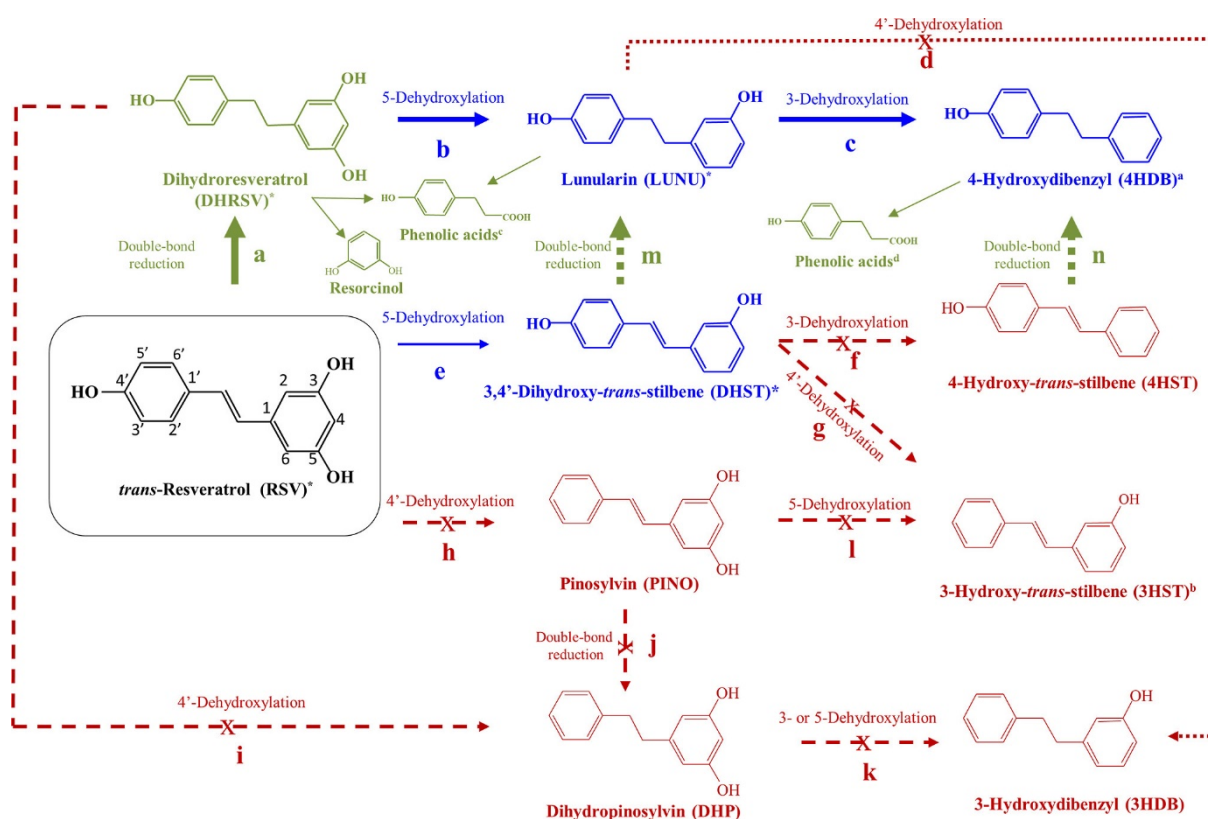


Figure 11. Proposed catabolism of resveratrol (RSV) by the human gut microbiota. Blue arrows and metabolites (LUNU, DHST, and 4HDB) are only present in LUNU-producers. Green arrows and metabolites (DHRSV, phenolic acid derivatives, resorcinol, and the double-bond reduction in **m** and **n** steps) are present in both metabolotypes. Red arrows and metabolites (PINO, DHP, 3HST, 4HST, and 3HDB) are not present *in vivo* or *in vitro*. Thicker arrows designate more favoured catabolic steps. Straight arrows refer to *in vivo* steps that were also confirmed *in vitro*. Dashed arrows refer to steps explored and confirmed *in vitro*. *Some phase-II conjugates were tentatively identified. ^aPhase-II 4HDB conjugates (glucuronides and sulfates) have been assumed. ^b3HST was discarded. ^cPhenolic acids: 3'- and 4'-(hydroxyphenyl)acetic acids, 3-(3'-hydroxyphenyl)propanoic, and 3-(4'-hydroxyphenyl)propanoic acids, 3- and 4-hydroxybenzoic acids, and resorcinol. ^dPhenolic acids: 4'-(hydroxyphenyl)acetic acid, 3-(4'-hydroxyphenyl)propanoic acid, and 4-hydroxybenzoic acid (from Iglesias-Aguirre et al., 2022).

Bode et al. (2013) found an association between dihydroresveratrol production and an intestinal microbiota enriched in *Slackia equolifaciens* and *Adlercreutzia equolifaciens*. Conversely, the non-production of dihydroresveratrol was associated with a greater presence of *Bacteroidetes*, *Actinobacteria*, *Verrucomicrobia* and *Cyanobacteria* (Bode et al., 2013). Iglesias-Aguirre et al. (2022) have recently described the consistent observation of two metabotypes associated with resveratrol catabolism: 195 healthy individuals have been clusterized as lunularin producers and non-producers (*cluster production*). In the studied population, 74.4% of individuals were lunularin producers and among them, high, medium and low producers were defined. In addition, lunularin producers selectively excreted two additional catabolites, namely 4-hydroxydibenzyl and 3,4'-dihydroxy-*trans*-stilbene, similarly to what Bode et al. (2013) previously observed. The lunularin non-producer metabotype was significantly more prevalent in females, but independent of individuals' BMI and age (Iglesias-Aguirre et al., 2022). These results are interesting and further research confirming lunularin metabotypes, the role of gut microbiota composition in its production, and the possible impact on health, are needed.

Other (poly)phenols

Concerning other (poly)phenol sub-classes, it is worth mentioning IIV in the metabolism of hop prenylflavonoids, particularly isoxanthohumol. Isoxanthohumol microbial catabolism allows the classification of individuals into poor, moderate and strong producers (*continuous production*) of 8-prenylnaringenin (Bolca et al., 2009; Bolca et al., 2007) (Figure 12a) These three quantitative metabotypes could be linked to differences in gut microbiota composition. Regarding IIV in alkylresorcinol and oleuropein metabolism, sex may influence it, based on available studies (Bock et al., 2013; Söderholm et al., 2009). In particular, for both classes, *continuous production* of catabolites was observed, with women presenting higher AUCs for the two main end products of alkylresorcinol catabolism, namely 3,5-dihydroxybenzoic acid and 3-(3',5'-dihydroxyphenyl)propanoic acid (Söderholm et al., 2009). On the contrary, men showed higher AUCs for conjugated metabolites of hydroxytyrosol after intake of an olive leaf extract, independently of the oleuropein dose and the administration format (de Bock et al., 2013). Last, avenanthramides, (poly)phenolic alkaloids representing the unique bioactive phytochemical signature of whole-grain oat, can be converted into dihydro-avenanthramides by the gut microbiota, but this transformation may not be completed in all individuals (*cluster*

production) since it depends on the presence of *Faecalibacterium prausnitzii* (Wang et al., 2021) (Figure 12b). This result was achieved in a small sample set (13 healthy subjects), but it was supported by both human fecal fermentations and gnotobiotic mice experiments (Wang et al., 2021).

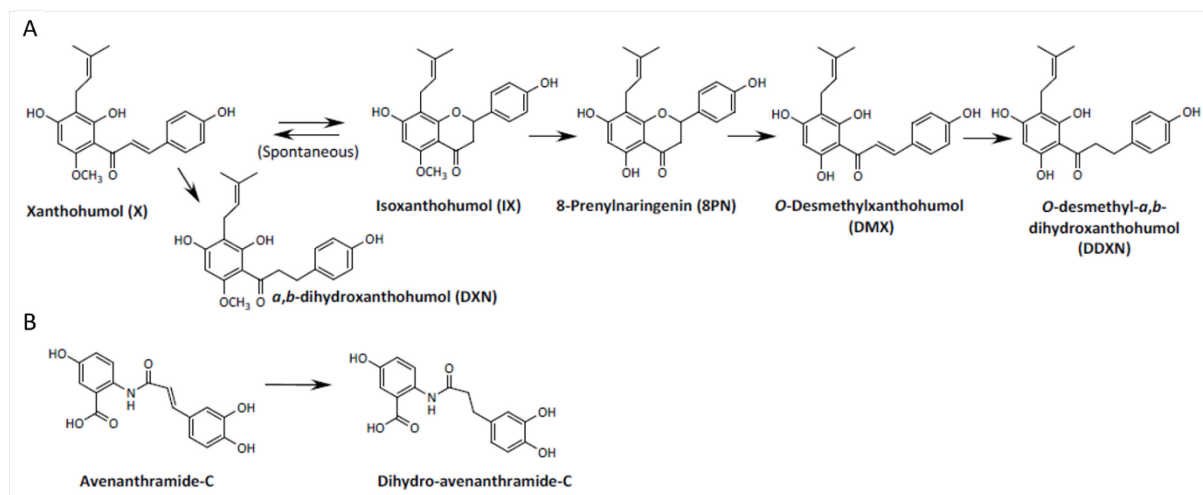


Figure 12. Gut microbiota conversion steps of (A) (iso)xanthohumol and (B) avenanthramide-C (modified from Iglesias-Aguirre et al., 2021).

1.3.3. Types and determinants of inter-individual variability in (poly)phenol ADME

Two major types of IIV in the bioavailability and metabolism of (poly)phenols have been observed: continuous or cluster-based metabolite production. The most common patterns of IIV are associated with *continuous production* of metabolites (all the subjects produce the same metabolites but in different amounts), where some groups of low, medium and/or high excretors can be identified using various criteria. These patterns have been reported for all flavonoids, phenolic acids, lignans, prenylflavonoids, alkylresorcinols, and hydroxytyrosol. *Cluster production* can be achieved discriminating subjects based on their different ability of producing or not specific metabolites (qualitative differences, producers vs. non-producers), as in the case of ellagitannins -urolithins-, isoflavones -equol and O-DMA-, resveratrol -lunularin-, or avenanthramides -dihydro-avenanthramides-, or based on quali-quantitative metabotypes (when subjects produce different proportions of the same metabolites), as defined for flavan-3-ols -PVLs and 3'-HPPA-, flavanones -ratio phenolic acids/phase II conjugates-, and isoflavones -daidzein, genistein and their colonic metabolites-. Nevertheless, we should be aware of the potential lack of relevance of these metabotypes in the future, as

they may depend on instrumental limits of detection, or the statistical approaches followed for a specific sample population.

Although inter-individual differences in the bioavailability and metabolism of phenolic compounds are frequently observed and reported, the factors underlying these differences are mainly undefined, particularly for some sub-classes of flavonoids and phenolic acids. An influence of genetic polymorphisms in phase I/II enzymes and transporters has been reported for flavanone metabolites (Bourian et al., 1999; Fraga et al., 2022; Nishioka et al., 2021), and suggested for flavan-3-ol metabolites (Anesi et al., 2019; Cortés-Martín et al., 2019; Mena et al., 2022), based on the observation of different excretion patterns among individuals between sulfate and glucuronide derivatives. In this latter case, different data pre-treatment methods proved fundamental to unravel inter-individual differences in the production of phase II flavan-3-ol metabolites (Mena et al., 2022). These results suggested that diverse pre-treatments, as well as clustering methods (density- versus distance-based), are fundamental to explore inter-individual differences and their potential causes (Mena et al., 2022).

Gut microbiota composition emerged as the primary determinant for IIV in the metabolism and bioavailability of isoflavones, ellagitannins, and lignans. The microbiota undoubtedly plays a key role also in the inter-person differences in the production of gut microbiota-derived catabolites of all other flavonoid classes, phenolic acids, stilbenes and other minor (poly)phenol sub-classes, but the specific bacteria and conditions responsible for the observed differences still have to be clarified. Faecal fermentation studies may be of help in classifying individuals according to the microbial metabolism of specific phenolics, as recently done for flavan-3-ol monomers and avenanthramides (Li et al., 2022; Liu et al., 2022; Wang et al., 2021). Sex, age, ethnicity, BMI and nutritional status, physical activity, dietary habits, and (patho)physiological status may condition the metabolism of some phenolic classes, but, in general, they do not seem to influence too much (poly)phenol metabolism. Data is scattered mainly for some classes of phenolics. For instance, for ellagitannins, age, sex, nutritional and health status have been described to be relevant sources of IIV in the production of urolithins (Cortés-Martín, Villalba, et al., 2018; González-Sarrías et al., 2017; Selma et al., 2018), while ethnicity may be involved in a different production of equol/O-DMA from isoflavones (Song et al., 2006). Sex may also drive the metabolism of flavanones (Hernández-Prieto, Fernández, et al., 2023; Hernández-Prieto, Garre, et al., 2023). Studies comparing different classes of

phenolic metabolites may help to better understand the drivers of the inter-person variation in (poly)phenol ADME. An example could be the work by Hidalgo-Liberona et al. (2020), who showed that a healthy intestinal barrier favours phenolic bioavailability compared to impaired intestinal permeability.

IIV is usually assessed in relation to individual phenolic sub-classes and hardly ever takes into account the whole spectrum of phenolic metabolites produced from a foodstuff or a diet, precluding the investigation of different patterns of (poly)phenol metabolism and limiting the possibility of drawing sound conclusions on its determinants. Studies that consider many (poly)phenol sub-classes and assess IIV in the production of their metabolites/catabolites, trying to define broader phenolic metabotypes and the determinants involved, are missing. Just a few examples have been published in the literature, focused on wine phenolic metabolites, where volunteers were clustered on the amount of phenolics excreted beyond the metabolic pathway of a single family of dietary (poly)phenols (Belda et al., 2021; Esteban-Fernández et al., 2020). Future studies, designed to comprehensively assess the metabolism of (poly)phenols, collecting as much information as possible about individuals (dietary and lifestyle habits, microbiota composition, genetics data, etc.) and considering the whole set of phenolic metabolites found in biofluids, may help to identify and define broader phenolic metabotypes and their determinants. The actual relevance of these phenolic metabotypes will then have to be determined through specific associations with health-related outcomes. For this purpose, the use of data-driven approaches trying to explain IIV can be very useful when dealing with complex datasets. In that sense, machine learning, like in the case of Hernández-Prieto and colleagues (Hernández-Prieto, Fernández, et al., 2023; Hernández-Prieto, Garre, et al., 2023), and other statistics or artificial intelligence approaches are of interest. They may be particularly relevant when dealing with data from large intervention trials with massive data collection. Lastly, data reporting is vital to properly demonstrating and handling IIV. Due to the lack of guidelines in the field, the COST Action POSITIVE provided some recommendations for better designing and reporting studies dealing with IIV (Nikolic et al., 2019). They also developed the “POSITIVE quality index”, a valid, reliable, and responsive score including 11 reporting criteria to assist the research community while describing IIV. The use of this index is quite anecdotal to date, but the adherence to it is encouraged to improve the quality of the evidence published.

1.4. Gut-mediated phenolic metabotypes in the context of personalized nutrition

(Poly)phenolic compounds and gut microbiome: new opportunities for personalized nutrition

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Abstract

For decades, (poly)phenols have been linked to cardiometabolic health, but population heterogeneity limits their apparent efficacy and the development of tailored, practical protocols in dietary interventions. This heterogeneity is likely determined by the existence of different metabotypes, sub-populations of individuals metabolizing some classes of (poly)phenols differently. The gut microbiota plays a major role in this process. The impact of microbiota-related phenolic metabotypes on cardiometabolic health is becoming evident, although the picture is still incomplete, and data are absent for some classes of (poly)phenols. The lack of a complete understanding of the main microbial actors involved in the process complicates the picture. Elucidation of the mechanisms behind phenolic metabotypes requires novel experimental designs that can dissect the inter-individual variability. This paper, in addition to providing an overview on the current state-of-the-art, proposes wider metabotyping approaches as a means of paving the way towards effective personalized nutrition with dietary (poly)phenols.

Keywords

(Poly)phenols, cardiometabolic health, metabotype, gut microbiota, personalized nutrition

1.4.1. Introduction

Modern societies struggle with the economic and social consequences of the rise in CMD, a cluster of metabolic dysfunctions (e.g., dyslipidemia, hypertension, insulin resistance, impaired glucose tolerance, etc.) that may lead to systemic impairments like CVD and type 2 diabetes (Kirk & Klein, 2009). Unhealthy dietary habits, including excessive consumption of free sugars, saturated fats, and sodium, and low intake of vegetables and fruits, among others, are recognized as key determinants of the onset of CMD, while the promotion of healthy diets represents a cornerstone for public health policies. Data suggest that more than 30% of all deaths could be prevented through dietary changes, particularly by increasing the consumption of plant-based foods (Mozaffarian et al., 2016; Rienks et al., 2017). Indeed, adequate nutrition has been established as the most important prevention factor in CMD (Casas et al., 2018), as healthy dietary patterns supply all the necessary nutrients for the human body and promote health. These benefits have been related to the direct action of these nutrients and the proliferation of a healthy microbiota in the human intestine (Conlon & Bird, 2015). This is relevant as several indirect factors are associated with cardiometabolic risk, namely lipid metabolism including cholesterol transport, SCFA production, and vascular inflammation, which are known to be mediated by the gut microbiota.

Extensive research is shedding light on the health benefits of plant-based diets and specific plant foods. Besides providing energy, fiber and essential micronutrients, plant-based foods are rich sources of a variety of phytochemicals with well-known health-promoting effects, the so-called plant bioactives (Liu, 2013). Some bioactive phytochemicals such as (poly)phenols, carotenoids, phytosterols, glucosinolates, alkaloids, thiosulfinates, and alkylresorcinols may promote health and well-being by preventing obesity, type 2 diabetes, and CVD (Bondonno et al., 2019; R. H. Liu, 2013; Rienks et al., 2017, 2018). Among them, (poly)phenols are the most diverse category of plant bioactive compounds, widespread in fruits, vegetables, cereals, and beverages including fruit juices, wine, coffee, beer, and tea. They constitute a heterogeneous family, and are classified into flavonoids [flavonols, flavan-3-ols (both monomers and PACs), flavanones, flavones, isoflavones, and anthocyanins, and non-flavonoids including phenolic acids, hydrolysable tannins, lignans, and stilbenes].

Growing evidence from clinical trials and cohort studies suggests that an increased intake of (poly)phenols may reduce the risk of CMD and obesity (Del Rio et al., 2013; Rodriguez-Mateos

et al., 2014; Tresserra-Rimbau et al., 2014). (Poly)phenol-rich diets reduce blood pressure and improve blood lipid profile, avoid endothelial dysfunction through a nitric-oxide mediated decrease of pro-inflammatory cytokines, inhibit platelet function and aggregation in blood vessels, prevent insulin resistance through pancreatic β -cells protection, control glycemic response lowering blood glucose and transport, and avoid low-grade inflammation, among other features (Curtis et al., 2019; Martínez-López et al., 2019). However, these beneficial effects may vary among individuals due to differences in gut microbiota composition and functionality, among other factors, defining the singularity of each human being.

1.4.2. (Poly)phenols, gut microbiota, and cardiometabolic diseases: a complex relationship

Despite the conspicuous body of evidence, the relationship between (poly)phenol intake and cardiometabolic prevention is enigmatic. A wide variability has been highlighted within and between studies, undermining the significance of the role of (poly)phenols in CMD prevention. Apart from the expected variability due to the administration procedure, the response to dietary (poly)phenols has been demonstrated to be heterogeneous among subjects and populations, leading to inconclusive results. Consequently, the role of (poly)phenols in cardiometabolic health promotion has not yet been demonstrated consistently (Cassidy & Minihane, 2017; Gibney et al., 2019). It is clear that the studies attempting to link (poly)phenols with the prevention of CMD have not taken all the relevant variables into account. One or more pieces of the puzzle are still missing.

(Poly)phenol bioavailability and metabolism: the role of gut microbiome

The heterogeneity observed in response to (poly)phenol consumption can be associated primarily with inter-individual differences in their bioavailability (Morand et al., 2020). These differences are attributed to several factors, including genetic background, gut microbiome, sex, age, medication, and lifestyle habits such as diet, smoking, and physical activity (Figure 13). There is, however, a growing body of evidence indicating that the gut microbiome plays a key role in (poly)phenol metabolism and absorption and is believed to underpin much of the inter-individual variation observed in their bioavailability (Morand et al., 2020).

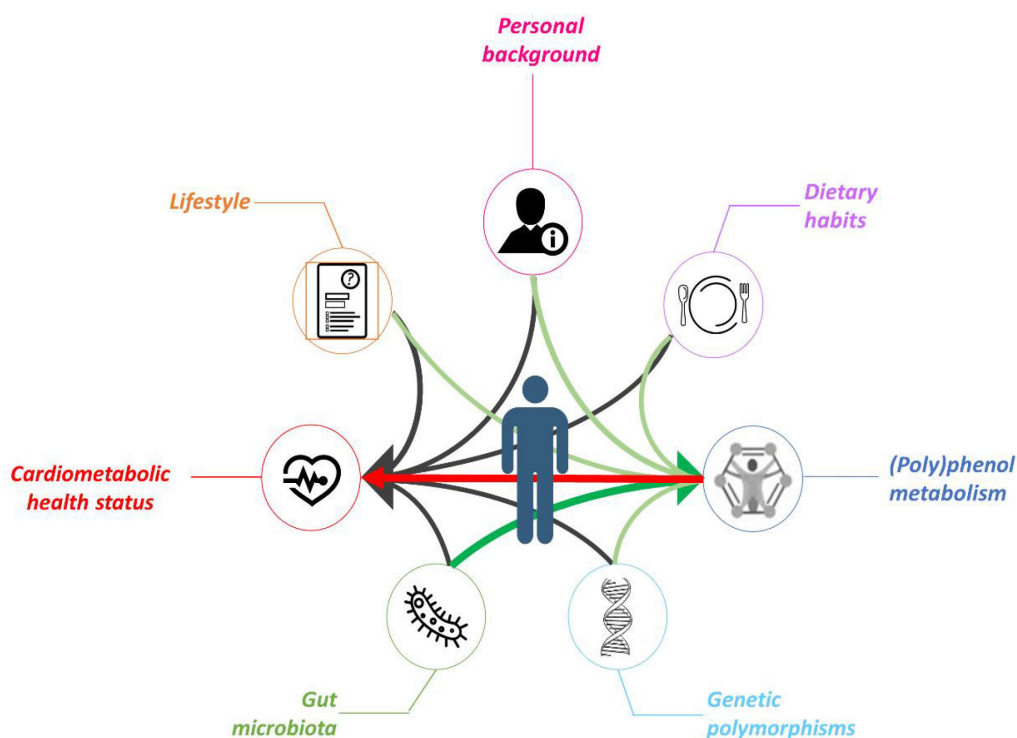


Figure 13. All the factors reported in this figure directly influence cardiometabolic health (black arrows). (Poly)phenol metabolism has an important role in cardiometabolic health (red arrow) and is influenced by other factors, especially the gut microbiota (green arrows).

Ingested (poly)phenols are present in foods mainly as glycosides and esterified forms, and while they can be absorbed to some extent in the upper gastrointestinal tract, substantial amounts nonetheless arrive at the colon, where they encounter the resident microbiota. The microbiota can cleave conjugated moieties releasing aglycones and perform ring-fission, dehydroxylation, and other reactions, transforming the native (poly)phenolic compounds into a plethora of low molecular weight catabolites that can be absorbed by colonocytes and subjected to phase II metabolism by colonocytes and hepatocytes before entering the systemic circulation. Some phenolics originating from microbial catabolism exhibit higher bioactivity than their parent compounds. This is the case with dihydroresveratrol (resveratrol catabolite) and equol (daidzein catabolite) (Bohn, 2014). However, the array of gut microbial metabolites is highly variable between individuals, due to the diversity of bacterial species and strains comprising the colonic microbiota.

The relationship between gut microbiota and (poly)phenols is bidirectional: gut microbiota is involved in the bioconversion of (poly)phenols, affecting their bioavailability, metabolism, and bioactivity, while phenolic compounds can positively modulate the colonic microbial composition and function. (Poly)phenols can affect the intestinal ecology and preserve the gut microbial balance exerting (i) a prebiotic effect by enhancing the growth and establishment of

probiotic bacterial families such as Bifidobacteriaceae and Lactobacillaceae, and (ii) an antimicrobial activity by inhibiting pathogenic bacteria such as *Escherichia coli*, *Clostridium perfringens*, *Clostridium histolyticum*, and *Helicobacter pylori* (Aravind et al., 2021). The concept of the “three P’s for gut health”, comprising probiotics, prebiotics, and (poly)phenols, has been proposed, and the classical concept of “prebiotics”, restricted to certain dietary carbohydrates, has been re-evaluated (Espín et al., 2017).

A “prebiotic-like” effect has been reported for some phenolic compounds and some (poly)phenol-derived gut microbial metabolites using both *in vitro* assays with human gut microbiota and *in vivo* preclinical studies and clinical trials, with phenolic compounds and (poly)phenol-rich foods such as grape seed, wine, cocoa, cranberry, green tea, blueberry, nuts, and pomegranate (Espín et al., 2017). (Poly)phenols can modify the gut microbiota: (i) favouring specific gut microorganisms that may provide health benefits to the host, such as *Akkermansia muciniphila*, *Faecalibacterium prausnitzii*, *Enterococcus*, *Prevotella*, *Roseburia*, and *Bacteroides* spp.; and (ii) reducing the *Firmicutes/Bacteroidetes* ratio, which is higher in obese and metabolic syndrome subjects (Cardona et al., 2013). A recent study shows that (poly)phenol intake during early life in mice promotes *Akkermansia* growth and host colonic barrier improvement in association with an increase in *Lactobacillus*-secreted lactate, suggesting that (poly)phenol consumption may be beneficial also in early life (Lu et al., 2021).

Handling population-wise variability in (poly)phenol metabolism: metabolotypes

Since (poly)phenols are extensively metabolized by the gut microbiota, inter-individual differences in bacteria-converting species can contribute to inter-person variations. This results in an extensive variability in the concentration of phenolic metabolites in circulation, as has been demonstrated for: (i) flavanones, with the production of several phenolic acids (Vallejo et al., 2010); (ii) hop prenylflavonoids, with the production of 8-prenylnaringenin (Bolca et al., 2009); (iii) lignans, with the production of enterolactone (Rowland et al., 2000); and (iv) flavan-3-ols, with the production of PVLs and 3'-HPPAs (Mena et al., 2018). Phenotypical differences can also be related to the selective production of specific metabolites, like equol (soy isoflavone daidzein derivative), dihydro-avenanthramides (oat avenanthramides derivatives), or urolithins (ellagic acid/ellagitannin derivatives) (González-Sarriás, García-Villalba, et al., 2017; Setchell & Clerici, 2010; Turrini et al., 2019; Wang et al., 2021).

The different production patterns of gut microbiota-derived (poly)phenol metabolites can be related to the existence of metabolic phenotypes (namely “metabotypes”) in the population. According to earlier research (Espín et al., 2017), the term “gut metabotype” refers to a metabolic phenotype defined by: (i) the existence of specific gut microbial metabolites characterizing the metabolism of the parent phenolic compound; and (ii) the associated gut microbiota in terms of composition and activity. This definition, based on a qualitative criterion (binary response of production versus non-production), is of interest and fits well some metabolites (e.g., equol, dihydro-avenanthramides, and urolithins). However, it may be restrictive when addressing the inter-individual variation in the metabolism of most phenolics, characterized by production gradients. A broader meaning, based on a quali-quantitative criterion, has been proposed by our research group when considering the metabolism of green tea and cranberry flavan-3-ols (Mena et al., 2018, 2022). This approach for “phenolic metabotypes” is based on the metabotype concept commonly accepted within the nutrition field as “subgroups of individuals sharing the same metabolic profile” (Everett et al., 2019). Undeniably, a wide concept of gut-mediated phenolic metabotypes may also assist in addressing the inter-individual variability in the production of (poly)phenol-derived gut microbial metabolites when the subjects produce all or most of the phenolic metabolites of a catabolic pathway, but in different proportions, as it happens for flavan-3-ols and other classes of dietary (poly)phenols (Bolca et al., 2007; Mena et al., 2018, 2022; Rowland et al., 2000; Stevens et al., 2019) (Figure 14).

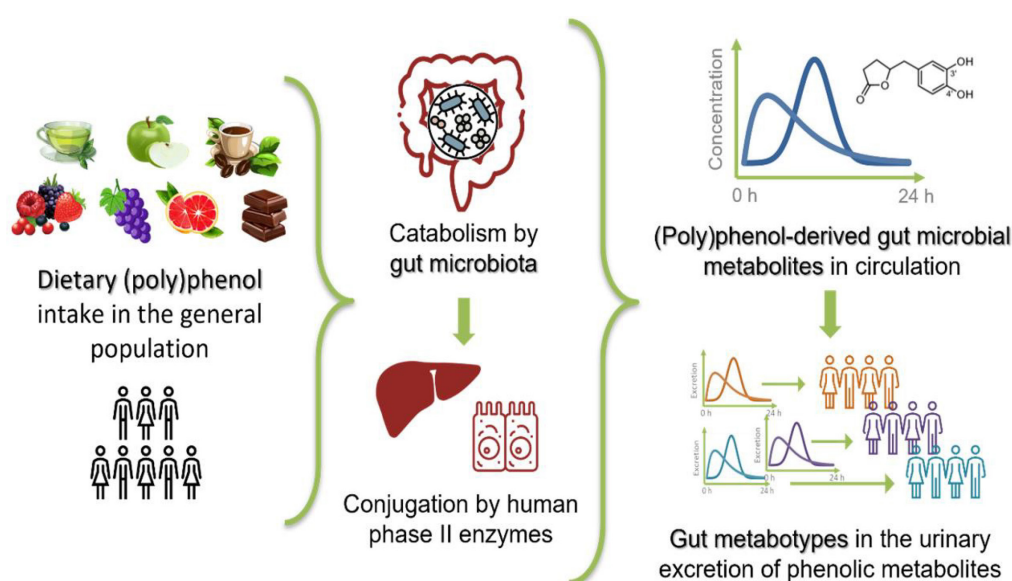


Figure 14. (Poly)phenol intake and metabolism in the general population and metabotype identification according to the excretion of gut microbial metabolites.

The stratification of individuals according to their qualitative phenolic profile has been assessed for the metabolism of ellagitannins/ellagic acid and isoflavones. Subjects can be classified into three urolithin metabotypes (UMs) according to the excretion of urolithins after consumption of ellagitannins or ellagic acid-rich foods, mainly walnuts and pomegranate, but also berries including raspberries and strawberries. UM-A is characterized by the excretion of Uro-A and its phase II conjugates. UM-B produces conjugates of IsoUro-A and Uro-B in addition to Uro-A, while UM-0 does not produce Uro-A, IsoUro-A, or Uro-B derivatives (Tomás-Barberán et al., 2017). UM-0 shows lower microbiome diversity and richness than UM-A and UM-B. A study has reported that 3-day walnut consumption already modulates the gut microbiota in a UM-dependent manner and boosts the production of SCFAs (García-Mantrana et al., 2019). So far, two urolithin-producing species, *Gordonibacter urolithinifaciens* and *G. pamelaee*, belonging to the Coriobacteriaceae family, have been isolated from human feces. The Coriobacteriia class is increased in UM-B versus UM-A and UM-0 and is positively associated with BMI and cardiometabolic risk factors (Iglesias-Aguirre et al., 2021).

Regarding the metabolism of soy isoflavone daidzein, equol-producing and *O*-DMA-producing metabotypes have been identified. These metabotypes are unrelated; the capacity to harbour equol-producing bacteria is not associated with the capacity to favour *O*-DMA-producing bacteria. Caucasian and Asian populations display a distinct distribution of these metabotypes, and this difference cannot be shortened by regular consumption of high-isoflavone-containing products (Cortés-Martín et al., 2020). The equol metabotypes show a different abundance of equol-producing bacteria and a different microbial composition (Iglesias-Aguirre et al., 2021). More than ten gut microbes are involved in equol production, including *Slackia equolifaciens*, *Slackia isoflavoniconvertens*, and *Adlercreutzia equolifaciens*. Reports regarding gut microbes able to produce *O*-DMA are less abundant.

Metabotyping has also been attempted for flavan-3-ols, the main dietary (poly)phenols. However, the picture here requires a comprehensive metabotype concept that can handle the heterogeneity of structures characterizing this subclass of flavonoids and the complexity of their catabolic pathways. Putative metabotypes have been proposed *in vivo* based on a different quali-quantitative urinary production of PVLs and 3'-HPPAs after consumption of (epi)gallocatechin-rich green tea. Three metabotypes have been identified: metabotype 1 characterized by high excretion of triHPVLs and diHPVLs and reduced excretion of 3'-HPPAs,

metabotype 2 distinguished by a medium excretion of diHPVLs and a limited excretion of triHPVLs and 3'-HPPAs, and metabotype 3 characterized by limited production of PVLs and high amounts of 3'-HPPAs (Mena et al., 2018). Further studies reported a similar picture, although the precursor flavan-3-ols were different from those in green tea; low and high producers of flavan-3-ols colonic metabolites have been observed in two different studies, one performed *in vitro* fermenting pure (-)-epicatechin and the second one *in vivo* administering cranberry products (Liu et al., 2022; Mena et al., 2022). In the *in vitro* study, anaerobic incubations of (-)-epicatechin with fecal inocula from 24 healthy individuals revealed different catabolic patterns, with some individuals able to convert 5-(3',4'-dihydroxyphenyl)- γ -valerolactone into 5-(3'-hydroxyphenyl)- γ -valerolactone and 3'-HPPA at a faster rate compared to others (Liu et al., 2022). This result was also reflected in the urinary excretion of flavan-3-ol colonic metabolites in different human studies with cranberry products (Mena et al., 2022), as can be observed in the Loading Plot of the Principal Component Analysis (PCA) presented in Figure 15.

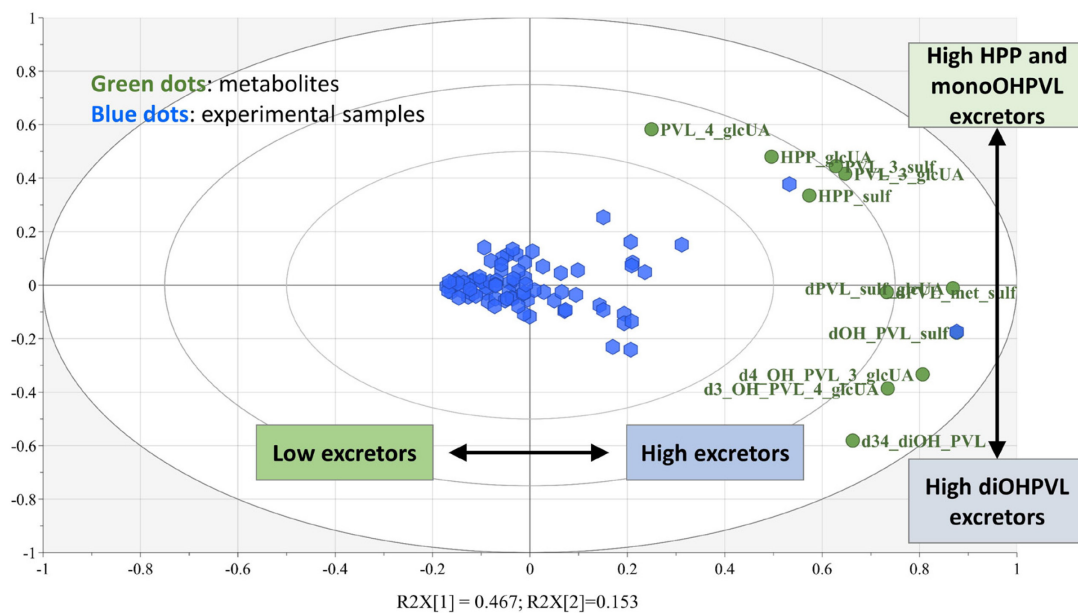


Figure 15. Different profiles in the urinary excretion of flavan-3-ol colonic metabolites after consumption of cranberry. PCA figure modified from Mena et al. (2021). Abbreviations: PVL_3_sulf: 5-phenyl- γ -valerolactone-3'-sulfate, PVL_3_glcUA: 5-phenyl- γ -valerolactone-3'-glucuronide; PVL_4_glcUA: 5-phenyl- γ -valerolactone-4'-glucuronide; d34_diOH_PVL: 5-(3',4'-dihydroxyphenyl)- γ -valerolactone; dOH_PVL_sulf: (hydroxyphenyl)- γ -valerolactone-sulfate (3',4' isomers); d4_OH_PVL_3_glcUA: 5-(4'-hydroxyphenyl)- γ -valerolactone-3'-glucuronide; d3_OH_PVL_4_glcUA: 5-(3'-hydroxyphenyl)- γ -valerolactone-4'-glucuronide; dPVL_met_sulf: 5-phenyl- γ -valerolactone-methoxy-sulfate isomer (3',4'); dPVL_sulf_glc: 5-phenyl- γ -valerolactone-sulfate-glucuronide isomer (3',4'); HPP_sulf: 3-(phenyl)propanoic acid-sulfate; HPP_glcUA: 3-(phenyl)propanoic acid-glucuronide, diOHPVL = 5-(3',4'-dihydroxyphenyl)- γ -valerolactone derivatives; monoOHPVL: 5-(hydroxyphenyl)- γ -valerolactone derivatives, both 3' and 4' isomers; HPP: 3-(hydroxyphenyl)propanoic acid derivatives.

Nevertheless, despite the robustness of the evidence for colonic metabolotypes of flavan-3-ols, further ad hoc confirmatory research is needed to consolidate this result and fully understand

what gut microbiota species lie behind these differences and which may be their health-related consequences. Unfortunately, information on specific bacterial strains responsible for the catabolism of flavan-3-ols into PVLs and low molecular weight phenolics is very scarce. *Lactobacillus plantarum* IFPL935, *Eggerthella lenta*, *Adlercreutzia equolifaciens*, and *Flavonifractor plautii* are currently the only bacteria identified as being able to convert flavan-3-ols into 5-(3',4'-dihydroxyphenyl)- γ -valerolactone and 5-(3'-hydroxyphenyl)- γ -valerolactone (Kutschera et al., 2011; Sánchez-Patán et al., 2012; Takagaki & Nanjo, 2013). Moreover, no microorganisms responsible for further conversion into PVAs and PPAs have yet been identified.

Metabotypes based on a quantitative criterion have been reported for the microbiota-derived metabolites of flavanones, hop prenylflavonoids and lignans. Indeed, it has been observed that among the human population, three different metabotypes arise, and they can be classified in three quantitative metabotypes: poor, moderate, and strong producers of 8-prenylnaringenin (for isoxanthohumol) and enterolactone and enterodiols (for lignans) (Bolca et al., 2007; Senizza et al., 2020; Vallejo et al., 2010).

Some studies have explored the possibility of inducing a switch in the metabotypes by increasing the intake of specific (poly)phenols. Long-term intake of isoflavones increases the level of equol production in equol producers, likely due to a metabolic adaptation of the gut microbiota. Nonetheless, boosting the ability to produce equol in non-equol producers has shown to be impractical. A study found that long-term intake of a high dose of isoflavones did not result in such a change (Védrine et al., 2006). In contrast, another, albeit low-powered study, reported that three among five non-equol producer women acquired the ability to produce equol after two weeks of soymilk consumption (Lu et al., 1996). An explanation for these contrasting results might be that the microbial species involved in equol production differ among individuals, while the absence of equol-producing bacteria in the colon impairs the capability to produce equol. The percentage of equol producers has been estimated to be around 30% and 50%-60% in the Caucasian and Asian populations, respectively, suggesting that equol production is linked to ethnic background, which covers genetic background, gut microbiota composition, and dietary habits, including long-term isoflavone consumption (Iglesias-Aguirre et al., 2021).

Regarding UMs, evidence suggests that some individuals included in UM-0 can be converted to UM-A or UM-B producers after a long-term/high-dose intake of ellagitannins and ellagic acid, the so-called “UM-0 responders” (González-Sarrías et al., 2017). Moreover, the distribution of UM-A and UM-B is critically affected by aging, while the proportions of individuals within UM-0 (about 10% of the total) remain constant from 5 to 90 years of age. At an early age, the proportion of UM-A accounts for about 85% of the population, progressively decreasing to 55% from 40 to 90 years of age, concomitant with an increment of UM-B from 15% up to 45%. The conversion from UM-A to UM-B happens mostly between 25 to 35 years of age; thereafter, the proportion of UM-A and UM-B (55% and 45%, respectively) remains more or less constant (Iglesias-Aguirre et al., 2021).

Impact of metabolotypes on cardiometabolic health

To date, the possible effects of (poly)phenols on individual human health have not been fully identified. These could be due to: (i) the metabolites deriving from gut microbiota catabolism and/or phase II conjugation; and/or (ii) the gut microbial ecology associated with (poly)phenol metabolism, among other factors (Iglesias-Aguirre et al., 2021). Moreover, recent evidence indicates that the gut microbiota, independently and/or interactively with dietary intake, is a target for reducing CVD risk through its effects on cardiometabolic risk factors. Dietary (poly)phenols may be a source for these interactive effects, and clustering of subjects into known metabolotypes (i.e., metabotyping) could be a strategy to explain, at least in part, the different responses of individuals to (poly)phenol intake.

There is evidence of the relationship between cardiovascular risk and phenotypes in the metabolization of ellagitannins and isoflavones, paving the way towards using metabolotypes as cardiometabolic risk biomarkers and biomarkers of the potential (poly)phenol health effects mediated through gut microbiota (Espín et al., 2017). In the case of ellagitannins, UM-A seems to be a protective metabolotype, while UM-B seems to be a dysbiotic-prone metabolotype associated with cardiometabolic impairments. The UM explains individual differences not only in baseline cardiovascular risk but also in the cardioprotective effect upon pomegranate consumption (González-Sarrías et al., 2017). In particular, a study described an improvement in cardiometabolic biomarkers after pomegranate consumption only in overweight-obese subjects belonging to UM-B, suggesting a differential response to ellagitannin/ellagic acid intake according to the metabolic phenotype (González-Sarrías et al., 2017).

Regarding isoflavones, an increasing number of studies associate daidzein-related metabolites with cardiovascular risk, suggesting that equol and/or *O*-DMA producers may have a lower risk than nonproducers (Espín et al., 2017; Frankenfeld, 2017). Acute vascular benefits have been reported after administering synthetic equol only to men with an equol-producer metabolite, suggesting that the microbial ecology associated with the equol-producing capacity is crucial for determining health benefits (Hazim et al., 2016). In contrast, another study described a significant improvement of cardiometabolic risk biomarkers after chronic daily oral intake of *S*-equol, particularly in equol non-producer females (Usui et al., 2013). Overall, it is unclear whether the presence of specific microbial communities associated with equol and/or *O*-DMA production is involved in the effects exerted by these gut metabolites. Therefore, more intervention studies that stratify individuals according to their metabolite are necessary to explain the effects exerted by dietary isoflavones and assess if these protective effects are produced by the metabolites associated or not with the gut microbiota.

1.4.3. The need of metabolotyping to pursue personalized nutrition

Recommendations promoted at a population level in a “one-size-fits-all approach” for plant-based food consumption do not ensure that everyone is adequately exposed to and benefits from the protective features of plant bioactives. Evidence indicates that personalized nutritional recommendations may result in improved dietary behaviors. In this context, metabolotyping (grouping individuals with comparable metabolic/phenotypic profiles) could pave the way forward in terms of personalized and targeted nutrition, improving health at a population level (Hillesheim & Brennan, 2020). Using metabolites to identify subgroups responding differentially to dietary interventions and to examine associations with cardiometabolic risk factors is turning into reality. The use of metabolites in longitudinal studies demonstrates that they can be associated with cardiometabolic risk factors, whereas application in clinical studies serves to identify metabolites with differential responses (Hillesheim & Brennan, 2020).

Metabolotyping may also be a key success factor in personalized approaches when considering (poly)phenols, providing tailor-made nutritional recommendations to lower disease risks. This concept mirrors the idea of precision medicine already developed to treat human diseases. A conspicuous corpus of literature indicates that there exists a wide inter-individual variability

influencing the response of patients to treatments (including drugs) and prevention strategies, mostly due to genetic and environmental factors. Therefore, there is a need to develop a precision medicine able to take into account this variability and to suggest a subject-specific approach to improve the benefits and reduce the harm of treatments (Ashley, 2016). Similarly, individuals' stratification according to their (poly)phenol-producer metabotypes is necessary for intervention trials as specific metabotypes may produce the metabolites responsible for differential health effects. (Poly)phenol-metabolizing phenotypes can reflect different gut microbiota composition and metabolic status and could be biomarkers of health effects of (poly)phenols grounded on differential responses. Therefore, the potential application of metabotyping may be the evaluation of (poly)phenol effect on cardiometabolic health, while considering factors of inter-individual variability. Nonetheless, some points need to be addressed to guide the provision of effective strategies for the prevention of CMD:

1. Approaches focused on individual classes of (poly)phenols and their related phenolic metabolites are reductionist. Although it is important to attain fundamental knowledge and develop the field of study, these restricted approaches are hardly translated into effective nutritional guidelines.
2. Metabotyping studies are both challenging and time-consuming as they require screening of participants and targeted recruitment for a particular phenotypic characteristic. Moreover, they must guarantee that they are adequately powered to evaluate the effect of dietary (poly)phenols both within and between specific population groups.
3. Although metabotyping is key for targeted nutrition of population subgroups, sound tailored dietary advice with (poly)phenol-containing foods requires a more holistic view, including individual characteristics (precision nutrition).
4. The potential of metabotypes to predict cardiometabolic risk factors is currently promising. Beyond classical group analysis (metabotype x response), machine-learning algorithms can integrate multiple individual datasets to develop robust prediction models for the response to a dietary intervention.
5. Studies on the metabotype-wise mechanisms of action are completely lacking. In this sense, a must-do step would be to deploy omics studies to correlate the (poly)phenol effects on CMD

to specific markers of effect in each metabotype. This would help the comprehension of the biochemical basis underlying personalized nutrition.

Overcoming these five limitations with appropriate study designs and analytical approaches would lead to conceptual and methodological progress. In this sense, the development and application of novel approaches may drive the success of strategies addressed to improve cardiometabolic health at an individual level based on the differential response to dietary (poly)phenols. Future knowledge of phenolic metabotypes will favour a better understanding of the gut microbiome and its interactions with host health, leading to new opportunities for personalized nutrition in the framework of plant-based diets. Furthermore, a comprehensive understanding of the role of specific gut-derived metabolites and gut microorganisms can lead to the development of preventive/therapeutic strategies that, according to the principles of precision medicine, could be applied in tailored interventions to increment or reduce the bioavailability of such metabolites or drive gut microbiota composition in specific populations, aiming to improve their cardiometabolic health.

Authors' contributions

Made substantial contributions to conception and design of the study: Mena P, Del Rio D

Made substantial contribution to manuscript organization and writing: Narduzzi L, Mena P

Contributed equally to the design of the figures: Narduzzi L, Tosi N, and Mignogna C.

Made substantial contribution to the description of the metabotypes: Favari C, Tosi N, Mena P

Contributed to describe the role of gut microbiome: Tosi N, Mignogna C, and Agullo V.

Critically revised the manuscript: Agullo V, Favari C, Narduzzi L, Crozier A, Del Rio D, Mena P

Availability of data and materials

Not applicable.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Chapter 2. Aim of the thesis

The aim of this Doctoral Thesis was to investigate the variability in (poly)phenol bioavailability and metabolism applying multiple approaches, to gain new and further insights in the field of nutrition research focused on dietary bioactive phenolics. Exploring the heterogeneity in (poly)phenol ADME is key to unveiling the health-promoting activity of phenolic metabolites associated with the consumption of (poly)phenol-rich foods.

Different sources of phenolic compounds were considered: pure compounds, food extracts, freeze-dried powders, and (poly)phenol-rich foods/beverages. Moreover, two distinct factors contributing to the variation in (poly)phenol bioavailability and metabolism were analyzed: (1) endogenous/host-related factors, including genetic background and gut microbiota, and (2) chemical/structural properties, namely the native compound structure, which could affect the bioaccessibility and the colonic microbial catabolism.

Chapter 3. General methodology

Four different approaches were applied to achieve the objective previously stated in this dissertation: the first three approaches are experimental designs based on targeted metabolomics, while the fourth is a systematic review (Figure 16).

(1) An *in vitro* study using a human fermentation model:

*Optimization of a protocol for the *in vitro* colonic fermentation using flavan-3-ols*

A protocol of *in vitro* colonic fermentation was optimized to evaluate the influence of the flavan-3-ol structure on the production of phenolic catabolites from a qualitative, quantitative and kinetic point of view. Three pure compounds were used: (–)-epicatechin and procyanidin dimers B2 and A2. The protocol optimization was achieved by extending the incubation time until 48 hours and monitoring incubation conditions, namely pH and faecal microbiota composition. Both upstream and downstream catabolites were identified and quantified through ultra-high performance liquid chromatography coupled with tandem mass spectrometry (uHPLC-MS/MS).

(2) An acute human intervention study:

Influence of the flavan-3-ol structure on the quali-quantitative production of phenolic metabolites after acute consumption of different plant extracts by healthy subjects

An acute, randomized, double-blind, cross-over intervention trial was carried out to evaluate the influence of the flavan-3-ol structure on the production of phenolic metabolites from a quali-quantitative point of view. Three different plant extracts rich in flavan-3-ols were consumed by healthy subjects: green tea extract, grape seed extract with high monomeric content, and grape seed extract with high procyanidin content. Urine samples (24-hour and 48-hour urine) were collected and analyzed to identify and quantify flavan-3-ol metabolites, both SRFMs and RFMs, through uHPLC-MS/MS.

(3) A chronic human intervention study:

Unravelling phenolic metabotypes in the frame of the COMBAT study, a randomized, controlled trial with cranberry supplementation

Plasma and spot urine samples from a 12-week randomized, double-blind, placebo-controlled parallel intervention trial were analyzed through uHPLC-MS/MS to investigate inter-group (treatment-driven) and inter-individual (mainly gut microbiota-driven) differences in the levels of phenolic metabolites. The existence of urinary metabotypes related to dietary

(poly)phenols was also explored. The intervention consisted of the intake of freeze-dried cranberry powder in healthy older adults. Both SRFMs and RFMs were monitored, and a metabotyping approach based on PCA clusters was applied to handle the complexity of (poly)phenol metabolism in free-living conditions.

(4) A systematic review of human studies:

The impact of genetic variations on the absorption, distribution, metabolism, and excretion of dietary (poly)phenols: a critical systematic review

A critical systematic review of human studies was carried out to evaluate the influence of genetic variations on the variability in the bioavailability and metabolism of dietary (poly)phenols. In particular, the association between single nucleotide polymorphisms (SNPs) in genes involved in (poly)phenol ADME and the inter-individual variability in the concentration/amount of (poly)phenol metabolites in plasma or urine was analyzed. Both experimental studies investigating dietary interventions with (poly)phenols, administered as pure compounds, extracts, powders or foods, and observational studies dealing with exposure to dietary (poly)phenols were considered.

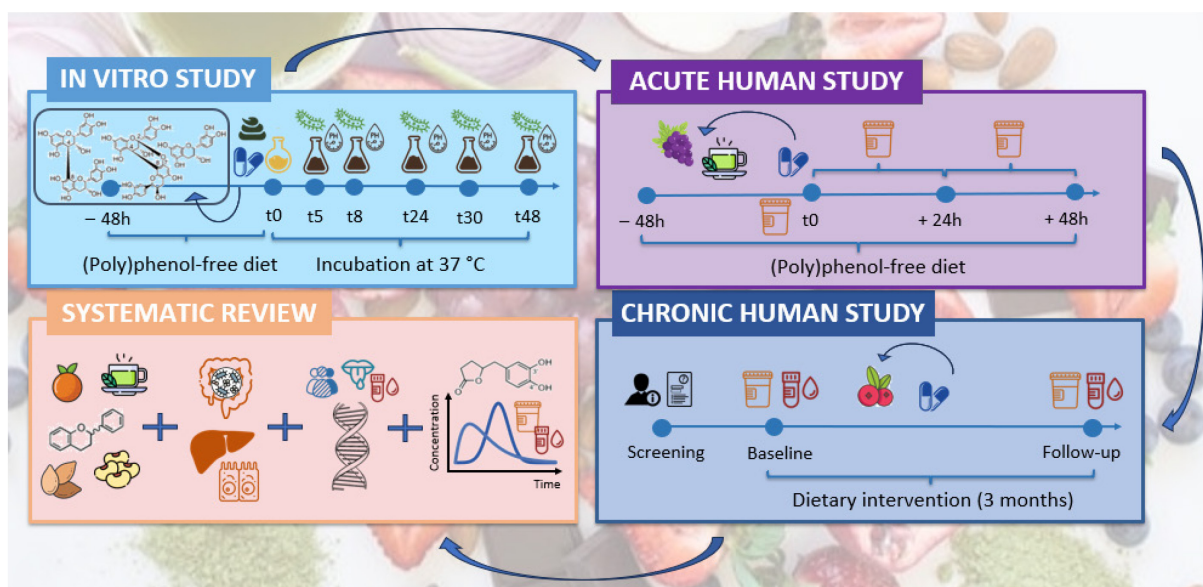


Figure 16. Approaches used in this Doctoral Thesis to investigate the variability in (poly)phenol bioavailability and metabolism.

Chapter 4. Selected studies

4.1. An *in vitro* study using a human fermentation model

Optimization of a protocol for the in vitro colonic fermentation using flavan-3-ols

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Abstract

Scope: This study aims to optimize a validated protocol of *in vitro* faecal fermentation of flavan-3-ols through the extension of the incubation time until 48 h and the monitoring of fermentation conditions (pH and faecal microbiota composition).

Results: Three flavan-3-ols, (–)-epicatechin (EC) and procyanidin (PC) dimers B2 and A2, were fermented *in vitro* for 48 h using human faecal microbiota. A total of 14 catabolites belonging to the gut microbiota degradation pathway of flavan-3-ols were quantified in 5 different incubation times. EC had the highest molar mass recovery, followed by PC_B2, which showed the richest catabolic profile, being qualitatively similar to EC. The substrates were characterized by different catabolic rates. These results confirmed that flavan-3-ol polymerization and the addition of an ether linkage in A-type PCs increase the steric hindrance and stiffness of the molecule, with a reduction of bioaccessibility and a delayed catabolism. The extension of fermentation time until 48 h and the monitoring of 5 different incubation times resulted in a more comprehensive view on flavan-3-ol kinetics and allowed to quantify small phenolic acids. The monitoring of the fermentation conditions confirmed the stability of the incubation environment, with a pH around 5-6 and a balanced microbial composition. This composition exhibited only minor fluctuations in terms of relative abundance, determined by the incubation time and not depending on the fermented substrate.

Conclusion: The optimization of the *in vitro* fermentation protocol confirmed the effect of flavan-3-ol structure on microbial catabolism from a quantitative, qualitative and kinetic point of view. This study opens new perspectives to further elucidate the gut microbiota catabolism of flavan-3-ols, following the production and kinetics of both upstream and downstream catabolites, and monitoring environment parameters such as pH and microbial composition.

Keywords

Flavan-3-ols, *in vitro* fermentation, gut microbiota, colonic catabolism

4.1.1. Introduction

Flavan-3-ols are the most consumed and ubiquitous flavonoids, being dark chocolate, green tea, nuts, and some fruits the principal sources in the Western diet [1,2]. Flavan-3-ols are the most complex class of flavonoids from a structural point of view and are the only flavonoids that occur in nature as aglycones [3,4]. They can be in a monomeric form or in an oligo/polymeric form, the latter being named proanthocyanidins (PACs) or condensed tannins. The monomeric structure is characterized by two aromatic rings (A- and B-ring) and by a pyran ring (C-ring) condensed with the A-ring. C-ring is hydroxylated in position 3 and can be esterified in that position with gallic acid, giving origin to galloylated flavan-3-ols. A-ring presents two hydroxyl (-OH) groups in positions 5 and 7, while hydroxylation of B-ring varies giving origin to different types of monomers and PACs: (1) (epi)afzelechins, which have one -OH group in position 4', and their polymerization produces propelargonidins; (2) (epi)catechins, presenting two -OH groups in positions 3' and 4', giving origin to procyanidins (PCs); (3) (epi)gallocatechins, characterized by three -OH groups in positions 3', 4' and 5', respectively, and giving rise to prodelphinidins. (Epi)catechins and PCs are the most common flavonoids in the diet, while (epi)gallocatechins characterize green tea [3–5]. PCs are divided in two groups according to the number of linkages between monomeric units: B-type PCs are characterized by C4–C8 or C4–C6 linkage, while A-type PCs have an additional ether linkage between the C2 of a monomer and the oxygen-bearing C5 or C7 in the A-ring of the adjacent unit [5]. While B-type PCs are present in different foods such as cocoa, fruits, nuts, and legumes, A-type PCs are less common and occur mainly in American cranberries [3–5].

After ingestion, only a minor part of monomeric flavan-3-ols (30% of (epi)catechins and 10% of (epi)gallocatechins) and traces of PACs are absorbed in the upper gastrointestinal tract. Unabsorbed flavan-3-ols reach the colon, where they are extensively catabolized by the gut microbiota, yielding a plethora of small microbial catabolites, which are finally absorbed and conjugated by phase II human enzymes in colonocytes and hepatocytes [3–5]. The catabolic pathway of B-type PCs can follow four different routes: (1) interflavan cleavage, releasing (epi)catechin monomers, (2) degradation of the lower unit, giving origin to phenyl- γ -valerolactones (PVLs), (3) degradation of the upper unit, yielding 3',4'-dihydroxyphenylacetic acids, and (4) simultaneous degradation of the upper and lower units, yielding 5-(2',4'-dihydroxyphenyl)-2-ene-valeric acid [5]. Monomeric units undergo C-ring opening, giving

origin to diphenylpropan-2-ol derivatives (DPPOLs), and A-ring fission, yielding PVLs, which can be hydrolyzed and converted into phenylvaleric acids (PVAs). DPPOLs, PVAs and PVLs are peculiar flavan-3-ol catabolites, and the last two are known as 5C-RFCs (ring fission catabolites with a 5-carbon chain) [5]. Finally, 5C-RFCs can be converted into small microbial catabolites through different steps of β - and/or α - oxidation of the side chain: (1) 3C-RFCs, namely phenylpropanoic acids (PPAs), (2) 2C-RFCs, namely phenylacetic acids (PAAs), (3) 1C-RFCs, including benzoic acids (BAs) and benzaldehydes (BALs), originated by the reduction of BAs. Finally, benzene derivatives (BZs) can originate from the decarboxylation of BAs.

Consumption of flavan-3-ols has been associated with risk reduction of several chronic diseases, including cardiometabolic and neurodegenerative diseases [6–9]. Microbial catabolites and their phase II conjugates are the circulating molecules that can potentially have beneficial effects on different cellular and tissue targets [10–13]. The native structure of flavan-3-ols, including the degree of polymerization and the type of subunit linkages, can influence the type and amount of gut microbiota catabolites, having an impact on health effects [14]. Polymerization of flavan-3-ols increases the steric hindrance of the molecule, limiting the access to the C-ring by the gut microbiota, with a reduction of bioaccessibility and a delayed catabolism [14–17]. A reduction of microbial catabolism also occurs with A-type PACs with respect to B-type, since the additional ether linkage makes the interflavan linkage stiffer [5,14]. Other factors influencing (poly)phenol degradation at colonic level are some physiological properties of the colonic environment, such as pH, bile salts, volume of liquid, and the composition and activity of the gut microbiota [18–21].

Information on bacterial strains and enzymes involved in the bioconversion of flavan-3-ols is very limited. Production of PVLs was positively correlated with members of Clostridia and Actinobacteria, and the genus *Propionibacterium* [5]. The relationship between (poly)phenols, in particular flavan-3-ols, and gut microbiota is bidirectional: (poly)phenols can modulate the activity and composition of the gut microbiota, and the gut microbiota can catabolize them influencing their bioavailability and bioefficacy [22]. In particular, (poly)phenols have shown antimicrobial properties against potential pathogens (*Clostridium perfringens*, *Clostridium difficile*, and certain Gram-negative *Bacteroides*) and probiotic effects, up-regulating some commensal and beneficial bacteria [23–27]. In detail, (epi)catechins have shown to increase the growth of the *Clostridium Coccoides-Eubacterium rectale* group, PACs have been associated to the growth of health promoting bacteria (*Lactobacillus*, *Bifidobacterium*,

Akkemansia muciniphila), and to a decrease of the ratio Firmicutes/Bacteroides, and red wine (poly)phenols have demonstrated to enhance the abundance of beneficial bacteria (*Bifidobacterium*, *Lactobacillus*, *Faecalibacterium prausnitzii*) at the expense of *Escherichia coli* and *Enterobacter cloacae* [28–30]. So, the protective effects deriving from the intake of flavan-3-ol-rich foods can depend not only on the production of phenolic catabolites, but also on the microbiota-modulating effects that have been recently attributed to (poly)phenols.

The present study aims to optimize a validated protocol of *in vitro* colonic fermentation used by Di Pede et al. [14] to mimic the colonic bioconversion of undigested compounds, particularly phytochemicals. The optimization of the model was performed using three different flavan-3-ol substrates and was reached through the extension of the incubation time and the monitoring of fermentation conditions, namely pH and faecal microbiota composition.

4.1.2. Materials and methods

Materials

All reagents and solvents were of analytical grade and were purchased from VWR International (Milan, Italy), unless otherwise indicated. Ultrapure water from MilliQ system (Millipore, Bedford, MA, USA) was used throughout the experiment. Formic acid, bile salts, soluble starch, (+)-arabinogalactan, tryptone, yeast extract, xylan from birchwood, L-cysteine hydrochloride monohydrate, guar gum, inulin, Tween 80, buffered peptone water, Dulbecco's phosphate buffer saline (PBS), casein sodium salt from bovine milk, pectin from citrus fruits, mucin from porcine stomach-type III, CaCl_2 , KCl, NaCl, NaHCO_3 , anhydrous K_2HPO_4 , KH_2PO_4 , MgSO_4 monohydrate, FeSO_4 heptahydrate, resazurin redox indicator, (–)-epicatechin, phenylacetic acid, 4'-hydroxyphenylacetic acid, 3'-hydroxyphenylacetic acid, 3',4'-dihydroxyphenylacetic acid, 3-phenylpropanoic acid, 3-(4'-hydroxyphenyl)propanoic acid, 3-(3'-hydroxyphenyl)propanoic acid, 3-(3',4'-dihydroxyphenyl)propanoic acid, benzoic acid, 4-hydroxybenzoic acid, 3-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 1,3,5-trihydroxybenzene, 3,4-dihydroxybenzaldehyde, and 4-hydroxybenzaldehyde were purchased from Sigma-Aldrich (St Louis, MO, USA). Dimer A2 and dimer B2 were purchased from Extrasynthese (Genay Cedex, France). 5-(4'-Hydroxyphenyl)-valerolactone, 5-(3'-hydroxyphenyl)-valerolactone, and 5-(3',4'-dihydroxyphenyl)- γ -valerolactone were

synthesized in house and kindly supplied by Prof. Curti using the synthetic strategy previously reported [31].

***In vitro* faecal fermentation substrates**

Three flavan-3-ols, including (–)-epicatechin (EC), procyanidin B2 (PC_B2) and procyanidin A2 (PC_A2), were used individually as substrates for microbial catabolism using an *in vitro* faecal fermentation model. PC_B2 is a B-type dimer containing two units of (–)-epicatechin having a bond between positions 4 and 8' in a β -configuration. Procyanidin A2 is a A-type dimer made of a unit of (–)-epicatechin and a unit of (+)-epicatechin joined by a bond between positions 4 and 8' in a β -configuration, and an additional ether bond between positions 2 and 7 in a β -configuration.

***In vitro* colonic fermentation procedure**

In vitro colonic fermentation was performed following validated protocols, with some modifications [14,32–35]. The growth medium (1 L) was prepared as previously reported [33–36], aliquoted and sterilized at 121 °C for 15 min in glass vessels (12 mL) before sample preparation. Fresh faecal samples were collected from three healthy donors with no previous intestinal diseases and who had not been treated with antibiotics for the previous 3 months. The Ethics Committee of Area Vasta Emilia Nord (AVEN) approved the collection and use of the faecal slurries (protocol no. 796/2018/sper/unipr) and all the donors provided informed consent for the collection of faecal slurries. Donors followed a strict and controlled diet lacking (poly)phenol-containing foods for 2 days prior to faecal collection. Faeces were immediately stored in anaerobic jars containing an anaerobic atmosphere generation bag to create an oxygen-free environment. An equal mass of faeces from each donor was weighed, diluted with Dulbecco's phosphate buffer saline (1%, w/v) and homogenized to obtain a 10% (w/w) slurry to be used as the inoculum for fermentation. Parent compounds were dissolved in an aqueous solution, and suspensions were left for 2 h at room temperature under constant magnetic stirring. Parent compounds were fermented at a final concentration of 75 $\mu\text{mol/L}$. This concentration is in line with the concentration of flavan-3-ols found in the ileal fluid of ileostomy patients consuming dietary amounts of flavan-3-ols [37,38]. In each fermentation batch, 45% of the growth medium, 45% of faecal slurry, and 10% of substrate suspension were added to reach a total fermentation volume of 4 mL. The faecal slurry and the aqueous

substrate suspension were added into the vessel containing growth medium, which were rapidly sealed and flushed with N₂ to create anaerobiosis. Vessels were incubated at 37 °C at 200 strokes min⁻¹ in a Dubnoff bath (JULABO, Seelbach Germany). Samples were collected prior starting the faecal fermentation (0 h) and after different incubation times: 5 h, 8 h, 24 h, 30 h, 48h. Blank samples, containing the culture medium and the faecal slurry, without substrate, as well as abiotic control samples, containing the culture medium and the substrate, without faecal slurry, were prepared and incubated as previously reported [33–36].

With respect to validated protocol, the incubation period has been increased. The extension of the incubation time beyond 24 h was obtained with some additional steps after 24 h and 30 h for samples incubated for 30 h and 48 h, respectively. In particular, batches were degassed with a sterile syringe to reduce the pressure caused by the microbiota fermentation. Vessels were flushed with N₂ to maintain an anaerobic environment, and sterilized fresh growth medium (1 mL) was added prior re-flushing N₂. To monitor the fermentation conditions, pH was monitored at every fermentation time, prior stopping the microbiota activity, using a pH meter (Mettler-Toledo S.p.A., Milan, Italy). Finally, after the incubation period and pH measurements, microbial catabolism was stopped adding 10% v/v of acetonitrile, for (poly)phenol catabolite analysis, or adding an inactivating liquid (0.7 mL) to an aliquot (1 mL) of fermented sample, to analyze microbiota composition. Samples were frozen (–80 °C) until extraction and analysis. All experiments were carried out in triplicate.

Faecal metabolite extraction

Native flavan-3-ols and phenolic catabolites generated during the *in vitro* faecal fermentation were extracted adopting the method previously reported by Di Pede et al. [14], with slight modifications. An aliquot of 300 µL of each fermented sample was extracted using 1200 µL of acidified methanol (0.1% v/v formic acid). The solution was vortexed for 2 min, sonicated for 10 min in an ultrasonic bath, vortexed for 2 min and re-sonicated for 5 min. Samples were centrifuged (Centrisart A-14C Refrigerated Micro-Centrifuge and Rotor YCSR-A1C, Sartorius Lab Instruments GmbH and Co. KG, Goettingen, Germany) at 14 460 × *g* for 10 min, and the organic extract was transferred into a clean microfuge tube. After the first extraction, the residual pellet of the fermented samples was re-extracted following the same procedure, using 500 µL of the same solvent. Finally, supernatants were pooled. An aliquot of 1 mL of supernatant was dried under vacuum by centrifugal vacuum concentrator (SpeedVac Savant

SPD121P, Thermo Fisher Scientific Inc., San José, CA, USA), and the dried pellet was re-suspended with 200 μ L of acidified methanol (0.1% v/v formic acid), vortexed 2 min, sonicated 10 min and finally centrifuged 5 min at $14\,460 \times g$ at 4°C. The concentrated samples, as well as the remaining supernatant, were analyzed by ultra-high performance liquid chromatography coupled with tandem mass spectrometry (uHPLC-MS/MS).

UHPLC-ESI-MS/MS Analysis

Faecal extracts were analyzed using a uHPLC DIONEX Ultimate 3000 fitted with a TSQ Vantage triple quadrupole mass spectrometer (Thermo Fisher Scientific Inc., San Jose, CA, USA) equipped with a heated-electrospray ionization source (H-ESI; Thermo Fisher Scientific Inc.). Separation was carried out by means of a Kinetex Evo C18 column (100 x 2.1 mm; 2.6 μ m particle size; Phenomenex, CA, USA), installed with a precolumn cartridge (Phenomenex). Mobile phase, pumped at a flow rate of 0.4 mL/min, consisted of a mixture of acidified water (0.01% v/v formic acid) (solvent A) and acidified acetonitrile (0.01% v/v formic acid) (solvent B). Following 0.5 min of 5% solvent B in A, the proportion of B was increased linearly to 40% over a period of 7 min. Solvent B was increased again to 80% in 1 min, maintained for 2 min and then the start conditions were re-established in 0.5 min and maintained for 3 min to re-equilibrate the column (total run: 14 min). The ESI source interface was set to a capillary temperature of 275 °C and the source heater temperature was 250 °C. The sheath gas (N_2) flow rate was set at 40 (arbitrary units), the auxiliary gas (N_2) flow rate at 5 and the sweep gas flow was set at 15 (arbitrary units). The source voltage was 3 kV, the capillary voltage was -9 V and tube lens voltage was -53 V. Chromatographic and ionization parameters were based on the method of Brindani et al. [39]. A total of 40 compounds (3 parent compounds and 37 gut microbiota catabolites) were monitored through selective reaction monitoring (SRM) mode (Table S1 in Supplementary Information). Quantification was performed with calibration curves of standards, when available, or using the most structurally similar compound. Data processing was performed using Xcalibur software version 2.1 (Thermo Scientific Inc., Waltham, MA, USA).

Data and statistical analysis

Results were expressed as mean values $\pm$ standard deviation (SD). In accordance with previous studies [14,40], molar mass recoveries for catabolites were expressed as percentage (%) with

respect to the incubated concentration of parent compound (75 $\mu\text{mol/L}$) to compare the different fermented substrates. Molar mass recovery in the production of total catabolites was calculated as the sum of single molar mass recoveries of catabolites. One-way ANOVA with Tukey's *post-hoc* test was applied to detect differences in molar mass recoveries of the catabolites produced from faecal fermentation of different substrates over the same incubation period (T0, T5, T8, T24, T30, T48), and to detect differences in the molar mass recovery of catabolites produced from faecal incubation of each substrate among different incubation periods (T0, T5, T8, T24, T30, T48). Differences were considered significant at p value < 0.05 . ANOVA was performed using IBM SPSS Statistics version 26 (IBM, Chicago, IL, USA).

Principal Component Analysis (PCA) was applied to explore differences in the behavior of the substrates in the *in vitro* colonic environment and in the appearance of catabolites over the fecal fermentation. PCA was carried out considering molar mass recoveries of all microbial catabolites before incubation (T0) and during the different incubation times (T5, T8, T24, T30, T48). Data were pre-treated with mean-centering plus Unit Variance (UV) scaling, also called autoscaling. Centering converted all the data to variations around zero instead of around the mean of the data, and UV scaling used the standard deviation as scaling factor. Two principal components (PCs) were set. The parameters used to assess the quality of the model and subsequent data interpretability were R^2X and Q^2 , namely the model fit (or explained variation) and the predictive ability, respectively. PCA was performed using SIMCA 16.0.1 software (Sartorius Stedim Data Analytics, Umea, Sweden).

Fecal bacterial DNA extraction, 16S rRNA Gene PCR amplification and sequencing

Fecal fermentations were collected and stored at -80°C until microbiota analysis. Bacterial DNA was extracted using a QIAamp Fast DNA Stool Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. Partial 16S rRNA gene sequences were amplified from extracted DNA using primer pair Probio_Uni and/Probio_Rev, targeting the V3 region of the 16S rRNA gene sequence (Milani et al., 2013). Illumina adapter overhang nucleotide sequences were added to the partial 16S rRNA gene-specific amplicons, which were further processed by the 16S Metagenomic Sequencing Library Preparation Protocol (Part #15044223 Rev. B – Illumina). Amplifications were carried out using a Verity Thermocycler (Applied Biosystems). The integrity of the PCR amplicons was analyzed by electrophoresis on a 2200 TapeStation

Instrument (Agilent Technologies, USA). DNA products obtained following PCR-mediated amplification of the 16S rRNA gene sequences were purified by a magnetic purification step involving the Agencourt AMPure XP DNA purification beads (Beckman Coulter Genomics GmbH, Bernried, Germany) to remove primer dimers. A fluorimetric Qubit quantification system determined the DNA concentration of the amplified sequence library (Life Technologies, USA). Amplicons were diluted to a concentration of 4 nM, and 5 µL quantities of each diluted DNA amplicon sample were mixed to prepare the pooled final Library. Sequencing was performed using an Illumina MiSeq sequencer with MiSeq Reagent Kit v3 chemicals.

Bioinformatics analysis

The fastq files obtained from sequencing were processed using QIIME2 (PMID: 31341288, PMID: 20383131) as previously described (PMID: 23869230). Paired-end reads were merged, and sequences that had passed quality control with a length between 140 and 400 bp, mean sequence quality score >25, and with truncation of a sequence at the first base if a low-quality rolling 10 bp window was found. Sequences with mismatched forward and/or reverse primers were removed.

In order to calculate downstream diversity measures (alpha and beta diversity indices), 16S rRNA amplicon sequence variants (ASVs) were defined at 100 % sequence homology using DADA2 (PMID: 27214047). ASVs not encompassing at least 2 sequences of the same sample were removed. Notably, this approach allows highly distinctive taxonomic classification at single nucleotide accuracy (PMID: 27214047). All reads were classified to the lowest possible taxonomic rank using QIIME2 (PMID: 31341288, PMID: 20383131) and a reference dataset from the SILVA database version 123 (PMID: 23193283). Similarities between samples (beta-diversity) were calculated by weighted uniFrac (PMID: 16332807). Similarity scores were calculated as values between 0 and 1. PCoA (Principal Coordinates Analysis) representations of beta-diversity were performed using QIIME2 (PMID: 31341288, PMID: 20383131).

4.1.3. Results and discussion

Identification and quantification of microbial catabolites

Three substrates, namely EC, PC_B2 and PC_A2, were incubated with faecal material for 5, 8, 24, 30 and 48 h at a concentration of 75 µmol/L. After 5 h faecal fermentation, the decrease of the parent compound was 72% for PC_A2 and 100% for PC_B2 and EC, in line with the data

obtained by Di Pede et al. [14]. This confirms that the degradation of PC_B2 and EC is more rapid than the catabolism of PC_A2 due to differences in structure and chemical linkages. A total of 37 microbial catabolites were monitored, based on the available bioconversion pattern for monomeric and dimeric flavan-3-ols [3,41–43], and on previous *in vitro* fermentation studies [14,15,34,35,40,44–53]. A total of 14 catabolites were identified and quantified in samples after faecal fermentation. In blank control samples, consisting of growth medium and faecal material without flavan-3-ol substrates, small amounts of phenolic catabolites were detected, in particular 3-HPPA, 4-HBA and 4-HBAL. These quantities were subtracted from the amounts found in substrate-containing fermented samples at each incubation time. In abiotic control samples, consisting of substrates and growth medium without the faecal material, no more than negligible amounts of catabolites were detected. These data indicate that the (poly)phenol-free diet produced almost blank faeces, and that the incubation process did not influence the production of the quantified catabolites.

The quantified earlier catabolites presented different chemical structures: monomers (N=1), originated from the fission of the interflavan bond of dimers, fission dimers (N=2), presenting a C-ring opening of one monomeric unit, and diphenylpropan-2-ols (DPPOLs, N=2), derived from the C-ring fission of monomers. After native structure modification, flavan-3-ols are further catabolized by colonic microbiota into lower molecular weight compounds, including phenyl- γ -valerolactones (PVLs, N=2), derived from the A-ring fission of DPPOLs or possibly from the degradation of the lower unit of dimers, phenylvaleric acids (PVAs, N=3), resulting from the hydrolysis of the lactone ring of PVLs, phenylpropanoic acids (PPAs, N=1), originated from the β -oxidation of PVAs, phenylacetic acids (PAAs, N=1), arising from the α -oxidation of PPAs or from the degradation of the upper unit of dimers, benzoic acids (BAs, N=1), originating from the β -oxidation of PPAs or the α -oxidation of PAAs, and benzaldehydes (BALs, N=1), obtained after the reduction of the carboxyl group of BAs.

Comparing the substrates, PC_B2 was characterized by the richest catabolic profile from a qualitative point of view, resulting in the production of 13 metabolites, followed by EC, accounting for 10 metabolites. From a quantitative point of view, EC was the substrate leading to the richest production, being the most catabolized parent compound, followed by PC_B2. PC_A2 was characterized by the poorest profile both from a qualitative and quantitative point of view, displaying only 5 catabolites and having the lowest concentration of quantified compounds. Analyzing the kinetics, the peak of catabolites was obtained after 8 and 24 h of

incubation for EC and PCs, respectively, reflecting their catabolic rate and the different hindrance to degradation between monomers and dimers, in accordance with existing evidence [14–17]. As shown by Di Pede et al. [14], PVLs were the principal class of catabolites for EC and PC_B2, while fission dimer was the characterizing catabolite for PC_A2. Production of PVLs reached its maximum value at 8 h for EC, at 24 h for PC_B2, and at 48 h for PC_A2, suggesting that also PC_B2 and PC_A2 have different catabolic rates. In this case, the extension of the fermentation time until 48 h and the addition of time 8 allowed to have a more comprehensive view on flavan-3-ol catabolism from a kinetic point of view.

Catabolism and molar mass recoveries for microbial catabolites

Based on the incubated concentration of parent compound (75 $\mu\text{mol/L}$), the molar mass recovery of each microbial catabolite was calculated, and comparisons were made among different substrates at the same fermentation time, and among different incubation periods for the same substrate (Table 1). EC was produced in small amounts only after 5 h incubation of PC_B2, indicating that the cleavage of the interflavan bond is a minor pathway (<10%) for B-type PCs, as observed in other studies [14,15]. Fission catabolites, derived from the cleavage of one C ring of PCs, were produced from both dimers, but in different quantities: 1fission A2 was the principal catabolite of PC_A2, reaching a molar mass recovery of 53% at 24 h, while for PC_B2 the fission dimer (1fission B2) was a minor catabolite produced until 8 h, with a second peak at 48 h. PC_B2 fission catabolite presenting two cleavages was not identified, differently from the results obtained by Di Pede et al. [14]. DPPOLs in their mono- and dihydroxylated forms on the B-ring appeared only after 5 and 8 h incubation of EC and PC_B2, as expected [14,15,47]. As noticed for 1fission_B2, a second peak of OH-DPPOLs appeared at 48 h, suggesting that upstream catabolites, normally produced in the first fermentation hours, could also be generated after 30 h for B-type PCs. This could be due to a recovery of microbial catabolism after the microbiota-driven release of PCs that were interacting with components of the fermentation medium, such as proteins. The presence of proteins in the donor faeces is expected because volunteers following a (poly)phenol-free diet are induced to eat less plant-based foods, rich in fibers and/or carbohydrates, and more animal foods, which are relevant sources of proteins. It is well-known that tannins, including PACs, interact with proteins in the gut lumen, limiting the access to the C-ring by the colonic microbiota and slowing down their catabolism [54].

Table 1. Molar mass recoveries (%) for microbial catabolites produced after 5, 8, 24, 30 and 48 h faecal fermentation of (-)-epicatechin (EC), procyanidin dimer B2 (PC_B2) and procyanidin dimer A2 (PC_A2). Molar mass recoveries are expressed as percentage with respect to the incubated concentration of parent compound (75 μ mol/L). Data are expressed as mean $\pm$ SD. Different lower case letters indicate significant differences comparing the same fermented substrate after different incubation periods (5, 8, 24, 30, 48 h) ($p < 0.05$). Different upper case letters indicate significant differences among different fermented parent compounds (EC, PC_B2, PC_A2) considering the same incubation period ($p < 0.05$).

Catabolites	Time (h)	Substrates		
		EC	PC_B2	PC_A2
(-)-Epicatechin (EC)	5	-	0.5 $\pm$ 0.0	-
	8	-	-	-
	24	-	-	-
	30	-	-	-
	48	-	-	-
1 Fission Dimer A2 (1fission A2)	5	-	-	48.1 $\pm$ 0.5 ^a
	8	-	-	49.8 $\pm$ 1.7 ^a
	24	-	-	53.0 $\pm$ 2.1 ^a
	30	-	-	36.1 $\pm$ 4.8 ^b
	48	-	-	35.0 $\pm$ 4.0 ^b
1 Fission Dimer B2 (1fission B2)	5	-	0.3 $\pm$ 0.1 ^a	-
	8	-	0.2 $\pm$ 0.0 ^b	-
	24	-	-	-
	30	-	-	-
	48	-	0.4 $\pm$ 0.1 ^a	-
1-(3',4'-Dihydroxyphenyl)-3-(2'',4'',6''-trihydroxyphenyl)-propan-2-ol (3',4'-DiOH-DPPOL)	5	3.6 $\pm$ 0.9 ^A	1.9 $\pm$ 0.9 ^{aA}	-
	8	-	0.2 $\pm$ 0.0 ^b	-
	24	-	-	-
	30	-	-	-
	48	-	-	-
1-(Hydroxyphenyl)-3-(2'',4'',6''-trihydroxyphenyl)-propan-2-ol (OH-DPPOL)	5	20.3 $\pm$ 2.6 ^{aA}	14.6 $\pm$ 1.6 ^{aB}	-
	8	1.9 $\pm$ 0.1 ^{bA}	0.5 $\pm$ 0.1 ^{cB}	-
	24	-	-	-
	30	-	-	-
	48	-	5.2 $\pm$ 0.6 ^b	-
5-(3',4'-Dihydroxyphenyl)- γ -valerolactone (3',4'-DiHPVL)	5	36.1 $\pm$ 5.9 ^{aA}	33.5 $\pm$ 5.2 ^{aA}	0.4 $\pm$ 0.0 ^{cB}
	8	40.3 $\pm$ 2.5 ^{aA}	37.3 $\pm$ 1.1 ^{aA}	0.5 $\pm$ 0.0 ^{cB}
	24	32.5 $\pm$ 1.5 ^{aA}	31.3 $\pm$ 1.5 ^{aA}	0.8 $\pm$ 0.1 ^{bB}
	30	19.6 $\pm$ 1.3 ^{bA}	13.8 $\pm$ 0.9 ^{bB}	1.0 $\pm$ 0.1 ^{abC}
	48	16.9 $\pm$ 3.3 ^{bA}	13.2 $\pm$ 0.7 ^{bA}	1.1 $\pm$ 0.1 ^{aB}
5-(3'-Hydroxyphenyl)- γ -valerolactone (3'-HPVL)	5	16.2 $\pm$ 2.6 ^{cA}	16.1 $\pm$ 1.9 ^{dA}	-
	8	70.1 $\pm$ 1.8 ^{abA}	58.6 $\pm$ 0.5 ^{bB}	-
	24	73.2 $\pm$ 3.0 ^{aA}	66.7 $\pm$ 4.9 ^{aA}	0.5 $\pm$ 0.1 ^{bB}
	30	58.2 $\pm$ 3.2 ^{bA}	39.0 $\pm$ 0.2 ^{cB}	0.6 $\pm$ 0.1 ^{bC}
	48	49.9 $\pm$ 9.3 ^{bA}	18.9 $\pm$ 1.1 ^{dB}	1.0 $\pm$ 0.3 ^{aC}
5-(3',4'-Dihydroxyphenyl)valeric acid (3',4'-DiHPVA)	5	0.1 $\pm$ 0.0 ^b	-	-
	8	0.2 $\pm$ 0.0 ^{aA}	0.2 $\pm$ 0.0 ^{bA}	-
	24	0.3 $\pm$ 0.0 ^{aA}	0.3 $\pm$ 0.0 ^{aA}	-
	30	0.3 $\pm$ 0.0 ^{aA}	0.1 $\pm$ 0.0 ^{cB}	-
	48	-	-	-

4-Hydroxy-5-(hydroxyphenyl)valeric acid (4-OH-HPVA)	5	1.6±0.3 ^{c A}	1.3±0.4 ^{d A}	-
	8	4.8±0.0 ^{b A}	3.9±0.2 ^{b B}	-
	24	8.4±0.6 ^{a A}	7.6±0.3 ^{a A}	-
	30	7.2±0.2 ^{a A}	3.7±0.4 ^{b B}	-
	48	4.7±0.8 ^{b A}	2.8±0.2 ^{c B}	-
5-(3'-Hydroxyphenyl)valeric acid (3'-HPVA)	5	-	-	-
	8	-	-	-
	24	1.5±0.1 ^{a A}	1.1±0.1 ^{a B}	-
	30	1.3±0.3 ^{a A}	0.9±0.2 ^{b A}	-
	48	1.4±0.3 ^a	-	-
3-(3'-Hydroxyphenyl)propanoic acid (3'-HPPA)	5	1.4±0.1 ^{b C}	2.2±0.3 ^{b B}	6.5±0.3 ^{bc A}
	8	3.2±0.5 ^{a B}	4.0±0.4 ^{b B}	9.1±0.9 ^{ab A}
	24	1.8±0.4 ^{b B}	1.4±0.5 ^{b B}	10.8±3.0 ^{a A}
	30	1.9±0.5 ^{b B}	4.3±1.5 ^{b B}	8.3±1.4 ^{abc A}
	48	2.4±0.1 ^{ab C}	14.0±2.5 ^{a A}	4.3±0.1 ^{c B}
3',4'-Dihydroxyphenylacetic acid (3',4'-DiHPAA)	5	-	3.6±0.8 ^a	-
	8	-	2.5±0.4 ^b	-
	24	-	-	-
	30	-	-	-
	48	-	-	-
4-Hydroxybenzoic acid (4-HBA)	5	-	-	-
	8	-	-	-
	24	-	-	-
	30	2.5±0.7 ^a	-	-
	48	2.0±0.1 ^{a A}	0.7±0.1 ^B	0.6±0.2 ^B
4-Hydroxybenzaldehyde (4-HBAL)	5	-	-	-
	8	-	0.2±0.0 ^b	-
	24	-	0.4±0.1 ^a	-
	30	0.9±0.0.3 ^a	-	-
	48	0.5±0.2 ^b	-	-

As regards 5C-RFCs, PVLs were found to be the most important catabolites for EC and PC_B2, and minor catabolites for PC_A2, confirming that A-type PC are more resistant to microbial catabolism, in particular for the A-ring fission step [5,14,40]. The presence of an additional ether bond in A-type PCs makes their structure stiffer and less subjected to degradation. 3',4'-DiHPVL was the main catabolite for EC and PC_B2, and it reached its maximum recovery at 8 h for EC, 24 h for PC_B2, and 48 h for PC_A2. This indicates that the substrates have a different degradation rate, due to differences in the steric hindrance and structure stiffness [14,15,17,40]. Similarly to 3',4'-diHPVL, 3'-HPVL showed a different kinetics according to the substrate: it reached its production peak after 24 h incubation for EC and PC_B2, and at 48 h for PC_A2. These results indicate that the catabolism of PC_A2 increases after 24 h, giving importance to the extension of the incubation time until 48 h. Moreover, the maximum yield of 3',4'-diHPVL detected at 8 h suggests that the addition of this fermentation time could be crucial in the study of the degradation pathways of monomers. The delayed peak of 3'-HPVL

compared to 3',4'-diHPVL was observed also in other studies [14,40,55], and reflects the timing of the catabolism pathway, since 3'-monohydroxylated catabolites derive from 4'-dehydroxylation of 3',4'-dihydroxylated precursors. Three different PVAs were quantified after EC and PC_B2 faecal incubation, and their kinetics and relative abundance was in accordance with the data obtained by Di Pede et al. [14]. These PVAs were detected in other studies investigating the *in vitro* microbial conversion of flavan-3-ol monomers and dimers [15,40,45,56]. All PVAs reached their maximum recovery at 24 h for both substrates. 4-OH-HPVA was the principal PVA, followed by 3'-HPVA, while 3',4'-DiHPVA was the minor one. This confirms that 4-OH-PVAs, generated by the hydrolysis of PVLs, are more abundant than their corresponding 4-dehydroxylated forms [5]. Moreover, the abundance of 3'-hydroxyl derivatives of PVLs and PPAs suggests that the 4'-hydroxyl group is removed more readily and easily than the 3'-hydroxyl group from dihydroxylated precursors, as observed in other studies [15,40,45,55,56].

Considering small phenolic acids, 3'-HPPA was quantified in all incubation samples. This compound showed a biphasic behavior for EC and PC_B2, reaching its peak both at 8 h and at 48 h, suggesting that it could derive from two different pathways, namely the β -oxidation of 3'-monohydroxylated PVA, and the 4'-dehydroxylation of 3',4'-dihydroxylated PPAs, which has not been quantified here but was reported in other studies [45,56]. The monitoring of 5 different fermentation times has allowed to understand properly the kinetics of 3'-HPPA. 3'-HPPA was identified as a characterizing metabolite for PC_A2, reaching a recovery of 11% at 24 h, in accordance with other studies [14,40]. The extension of the incubation time until 48 h and the addition of T8 allowed to quantify other small phenolic acids, including 3',4'-diHPAA, 4-HBA and 4-HBAL, which were not detected by Di Pede et al. [14]. In line with existing evidence [40,45,55], 3',4'-diHPAA resulted to be a catabolite specific for PC_B2, being quantified after 5 and 8 h of fermentation, and reaching its maximum recovery at 5 h. These data suggest that this compound arises from the quick degradation of the upper unit of B-type dimers. Small quantities of 4-HBA were found at 48 h for all substrates and at 30 h for EC, suggesting a delayed production, as expected for final catabolites [43]. A study by Sánchez-Patán et al. [55] confirmed the production of 4-HBA as end-product of grape seed flavan-3-ol fermentation. 4-HBA and its dehydroxylated derivative benzoic acid are precursors of 4'-hydroxyhippuric acid and hippuric acid, respectively, which are important *in vivo* glycine-conjugated metabolites of flavan-3-ols [43]. Finally, 4-HBAL was quantified for EC and PC_B2

after different incubation times: for EC, it appeared after 30 and 48 h incubation, and for PC_B2 it was generated after 8 and 24 h, suggesting a different kinetics for monomers and PACs. This is the first time that 4-HBAL is reported in *in vitro* fermentation study using flavan-3-ols.

Assessment of the differences in microbial catabolism of the incubated flavan-3-ols

Unsupervised multivariate analysis (PCA) was applied to explore differences in the behavior of the substrates in the *in vitro* colonic environment and in the time of appearance of catabolites over the fecal fermentation period (Figure 1). Two principal components (PCs) explained up to 62.9% of the total variability, with PC1 and PC2 explaining 36.4% and 26.5%, respectively. The model predicted 20.4% of the total variability, and the predicted power was mainly determined by PC1 (15.7%). PC1 was positively loaded mainly by metabolites characterizing PC_A2, namely 1fission A2 and 3'-HPPA, and was negatively loaded by RFCs characterizing EC and PC_B2, including PVLs, PVAs, 4-HBA, and 4-HBAL. PC2 was positively loaded by early produced catabolites characterizing EC and PC_B2 in the first fermentation times (T5 and T8), including EC, 1fission B2, DPPOLs, 3',4'-DiHPVL and 3',4'-DiHPAA. Considering the score plot with PC1 versus PC2, observations were discriminated by PC1 according to the substrate, and by PC2 according to the incubation time. PC_A2 observations at all incubation times lied in the same quadrant (positive PC1 and negative PC2) and clustered together. This confirms that PC_A2 is subjected to a different catabolism than EC and PC_B2 and suggests that the catabolic profile of this substrate does not change significantly during the 48-h incubation period. On the contrary, EC and PC_B2 observations clustered together according to the incubation time: for early time points (T5 and T8), observations lied in the upper quadrants (positive PC2), while for later times, including T24, T30 and T48, observations lied in the bottom left quadrants (negative PC1 and PC2), with the exception of T48 for PC_B2, located in the upper right quadrant (positive PC1 and PC2). This indicates that EC and PC_B2 are catabolized in a similar way, and the catabolic profile is driven mainly by the incubation time. The only difference between the incubated substrates is the kinetics: PC_B2 showed a slower catabolism, resulting in the shifting of T5 and T8 in the upper part of the quadrants compared to EC at the same incubation time. After 5 h of fermentation, the profile of EC and PC_B2 was characterized by DPPOLs, 3',4'-DiHPVL and catabolites specific for PC_B2, namely EC, 1fission B2 and 3',4'-DiHPAA. 3',4'-DiHPVL characterized also the profile obtained after 8 h incubation of both substrates. On the contrary, in late fermentation times (T24, T30, T48), the most relevant

catabolites were low molecular weight compounds such as 3'-HPVL, PVAs, 3'-HPPA, 4-HBA, and 4-HBAL.

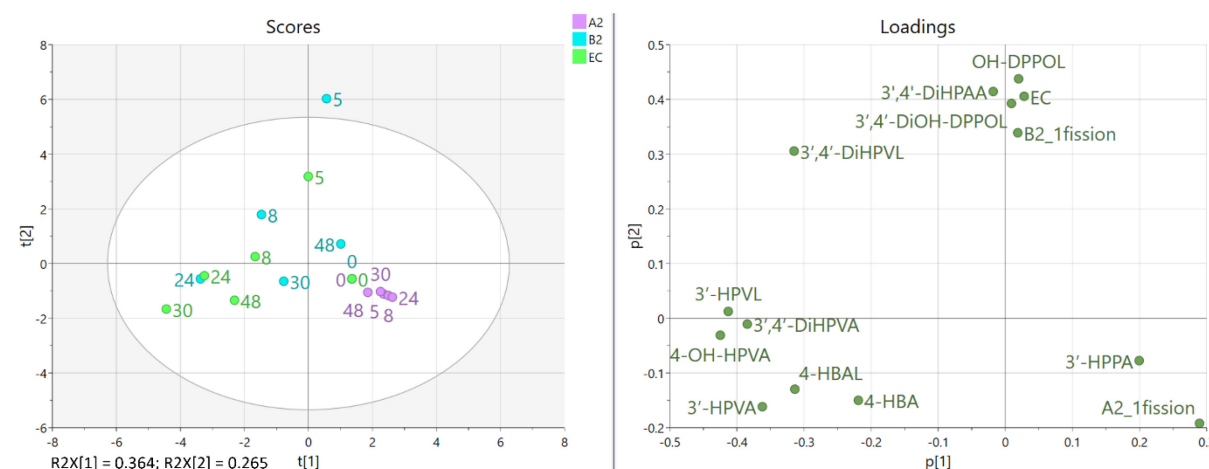


Figure 1. Score and loading plots resulting after applying PCA on autoscaled data. Observations represent molar mass recoveries for microbial metabolites at each fermentation time (T0, T5, T8, T24, T30, T48) for the substrates procyanidin dimer A2, procyanidin dimer B2 and (–)-epicatechin (EC). A2_1fission: 1 fission dimer A2; B2_1fission: 1 fission dimer B2; 3',4'-diOH-DPPOL: 1-(3',4'-dihydroxyphenyl)-3-(2'',4'',6''-trihydroxyphenyl)-propan-2-ol; OH-DPPOL: 1-(hydroxyphenyl)-3-(2'',4'',6''-trihydroxyphenyl)-propan-2-ol; 3',4'-diHPVL: 5-(3',4'-dihydroxyphenyl)- γ -valerolactone; 3'-HPVL: 5-(3'-hydroxyphenyl)- γ -valerolactone; 3',4'-diHPVA: 5-(3',4'-dihydroxyphenyl)valeric acid; 4-OH-HPVA: 4-hydroxy-5-(hydroxyphenyl)valeric acid; 3'-HPVA: 5-(3'-hydroxyphenyl)valeric acid; 3'-HPPA: 3-(3'-hydroxyphenyl)propanoic acid; 3',4'-diHPAA: 3',4'-dihydroxyphenylacetic acid; 4-HBA: 4-hydroxybenzoic acid; 4-HBAL: 4-hydroxybenzaldehyde.

Assessment of the stability of the fermentation environment: measurement of pH

The monitoring of the fermentation conditions was reached through pH measurements and faecal microbiota composition analysis in the different incubation times (T0, T5, T8, T24, T30, T48). The microbial fermentation activity had a similar effect on the acid-base balance for each substrate: pH ranged from 7 to 8 at T0, and decreased after 5 h of fermentation, reaching a value between 5 and 6, remaining stable until 48 h, with only slight fluctuations (Figure 2). This decrease was probably due to the formation of microbial catabolites, such as phenolic acids, lactic acid, and short chain fatty acids (SCFAs), namely acetic, propionic and butyric acid [57–59]. Low molecular weight phenolic acids are mainly produced through fermentation of the native incubated (poly)phenol substrates, although a minor part can arise from the microbial catabolism of aromatic amino acids, present in the growth medium [60], as well as possibly contained in donor faeces because of the low (poly)phenol diet. SCFAs would arise from the fermentation of fermentable dietary fibers added to the growth medium, such as arabinogalactan, xylan, guar gum, inulin, and pectin [61], and, to a lesser extent, from the catabolism of amino acids derived from proteins contained in donor faeces and medium

proteins, including tryptone, yeast extract, peptone, casein, and mucin [60]. It is known that pectin fermentation produces acetate, while inulin increases the molar proportion of butyrate [58]. The saccharolytic activity of the faecal microbiota reproduced the colonic environment, where fibers are fermented maintaining the pH around 5.5 [18,57,58]. This confirms that the *in vitro* fermentation environment mimics colonic features and is optimal for the microbial activity. Moreover, the decrease of pH in blank controls suggests that fibers and amino acids present in the medium and faeces may be main source of organic acids responsible for environment acidification, rather than (poly)phenols.

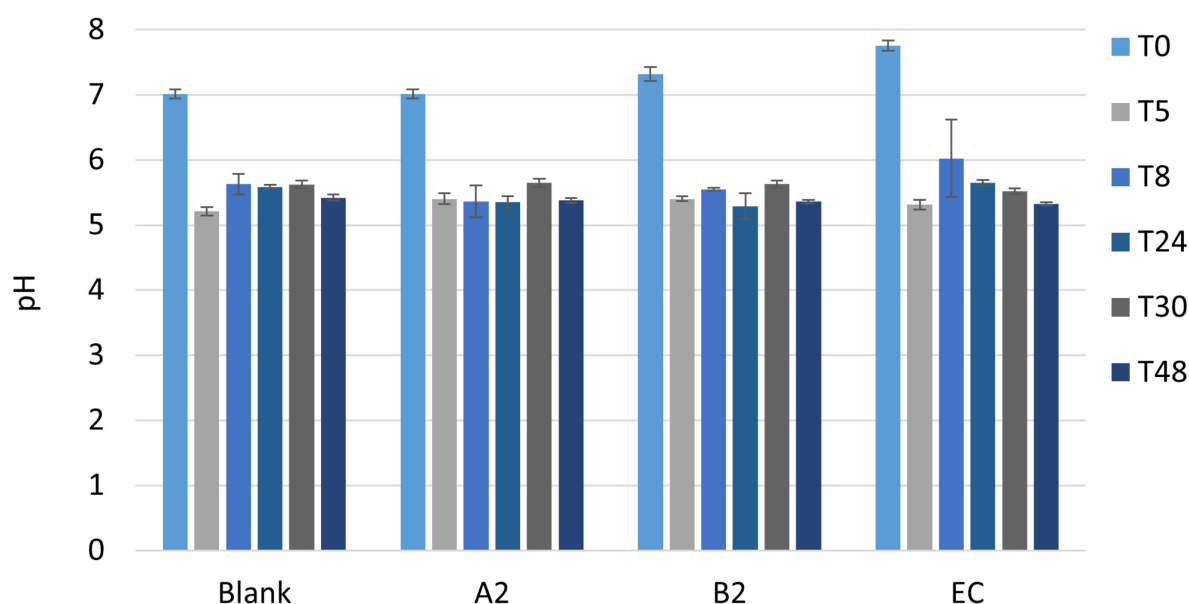


Figure 2. Monitoring of pH through each fermentation time (T0, T5, T8, T24, T30, T48) in blank controls and fermented samples for the substrates procyanidin dimer A2, procyanidin dimer B2 and (–)-epicatechin (EC).

Assessment of the stability of the fermentation environment: analysis of the microbiota composition

Fermentation of (poly)phenols and dietary fibers can also have an effect on the composition and activity of the gut microbiota [18,62]. Moreover, a specific microbiota composition is crucial to reach a good fermentation activity. In particular, the healthy gut ecosystem is characterized by the presence of anaerobic bacteria belonging to Bacteroidetes and Firmicutes phyla, contributing to more than 90% of the total bacterial species. Other minor phyla are Proteobacteria, Actinobacteria and Verrucomicrobia [18]. These taxa have been identified by analyzing the microbial composition of fermented samples and blank controls at T0, T8, T24, T30 and T48, in terms of phyla, families and genera using a 16S-rRNA microbial profiling approach. As expected, the main phyla were Bacteroidetes and Firmicutes and the

Firmicutes/Bacteroidetes ratio ranged from 0.4 to 1.6, maintaining itself in a physiological concentration range (0.1-10) (Figure 3A) [63]. Proteobacteria increased at T8 both in fermented samples and in controls, and this increase was mainly due to the genera *Sutterella* and *Escherichia-Shigella*, belonging to the Enterobacteriaceae family. This indicates that some facultative anaerobes (*Escherichia-Shigella*) and microaerophiles (*Sutterella*) commonly present in the intestine were able to grow, probably during the first early step of the fermentation, prior recreating the anaerobic conditions. However, their abundance remained low, ranging from 2 to 5%, and did not compromise the microbial balance. Only a sample, namely PC_B2 fermented for 48 h, showed a percentage of Proteobacteria of 10%, mainly determined by a spread of Enterobacteriaceae. Moreover, this sample, along with PC_B2 incubated for 30 h, showed an inversion of Firmicutes/Bacteroidetes ratio, with Firmicutes more abundant than Bacteroidetes. This was mainly due to an increase of Clostridiaceae 1 and Acidaminococcaceae, and a decrease of Prevotellaceae, which are known to have beneficial effects and are associated to SCFA production [63].

As happens for Proteobacteria, Actinobacteria increased at T8 due to a spread of Coriobacteriaceae, except for PC_B2 fermented for 48 h, which was characterized by an increase of *Bifidobacterium* genus. These data are interesting since three species belonging to the Coriobacteriaceae family, including *Slackia equolifaciens* JCM 16059T, *Adlercreutzia equolifaciens* JCM 14793T, and three strains of *Eggerthella lenta* (*E. lenta* CAT-1, *E. lenta* Rk3 and *E. lenta* SDG-1) are able to cleave the C-ring of monomeric flavan-3-ols forming DPPOLs. *E. lenta* CAT-1 and *A. equolifaciens* JCM 14793T can also dehydroxylate flavan-3-ols and 5C-RFCs at the B-ring, usually in 4' position. Two strains of *Flavonifractor plautii* (*F. plautii* DSM 6740 and *F. plautii* Ak2), a member of the Ruminococcaceae family, can further degrade the C-ring fission products, catalyzing the A-ring cleavage and the hydrolysis of the lactone ring, giving origin to PVLs and PVAs [5,64]. The Ruminococcaceae family was one of the principal families and remained stable during the incubation times. Other important families were Bacteroidaceae, Prevotellaceae and Lachnospiraceae, the latter increasing after 24 h fermentation (Figure 3B). The principal genera were *Bacteroides*, *Prevotella* 9, *Prevotellaceae* NK3B31 group, *Faecalibacterium*, and *Ruminococcaceae* UCG-002 (Figure 3C). Interestingly, while *Prevotella* 9 and *Prevotellaceae* NK3B31 decreased after 24 h fermentation, *Paraprevotella* increased, leaving the abundance of Prevotellaceae family almost unchanged.

Moreover, *Faecalibacterium* decreased from 8 h fermentation till the 48 h *in vitro* fermentation.

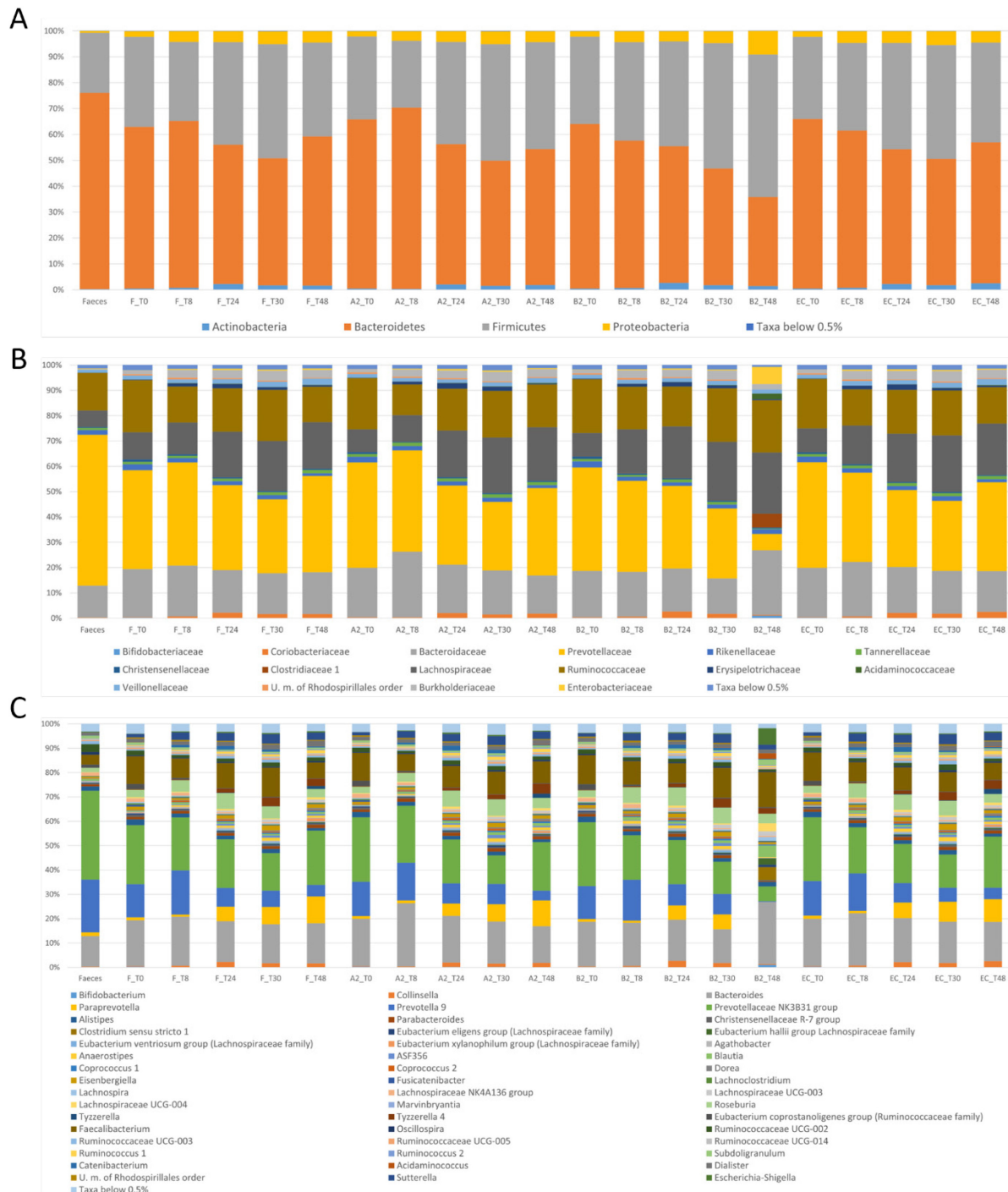


Figure 3. Composition of the faecal microbiota in a frozen faecal sample (faeces) and at each fermentation time (T0, T8, T24, T30, T48) in blank controls (F) and in fermented samples for the substrates procyanidin dimer A2, procyanidin dimer B2 and (–)-epicatechin (EC). The composition is analyzed in terms of (A) phyla, (B) families and (C) genera.

As confirmed by PCoA results (Figure 4), the microbiota composition in terms of genera was similar in all samples except for PC_B2 incubated for 48 h. This aspect needs to be further

investigated in future studies. Moreover, the factor that mostly contributed to sample variability seemed to be the incubation time, suggesting that the presence and type of substrate did not significantly impact the relative abundance of microbial genera.

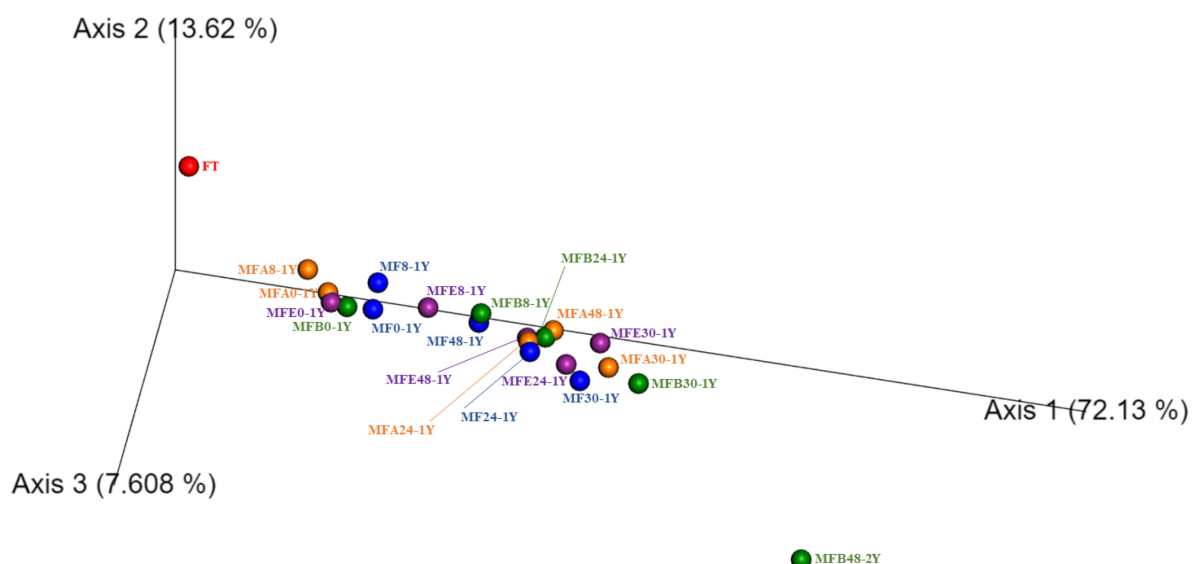


Figure 4. PCoA representing the distribution of observations according to the microbiota composition in terms of relative abundance of genera. Observations include a frozen faecal sample (faeces), blank controls at each fermentation time (MF0, MF8, MF24, MF30, MF48), and fermented samples at each fermentation time for the substrates procyanidin dimer A2 (MFA0, MFA8, MFA24, MFA30, MFA48), procyanidin dimer B2 (MFB0, MFB8, MFB24, MFB30, MFB48) and (–)-epicatechin (MFE0, MFE8, MFE24, MFE30, MFE48).

4.1.4. Conclusions

The optimization of the *in vitro* fermentation protocol confirmed the effect of flavan-3-ol structure on microbial catabolism from a quantitative, qualitative and kinetic point of view. The results showed that flavan-3-ol polymerization and the addition of an ether linkage in A-type PCs increased the steric hindrance and stiffness of the molecule, limiting the access to the C and the A-ring fission by the gut microbiota, with a reduction of bioaccessibility and a delay in native structure catabolism. The extension of fermentation time until 48 h and the monitoring of 5 different incubation times resulted in a more comprehensive view of flavan-3-ol kinetics and allowed to quantify small phenolic acids, including a catabolite specific of PC_B2 and two downstream catabolites, one never reported in a flavan-3-ol fermentation study before. The monitoring of fermentation conditions confirmed the stability of the incubation environment, with the pH maintained between 5 and 6, and a well-balanced microbial composition. Microbiota composition exhibited only minor fluctuations in terms of relative

abundance at the phylum, family, and genus levels. These fluctuations were primarily influenced by incubation time and appeared independent of the fermented substrate type. The enhancement of this *in vitro* fermentation procedure till 48 h will allow to better follow the microbial production and kinetics of both upstream and downstream catabolites of (poly)phenols.

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Authors' contributions

LB and PM designed the study; NT conducted the experiments; NT performed sample and data analysis; GA performed microbial DNA extractions; LM performed bioinformatic and statistical microbiota analyses; FT supervised microbiota analysis; LB and PM supervised analysis; NT drafted the manuscript; LB, PM, MV and DDR edited the manuscript. All authors critically read and approved the final version of the manuscript.

Data Availability Statement

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

Declaration of interest statement

The authors declare no conflict of interest.

Electronic Supplementary Information (ESI)

Supplementary Table 1. Chromatographic and spectrometric properties of the monitored parent flavan-3-ols and their gut microbiota catabolites. Nomenclature of catabolites is reported as proposed by Kay et al. [65]. Abbreviations are reported under brackets. RT means retention time; m/z means mass to charge ratio; CE means collision energy; STD means standard used for quantification; n.d. means targeted but not detected; n.q. means detected but not quantified.

Compound (Abbreviation)	RT (min)	Parent ion (M - H) ⁻ (m/z)	S-lens	Quantifier		Qualifier		STD
				Product ion (m/z)	CE (V)	Product ion (m/z)	CE (V)	
Parent compounds								
Procyanidin dimer A2 (PC_A2)	4.85	575	131	289	25	449	25	PC A2
Procyanidin dimer B2 (PC_B2)	4.12	577	131	289	27	407	25	PC B2
(-)-Epicatechin (EC)	4.21	289	112	245	18	203	23	EC
Fission catabolites								
1 Fission Dimer A2 (A2_1fission)	5.10	577	131	577	25	291	25	PC A2
1 Fission Dimer B2 (B2_1fission)	4.73	579	131	291	25	289	25	PC B2
2 Fission Dimer A2 (A2_2fission)	-	579	-	-	-	-	-	n.d.
2 Fission Dimer B2 (B2_2fission)	-	581	131	581	25	291	25	n.d.
Diphenylpropan-2-ol derivatives (DPPOLs)								
1-(3',4'-Dihydroxyphenyl)-3-(2'',4'',6''-trihydroxyphenyl)-propan-2-ol (3',4'-DiOH-DPPOL)	4.21	291	98	123	30	247	16	3',4'-diHPVL
1-(Hydroxyphenyl)-3-(2'',4'',6''-trihydroxyphenyl)-propan-2-ol (OH-DPPOL)	4.77	275	98	231	16	191	30	3',4'-diHPVL
Phenyl-γ-valerolactones (PVLs)								
5-(3',4'-Dihydroxyphenyl)-γ-valerolactone (3',4'-DiHPVL)	4.25	207	81	163	18	122	21	3',4'-diHPVL
5-(4'-Hydroxyphenyl)-γ-valerolactone (4'-HPVL)	-	191	67	147	16	106	31	n.d.
5-(3'-Hydroxyphenyl)-γ-valerolactone (3'-HPVL)	5.01	191	67	147	16	106	31	3'-HPVL
5-Phenyl-γ-valerolactone (PVL)	-	175	-	-	-	-	-	n.d.
Phenylvaleric acids (PVAs)								

4-Hydroxy-5-(3',4'-Dihydroxyphenyl)valeric (4-OH-3',4'-DiHPVA)	-	225	-	-	-	-	-	n.d.
5-(3',4'-Dihydroxyphenyl)valeric acid (3',4'-DiHPVA)	5.02	209	63	191	15	165	12	3',4'-diHPVL
4-Hydroxy-5-(hydroxyphenyl)valeric acid (4-OH-HPVA)	4.30	209	63	147	20	101	20	3',4'-diHPVL
5-(4'-Hydroxyphenyl)valeric acid (4'-HPVA)	-	193	71	147	30	175	30	n.d.
5-(3'-Hydroxyphenyl)valeric acid (3'-HPVA)	5.50	193	71	147	30	175	30	3'-HPVL
4-Hydroxy-5-(phenyl)valeric acid (4-OH-PVA)	5.41	193	-	-	-	-	-	n.d.
5-Phenylvaleric acid (PVA)	-	177	-	-	-	-	-	n.d.
Phenylpropanoic acids (PPAs)								
3-(4'-Hydroxy-3'-methoxyphenyl)propanoic acid (3'-MeO-4'-HPPA)	-	195	-	-	-	-	-	n.d.
3-(3',4'-Dihydroxyphenyl)propanoic acid (3',4'-DiHPPA)	3.16	181	64	137	14	109	18	n.d.
2-Hydroxy-3-(hydroxyphenyl)propanoic acid (2-OH-HPPA)	-	181	-	-	-	-	-	n.d.
3-(4'-Hydroxyphenyl)propanoic acid (4'-HPPA)	4.26	165	64	121	13	93	14	n.d.
3-(3'-Hydroxyphenyl)propanoic acid (3'-HPPA)	4.45	165	64	121	13	119	18	3'-HPPA
2-Hydroxy-3-(phenyl)propanoic acid (2-OH-PPA)	-	165	-	-	-	-	-	n.d.
3-Phenylpropanoic acid (PPA)	5.60	149	60	149	10	105	13	n.q.
Phenylacetic acids (PAAs)								
3',4'-Dihydroxyphenylacetic acid (3',4'-DiHPAA)	1.80	167	35	123	13	122	28	3',4'-DiHPAA
4'-Hydroxyphenylacetic acid (4'-HPAA)	3.20	151	75	107	13	-	-	n.d.
3'-Hydroxyphenylacetic acid (3'-HPAA)	3.77	151	75	107	13	-	-	n.d.
Phenylacetic acid (PAA)	-	135	40	91	10	135	10	n.q.
Benzoic acids (BAs)								
3,4-Dihydroxybenzoic acid (3,4-DiHBA)	1.54	153	74	109	16	108	26	n.q.
4-Hydroxybenzoic acid (4-HBA)	2.58	137	72	93	16	-	-	4-HBA
3-Hydroxybenzoic acid (3-HBA)	3.63	137	72	93	16	-	-	n.d.
Benzoic acid (BA)	4.96	121	68	77	13	121	10	n.d.
Benzaldehydes (BALs)								
3,4-Dihydroxybenzaldehyde (3,4-DiHBAL)	2.30	137	72	136	22	108	26	n.d.
4-Hydroxybenzaldehyde (4-HBAL)	3.61	121	68	92	26	120	20	4-HBAL
Benzyl alcohols (BOHs)								
Hydroxybenzyl alcohol (OH-BOH)	-	123	-	-	-	-	-	n.d.
Benzene derivatives (BZs)								
Benzene-1,3,5-triol (1,3,5-TriOH-BZ)	0.85	125	62	125	10	57	17	n.d.
Benzene-1,2-diol (1,2-DiOH-BZ)	-	109	-	-	-	-	-	n.d.

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4.2. An acute human intervention study

Influence of the flavan-3-ol structure on the quali-quantitative production of phenolic metabolites after acute consumption of different plant extracts by healthy subjects

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Abstract

Flavan-3-ols are the most consumed flavonoids in Western diets. Evidence suggests that circulating flavan-3-ol metabolites may act as mediators of their beneficial effects on health. The structural properties of parent flavan-3-ols may affect their metabolism, with a putative impact on biological response. This study aims to evaluate the influence of the flavan-3-ol structure on the quali-quantitative urinary excretion of phenolic metabolites. A double-blind, randomized, cross-over, acute intervention trial was performed in 11 healthy subjects consuming 1 mol of total flavan-3-ols from three different sources: green tea extract (GT), grape seed extract with high monomeric content (GSM), and grape seed extract with high procyanidin content (GSP). Baseline spot urine and the urine produced during the 24 hours (48 hours for 6 subjects) after the treatment were collected. Urine samples were analyzed by μ SPE and uHPLC-MS/MS. A total of 75 flavan-3-ol metabolites were quantified in urine samples, with phenyl- γ -valerolactones being the main class. Flavan-3-ols from GT had the highest bioavailability, while flavan-3-ols from GSP had the lowest value. Treatment with GT was characterized by (epi)gallocatechin derivatives, trihydroxylated and 3',5'-dihydroxylated phenyl- γ -valerolactones, trihydroxylated benzoic acids, benzene-triols, and 4'-hydroxycinnamic acids; GSM was characterized by (epi)catechins, monohydroxylated and 3',4'-dihydroxylated phenyl- γ -valerolactones, phenylvaleric acids, phenylpropanoic acids, and benzene-diols; GSP by cinnamic acids, phenylacetic acids, benzoic acids, and benzaldehydes. Moreover, the endogenous production of phenolic metabolites was evaluated, suggesting that small phenolic acids and benzene derivatives may determine biases in the bioavailability estimates. This work suggests that the flavan-3-ol structure influences the bioavailability and metabolism of this (poly)phenol class, being key in the investigation of the health effects deriving from the intake of (poly)phenol-rich foods.

Keywords

Flavan-3-ols, phenolic metabolites; gut microbiota; metabolism; phenyl- γ -valerolactones

4.2.1. Introduction

Flavan-3-ols are the most consumed flavonoids in Western diets across all age groups [1,2]. Their contribution to flavonoid intake (≈ 370 mg/day) is similar in Mediterranean and non-Mediterranean countries, reaching 75%, with a different contribution of flavan-3-ol monomers and proanthocyanidins (PACs): in Mediterranean countries, PACs contribute with 59% and flavan-3-ol monomers with only 13%, whereas in non-Mediterranean regions, PACs and flavan-3-ol monomers contribute with 48 and 24%, respectively [1]. Flavan-3-ols are characteristic compounds of several plant-based foods and beverages, such as green tea, cocoa, dark chocolate, red wine, nuts, legumes, pome fruits, stone fruits, grapes, and berries [3]. They are the unique flavonoid subclass that does not exist in planta in a glycosidic form, and they are the most complex group, ranging from simple monomers to oligomeric and polymeric PACs of up to 190 units [3–5]. The monomeric structure is characterized by two aromatic rings (A-ring and B-ring), a pyran ring (C-ring) condensed with A-ring, and a characteristic hydroxyl group in position 3 that can be esterified with gallic acid. B-ring can present a variable number of hydroxyl groups in different positions, giving origin to (epi)afzelechins (OH in 3' or 4'), (epi)catechins (OH in 3' and 4'), the most common monomers, and (epi)gallocatechins (OH in 3', 4' and 5') [3–5]. (Epi)gallocatechins and flavan-3-ol monomers esterified with gallic acid are characteristic of green tea [6]. PACs consisting exclusively of (epi)catechin units are called procyanidins (PCs) and are the most abundant type in plants, while prodelfinidins (PDs) are defined as PACs containing at least one unit of (epi)gallocatechin. The most common PCs are B-type PCs, formed by oxidative coupling between the C-4 of the heterocycle and the C-6 or C-8 positions of the adjacent unit, while A-type PCs contain an additional C2-O-C-5 or C2-O-C-7 ether linkage and exist only in a limited number of foods such as cranberries, persimmon, peanuts, plums, avocados, and cinnamon [3].

When ingested, monomeric flavan-3-ols can be absorbed in the small intestine and then conjugated by phase II enzymes present in enterocytes and hepatocytes, generating O-methylated, sulfated, and glucuronidated forms, named structurally-related flavan-3-ol metabolites (SRFMs), which are finally excreted through urine [4,5,7]. Monomers with a 3-O-galloyl moiety are resistant to phase II metabolism and circulate as native forms in the bloodstream, from which they are removed by the liver and returned to the small intestine in the bile [4,6]. However, most flavan-3-ol monomers (70% of (epi)catechins and 90% of

(epi)gallo catechins) and almost all PACs (>90%) are not absorbed in the upper gastrointestinal tract and reach the colon, where they are extensively metabolized by the human gut microbiota and converted to smaller catabolites, named ring-fission metabolites (RFMs). Galloylated flavan-3-ols need to be hydrolyzed by microbial esterases to be further catabolized [8]. The released gallic acid moieties can be absorbed and undergo phase II metabolism, or they can be subjected to microbial bioconversion (decarboxylation and subsequent dehydroxylation), giving origin to pyrogallol and catechol derivatives [9–12]. B-type PACs can follow 4 different pathways: (1) interflavan cleavage (minor pathway, <10%), releasing (epi)catechin monomers, (2) degradation of the lower unit, yielding phenyl- γ -valerolactones (PVLs), (3) degradation of the upper unit, yielding 3',4'-dihydroxyphenylacetic acid, and (4) simultaneous degradation of the upper and lower units, giving origin to 5-(2',4'-dihydroxyphenyl)-2-ene-valeric acid, an exclusive PAC metabolite [3]. The monomeric units deriving from undigested or hydrolyzed (epi)(gallo)catechins and cleaved PACs undergo C-ring opening, yielding diphenylpropan-2-ol derivatives (DPPOLs), and A-ring fission, giving origin to PVLs, which can be further converted into phenylvaleric acids (PVAs). PVAs and PVLs, also known as 5C-RFMs (ring fission metabolites with a 5-carbon chain), are characteristic flavan-3-ol metabolites and have been identified as biomarkers of flavan-3-ol intake [3,13,14]. Other circulating microbial metabolites derive from 5C-RFMs through β - and/or α - oxidation steps involving the side chain: among them, low molecular weight phenolic acids (3/2/1C-RFMs), including phenylpropanoic, phenylacetic, cinnamic, benzoic and hippuric acids, and benzaldehyde and benzene derivatives [7,15,16]. RFMs are easily absorbed in the colon and can undergo phase II metabolism in colonocytes and hepatocytes before being excreted in urine. Quantitative estimates of the extent of ring fission of the flavan-3-ol skeleton are difficult to assess because some urinary phenolic acids can also derive from human and/or microbial metabolism of drugs, endogenous compounds such as catecholamines, and other dietary components (aromatic amino acids, tocopherols, food additives) [7,17,18].

The dietary intake of flavan-3-ols has been associated with several beneficial effects on human health, especially on cardiometabolic diseases [19]. This protective action is not associated with the flavan-3-ol native structure, but with the bioactivity of the circulating metabolites, which are the molecules potentially available to target tissues. SRFMs and RFMs can act as mediators of diet-induced effects on health, being responsible for the beneficial properties attributed to flavan-3-ols [3,20–22]. The structural properties of parent flavan-3-ols, including

the degree of polymerization, the type of subunit linkages, the extent of B-ring hydroxylation, and the presence of galloyl moieties, may affect the native compound-colonic microbiota interaction and the subsequent microbial catabolism, representing a key aspect of the protective effects associated with the intake of these phytochemicals. In particular, the degree of polymerization, the type of subunit linkages, and the extent of B-ring hydroxylation can influence the bioavailability, (epi)catechins being more bioavailable than (epi)gallocatechins and PACs, and B-type PACs being more available than A-type PACs [23–28]. The native structure can also influence the metabolite profile: for example, (epi)gallocatechins and galloylated (epi)(gallo)catechins yield specific metabolites such as trihydroxylated and 3',5'-dihydroxylated derivatives, and PAC catabolism gives origin to a plethora of 3',4'-dihydroxylated or monohydroxylated phenolic acids [7,25,28–34]. Moreover, pharmacokinetics can be influenced, with a delayed metabolism for PACs, due to their steric hindrance limiting the access to the C-ring by the colonic microbiota, and for galloylated monomers, which need to be hydrolyzed by gut microbiota enzymes to be further catabolized.

The aim of the present study was to evaluate the influence of the flavan-3-ol structure, in particular the degree of polymerization and the monomer structure (extent of B-ring hydroxylation and presence of galloyl moieties) on the bioavailability and metabolite profile of this (poly)phenol class in a human intervention trial. In particular, the primary outcome was the assessment of the urinary excretion of phenolic metabolites after the consumption of three different plant extracts used as flavan-3-ol sources. Secondary outcomes are methodological considerations about (1) the consistency between the results obtained using the volume of 24 h urine (amount of metabolites) and the urinary creatinine concentration (concentration of metabolites per unit of creatinine), to evaluate the accuracy and reliability of creatinine-corrected measures of total urinary (poly)phenol metabolites, and (2) the contribution of endogenous metabolites to the pool of circulating flavan-3-ol metabolites, in order to assess the impact of non-phytochemical metabolism on the phenolic metabolic profile, and to avoid biases in the bioavailability estimates. For this purpose, an evaluation of the excretion of low molecular weight phenolic metabolites was performed in baseline spot urine. Moreover, the excretion of specific flavan-3-ol metabolites, including SRFMs and 5C-RFMs, allowed to check for volunteers' dietary compliance along with the dietary record.

4.2.2. Materials and methods

Materials

All chemicals and solvents used in this study were of analytical grade and are indicated in Supplementary Information S1.1.

Participants

Eleven healthy participants, aged 18-70 years, with a body mass index (BMI) of 18.5-30 kg/m², were recruited through advertising at the university, at the hospital and in public places in Parma. Exclusion criteria included history or presence of cancer, metabolic, cardiovascular, endocrine and/or inflammatory disease; chronic/systemic illnesses; history of surgeries of liver, kidney or gastrointestinal tract; immunodeficiency, anemia or other blood disorders; anxiety, depression, psychological problems; eating disorders, food intolerances or allergies; regular consumption of medication and antibiotic therapy within the last 3 months; intense physical activity. Participants were also excluded if they were pregnant, lactating, or contemplating pregnancy. These criteria were set to avoid likely confounding factors and were in line with similar works in the field [35,36]. The initial aim of the study was to enroll fifty participants, but the recruitment was interrupted because of Covid-19 pandemic. The original sample size was calculated basing on studies with a similar aim [35,37].

Food products

The food products used in the study were plant extracts in powder obtained from Indena S.p.a. and ACTIV'INSIDE. Three plant extracts were chosen as sources of flavan-3-ols of different structure: (1) green tea extract (GT), rich in trihydroxylated and galloylated flavan-3-ol monomers (Green tea dry decaffeinated extract (poly)phenols - Greenselect®, Indena S.p.a.), (2) grape seed extract with high monomeric content (GSM), rich in dihydroxylated, monomeric flavan-3-ols (Grape seed inside ex 30M, ACTIV'INSIDE), and (3) grape seed extract with high procyanidin content (GSP), rich in dihydroxylated, oligomeric flavan-3-ols (Grape seed oligomers, Indena S.p.a).

Intervention study

The study was approved by the Research Ethics Committee (REC) of Area Vasta Emilia Nord in March 2019 and was registered on ClinicalTrials.gov website (NCT04124016). The study was conducted in accordance with the guidelines stated in the current revision of the Declaration of Helsinki and participants gave their informed written consent before enrollment. The study was a double-blind, randomized, cross-over, acute intervention trial, consisting of 3 consecutive treatments with a washout period of at least one week. The intervention involved the intake of three different plant extracts in a dose providing 1 mole of total flavan-3-ols, which is in line with the daily intake of these (poly)phenol class in European populations and is compliant with the Scientific Opinion of EFSA on the safety of green tea catechins [1,38]. The dose given to volunteers was the following: 624 mg for GT, 472 mg for GSM, and 761 mg for GSP. Since the study was interrupted because of Covid-19 pandemic, only 2 volunteers completed the treatments (GT, GSM and GSP in a random order). Five volunteers were subjected to two treatments (3 subjects consumed GT and GSM, 1 subject consumed GT and GSP, and 1 consumed GSM and GSP), and 4 volunteers were subjected to one treatment (2 subjects consumed GSM, and 2 consumed GSP). To sum up, a total of 20 treatments were carried out: 6 subjects consumed GT, 9 subjects consumed GSM, and 6 subjects consumed GSP. The morning of each treatment, after fasting baseline urine collection (spot sample at 0 h, t₀), volunteers received a standardized breakfast and the dose of one of the food extracts. Breakfast consisted of a pre-packaged, (poly)phenol-free, semisolid meal (Risolat vaniglia, 1% fat, FAGE®), and the extract powder was mixed with it. Participants collected all the urine produced in the 24 hours after the intervention (24 h urine). Six subjects continued the collection for the following 24 hours (48-h subgroup): among them, 4 subjects consumed GT, 4 consumed GSM, and 3 consumed GSP, for a total of 11 treatments. Volunteers were asked to follow a (poly)phenol-free diet for 2 days prior to the intervention and during urine collection (24 or 48 hours after the extract intake). To check dietary compliance, a 3/4-days dietary record of the 2 days prior to each sampling day and of the sampling day(s) was used.

Food product analysis

Each food product was extracted and analyzed in triplicate. Briefly, 50 mg of powder were added with 1 mL of 80% aqueous methanol acidified with formic acid (0.1%). The solution was vortexed for 1 minute, sonicated for 20 min at 25°C, vortexed for 1 minute, and centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant was collected. The pellet was re-extracted twice

using 0.5 mL of the same solvent, following the same procedure, and the three supernatants were pooled. Three different dilutions with water 0.1% formic acid were performed (1:5, 1:20, 1:200) before analysis. The identification and quantification of the flavan-3-ols present in the extracts was performed by ultra-high performance liquid chromatography coupled with mass spectrometry (uHPLC-MS). Details are described in Supplementary Information S1.2. Flavan-3-ol monomers, prodelphinidin dimers and trimers, and procyanidins up to a degree of polymerization (DP) of 20 were monitored, reaching a total of 57 compounds (Table S1 and Table S2 in Supplementary Information). Chromatograms, mass spectral data and data processing were performed using Xcalibur software 2.1 (Thermo Fisher Scientific Inc.). A total of 42 flavan-3-ol monomers and oligomers with a $DP \leq 5$ were monitored, and their identification was carried out by comparison of the retention time with in-house synthesized standards and/or with MS^2 and MS^3 fragmentation patterns. Quantification was performed using calibration curves built with pure standards, when available, or using the curves built with the most structurally similar compound. For PCs with a DP between 3 and 20, the chromatographic separation of the different isomers was not optimal (not fully resolved peaks), so that the peak generated through the selection of a specific m/z from the total ion current (TIC) in a specific range of retention time was considered to obtain a semi-quantification (Table S2 in Supplementary Information). For PCs with a $DP > 5$, the pentamer E1 was used as standard. Because of their high molecular weight (>2000 Da), some PACs have been identified and quantified using double charged ions $[M-2H]^{2-}$ or triple charged ions $[M-3H]^{3-}$. Concentration of flavan-3-ols in the extracts was expressed as $\mu\text{mol/g}$ and mg/g , and data were expressed as mean values $\pm$ standard deviation (SD).

Urine sample analysis

Urinary extraction of phenolic metabolites was performed using microelution solid phase extraction (μSPE) according to validated protocols, with some modifications [39–41]. Briefly, urine samples (100 μl) were diluted with water 0.1% formic acid (1:5). Another sample dilution (350 μl) was performed with water 4% phosphoric acid to reduce phenolic-protein interactions. Each sample (400 μl) was loaded on a 96 well μSPE plate, washed with water (200 μl) and water 0.2% acetic acid (200 μl), and finally eluted in three steps with methanol 0.1% formic acid and 10 nmol/L ammonium formate solution (30 μl for each elution step). Then, samples have been analyzed with ultra-high performance liquid chromatography coupled with

tandem mass spectrometry (uHPLC-MS/MS) to identify and quantify phenolic metabolites. Details are described in Supplementary Information S1.3.

Up to 126 compounds were simultaneously monitored in selective reaction monitoring (SRM) mode (Table S3 in Supplementary Information). Chromatograms, mass spectral data and data processing were performed using Xcalibur software 2.1 (Thermo Fisher Scientific Inc.). Metabolite identification was carried out by comparison of the retention time with in-house synthesized standards and/or MS/MS fragmentation patterns. The nomenclature used for (poly)phenol catabolites follows the current recommendations [42]. Quantification was performed with calibration curves of standards, when available; when not available, compounds were quantified with the most structurally similar compound. Urinary excretion of phenolic metabolites in 24 and 48 hours was expressed in two ways: (1) as amount of phenolic metabolites excreted (μmol), which was calculated multiplying the metabolite concentration ($\mu\text{mol/L}$) by the volume of urine produced in 24 or 48 hours, and (2) as concentration of phenolic metabolites per unit of creatinine excreted ($\mu\text{mol/mol}$ urinary creatinine), which was calculated dividing the metabolite concentration ($\mu\text{mol/L}$) by creatinine concentration (mol/L) in order to normalize the data. Urinary excretion at baseline was expressed as concentration of metabolites per unit of creatinine ($\mu\text{mol/mol}$ urinary creatinine). Sums of urinary metabolites belonging to the same aglycone moiety were calculated, defining 22 different classes, that were referred to as colonic classes.

Creatinine analysis

Urinary creatinine analysis was performed with ultra-high performance liquid chromatography coupled with tandem mass spectrometry (uHPLC-MS/MS) after dilution of samples with water 0.1% formic acid (1:1000). Details are described in Supplementary Information S1.4.

Statistical analysis on urine data

All data were expressed as mean values $\pm$ standard error of the mean (SEM). Univariate statistical analyses were performed using IBM SPSS Statistics version 26 (IBM, Chicago, IL, USA), and unsupervised multivariate analyses were performed using SIMCA 16.0.1 software (Sartorius Stedim Data Analytics, Umea, Sweden). Univariate statistical techniques were used to assess the differences in the excreted amounts of colonic classes and in the total excreted amounts of phenolic metabolites among treatments both at 24 h and 48 h. The normality of

data distribution within each treatment was checked through the Kolmogorov–Smirnov test. For normally distributed variables, one-way analysis of variance (ANOVA) was performed to compare the mean concentrations among treatment groups. Data homoscedasticity was checked through Levene’s test. *Post-hoc* analysis was conducted using *post hoc* Tukey test, since variances in treatment groups were equal (homoscedastic variables). For non-normally distributed variables, non-parametric Kruskal–Wallis test with *post hoc* pairwise multiple comparison and Bonferroni correction was used. Differences were considered significant at p value < 0.05 . Correlation analysis was performed to assess the relationship between the metabolic phenolic profile (excretion of colonic classes and total excretion of phenolic metabolites) obtained considering the excreted amounts of metabolites and the profile obtained considering the excreted concentrations of metabolites per creatinine unit. The normality of data distribution was checked through the Kolmogorov–Smirnov test. Pearson’s and Spearman’s correlation coefficients were used for normally and non-normally distributed variables, respectively. Coefficients were considered significant at p value < 0.05 . Comparisons between concentrations in 24 h urine and in spot urine were done by dependent sample Student t test for normally distributed variables and Wilcoxon test for non-normally distributed variables. Differences were considered significant at p value < 0.05 .

Principal Component Analysis (PCA) was used to assess the differences in the phenolic profiles among treatments (GT, GSM, GSP) and times (24 and 48 h), considering the excreted amounts of colonic classes as variables, and including all observations (subjects’ excretion values at 24 h and 48 h). Data were pre-treated with mean-centering and UV-scaling, and three principal components (PCs) were set (PC1, PC2, PC3). Centering converted all the data to variations around zero instead of around the mean of the data; UV scaling used standard deviation as scaling factor to give all variables equal weight. The parameters used to assess the quality of each model and subsequent data interpretability were R^2X and Q^2 , namely the model fit (or explained variation) and the predictive ability, respectively. PCA was performed using SIMCA 16.0.1 software (Sartorius Stedim Data Analytics, Umea, Sweden).

4.2.3. Results

Characterization of the flavan-3-ol content and composition of the plant extracts

A total of 37 flavan-3-ol monomers and oligomers with a $DP \leq 5$ were identified and quantified in the plant extracts. These compounds include several classes: (1) 3 (epi)catechins ((E)Cs), namely (+)-catechin (C), (-)-epicatechin (EC) and (-)-epicatechin-3-O-gallate (ECG), (2) 3 (epi)gallocatechins ((E)GCs), namely (+)-gallocatechin (GC), (-)-epigallocatechin (EGC) and (-)-epigallocatechin-3-O-gallate (EGCG), (3) 9 B-type PC dimers, composed of (E)C and/or (E)CG units, (4) 7 B-type PD dimers, with at least one monomer of (E)GC or (E)GCG, (5) 2 A-type PD dimers, composed of (E)GC and (E)GCG units, (6) 8 B-type PC trimers, including trimers composed of (E)C and/or (E)CG units, (7) 3 B-type PC tetramers, composed of (E)C units, and (8) 2 B-type PC pentamers, composed of (E)C units. PD trimers were searched but not identified. A total of 20 compounds were quantified in GT, including 3 (E)Cs, 3 (E)GCs, 5 PC dimers, and 9 PD dimers (7 B-type and 2 A-type dimers); 17 compounds were quantified in GSM, including 2 (E)Cs, 7 PC dimers, 6 PC trimers, and 2 PC tetramers; 20 compounds were quantified in GSP, including 3 (E)Cs, 6 PC dimers, 6 PC trimers, 3 PC tetramers, and 2 PC pentamers. Moreover, 15 PCs with a DP between 6 and 20 were semi-quantified in grape seed extracts (GSs), of which 6 in GSM and 15 in GSP. The dimer composition of the extracts was consistent with the monomer composition in terms of unit structure, as expected. Regarding flavan-3-ol molar composition of the extracts, GT is composed of monomers, both (E)Cs (28% of total flavan-3-ol moles) and (E)GCs (67%), and a minor amount of dimers, both PCs (B-type, 2%) and PDs (A- and B-type, 4%), while GSs are composed of (E)Cs (68% for GSM and 52% for GSP) and PCs with a different DP (below 11 for GSM and up to 20 for GSP). The relative amount of dimers is the same in GSM and GSP (22%), while trimers are more abundant in GSP (16%) than in GSM (8%), along with oligomers with a higher DP (2% in GSM and 9% in GSP). Polymers ($DP > 10$) are present only in traces in GSM and are higher in GSP (2%) (Table S4).

GT is the extract richest in total flavan-3-ols and in flavan-3-ol monomers from a quantitative point of view, and richest in flavan-3-ol monomers and dimers on a qualitative basis, since it contains (E)GCs and PDs along with (E)C and PCs. However, it is the poorest in PAC content, being composed only by a small quantity of dimers, most of which (70%) are esterified with at least one gallic acid moiety. The most abundant monomer is EGCG (50% of total monomers, considering molar quantities), followed by EGC (18%) and ECG (18%), with a smaller contribution of EC (9%), C (2%) and GC (2%). The total amount of monomers (6.3 mg/g) is in line with the information given by the manufacturer (7.3 mg/g) (Table S4), and the flavan-3-ol composition is consistent with the literature [43,44]. GSM is the extract richest in (E)Cs and

dimers. Almost half of dimers (41%) are esterified with at least one gallic acid moiety. The most abundant monomers are C and EC, and the main dimers are composed of 2 (epi)catechin units, followed by dimers composed of one (E)C unit and one (E)CG unit. ECG and dimers with two ECG units are present in minor amounts, as well as trimers and tetramers. Trimers with 3 (E)C units are the most common, followed by trimers with one unit of (E)CG, while trimers with two or three units of (E)CG are less present. This is consistent with the composition in flavan-3-ol monomers. PCs with a DP between 4 and 11 are present in small quantities. The total amount of monomers (3.1 mg/g) and dimers (2.0 mg/g) is in line with the information given by the manufacturer (monomers 3.7 mg/g; dimers 1.8 mg/g) (Table S4), and the flavan-3-ol composition is consistent with the literature [45,46]. GSP is the extract poorest in total flavan-3-ols but richest in high molecular weight oligomers ($4 \leq DP \leq 10$) and polymers ($10 < DP \leq 20$). This is in line with the information given by the manufacturer (Table S4). The amount of trimers is similar to GSM, and the relative composition in flavan-3-ol monomers, dimers and trimers is similar to the composition of GSM, as expected considering the common origin of the extracts (grape seed). The amount of PCs in GSM and GSP was calculated in a semi-quantitative way and resulted underestimated compared to the information given by the manufacturer, which used gel permeation chromatography (GPC) to quantify oligomers and polymers. In particular, the amount of PCs in GSM is about 71% of the value indicated in the technical sheet (3.4 mg/g and 4.8 mg/g, respectively), while the amount of PCs in GSP is about 24% of the value given by the manufacturer (2.1 mg/g and 8.6 mg/g, respectively) (Table S4). This was expected for several reasons: (1) HPLC methods with C18 columns are inefficient and need to be improved to separate flavan-3-ols with a high DP ($DP \geq 4$) [46]; (2) HPLC methods under reversed phase conditions follow a different fractionation technique than GPC methods, resulting in possible different outcomes [46]; (3) since separation with HPLC was not optimal, quantification of PCs with a DP between 3 and 20 was performed in a semi-quantitative way, considering the peak generated through the selection of a specific m/z from the total ion current (TIC) in a specific range of retention time; (4) due to the absence of pure PC compounds with a $DP > 5$, quantification of PCs with a DP between 6 and 20 was performed using the pentamer [(E)C]₅ as standard, resulting in a putative quantification bias due to different ionization efficiencies.

Characterization of the metabolite profile derived from flavan-3-ols

A comprehensive quantification of flavan-3-ol metabolites was performed in urine (Table 1).

Table 1. Total 24 h and 48 h urinary excretion (μmol) of individual flavan-3-ol metabolites and total metabolites (with and without hippuric acid derivatives) after treatment with green tea extract (GT), grape seed extract rich in monomers (GSM) and grape seed extract rich in procyanidins (GSP). Data are indicated as mean $\pm$ SEM.

Urinary flavan-3-ol metabolite	Total 24 h urinary excretion (μmol)			Total 48 h urinary excretion (μmol)		
	GT treatment	GSM treatment	GSP treatment	GT treatment	GSM treatment	GSP treatment
Structurally-related flavan-3-ol metabolites						
(Epi)gallocatechin-glucuronide	0.33 $\pm$ 0.07	-	-	0.39 $\pm$ 0.06	-	-
Methoxy-(epi)gallocatechin-sulfate	0.03 $\pm$ 0.00	-	-	0.03 $\pm$ 0.01	-	-
Methoxy-(epi)gallocatechin-glucuronide	0.30 $\pm$ 0.03	-	-	0.27 $\pm$ 0.09	-	-
(Epi)catechin-sulfate	0.23 $\pm$ 0.03	0.87 $\pm$ 0.12	0.15 $\pm$ 0.03	0.14 $\pm$ 0.05	0.85 $\pm$ 0.12	0.17 $\pm$ 0.04
(Epi)catechin-glucuronide isomer 1	0.03 $\pm$ 0.02	0.64 $\pm$ 0.12	-	0.04 $\pm$ 0.04	0.64 $\pm$ 0.21	-
(Epi)catechin-glucuronide isomer 2	1.20 $\pm$ 0.20	4.28 $\pm$ 0.60	0.47 $\pm$ 0.09	1.30 $\pm$ 0.17	4.25 $\pm$ 1.37	0.50 $\pm$ 0.04
Methoxy-(epi)catechin-sulfate	0.18 $\pm$ 0.02	0.66 $\pm$ 0.05	0.12 $\pm$ 0.02	0.15 $\pm$ 0.01	0.69 $\pm$ 0.08	0.15 $\pm$ 0.03
Methoxy-(epi)catechin-glucuronide isomer 1	0.01 $\pm$ 0.01	0.47 $\pm$ 0.06	0.07 $\pm$ 0.01	-	0.49 $\pm$ 0.14	0.05 $\pm$ 0.02
Methoxy-(epi)catechin-glucuronide isomer 2	0.01 $\pm$ 0.01	0.96 $\pm$ 0.10	0.14 $\pm$ 0.02	-	0.96 $\pm$ 0.23	0.17 $\pm$ 0.01
Phenyl-γ-valerolactones						
5-(3',4',5'-Trihydroxyphenyl)- γ -valerolactone	0.66 $\pm$ 0.59	-	-	0.55 $\pm$ 0.55	-	-
5-(Dihydroxyphenyl)- γ -valerolactone-sulfate (3',4',5' isomers)	7.64 $\pm$ 3.43	-	-	9.15 $\pm$ 6.15	-	-
5-(Dihydroxyphenyl)- γ -valerolactone-glucuronide (3',4',5') isomer 1	2.28 $\pm$ 1.30	-	-	3.09 $\pm$ 2.22	-	-
5-(Dihydroxyphenyl)- γ -valerolactone-glucuronide (3',4',5') isomer 2	22.30 $\pm$ 9.12	-	-	25.22 $\pm$ 16.21	-	-

5-(Hydroxyphenyl)- γ -valerolactone-methoxy-sulfate (3',4',5') isomer 1	20.03±9.24	0.01±0.01	-	44.40±29.74	0.03±0.03	-
5-(Hydroxyphenyl)- γ -valerolactone-methoxy-sulfate (3',4',5') isomer 2	5.27±2.64	-	-	6.31±4.42	-	-
5-(Hydroxyphenyl)- γ -valerolactone-methoxy-glucuronide (3',4',5') isomer 1	1.83±0.41	-	-	2.14±0.91	-	-
5-(Hydroxyphenyl)- γ -valerolactone-methoxy-glucuronide (3',4',5') isomer 2	3.46±1.58	-	-	4.36±2.99	-	-
5-(Hydroxyphenyl)- γ -valerolactone-sulfate-glucuronide (3',4',5' isomers)	0.34±0.12	-	-	0.48±0.17	-	-
5-(3',5'-Dihydroxyphenyl)- γ -valerolactone	1.83±0.43	-	-	2.80±0.62	-	-
5-(Hydroxyphenyl)- γ -valerolactone-sulfate (3',5' isomers)	95.38±21.57	0.22±0.10	0.14±0.09	139.95±31.11	0.28±0.12	0.08±0.08
5-(Hydroxyphenyl)- γ -valerolactone-glucuronide (3',5' isomers)	51.31±13.57	-	-	70.02±18.38	-	-
5-(3',4'-Dihydroxyphenyl)- γ -valerolactone	0.75±0.05	1.99±0.38	0.69±0.14	0.62±0.08	1.84±0.32	0.56±0.29
5-(Hydroxyphenyl)- γ -valerolactone-sulfate (3',4' isomers)	59.15±8.76	119.54±15.71	53.63±6.12	70.56±9.94	155.81±13.68	70.57±11.97
5-(Hydroxyphenyl)- γ -valerolactone-glucuronide (3',4' isomers)	50.44±5.13	103.24±11.77	38.19±3.97	59.76±4.47	101.67±6.79	43.90±5.51
5-Phenyl- γ -valerolactone-methoxy-sulfate (3',4' isomers)	1.40±0.34	2.44±0.34	0.85±0.08	1.88±0.44	3.59±0.43	1.47±0.30
5-Phenyl- γ -valerolactone-methoxy-glucuronide (3',4' isomers)	1.97±0.47	7.88±1.36	2.61±0.24	2.57±0.36	9.46±2.26	3.08±0.47
5-(3'-Hydroxyphenyl)- γ -valerolactone	0.53±0.31	1.44±0.59	0.21±0.17	0.44 ±0.44	1.43±0.90	0.43 ±0.43
5-Phenyl- γ -valerolactone-sulfate (3'/4' isomers)	14.26±5.97	32.61±13.72	6.67±4.46	13.92±10.57	45.64±29.21	17.53±14.66
5-Phenyl- γ -valerolactone-4'-glucuronide	0.74±0.14	0.41±0.12	0.12±0.04	0.83±0.08	0.77±0.34	0.12±0.02
5-Phenyl- γ -valerolactone-3'-glucuronide	11.66±5.10	24.05±8.91	4.55±3.04	12.27±9.19	30.22±18.31	11.65±9.11

Phenylvaleric acids						
4-Hydroxy-5-(phenyl)valeric acid-glucuronide (3',4' isomers)	0.68±0.36	2.28±0.82	0.26±0.20	0.54±0.50	2.63±1.40	0.63±0.63
4-Hydroxy-5-(3',4'-dihydroxyphenyl)valeric acid	1.52±0.21	1.54±0.14	1.26±0.14	1.57±0.22	1.59±0.11	1.84±0.33
4-Hydroxy-5-(hydroxyphenyl)valeric acid-sulfate (3',4' isomers)	0.03±0.01	0.01±0.00	0.01±0.01	0.04±0.02	0.02±0.02	0.06±0.02
4-Hydroxy-5-(hydroxyphenyl)valeric acid-glucuronide (3',4' isomers)	0.10±0.07	0.87±0.08	0.23 ±0.06	0.11±0.11	0.69±0.03	0.23±0.12
Cinnamic acids						
3',4'-Dihydroxycinnamic acid	0.90±0.07	1.10±0.13	0.89±0.21	1.45±0.09	1.76±0.42	1.22±0.22
4'-Hydroxycinnamic acid-3'-glucuronide	0.19±0.03	0.22±0.06	0.15±0.03	0.42±0.10	0.32±0.07	0.35±0.10
4'-Hydroxy-3'-methoxycinnamic acid	0.05±0.01	0.04±0.01	0.02±0.01	0.08±0.04	0.11±0.02	0.10±0.03
Methoxycinnamic acid-4'-sulfate	0.64±0.19	0.74±0.12	0.36±0.07	1.40±0.39	1.53±0.19	1.11±0.24
3'-Methoxycinnamic acid-4'-glucuronide	1.23±0.23	1.18±0.13	0.86±0.17	1.65±0.23	1.81±0.20	1.70±0.12
4'-Methoxycinnamic acid-3'-glucuronide	0.15±0.03	0.26±0.05	0.10±0.02	0.20±0.03	0.36±0.08	0.25±0.03
Feruloylglycine	1.25±0.35	1.42±0.36	0.77±0.10	2.34±0.79	2.07±0.35	2.32±0.77
4'-Hydroxycinnamic acid	0.04±0.01	0.02±0.01	0.02±0.01	0.04±0.01	0.04±0.01	0.03±0.01
3'-Hydroxycinnamic acid	0.38±0.10	0.30±0.05	0.24±0.04	0.72±0.29	0.50±0.11	0.61±0.17
Phenylpropanoic acids						
3-(Hydroxyphenyl)propanoic acid-sulfate (3',4' isomers)	0.90±0.38	1.58±0.72	0.52±0.14	2.29±1.06	3.07±1.25	1.42±0.52
3-(3'-Hydroxyphenyl)propanoic acid-4'-glucuronide	0.70±0.40	1.45±0.99	0.33±0.23	2.27±1.33	2.31±1.39	0.59±0.59

3-(4'-Hydroxyphenyl)propanoic acid-3'-glucuronide	4.33±1.12	5.13±1.60	2.87±0.64	8.22±2.97	7.31±2.59	5.23±2.14
3-(4'-Hydroxy-3'-methoxyphenyl)propanoic acid	0.17±0.05	0.20±0.04	0.11±0.04	0.36±0.08	0.34±0.09	0.41±0.15
3-(3'-Hydroxy-4'-methoxyphenyl)propanoic acid	0.12±0.06	0.30±0.18	0.07±0.04	0.31±0.18	0.44±0.08	0.14±0.12
3-(Methoxy-phenyl)propanoic acid-sulfate (3',4' isomers)	0.25±0.07	0.30±0.09	0.14±0.03	0.66±0.24	0.45±0.18	0.37±0.11
3-(3'-Methoxy-phenyl)propanoic acid-4'-glucuronide	0.14±0.04	0.21±0.04	0.12±0.03	0.30±0.11	0.20±0.04	0.35±0.13
3-(4'-Methoxy-phenyl)propanoic acid-3'-glucuronide	0.39±0.05	0.43±0.06	0.34±0.05	0.76±0.10	0.74±0.10	0.79±0.09
3-(3'-Hydroxyphenyl)propanoic acid	0.08±0.08	0.09±0.03	0.03±0.02	0.11±0.11	0.17±0.10	0.12±0.12
3-(Phenyl)propanoic acid-sulfate (3'/4' isomers)	0.55±0.31	1.05±0.25	0.33±0.11	1.87±1.33	2.24±0.57	0.73±0.18
3-(Phenyl)propanoic acid-4'-glucuronide	0.31±0.04	0.27±0.06	0.23±0.04	0.68±0.09	0.66±0.20	0.43±0.13
3-(Phenyl)propanoic acid-3'-glucuronide	0.60±0.26	0.32±0.09	0.10±0.05	0.94±0.53	0.72±0.23	0.14±0.14
Phenylacetic acids						
4'-Hydroxy-3'-methoxyphenylacetic acid	0.66±0.06	0.77±0.04	0.71±0.10	1.50±0.21	1.38±0.10	1.69±0.20
3'-Methoxy-phenylacetic acid-4'-sulfate	0.34±0.07	0.34±0.07	0.36±0.14	0.88±0.19	0.93±0.16	0.80±0.03
3'-Methoxy-phenylacetic acid-4'-glucuronide	0.35±0.19	0.70±0.47	0.16±0.11	1.09±0.63	1.11±0.66	0.36±0.23
4'-Hydroxyphenylacetic acid	7.59±1.31	6.33±1.51	6.41±1.64	18.63±3.12	8.55±0.70	11.41±2.09
3'-Hydroxyphenylacetic acid	5.23±2.15	4.32±1.05	4.14±0.77	7.99±3.51	6.24±1.42	9.25±3.26
Benzoic acids						
3,4,5-Trihydroxybenzoic acid	0.13±0.06	0.07±0.02	-	0.15±0.06	0.05±0.03	-

4-Methoxy-3,5-dihydroxybenzoic acid	0.55±0.14	0.47±0.08	0.06±0.06	0.60±0.24	0.37±0.22	-
3,4-Dihydroxybenzoic acid	0.35±0.08	0.28±0.03	0.31±0.08	0.75±0.21	0.58±0.06	0.54±0.02
4-Hydroxy-3-methoxybenzoic acid	15.04±4.46	10.35±2.48	10.94±2.60	17.14±5.56	10.24±2.72	16.19±6.55
Methoxybenzoic acid-sulfate (3,4 isomers)	12.55±3.17	11.70±2.55	11.02±1.90	20.98±5.88	19.37±2.77	20.61±3.21
3-Methoxybenzoic acid-4-glucuronide	21.39±2.73	17.74±1.52	15.81±2.04	26.33±3.62	19.11±2.63	24.09±1.98
4-Hydroxybenzoic acid	2.23±0.52	1.90±0.21	1.78±0.29	5.16±1.10	4.26±0.64	3.32±0.77
Benzoic acid-sulfate (3/4 isomers)	5.30±2.28	4.98±1.20	5.43±0.62	15.68±7.92	9.81±3.18	12.47±0.74
Hippuric acids						
4'-Hydroxyhippuric acid	8.32±2.99	6.25±1.26	4.82±0.67	17.60±7.85	13.28±4.89	9.46±0.06
Hippuric acid	25.63±2.36	31.17±3.90	25.25±5.77	39.98±2.07	48.88±11.41	35.04±5.67
Benzaldehydes						
4-Hydroxybenzaldehyde	0.25±0.03	0.36±0.08	0.30±0.05	0.55±0.19	0.50±0.10	0.59±0.21
Benzene derivatives						
2,6/2,3-Dihydroxybenzene-1-glucuronide	5.10±0.41	0.36±0.04	0.15±0.05	4.66±0.67	0.25±0.07	0.10±0.04
2-Hydroxy-3/6-methoxybenzene-1-sulfate or 3-Hydroxy-2-methoxybenzene-1-sulfate	1.45±0.22	0.18±0.03	0.10±0.03	1.64±0.15	0.17±0.07	0.12±0.08
2-Hydroxy-benzene-1-sulfate	5.01±0.92	12.00±1.22	8.30±0.86	12.19±3.14	21.48±3.10	14.80±2.62
2-Hydroxy-4/5-methylbenzene-1-sulfate	0.33±0.03	0.63±0.07	0.41±0.05	0.64±0.12	1.07±0.20	0.69±0.09
Total flavan-3-ol metabolites	488.87±47.66	433.73±39.28	215.31±23.44	674.56±74.85	558.07±66.62	332.80±68.04
Total flavan-3-ol metabolites without hippuric acid derivatives	454.91±44.61	396.31±38.69	185.24±20.01	616.97±65.99	495.91±59.17	288.30±62.36

A total of 75 flavan-3-ol metabolites were quantified after extract consumption: 75 were detected after GT intake, while 62 and 59 were identified after GSM and GSP consumption, respectively. Metabolites belong to the metabolic pathway of flavan-3-ol monomers (both (E)Cs and (E)GCs) and PACs, and can be classified in the following phenolic groups: structurally-related flavan-3-ol metabolites (SRFMs, n=9), phenyl- γ -valerolactones (PVLs, n=21), phenylvaleric acids (PVAs, n=4), phenylpropanoic acids (PPAs, n=12), phenylacetic acids (PAAs, n=5), cinnamic acids (CAs, n=9), benzoic acids (BAs, n=8), hippuric acids (HAs, n=2), benzaldehydes (BALs, n=1), benzene derivatives (BZs, n=4). The metabolites with the highest excretion values both at 24 h and 48 h were dihydroxyphenyl- γ -valerolactones (3',5' isomers for GT and 3',4' isomers for GSM and GSP) in their monoconjugated forms, namely hydroxyphenyl- γ -valerolactone-sulfate (HPVL-S) and hydroxyphenyl- γ -valerolactone-glucuronide (HPVL-GlcUA). Excretion of HPVL-S (3',5' isomers for GT and 3',4' for GT, GSM and GSP) exceeded 50 μ mol in 24 h and 48 h after consumption of all extracts, while excretion of HPVL-GlcUA exceeded that value only after consumption of GT (3',5' and 3',4' isomers) and GSM (3',4' isomers). PVLs were the most represented class after flavan-3-ol intake (on average, 63.4% and 58.2% of all metabolites at 24 and 48 h, respectively), followed by BAs (14.6% at 24 h and 15.8% at 48 h), HAs (9.8% at 24 h and 11.0% at 48 h), PAAs (3.7% at 24 h and 4.9% at 48 h), BZs (3.2% at 24 h and 3.9% at 48 h), PPAs (2.3% at 24 h and 3.1% at 48 h) and CAs (1.2% at 24 h and 1.6% at 48 h). The contribution of the remaining phenolic classes (PVAs, BALs) was less important (<1%). The proportion of SRFMs, PVLs and PVAs was greater at 24 h than at 48 h, while the contribution of small molecular weight phenolic metabolites (PPAs, PAAs, CAs, BAs, HAs, BALs and BZs) on the overall excretion was greater at 48 h (Figure 1).

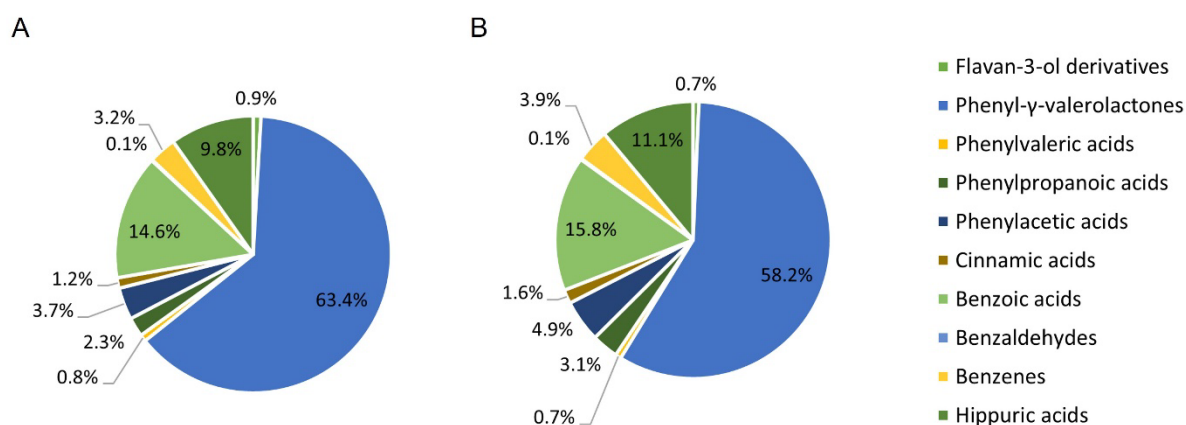


Figure 1. Distribution of classes of flavan-3-ol metabolites excreted in urine (A) during the 24 hours after the treatments and (B) during the 48 hours after the treatments.

Differences among treatments in the type and amount of phenolic metabolites excreted in 24 h and 48 h

The urinary profile of the different treatments was analyzed to detect differences in the production of classes of flavan-3-ol metabolites at 24 h and 48 h. In this case, compounds belonging to HAs, including hippuric acid and 4'-hydroxyhippuric acid, were excluded because they are not specific for phytochemical metabolism. Hippuric acid is formed in the liver by glycine conjugation of human and colonic benzoic acid, which can derive from sources other than (poly)phenols, such as aromatic amino acids (tyrosine, phenylalanine, tryptophan) and food additives [17,47–49]. Moreover, urinary hippuric acid levels correlate with hypertension, obesity, and age [50–52]. The distribution of phenolic classes without HAs in urine after treatment with GT (Figure 2A and 2B) and GSM (Figure 2C and 2D) was similar, with a major contribution of PVLs both at 24 h (77.9% and 74.1%, respectively) and at 48 h (73.2% and 70.7 %, respectively), followed by BAs. Treatment with GSM showed a higher percentage of SRFMs and PVAs than treatment with GT, both at 24 and 48 h. Treatment with GSP had a lower contribution of PVLs (58.1% at 24 h and 51.8% at 48 h) and SRFMs, and a greater contribution of small metabolites such as PAAs, CAs, BAs, and BZs compared with treatment with GSM (Figure 2E and 2F). In particular, BAs were very relevant, contributing for 24.5% at 24 h and 26.8% at 48 h.

To analyze more deeply flavan-3-ol metabolism, specific classes of flavan-3-ol metabolites were defined grouping metabolites according to their aglycon moiety and were referred to as colonic classes. This approach allowed to focus on colonic catabolism, grouping metabolites originating from the same microbial activities, including hydrolysis, ring cleavage, demethylation, dehydroxylation, oxidation, reduction, and decarboxylation [53]. A total of 22 colonic classes were defined: (epi)catechins ((E)Cs), (epi)gallocatechins ((E)GCs), 5-(hydroxyphenyl)- γ -valerolactones (HPVLs), 5-(3',4'-dihydroxyphenyl)- γ -valerolactones (3',4'-diHPVLs), 5-(3',5'-dihydroxyphenyl)- γ -valerolactones (3',5'-diHPVLs), 5-(3',4',5'-trihydroxyphenyl)- γ -valerolactones (3',4',5'-triHPVLs), 4-hydroxy-5-(hydroxyphenyl)valeric acids (4-OH-HPVAs), 4-hydroxy-5-(3',4'-dihydroxyphenyl)valeric acids (4-OH-3',4'-diHPVAs), 3-(hydroxyphenyl)propanoic acids (HPPAs), 3-(3',4'-dihydroxyphenyl)propanoic acids (3',4'-diHPPAs), 3'-hydroxyphenylacetic acids (3'-HPAAs), 4'-hydroxyphenylacetic acids (4'-HPAAs), 3',4'-dihydroxyphenylacetic acids (3',4'-diHPAAs), 3'-hydroxycinnamic acids (3'-HCAs), 4'-

hydroxycinnamic acids (4'-HCAs), 3',4'-dihydroxycinnamic acids (3',4'-diHCAs), hydroxybenzoic acids (HBAs), 3,4-dihydroxybenzoic acids (3,4-diHBAs), trihydroxybenzoic acids (3,4,5-triHBAs), hydroxybenzaldehydes (OH-BALs), 1,2-dihydroxybenzenes or catechols (1,2-diOH-BZs), 1,2,3-trihydroxybenzenes or pyrogallols (1,2,3-triOH-BZs). Class of hippuric acids (HAs) was not included in the evaluation of the differences between treatments, because it was supposed not to be relevant for the reasons explained above.

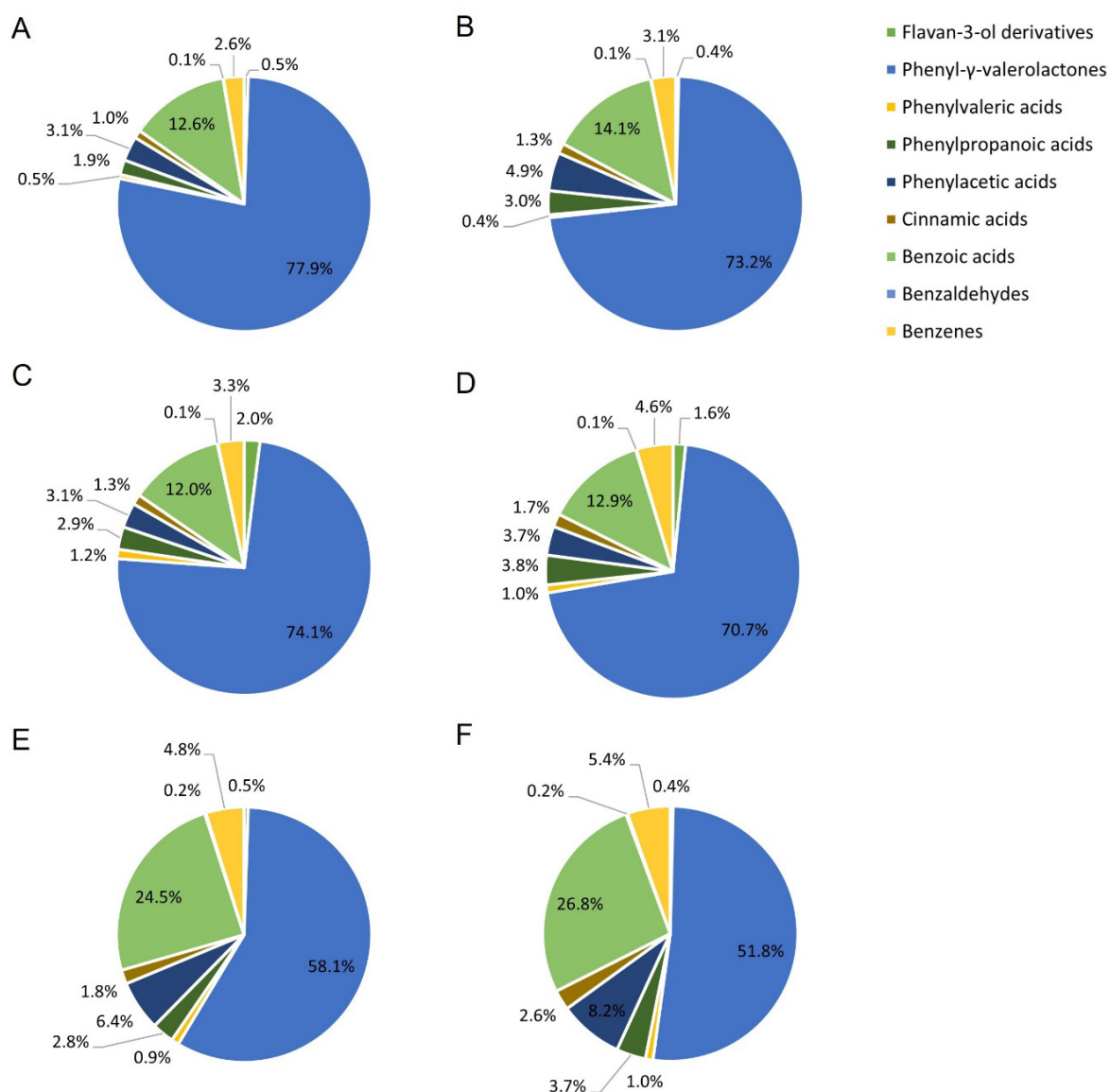


Figure 2. Distribution of classes of flavan-3-ol metabolites (without considering hippuric acids) excreted (A) during the 24 hours after treatment with green tea, (B) during the 48 hours after treatment with green tea, (C) during the 24 hours after treatment with grape seed extract rich in monomers, (D) during the 48 hours after treatment with grape seed extract rich in monomers, (E) during the 24 hours after treatment with grape seed extract rich in procyanidins, and (F) during the 48 hours after treatment with grape seed extract rich in procyanidins.

Differences among treatments in the excreted amount of colonic classes and total metabolites (with and without considering HAs) in 24 h and 48 h were evaluated (Table 2).

Table 2. Total 24 h and 48 h urinary excretion (μmol) of colonic classes of flavan-3-ol metabolites (including hippuric acid derivatives) and total metabolites (with and without hippuric acid derivatives) after treatment with green tea extract (GT), grape seed extract rich in monomers (GSM) and grape seed extract rich in procyanidins (GSP). Data are indicated as mean $\pm$ SEM. Different low case letters indicate statistically significant mean differences between treatments at 24 h and 48 h ($p < 0.05$).

Colonic class of flavan-3-ol metabolites	Total 24 h urinary excretion (μmol)			Total 48 h urinary excretion (μmol)		
	GT treatment	GSM treatment	GSP treatment	GT treatment	GSM treatment	GSP treatment
(Epi)(gallo)catechins ((E)GCs)	0.66 $\pm$ 0.09 ^a	0.00 $\pm$ 0.00 ^b	0.00 $\pm$ 0.00 ^b	0.69 $\pm$ 0.11 ^a	0.00 $\pm$ 0.00 ^b	0.00 $\pm$ 0.00 ^b
(Epi)catechins ((E)Cs)	1.67 $\pm$ 0.17 ^b	7.89 $\pm$ 0.90 ^a	0.96 $\pm$ 0.16 ^b	1.63 $\pm$ 0.15 ^b	7.89 $\pm$ 2.02 ^a	1.04 $\pm$ 0.01 ^b
3',4',5'- Trihydroxyphenyl- γ -valerolactones (3',4',5'-TriHPVLs)	64.99 $\pm$ 27.28 ^a	0.01 $\pm$ 0.01 ^b	0.00 $\pm$ 0.00 ^b	75.76 $\pm$ 47.27 ^a	0.01 $\pm$ 0.01 ^b	0.00 $\pm$ 0.00 ^b
3',5'-Dihydroxyphenyl- γ -valerolactones (3',5'-DiHPVLs)	148.52 $\pm$ 35.38 ^a	0.22 $\pm$ 0.10 ^b	0.14 $\pm$ 0.09 ^b	212.77 $\pm$ 49.82 ^a	0.28 $\pm$ 0.12 ^b	0.08 $\pm$ 0.08 ^b
3',4'-Dihydroxyphenyl- γ -valerolactones (3',4'-DiHPVLs)	113.71 $\pm$ 13.41 ^b	235.09 $\pm$ 28.30 ^a	95.95 $\pm$ 9.93 ^b	135.39 $\pm$ 14.91 ^b	272.37 $\pm$ 22.65 ^a	119.57 $\pm$ 17.14 ^b
Hydroxyphenyl- γ -valerolactones (HPVLs)	27.20 $\pm$ 11.35 ^a	58.51 $\pm$ 23.05 ^a	11.55 $\pm$ 7.66 ^a	27.45 $\pm$ 20.15 ^a	78.06 $\pm$ 48.29 ^a	29.73 $\pm$ 24.20 ^a
4-Hydroxy-3',4'-dihydroxyphenylvaleric acids (4-OH-3',4'-DiHPVAs)	1.64 $\pm$ 0.18 ^b	2.42 $\pm$ 0.19 ^a	1.50 $\pm$ 0.14 ^b	1.72 $\pm$ 0.15 ^a	2.31 $\pm$ 0.14 ^a	2.13 $\pm$ 0.23 ^a
4-Hydroxy-hydroxyphenylvaleric acids (4-OH-HPVAs)	0.68 $\pm$ 0.36 ^a	2.28 $\pm$ 0.82 ^a	0.26 $\pm$ 0.20 ^a	0.54 $\pm$ 0.50 ^a	2.63 $\pm$ 1.40 ^a	0.63 $\pm$ 0.63 ^a
3',4'-Hydroxycinnamic acids (3',4'-DiHCAs)	4.40 $\pm$ 0.63 ^a	4.95 $\pm$ 0.53 ^a	3.15 $\pm$ 0.45 ^a	7.54 $\pm$ 1.27 ^a	7.97 $\pm$ 0.62 ^a	7.05 $\pm$ 0.72 ^a
4'-Hydroxycinnamic acids (4'-HCAs)	0.04 $\pm$ 0.01 ^a	0.02 $\pm$ 0.01 ^a	0.02 $\pm$ 0.01 ^a	0.04 $\pm$ 0.01 ^a	0.04 $\pm$ 0.01 ^a	0.03 $\pm$ 0.01 ^a
3'-Hydroxycinnamic acids (3'-HCAs)	0.19 $\pm$ 0.05 ^a	0.15 $\pm$ 0.02 ^a	0.12 $\pm$ 0.02 ^a	0.36 $\pm$ 0.14 ^a	0.25 $\pm$ 0.05 ^a	0.30 $\pm$ 0.09 ^a
3',4'-Dihydroxyphenylpropanoic acids (3',4'-DiHPPAs)	7.00 $\pm$ 2.02 ^a	9.60 $\pm$ 3.32 ^a	4.49 $\pm$ 0.80 ^a	15.17 $\pm$ 5.56 ^a	14.85 $\pm$ 5.51 ^a	9.29 $\pm$ 2.96 ^a
Hydroxyphenylpropanoic acids (HPPAs)	1.54 $\pm$ 0.61 ^a	1.73 $\pm$ 0.36 ^a	0.70 $\pm$ 0.18 ^a	3.59 $\pm$ 1.98 ^a	3.78 $\pm$ 0.96 ^a	1.43 $\pm$ 0.42 ^a

3',4'-Hydroxyphenylacetic acids (3',4'-DiHPAAs)	1.35±0.31 ^a	1.82±0.56 ^a	1.23±0.23 ^a	3.46±1.00 ^a	3.41±0.75 ^a	2.85±0.42 ^a
4'-Hydroxyphenylacetic acids (4'-HPAAs)	7.59±1.31 ^a	6.33±1.51 ^a	6.41±1.64 ^a	18.63±3.12 ^a	8.55±0.70 ^a	11.41±2.09 ^a
3'-Hydroxyphenylacetic acids (3'-HPAAs)	5.23±2.15 ^a	4.32±1.05 ^a	4.14±0.77 ^a	7.99±3.51 ^a	6.24±1.42 ^a	9.25±3.26 ^a
3,4,5-Dihydroxybenzoic acids (3,4,5-TriHBAs)	0.68±0.17 ^a	0.53±0.09 ^a	0.06±0.06 ^b	0.75±0.26 ^a	0.42±0.25 ^b	0.00±0.00 ^b
3,4-Dihydroxybenzoic acids (3,4-DiHBAs)	49.33±9.82 ^a	40.06±5.25 ^a	38.09±4.25 ^a	65.20±14.69 ^a	49.30±6.36 ^a	61.43±11.49 ^a
Hydroxybenzoic acids (HBAs)	7.53±2.76 ^a	6.88±1.39 ^a	7.21±0.85 ^a	20.84±8.85 ^a	14.07±3.81 ^a	15.79±0.98 ^a
Hippuric acids (HAs)	33.95±4.03 ^a	37.42±3.97 ^a	30.07±6.42 ^a	57.59±9.70 ^a	62.16±11.79 ^a	44.50±5.70 ^a
Hydroxybenzaldehydes (OH-BALs)	0.525±0.03 ^a	0.36±0.08 ^a	0.30±0.05 ^a	0.55±0.19 ^a	0.50±0.10 ^a	0.59±0.21 ^a
1,2,3-Trihydroxybenzenes (1,2,3-TriOH-BZs)	6.55±0.48 ^a	0.54±0.05 ^b	0.25±0.07 ^b	6.30±0.63 ^a	0.42±0.14 ^b	0.23±0.12 ^b
1,2-Dihydroxybenzenes (1,2-DiOH-BZs)	5.34±0.93 ^b	12.62±1.25 ^a	8.71±0.89 ^b	12.84±3.24 ^a	22.55±3.19 ^a	15.49±2.71 ^a
Total flavan-3-ol metabolites	488.87±47.66 ^a	433.73±39.28 ^a	215.31±23.44 ^b	674.56±74.85 ^a	558.07±66.62 ^{ab}	332.80±68.04 ^b
Total flavan-3-ol metabolites without hippuric acid derivatives	454.91±44.61 ^a	396.31±38.69 ^a	185.24±20.01 ^b	616.97±65.99 ^a	495.91±59.17 ^{ab}	288.30±62.36 ^b

Considering 24 h excretion, treatment with GSM resulted in a statistically significant higher production of (E)Cs, 3',4'-diHPVLs, 4-OH-diHPVAs, and 1,2-diOH-BZs with respect to the other treatments; other classes such as HPVLs and 4-OH-HPVAs were excreted in higher amounts but the difference was not statistically significant because of the high inter-individual variability in their excretion. Treatment with GT resulted in a statistically significant higher excretion of (E)GCs, 3',5'-diHPVLs, 3',4',5'-triHPVLs, and 1,2,3-triOH-BZs with respect to the other treatments; 4'-HCAs were also excreted in higher amounts but the difference was not statistically significant. Treatments with GT and GSM were also characterized by a significantly higher excretion of 1,2,3-triHBAs and total metabolites (both with and without considering HAs), and by a higher but not significant excretion of HPPAs and 3',4'-diHPPAs with respect to GSP. Considering 48 h excretion, treatment with GSM resulted in a statistically significant higher production of (E)Cs and 3',4'-diHPVLs; other classes such as HPVLs, 4-OH-HPVAs and 1,2-diOH-BZs were excreted in higher amounts but the difference did not reach statistical significance. Treatment with GT resulted in a statistically significant higher excretion of (E)GCs, 3',5'-diHPVLs, 3',4',5'-triHPVLs, 3,4,5-triHBAs, 1,2,3-triOH-BZs with respect to the other treatments, as observed for 24 h excretion; other classes such as 4'-HPAAs and HBAs were excreted in higher amounts but the difference was not statistically significant. Treatment with GT was also characterized by a significantly higher excretion of total metabolites (both with and without considering HAs) with respect to treatment with GSP. The difference in total excretion was not significant with respect to GSM treatment, which showed a higher excretion with respect to GSP, though not significant. Finally, treatment with GSP resulted in a higher excretion of 3'-HPAAs and OH-BALs with respect to the other treatments, though not significant.

Differences among treatments in the overall metabolic profile and in flavan-3-ol bioavailability and kinetics

PCA was applied to evaluate qualitative differences in the overall phenolic profile among treatments and between 24 h and 48 h excretion (Figure 3). Colonic classes including HAs were considered as variables, and all observations were included (subjects' excretion values at 24 h and 48 h after each treatment). Data were auto-scaled to give them equal weight, since excretion amount of colonic classes was very heterogeneous, with very high values for HAs, 3,4-diHBAs and all PVLs (HPVLs and 3',4'-diHPVLs for all treatments, 3',5'-diHPVLs and 3',4',5'-

triHPVLs for GT). Three PCs explained 70.9% of the total variability. The first PC (PC1) described 40.0% of the observed variation, the second PC (PC2) explained 21.2% of the variability, and the third PC (PC3) described 9.7%. The model predicted 34.6% of the total variability, and the predictive power was mainly driven by PC1 (21.7%) and PC2 (12.7%). PC1 was positively loaded mainly by diHPVLs (both 3',4' and 3',5'), 4-OH-3',4'-diHPVAs, and mono- and dihydroxylated forms of small phenolic metabolites, including PPAs, CAs, PAAs, BAs, HAs, BALs, and BZs (Figure 3A). Considering the score plot with PC1 versus PC2, observations were discriminated by PC1 according to the excretion time (24 h versus 48 h): most of 24 h observations lied in the left quadrants (except for subject 3 for GT treatment, and subjects 3 and 2 for GSM treatment), while most of 48 h observations lied in the right quadrants (except for subject 10 for GT and GSM treatments, and subject 7 for GSP treatment). PC2 was positively loaded mainly by 3',4',5'-trihydroxylated, 3',5'-dihydroxylated and 4'-hydroxylated metabolites, including (E)GCs, 3',4',5'-triHPVLs, 3',5'-diHPVLs, 1,2,3-triOH-BZs, 3,4,5-triHBAs, 4'-HPAAs and 4'-HCAs. PC2 separated observations according to the treatment type, based on the hydroxylation extent of the flavan-3-ol B-ring (GT versus GSM and GSP, referred to as GSs): all GT observations lied in the top quadrants, while most of GS observations lied in the bottom quadrants (except for subject 7 at 24 h for GSP treatment). Considering the plots with PC1 versus PC3, PC3 was positively loaded by specific metabolites of flavan-3-ols, including SREMs and 5C-RFMs, and gallic acid, namely 3,4,5-triHBAs and 1,2,3-triOH-BZs (Figure 3B), and allowed to discriminate observations according to the treatment type, based on the degree of polymerization of native flavan-3-ols (GT/GSM versus GSP). In particular, considering the score plot, most of GT and GSM observations lied in the top quadrants (except for subjects 3 at 24 h for GT, subject 7 at 48 h for GT, subjects 2, 6 and 10 at 24 h for GSM, and subjects 3 and 10 at 48 h for GSM), while all GSP observations lied in the bottom quadrants. To better evaluate the influence of the native flavan-3-ol structure (extent of B-ring hydroxylation and degree of polymerization) on the urinary phenolic profile, the contribution of the treatment type to the variability observed was analyzed plotting PC2 versus PC3 (Figure 3C). Most of GT observations lied in the right quadrants (positive values for PC2), most of GSM observations lied in the top left quadrant (negative values for PC2 and positive values for PC3), and most of GSP observations lied in the bottom left quadrant (negative values for both PC2 and PC3). GT observations were characterized by a high excretion of 3',4',5'-trihydroxylated and 3',5'-dihydroxylated metabolites, including (E)GCs, 3',4',5'-triHPVLs, 3',5'-diHPVLs, 1,2,3-triOH-BZs, 3,4,5-triHBAs,

4'-HCAs and 4'-HPAAs. GSM observations were characterized by a high excretion of (E)Cs, 5C-RFMs, including 3',4'-diHPVLs, HPVLs, and 4-OH-HPVAs, and some small metabolites, namely 3',4'-diHPPAs and 1,2-diOH-BZs. HPPAs characterized both GT and GSM, allowing to discriminate those treatments from GSP. GSP observations were characterized by a high excretion of 3',4'-HCAs, 3'-HCAs, 3',4'-diHPAAs, 3'-HPAAs, HBAs, 3,4-diHBAs and OH-BALs. 4-OH-3',4'-DiHPVAs characterized both GSM and GSP treatments, differentiating them from GT.

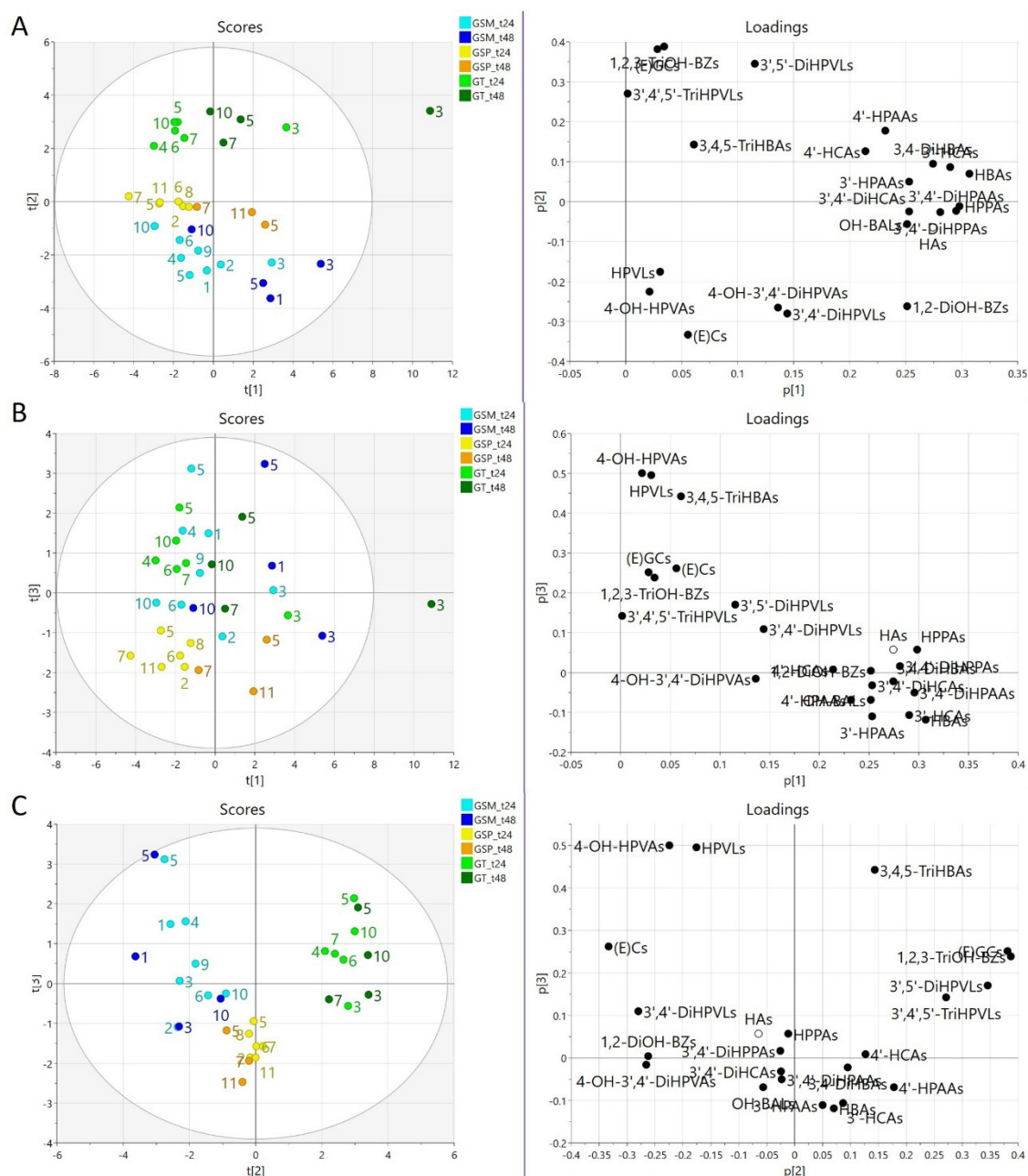


Figure 3. Score and loading plots resulting after applying PCA on autoscaled data for colonic classes and considering 3 PCs: (A) PC1 versus PC2, (B) PC1 versus PC3, (C) PC2 versus PC3. GT_t24, GSM_t24 and GSP_t24 indicate observations obtained from the analysis of 24 h urine collected after the intake of green tea extract (GT), grape seed extract rich in monomers (GSM) and grape seed extract rich in procyanidins (GSP), respectively; GT_t48, GSM_t48 and GSP_t48 indicate observations obtained from the analysis of 48 h urine collected after the intake of GT, GSM and GSP, respectively. The abbreviations used in the loading plot are indicated in Table 2.

To evaluate the kinetics of flavan-3-ol absorption, metabolism and excretion process, the percentage of flavan-3-ol metabolites produced, absorbed and excreted in the first 24 hours and in the second 24 hours after the intake of the food extracts was calculated for every treatment. Hippuric acid derivatives were not included in the calculations. GSM was the most rapidly metabolized extract, with 80% of metabolites excreted in the first 24 hours, while GSP was the most slowly metabolized, with 64% of metabolites excreted in the first day. For GT, 74% of metabolites were excreted in the first 24 hours (Figure 4). Evaluating flavan-3-ol bioavailability without considering hippuric acid derivatives, GT flavan-3-ols had the highest value, with the excretion of 45% and 62% of the molar amount of flavan-3-ol consumed at 24 h and 48 h, respectively. Also, GSM treatment had a high bioavailability, reaching 40% at 24 h and 50% at 48 h, while GSP flavan-3-ols had the lowest bioavailability, namely 19% at 24 h and 29% at 48 h. Particularly in this case, the collection of urine beyond the 24 hours is important to detect a relevant quantity of the metabolites produced.

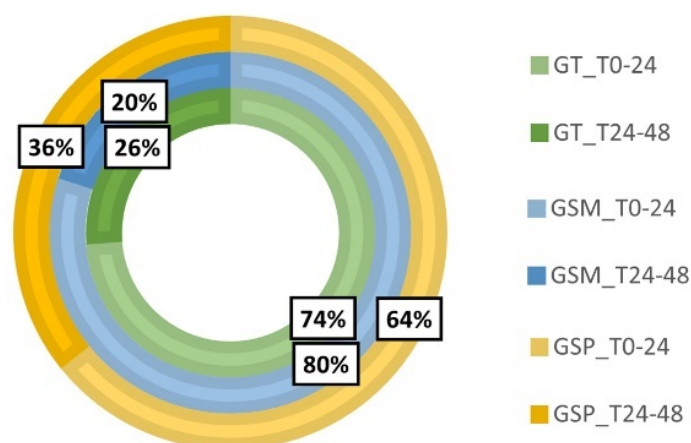


Figure 4. Percentage of total flavan-3-ol metabolites (without considering hippuric acid derivatives) excreted in urine during the first 24 hours (indicated as T0-24) and the second 24 hours (indicated as T24-48) after each treatment. GT indicates treatment with green tea extract, GSM indicates treatment with grape seed extract rich in monomers, and GSP indicates treatment with grape seed extract rich in procyanidins.

The bioavailability values for GT flavan-3-ols are in line with previous evidence [6,7,12,16,33,34,54]. The highest bioavailability for GT compared to GSs could be explained by several factors: (1) the presence of galloylated flavan-3-ol monomers, which are about 70% of native flavan-3-ols and from which 2 moles of metabolites can be generated (one from the metabolism of the flavan-3-ol moiety, and the other from gallic acid catabolism); (2) the presence of flavan-3-ol dimers that can potentially give origin to 2 moles of flavan-3-ol catabolites, which can increase to 3 or 4 if the presence of 1 or 2 galloyl moieties is considered (about 70% of dimers are galloylated); (3) the underestimate of the flavan-3-ol amount given

to volunteers, since dimers (not indicated in the technical sheet of the food extract) were not taken into consideration. Moreover, the estimation of the overall flavan-3-ol bioavailability could have been affected by biases in the quantification of phenolic metabolites, in particular some (E)(G)Cs, PVLs and PVAs, due to the absence of appropriate standards, and by the heterogeneous origin of some low molecular weight phenolic acids (PPAs, PAs, BAs, CAs), which are named as background metabolites since they are also produced by volunteers who have abstained from dietary sources of (poly)phenols [17]. In particular, these metabolites can derive from hepatic or gut microbiota metabolism of endogenous and exogenous compounds, such as aromatic amino acids (phenylalanine, tyrosine, tryptophan), catecholamines, drugs, tocopherols, cereal fiber (rich in cell wall bound hydroxycinnamates and diferulates) and food additives [17,55–57]. Since urine was collected after two days of polyphenol-free diet and while abstaining from dietary sources of phytochemicals and fiber, part of the detected phenolic acids could derive mainly from aromatic amino acids and catecholamines.

Methodological considerations arising from the use of urinary creatinine concentration

Beside 24 h and 48 h urine, fasting spot urine samples collected before the treatment (baseline) were analyzed using urinary creatinine concentration (mol/L) to normalize the data. Urinary creatinine concentration is commonly used in a ratio format to normalize analyte quantification in spot samples (with unknown volume) [58]. In this case, the analyte concentration ($\mu\text{mol/L}$) was divided by creatinine concentration (mol/L) to obtain the normalized concentration of the analyte in urine ($\mu\text{mol/mol creatinine}$). Evidence suggests that 24 h urine is the most reliable and not-invasive biological specimen for assessing (poly)phenol biomarkers [59]. However, this mode of collection is tedious and time-consuming for volunteers and may be prone to errors due to improper or incomplete collection. On the other hand, normalization of spot urine with creatinine may suffer from biases due to the variability of creatinine excretion rate according to demographics, age, sex, ethnicity, body mass index, fat-free mass, renal function, and dietary protein levels [60,61]. The comparison between 24 h volume and creatinine-corrected measures of total urinary (poly)phenol metabolites showed that the use of 24 h volume is more reliable to study the relationship between (poly)phenol intake and total urinary (poly)phenol excretion, used as biomarker of intake. However, the two methods are highly statistically correlated, and the use of creatinine normalization could be suitable for the purpose after adjusting for variables that modify

creatinine excretion (covariates) [61]. Data about excretion of colonic classes at baseline (t0) and during the day after the treatment (t24) were normalized with creatinine (Table 3).

First, the correlation between the measures of urinary colonic classes obtained using the volume of 24 h urine (μmol) and using the urinary creatinine concentration ($\mu\text{mol}/\text{mol}$ creatinine) was analyzed (Table S5). All colonic classes were strongly correlated, and the correlation was statistically significant ($p < 0.01$). The correlation coefficient was higher than 0.90 for (E)Cs, HPPAs, 3'-HPAAs, 3'-HPAAs, 3'-HCAs, 4'-HCAs, HBAs, 3,4,5-TriHBAs, OH-BALs and 1,2-diOH-BZs. The coefficient was higher than 0.99 for (E)GCs, HPVLs, 3',5'-diHPVLs, 3',4',5'-triHPVLs, 4-OH-HPVAs and 1,2,3-triOH-BZs, and it was between 0.78 and 0.90 for 3',4'-diHPVLs, 4-OH-diHPVAs, 3',4'-diHPPAs, 4'-HPAAs, 3',4'-diHPAAs, 3',4'-diHCAs, 3,4-diHBAs, HAs and total metabolites (with and without HAs). Secondly, the excretion of colonic classes in 24 h urine was compared with the excretion in fasting baseline spot urine to evaluate the contribution of endogenous metabolites to the pool of diet-derived flavan-3-ol catabolites, assessing the impact of non-phytochemical metabolism on the phenolic metabolic profile (Table 3). Moreover, the evaluation of the metabolic profile in baseline urine, in particular the excretion of specific flavan-3-ol metabolites, including SRFMs ((E)Cs, (E)GCs), and 5C-RFMs (3',4'-diHPVLs, 3',5'-diHPVLs, 3',4',5'-triHPVLs, 4-OH-HPVAs, 4-OH-3',4'-diHPVAs), allowed to check for volunteers' dietary compliance along with the dietary record. The baseline excretion of flavan-3-ol metabolites belonging to SRFMs, PVLs and PVAs was below 1.5% compared to 24 h excretion, suggesting that volunteers properly followed a (poly)phenol-free diet before the treatment and during the urinary collection.

The contribution of baseline metabolites, in particular small catabolites including 3/2/1-RFMs and benzene derivatives, to the pool of metabolites putatively deriving from the treatment was analyzed evaluating the proportion of their excretion in baseline spot urine compared to 24 h urine, calculated dividing the excretion value at T0 by the value at 24 h. Baseline excretion of HPPAs, HBAs, 3',4'-HCAs and HAs was 70% or higher of 24 h excretion; excretion of 4'-HPAAs, 3',4'-diHPAAs, 3'-HCAs, 4'-HCAs and 1,2-diOH-BZs was between 50 and 60%; excretion of 3',4'-diHPPAs, 3'-HPAAs and OH-BALs was between 30 and 40%. This suggests that relevant quantities of small metabolites derive from endogenous metabolism. On the contrary, very low relative values of excretion (<3%) in baseline urine compared to 24 h urine were obtained for 3,4-diHBAs, gallic acid metabolites (3,4,5-triHBAs and 1,2,3-triOH-BZs) and specific flavan-3-ol metabolites, indicating that these classes derive mainly from flavan-3-ol sources.

Table 3. Baseline spot and 24 h urinary excretion ($\mu\text{mol/mol}$ creatinine) of colonic classes of flavan-3-ol metabolites (including hippuric acid derivatives) after treatment with green tea extract (GT), grape seed extract rich in monomers (GSM) and grape seed extract rich in procyanidins (GSP). Data are indicated as mean $\pm$ SEM. Asterisks indicate statistically significant mean differences between baseline spot and 24 h urinary excretion after the same treatment (one asterisk for $p < 0.05$, two for $p < 0.01$).

Colonic class of flavan-3-ol metabolites	Baseline spot urinary excretion ($\mu\text{mol/creatinine}$)			Total 24 h urinary excretion ($\mu\text{mol/mol creatinine}$)		
	GT treatment	GSM treatment	GSP treatment	GT treatment	GSM treatment	GSP treatment
(Epi)(gallo)catechins ((E)GCs)	-	-	-	32.73 $\pm$ 4.28**	-	-
(Epi)catechins ((E)Cs)	-	0.05 $\pm$ 0.03	0.51 $\pm$ 0.46	84.29 $\pm$ 10.21**	453.06 $\pm$ 55.58*	55.07 $\pm$ 7.98*
3',4',5'- Trihydroxyphenyl- γ -valerolactones (3',4',5'-TriHPVLs)	0.60 $\pm$ 0.60	1.22 $\pm$ 1.22	-	3039.61 $\pm$ 1078.07*	0.33 $\pm$ 0.33	-
3',5'-Dihydroxyphenyl- γ -valerolactones (3',5'-DiHPVLs)	9.04 $\pm$ 3.78	2.67 $\pm$ 1.40	6.87 $\pm$ 3.74	7530.95 $\pm$ 1716.74**	14.11 $\pm$ 8.31	8.94 $\pm$ 5.84
3',4'-Dihydroxyphenyl- γ -valerolactones (3',4'-DiHPVLs)	129.15 $\pm$ 75.22	54.15 $\pm$ 9.33	46.09 $\pm$ 16.60	5613.87 $\pm$ 570.88*	13289.00 $\pm$ 1685.24*	5621.48 $\pm$ 600.31*
Hydroxyphenyl- γ -valerolactones (HPVLs)	11.64 $\pm$ 5.48	15.17 $\pm$ 7.64	13.26 $\pm$ 8.73	1392.38 $\pm$ 575.11*	3398.54 $\pm$ 1273.90*	595.69 $\pm$ 363.31*
4-Hydroxy-3',4'-dihydroxyphenylvaleric acids (4-OH-3',4'-DiHPVAs)	0.89 $\pm$ 0.35	0.65 $\pm$ 0.34	1.38 $\pm$ 1.02	81.94 $\pm$ 9.53**	142.51 $\pm$ 18.63*	89.89 $\pm$ 12.80*
4-Hydroxy-hydroxyphenylvaleric acids (4-OH-HPVAs)	-	-	-	34.01 $\pm$ 17.05	133.60 $\pm$ 47.89*	12.57 $\pm$ 9.80
3',4'-Hydroxycinnamic acids (3',4'-DiHCAs)	145.62 $\pm$ 40.49	161.28 $\pm$ 28.63	178.36 $\pm$ 49.79	218.19 $\pm$ 31.06	289.80 $\pm$ 41.98*	186.98 $\pm$ 31.72
4'-Hydroxycinnamic acids (4'-HCAs)	0.64 $\pm$ 0.22	0.87 $\pm$ 0.39	0.53 $\pm$ 0.25	1.76 $\pm$ 0.41*	1.08 $\pm$ 0.27	1.41 $\pm$ 0.48
3'-Hydroxycinnamic acids (3'-HCAs)	4.96 $\pm$ 1.28	5.38 $\pm$ 1.21	3.78 $\pm$ 0.77	9.53 $\pm$ 2.50*	8.70 $\pm$ 1.44*	7.14 $\pm$ 1.24*
3',4'-Dihydroxyphenylpropanoic acids (3',4'-DiHPPAs)	190.75 $\pm$ 98.42	142.40 $\pm$ 50.63	105.09 $\pm$ 34.35	340.69 $\pm$ 94.28*	525.93 $\pm$ 151.95*	263.91 $\pm$ 48.36
Hydroxyphenylpropanoic acids (HPPAs)	68.87 $\pm$ 26.35	54.17 $\pm$ 8.64	54.74 $\pm$ 23.15	76.36 $\pm$ 29.36	98.47 $\pm$ 20.09*	40.66 $\pm$ 9.25

3',4'-Hydroxyphenylacetic acids (3',4'-DiHPAAs)	39.69±10.14	34.61±4.32	45.73±10.94	65.65±14.05*	96.75±25.01*	71.24±12.09*
4'-Hydroxyphenylacetic acids (4'-HPAAs)	212.89±45.53	176.42±33.15	176.16±36.97	367.70±50.79*	372.93±108.22*	385.24±110.40*
3'-Hydroxyphenylacetic acids (3'-HPAAs)	112.89±76.81	57.17±8.69	75.01±20.26	259.71±103.09*	271.66±88.01*	244.65±45.34*
3,4,5-Dihydroxybenzoic acids (3,4,5-TriHBAs)	-	-	-	32.77±6.93**	29.07±4.84*	2.83±2.83
3,4-Dihydroxybenzoic acids (3,4-DiHBAs)	48.32±11.60	50.60±12.48	80.45±45.92	2459.83±481.47*	2281.42±304.18**	2217.28±227.98*
Hydroxybenzoic acids (HBAs)	336.44±114.54	373.30±94.47	365.31±76.84	372.35±132.01	393.81±87.73	424.47±54.23
Hippuric acids (HAs)	1434.79±294.81	1774.03±313.81	1599.54±414.77	1699.02±210.73	2198.48±299.75	1802.89±440.78
Hydroxybenzaldehydes (OH-BALs)	5.67±1.79	8.02±1.40	6.63±2.67	12.54±1.75*	22.10±6.16*	18.36±3.51
1,2,3-Trihydroxybenzenes (1,2,3-TriOH-BZs)	0.31±0.16	-	-	330.19±33.26**	31.00±3.90**	14.19±3.52**
1,2-Dihydroxybenzenes (1,2-DiOH-BZs)	156.79±39.42	360.51±89.34	257.69±97.85	268.30±48.36	727.89±77.22*	514.38±64.09

To further analyze the contribution of non-phytochemical metabolism on the phenolic metabolic profile, a statistical analysis was performed. Classes that resulted statistically higher in 24 h urine compared with baseline spot urine after all treatments were the following: (E)Cs, HPVLs, 3',4'-diHPVLs, 4-OH-3',4'-diHPVAs, 3'-HPAAs, 3',4'-diHPAAs, 3'-HCAs, 3,4-diHBAs, OH-BALs and 1,2,3-triOH-BZs. Classes that did not show differences in any treatment were HBAs and HAs. The remaining classes showed differences according to the type of treatment (GT or GSM): (E)GCs, 3',5'-diHPVLs, 3',4',5'-triHPVLs and 4'-HCAs were higher in 24 h urine after GT treatment, 4-OH-HPVAs, HPPAs, 3',4'-HCAs and 1,2-diOH-BZs were higher after GSM treatment, and 3',4'-diHPPAs, 4'-HPAAs and 3,4,5-triHBAs were higher after both treatments. To avoid biases in the estimation of urinary flavan-3-ol metabolites, the concentration of each colonic class and total metabolites in baseline urine has been subtracted from the corresponding concentration in 24 h urine. Then, the adjusted contribution of each colonic class to the total adjusted metabolite concentration was calculated (Figure 5).

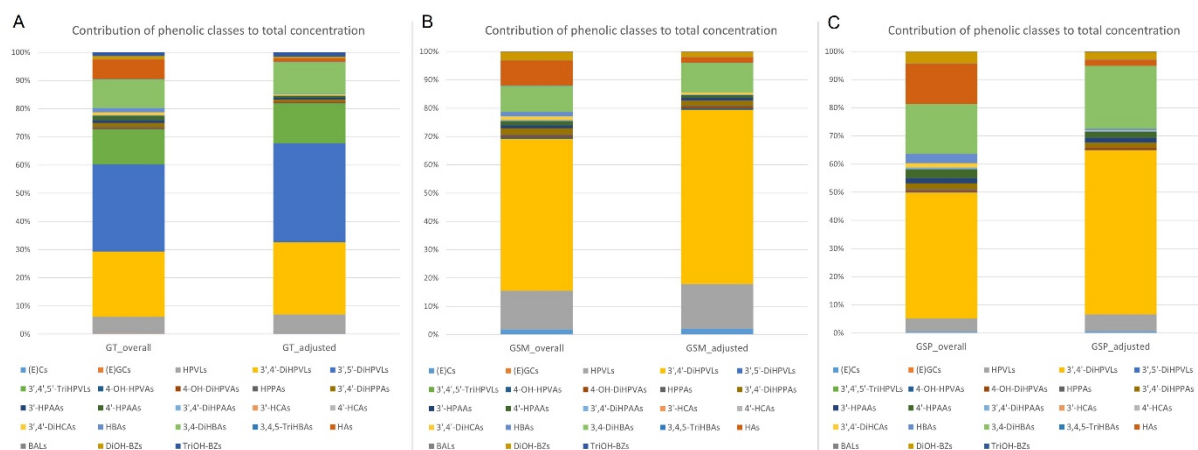


Figure 5. Contribution of each colonic class to the total metabolite concentration after each treatment, considering both the overall concentration values and the adjusted concentration values, obtained subtracting the overall concentration values for baseline concentration values in spot urine samples. GT indicates treatment with green tea extract, GSM indicates treatment with grape seed extract rich in monomers, and GSP indicates treatment with grape seed extract rich in procyanidins. The abbreviations for classes are indicated in Table 2.

As expected, the adjusted concentration of total metabolites was reduced for all treatments, reaching 88% for GT treatment, 87% for GSM, and 76% for GSP. The classes that contributed more to the reduction were HAs and HBAs for all treatments, and 3',4'-diHCAs and HPPAs for GT and GSP. Classes that increased their contribution after the correction for all treatments belong to the group of metabolites that are specific for flavan-3-ols, namely SRFMs, PVLs and PVAs. For GT, classes of metabolites specific for gallic acid, namely 1,2,3-TriOH-BZs and 3',4',5'-

TriHBAs, had a higher contribution considering the adjusted concentration values. On the contrary, 3',4'-DiHBAs increased their contribution after all treatments.

4.2.4. Discussion

The qualitative analysis of the food extracts showed that their overall flavan-3-ol composition was consistent with the scope of the study: GT was a good source of (E)GCs, galloylated monomers, and PD trimers; GSM was rich in (E)Cs and PC oligomers with a low DP; GSP was enriched in PC oligomers with a high DP and PC polymers. The information given by the manufacturer and indicated in the technical sheet (quantity of (E)Cs, (E)GCs, PC dimers and total PCs) was used to calculate the amount of plant extract to be given to volunteers in order to reach the intake of 1 mole of total flavan-3-ols. This quantity is in line with the daily intake of flavan-3-ols in European populations [1]. After each treatment, PVLs were the most abundant and richest class of metabolites, followed by BAs and PPAs from a quantitative and a qualitative point of view, respectively. Specific flavan-3-ol metabolites of microbial origin, including PVLs and PVAs, were far higher than SRFMs, which accounted for 0.5-3% of flavan-3-ol metabolites. Small phenolic metabolites (3/2/1-RFMs and BZs) contributed to flavan-3-ol bioavailability to a greater extent at 48 h compared to 24h, suggesting that their production is delayed, as expected based on the kinetics of flavan-3-ol colonic catabolism [3,7]. The urinary profile of phenolic classes is in line with existing evidence about the high urinary recovery in colon-derived catabolites after consumption of flavan-3-ol sources, including green tea, chocolate, grape pomace and grape seed [6,7,12,16,33,34,54].

As regards GT consumption, the excretion of (E)Cs was more than two times higher than (E)GCs, despite the latter were twice more abundant in the extract. This confirms that (E)GCs are less bioavailable than (E)Cs [7,25]. However, since no galloylated flavan-3-ols were detected in urine because of their biliary excretion, the bioavailability of flavan-3-ol monomers, and especially (E)GCs, was probably underestimated [7]. The principal metabolite was the sulfated form of 3',5'-diHPVL, followed by the sulfated form of its isomer 3',4'-diHPVL. The same trend was observed for the glucuronidated forms of diHPVLs, with 3',5'-diHPVL isomer present in slightly higher quantities than 3',4'-diHPVL. Gallic acid moieties released from galloylated flavan-3-ols underwent methylation or microbial catabolism (decarboxylation and subsequent dihydroxylation), giving origin to methoxy-gallic acid, pyrogallol and catechol, as shown in previous studies [9–12]. These metabolites were excreted mainly in the first day

after the treatment, suggesting that gallic acid is rapidly metabolized. GT consumption was associated with the richest metabolite excretion from a qualitative and quantitative point of view, both at 24 h and 48 h. This richness in the metabolic profile after GT consumption has been reported in the literature [62] and derives from the peculiar composition of green tea flavan-3-ols, which include 3',4',5'-trihydroxylated and galloylated forms along with the common 3',4'-dihydroxylated forms. The metabolic profile after GT treatment was similar to the one obtained after GSM intake, with the exception of SRFMs and PVAs, which had a greater contribution in GSM profile. (E)Cs and PVAs accounted for 2% and 1% of total flavan-3-ol metabolites, respectively, and their contribution decreased at 48 h, indicating that they are produced mainly in the first 24 h after the treatment, which is consistent with the metabolic pathway of flavan-3-ols. On the contrary, contribution of PVLs was similar at 24 and 48 h (74% and 71%, respectively), suggesting that their production continues until 48 h, as other studies have shown [3,54]. The same applies for PVLs deriving from GT flavan-3-ols. The lowest recovery in SRFMs for GT compared to GSM could be due to the lowest bioavailability of (E)GCs compared to (E)Cs, and to the abundance of galloylated monomers, which are absorbed intact and excreted through the biliary system [6,7]. The lowest excretion of monohydroxylated and 3',4'-dihydroxylated forms of PVAs for GT could be due to the lower excretion values of 3',4'-diHPVLs, which are their precursors. No 3',5'-dihydroxylated or 3',4',5'-trihydroxylated form of PVAs, putatively deriving from 3',5'-diHPVLs and 3',4',5'-triHPVLs, was ever detected. The metabolic profile after GSP intake differed from the others, being characterized by a lower percentage of PVLs and SRFMs, and a greater contribution of small metabolites (PAAs, CAs, BAs, BZs), with relevant amounts of BAs. This is in accordance with the urinary metabolic profile detected after consumption of grape pomace by Castello et al. [16]. The lower recovery in SRFMs for GSP compared to GSM was due to the lower content in monomers, while the lower recovery in microbial catabolites, especially PVLs, PVAs, PPAs, CAs and BZ, was probably due to the negative effect of polymerization on microbial catabolism. This phenomenon is extensively reported in literature [7,25,28–34] and could be explained with a reduced accessibility of PCs due to steric hindrance and to interactions with proteins in the gut lumen, which limit the access to the C-ring by the colonic microbiota [63]. Moreover, the antimicrobial properties of PCs could inhibit gut microbiota activity, reducing colonic degradation. These properties have been attributed to PC capacity to inhibit extracellular microbial enzymes, to form complexes with the substrates required for microbial growth, or to inhibit oxidative

phosphorylation [64]. After GSP treatment, PVLs accounted for 58% and 52% of total flavan-3-ol metabolites at 24 h and 48 h, respectively, while BAs contributed for 24% and 27%. Contribution of PVLs was substantially lower for GSP compared to GSM treatment. This could be due to the steric hindrance of PCs, negatively affecting the step of A-ring fission after C-ring cleavage. Small metabolites, in particular 2/1C-RFMs (PAAs, BAs, BALs), had a contribution two times higher after GSP intake compared with GSM both at 24 h and 48 h. The relevance of these catabolites was previously reported in literature both in *in vitro* studies and in human trials, after the consumption of PAC-rich sources such as cocoa [7,25,28–34]. PAAs and its derivatives (BAs, BALs, BZs) were excreted in similar quantities in the first and second day after GSP treatment, suggesting that they can derive from 2 metabolic pathways with a different timing: (1) the degradation of the upper unit of PACs, yielding rapidly 3',4'-diHPAA, which can undergo reactions of dehydroxylation, α -oxidation, reduction and decarboxylation, and (2) the degradation of the lower unit, yielding 3',4'-diHPVLs, which can undergo reactions of hydrolysis, β - and α -oxidation, reduction and decarboxylation, giving origin to PAAs and their catabolites in a delayed time [3,7]. A minor pathway yielding PAAs at a delayed time could be the interflavan cleavage, which releases (epi)catechin monomers that are catabolized to RFMs. Similarly to GT treatment, the contribution of small metabolites after grape seed intake, including PPAs, PAAs, CAs, BAs, BALs and BZs, was higher at 48 h compared to 24 h, suggesting that they mainly arise from 5C-RFM precursors [3,7].

To better evaluate the influence of flavan-3-ol structure on gut microbiota catabolism, excretion of colonic classes was considered from a qualitative and quantitative point of view. As expected, treatment with GT was characterized by the excretion of 3',5'-diHPVLs and trihydroxylated metabolites, including (E)GCs, 3',4',5'-triHPVLs, 3,4,5-triHBAs and 1,2,3-triOH-BZs. These metabolites derive from monomeric flavan-3-ols specific for green tea, most of which are 3',4',5'-trihydroxylated and 3-O-galloylated, and their presence is in accordance with other studies investigating green tea metabolism [6,9,10,12,25,54]. Also 4'-HPAAs and 4'-HCAs contributed to the specificity of GT flavan-3-ol metabolism, though not in a relevant way. Treatment with GSM was characterized by the excretion of (E)Cs, 1,2-diOH-BZs and 5C-RFMs, including HPVLs, 3',4'-diHPVLs, 4-OH-HPVAs and 4-OH-3',4'-diHPVAs. Except for 1,2-diOH-BZs, all of them are specific flavan-3-ols metabolites, suggesting that flavan-3-ol monomers are absorbed in small amounts in the upper gastrointestinal tract and largely catabolized by the colonic microbiota, which opens C-ring and rapidly breaks down A-ring, yielding PVLs and

PVAs. Moreover, HPPAs contributed to the specificity of GSM flavan-3-ol metabolism, and 3',4'-diHPPAs contributed to the specificity of both GSM and GT flavan-3-ols, though not in a significant way. This suggests that flavan-3-ol monomers are metabolized to PPAs in higher quantities than PCs. GSP treatment was characterized by small metabolites, particularly 3',4'-HCAs, 3'-HCAs, 3',4'-diHPAAs, 3'-HPAAs, HBAs, 3,4-diHBAs and OH-BALs. Class of 4-OH-3',4'-diHPVAs contributed to the specificity of GS metabolism, since it was not relevant for GT. This is probably due to the lower quantities of 3',4'-dihydroxylated precursors in green tea compared to grape seed. It is interesting to notice that 48 h excretion of monohydroxylated PPAs and CAs can discriminate GT and GSP treatment based on the hydroxyl position (3' for GSP and 4' for GT). The formation of 4'-HPAAs was observed in a study by Roowi et al. [12] in subjects with an ileostomy after the ingestion of green tea, indicating that this class of metabolites is produced in the body by additional routes unrelated to colonic catabolism. However, their high urinary excretion occurring after green tea consumption is likely to be partially derived from flavan-3-ol microbial degradation, and in particular from the catabolism and 3'-dehydroxylation of (E)GCs [7].

Considering the kinetics of flavan-3-ol metabolism, GSP showed the highest relative values of metabolites excreted in the second day after the treatment, suggesting that the steric hindrance and antimicrobial properties of PCs could slow gut microbiota catabolism. GT was metabolized more extensively than GSs both in the first and in the second day after the treatment. The higher recovery in RFMs in the first day for GT could be due to different reasons: (1) the greater proportion of monomers, which are rapidly catabolized, in spite of PC dimers and trimers, (2) the presence of 3',4',5'-trihydroxylated monomers, which are less bioavailable in the upper gastrointestinal tract and can reach the colon in higher amounts, (3) the greater proportion of galloylated flavan-3-ols, which reach the colon in good quantities due to scarce absorption in the upper gastrointestinal tract because of the presence of the galloyl moiety [4,7]. The significant proportion of galloylated monomers could also explain why a relevant excretion of PVLs was detected also in the second day after the treatment. Galloylated flavan-3-ols can undergo a delayed metabolism due to enterohepatic circulation, after being absorbed in the small intestine and excreted through the biliary system, and to the hydroxylation step necessary to release the flavan-3-ol moiety in the colon, after passing intact into the large intestine [4,7]. Evaluating flavan-3-ol bioavailability considering HAs, GT flavan-3-ols were the most bioavailable, reaching 49% at 24 h and 67% at 48 h, followed by GSM

flavan-3-ols, which reached 43% and 56%, respectively. As expected, GSP flavan-3-ols had the lowest bioavailability (19% at 24 h and 29% at 48 h). The contribution of SRFMs to the total bioavailability was substantially lower with respect to the data reported in the literature [62]. This could be due to the absence of appropriate standards for phase II metabolites of flavan-3-ols, leading to quantification biases. On the contrary, treatments with GT and GSM showed higher recoveries in gut microbiota metabolites compared to the evidence emerging from other studies [3,6,7,62]. This could be due to the presence of small background metabolites of non-phytochemical origin, deriving from the diet and endogenous metabolism, as confirmed analyzing their concentration in baseline urine.

After the assessment of the reliability and accuracy of creatinine-corrected 24 h excretion values of phenolic metabolites, a comparison between those values and the corresponding concentrations in baseline spot urine samples was performed, in order to identify colonic classes of background metabolites that could determine a bias in the bioavailability estimate. Small metabolites belonging to HPPAs, 3',4'-diHPPAs, 4'-HPAAs, 4'-HCAs, 3',4'-diHCAs, HBAs, 1,2,3-triOH-BZs, 1,2-diOH-BZs and HAs did not show statistically significant differences in baseline spot urine and 24 h urine for at least one treatment. These background metabolites were in accordance with those reported by the existing literature [7,17,55–57]. In particular, HBAs and HAs did not show significant differences in any treatment, indicating that their origin is very heterogeneous and does not depend on (poly)phenol metabolism (or at least not in relevant way). Other classes such as HPPAs, 4'-HCAs, 3',4'-diHCAs and 1,2-diOH-BZs showed differences only for a specific treatment (HPPAs, 3',4'-diHCAs and 1,2-diOH-BZs for GSM, and 4'-HCAs for GT), suggesting that they could be considered metabolites deriving from a flavan-3-ol source rich in monomers with a specific hydroxylation degree (3',4'-dihydroxylated or 3',4',5'-trihydroxylated flavan-3-ols). Finally, 3',4'-diHPPAs, 4'-HPAAs and 1,2,3-triOH-BZs were higher in 24 h urine only after GT and GSM intake, which were the treatments with a greater yield in total catabolites. This suggests that they could be considered metabolites of (poly)phenol origin after the intake of sources rich in monomers (partly galloylated). The remaining classes ((E)Cs, HPVLs, 3',4'-diHPVLs, 4-OH-3',4'-diHPVAs, 3'-HPAAs, 3',4'-diHPAAs, 3'-HCAs, 3,4-diHBAs, OH-BALs, 1,2,3-triOH-BZs) resulted statistically higher in 24 h urine for all treatments, suggesting that they are common flavan-3-ol metabolites originating from different sources. Among them, 3'-HPAAs, 3',4'-diHPAAs, 3'-HCAs, 3,4-diHBAs, and OH-BALs could be useful to characterize the treatment with GSP and in general the intake of PCs, also

when excluding endogenous production. Adjusting the 24 h excretion of flavan-3-ol metabolites for the baseline excretion, the overall contribution of endogenous metabolites accounted for 12% for GT, 13% for GSM and 24% for GSP treatment, and was mainly driven by HAs, HBAs, 3',4'-diHCAs and HPPAs, and to a lesser extent by OH-BALs, 1,2-DiOH-BZs, 3'-HCAs, 4'-HCAs, 3',4'-diHPAAs, 3'-HPAAs, 4'-HPAAs, and 3',4'-diHPPAs. The higher value for GSP may be due to the highest relative excretion of small metabolites after GSP intake compared with the other treatments. Specific metabolites of flavan-3-ols and gallic acid, along with the class of 3',4'-diHBAs, had a higher contribution to total excretion after the correction for baseline excretion, indicating that their endogenous production is negligible, as expected. It is interesting to notice that 3',4'-DiHBAs increased greatly their contribution after all treatments, suggesting that they are important metabolites deriving from flavan-3-ols, but they do not represent a discriminating class.

4.2.5. Conclusions

In conclusion, the treatment with different flavan-3-ol sources causes differences in the excretion of gut phenolic metabolites from a quantitative, qualitative and kinetic point of view. This indicates that the native structure influences flavan-3-ol bioavailability and microbial metabolism, suggesting that different flavan-3-ol-rich foods could originate different metabolic phenotypes with putatively different health effects. The study of flavan-3-ol bioavailability and metabolism is key to several objectives: (1) the identification of valuable biomarkers of flavan-3-ol intake to be used in intervention studies, (2) the study of flavan-3-ol mechanism of action in *in vitro* studies, using flavan-3-ol metabolites in their physiological circulating concentrations, and (3) the investigation on the inter-individual variability in flavan-3-ol metabolism and the associated metabotypes and biological response.

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Authors' contributions

DDR, FB, and PM contributed to the conception and design of the study; NT, RD, AR, and AB contributed to the day-to-day management of the clinical study; NT contributed to sample acquisition; NT, LB, CF, DDR, and PM contributed to the methodology; FB, DDR, and PM acquired funding; NT performed sample and data analysis and visualized the data; CF, LB, and PM supervised analysis; NT and PM wrote the manuscript. All the authors performed reviewing and editing of the article and consented to the final article.

Data Availability Statement

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

Declaration of interest statement

The authors declare no conflict of interest.

Electronic Supplementary Information (ESI)

S1.1 Materials

4-Hydroxybenzaldehyde, 3,4-dihydroxybenzaldehyde, 4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 3-(4'-hydroxyphenyl)propanoic acid, 3-(3'-hydroxyphenyl)propanoic acid, 3-(3',4'-dihydroxyphenyl)propanoic acid, 3-(4'-hydroxy-3'-methoxyphenyl)propanoic acid, 4'-hydroxy-3'-methoxycinnamic acid, 4'-hydroxyphenylacetic acid, 3'-hydroxyphenylacetic acid, 3',4'-dihydroxyphenylacetic acid, hippuric acid, (+)-catechin, and (–)-epicatechin were purchased from Sigma-Aldrich (St. Louis, MO, USA). 3'-Methoxycinnamic acid-4'-sulfate, 3'-methoxycinnamic acid-4'-glucuronide, 4'-methoxycinnamic acid-3'-sulfate, 4'-methoxycinnamic acid-3'-glucuronide, 3-(3'-methoxyphenyl)propanoic acid-4'-sulfate, 3-(4'-methoxyphenyl)propanoic acid-3'-glucuronide, 3-(4'-hydroxyphenyl)propanoic acid-3'-sulfate, 3-(4'-hydroxyphenyl)propanoic acid-3'-glucuronide, 3'-hydroxycinnamic acid-4'-glucuronide and 4'-hydroxycinnamic acid-3'-glucuronide were purchased from Toronto Research Chemicals (Toronto, ON, Canada). 4-Hydroxy-3-methoxybenzoic acid was purchased from Alfa Aesar (Thermo Fisher (Kandel) GmbH, Postfach, Karlsruhe, Germany), 4'-hydroxy-3'-phenylacetic acid and procyanidin B2 were purchased from Extrasynthese (Genay Cedex, France). 4'-Hydroxyhippuric acid was purchased from Bachem Ltd. (St Helens, UK). Prof. Alan Crozier (University of Glasgow) kindly supplied feruloylglycine. 3-Methoxybenzoic acid-4-glucuronide, 3-methoxybenzoic acid-4-sulfate, 4-methoxybenzoic acid-3-glucuronide, 4-hydroxybenzoic acid-3-sulfate, 4-hydroxybenzoic acid-3-glucuronide, benzoic acid-4-sulfate, benzoic acid-4-glucuronide were provided by Dr. Colin Kay (N.C. State University's Plants for Human Health Institute). 5-(3',4'-Dihydroxyphenyl)- γ -valerolactone, 5-(3',5'-dihydroxyphenyl)- γ -valerolactone, 5-(3',4',5'-trihydroxyphenyl)- γ -valerolactone, 5-phenyl- γ -valerolactone-3'-sulfate, 5-(3'-hydroxyphenyl)- γ -valerolactone-4'-sulfate, 5-(5'-hydroxyphenyl)- γ -valerolactone-3'-glucuronide were synthesized in house using the synthetic strategy previously outlined by Curti and colleagues for aglycones [1] and by Brindani and colleagues for phase II conjugates [2]. Ultrapure water from MilliQ system (Millipore, Bedford, MA, USA) was used throughout the experiment.

S1.2 Food product analysis

The identification and quantification of the flavan-3-ols present in the extracts was performed by ultra-high performance liquid chromatography coupled with mass spectrometry (uHPLC-MS), using an Accela uHPLC 1250 apparatus equipped with a Linear Ion Trap Mass Spectrometer (LIT-MS) (LTQ XL, Thermo Fisher Scientific Inc., San Jose, CA, USA), fitted with a heated-electrospray ionization source (H-ESI-II; Thermo Fisher Scientific Inc.). Separation was carried out by means of an Acquity HSS T3 column (100x 2.1 mm; 1.8 μ m particle size; Waters, Ireland) installed with a precolumn cartridge (Phenomenex, CA, USA). For uHPLC, mobile phase A was water containing 0.1% formic acid and mobile phase B was acetonitrile containing 0.1% formic acid. The gradient started with 5% B, keeping isocratic conditions for 0.5 min, reaching 51% B at 7 min and 80% B at 8 min, followed by 2 min at 80% B and then 4 min at the start conditions to re-equilibrate the column. The flow rate was set at 0.4 ml/min, the injection volume was 5 μ l, and the column was thermostatted at 40°C. Phenolic compounds were analyzed with a full-scan, data-dependent MS³ analysis in a negative ionization mode, monitoring all the negative ions with a mass to charge (m/z) value between 100 and 2000 Da. The H-ESI-II interface was set to a capillary temperature of 275 °C and the source heater temperature was 200 °C. The sheath gas (N₂) flow rate was set at 40 (arbitrary units) and the auxiliary gas (N₂) flow rate at 5. The source voltage was 4 kV, the capillary voltage was -42 V and tube lens voltage was -118 V. Ultra-high purity helium gas and a collision induced dissociation (CID) equal to 35 (arbitrary units) was used to obtain MS² and MS³ fragmentation.

S1.3 Urine sample analysis

Urine samples have been analyzed with ultra-high performance liquid chromatography coupled with tandem mass spectrometry (uHPLC-MS/MS) to identify and quantify phenolic metabolites. Samples were analyzed through UHPLC DIONEX Ultimate 3000 fitted with a TSQ Vantage Triple Quadrupole Mass Spectrometer (QqQ-MS) (Thermo Fisher Scientific Inc., San Jose, CA, USA), equipped with a heated-electrospray ionization source (H-ESI-II; Thermo Fisher Scientific Inc.). Separation was carried out by means of Kinetex Evo C18 column (100x 2.1 mm; 2.6 μ m particle size; Phenomenex, CA, USA) installed with a precolumn cartridge (Phenomenex, CA, USA). For uHPLC, mobile phase A was water containing 0.01% formic acid and mobile phase B was acetonitrile containing 0.01% formic acid. The gradient started with 5% B, keeping isocratic conditions for 0.5 min, reaching 95% B at 7 min, followed by 1 min at 95% B and then 4 min at the start conditions to re-equilibrate the column. The flow rate was

set at 0.4 ml/min, the injection volume was 5 μ l, and the column was thermostatted at 40°C. The MS worked in negative ionization mode with capillary temperature at 270°C, while the source was at 300°C. The sheath gas flow was 60 units, while auxiliary gas pressure was set to 10 units. The source voltage was 3 kV. Ultra-high purity argon gas was used for collision-induced dissociation (CID).

S1.4 Creatinine analysis

Diluted urine samples were analyzed through Accela uHPLC 1250 apparatus equipped with a Linear Ion Trap Mass Spectrometer (LIT-MS) (LTQ XL, Thermo Fisher Scientific Inc., San Jose, CA, USA), fitted with a heated-electrospray ionization source (H-ESI-II; Thermo Fisher Scientific Inc.). Separation was carried out by means of a XSelect HSS T3 column (50x 2.1 mm; 2.5 μ m particle size; Waters, Ireland) installed with a precolumn cartridge (Phenomenex, CA, USA). For uHPLC, mobile phase A was water containing 0.1% formic acid and mobile phase B was acetonitrile containing 0.1% formic acid. Chromatographic separation was performed in an isocratic mode keeping A and B at 50% for 2 min (duration of a single run). The flow rate was set at 0.2 ml/min, the injection volume was 5 μ l, and the column was thermostatted at 40°C. The H-ESI-II interface was set to a capillary temperature of 275 °C and the source heater temperature was 300 °C. The sheath gas (N₂) flow rate was set at 40 (arbitrary units) and the auxiliary gas (N₂) flow rate at 5. The source voltage was 4.5 kV, and the capillary voltage and tube lens voltage were +20 and +95 V, respectively. Ultra-high purity helium gas and a collision induced dissociation (CID) equal to 55 (arbitrary units) was used to obtain MS² fragmentation. Creatinine was analyzed with a full-scan, data-dependent MS² analysis in a positive ionization mode, monitoring all the ions with a m/z value between 35 and 200 Da. For identification and quantification, the molecular ion with m/z =114 and the fragment ion with m/z =86 were considered. Identification was carried out by comparison of the retention time with an in-house synthesized standard, and quantification was performed with calibration curve of the standard (concentration range: 0.1-100 μ mol/L). Chromatograms, mass spectral data and data processing were performed using Xcalibur software 2.1 (Thermo Fisher Scientific Inc.). Creatinine concentration in urine samples was expressed as mol/L.

Supplementary Table 1. Chromatographic and spectrometric properties of the monitored flavan-3-ol monomers and proanthocyanidins. Abbreviations for flavan-3-ol monomers are reported under brackets. MW means molecular weight; RT means retention time; m/z means mass to charge ratio; STD means standard used for quantification; $[M-H]^-$ and $[M-2H]^{2-}$ indicate single charged and double charged ions, respectively. PC refers to procyanidin and PD refers to prodelphinidin. If not indicated, proanthocyanidins are B-type.

Standard	RT (min)	MW (Da)	Molecular ion used for quantification	m/z	MS ² fragmentation (m/z)	STD
(+)-Catechin (C)	3.90	290	$[M-H]^-$	289	245, 205, 179	C
(-)-Epicatechin (EC)	4.48	290	$[M-H]^-$	289	245, 205, 179	EC
(+)-Gallocatechin (GC)	2.63	306	$[M-H]^-$	305	179, 221, 219, 261	EGC
(-)-Epigallocatechin (EGC)	3.54	306	$[M-H]^-$	305	179, 221, 219, 261	EGG
(-)-Epicatechin-3-O-gallate (ECG)	5.41	442	$[M-H]^-$	441	289, 169, 331	ECG
(-)-Epigallocatechin-3-O-gallate (EGCG)	4.41	458	$[M-H]^-$	457	169, 331, 305	EGCG
PC dimer B ₂ (EC-EC)	4.15	578	$[M-H]^-$	577	425, 451, 407, 289, 559	PC dimer B ₂
PC dimer (E)C-(E)C isomer 1	3.50	578	$[M-H]^-$	577	425, 451, 407, 559, 289	PC dimer B ₂
PC dimer (E)C-(E)C isomer 2	3.70	578	$[M-H]^-$	577	425, 451, 407, 559, 289	PC dimer B ₂
PC dimer (E)C-(E)CG isomer 1	4.63	730	$[M-H]^-$	729	577, 559, 603, 441, 407	PC dimer B ₂
PC dimer (E)C-(E)CG isomer 2	4.74	730	$[M-H]^-$	729	577, 559, 603, 441, 407	PC dimer B ₂
PC dimer (E)C-(E)CG isomer 3	4.82	730	$[M-H]^-$	729	577, 559, 603, 441, 407	PC dimer B ₂
PC dimer (E)C-(E)CG isomer 4	6.01	730	$[M-H]^-$	729	577, 711, 559, 407, 603	PC dimer B ₂
PC dimer (E)CG-(E)CG isomer 1	5.20	882	$[M-H]^-$	881	711, 559, 407, 577	PC dimer B ₂
PC dimer (E)CG-(E)CG isomer 2	6.30	882	$[M-H]^-$	881	711, 559, 407, 577	PC dimer B ₂
PD dimer (E)C-(E)GC	3.59	594	$[M-H]^-$	593	575, 441, 467, 423, 305	PC dimer B ₂
PD dimer (E)GC-(E)GC isomer 1	2.29	610	$[M-H]^-$	609	441, 423, 305, 483, 591	PC dimer B ₂
PD dimer (E)GC-(E)GC isomer 2	3.08	610	$[M-H]^-$	609	441, 423, 305, 483, 591	PC dimer B ₂
PD dimer (E)C-(E)GCG or (E)CG-(E)GC isomer 1	4.21	746	$[M-H]^-$	745	593, 575, 423, 619, 727	PC dimer B ₂

PD dimer (E)C-(E)GCG or (E)CG-(E)GC isomer 2	4.32	746	[M-H] ⁻	745	619, 577, 559, 407, 727	PC dimer B ₂
PD dimer (E)CG-(E)GCG	4.81	898	[M-H] ⁻	897	457, 169, 729, 305	PC dimer B ₂
PD dimer (E)GC-(E)GCG	-	764	[M-H] ⁻	763		PC dimer B ₂
PD dimer (E)GC-(E)GCG A-type isomer 1	3.29	762	[M-H] ⁻	761	591, 609, 423, 441	PC dimer B ₂
PD dimer (E)GC-(E)GCG A-type isomer 2	3.80	762	[M-H] ⁻	761	593, 575, 423, 609	PC dimer B ₂
PD dimer (E)GCG-(E)GCG	4.10	914	[M-H] ⁻	913	743, 591, 761, 573	PC dimer B ₂
PC trimer C ₁ (EC-EC-EC)	4.57	866	[M-H] ⁻	865	695, 739, 577, 713, 847	PC trimer C ₁
PC trimer (E)C-(E)C-(E)C isomer 1	2.14	866	[M-H] ⁻	865	695, 575, 577, 713, 739	PC trimer C ₁
PC trimer (E)C-(E)C-(E)C isomer 2	3.70	866	[M-H] ⁻	865	695, 739, 577, 713, 847	PC trimer C ₁
PC trimer (E)C-(E)C-(E)C isomer 3	3.96	866	[M-H] ⁻	865	695, 739, 577, 713, 847	PC trimer C ₁
PC trimer (E)C-(E)C-(E)C isomer 4	4.23	866	[M-H] ⁻	865	695, 577, 739, 713, 847	PC trimer C ₁
PC trimer (E)C-(E)C-(E)CG	5.72	1018	[M-H] ⁻	1017	891, 729, 847, 999	PC trimer C ₁
PC trimer (E)C-(E)CG-(E)CG	5.92	1170	[M-2H] ²⁻	584	508, 499, 575, 1043, 303	PC trimer C ₁
PC trimer (E)CG-(E)CG-(E)CG	6.13	1322	[M-2H] ²⁻	660	652, 893, 561, 477, 289	PC trimer C ₁
PD trimer (E)C-(E)C-(E)GC	-	882	[M-H] ⁻	881		PC trimer C ₁
PD trimer (E)C-(E)GC-(E)GC	-	898	[M-H] ⁻	897		PC trimer C ₁
PD trimer (E)GC-(E)GC-(E)GC	-	914	[M-H] ⁻	913		PC trimer C ₁
PD trimer (E)C-(E)GC-(E)GCG or (E)CG-(E)GC-(E)GC	-	1186	[M-2H] ²⁻	592		PC trimer C ₁
PC tetramer D ₁ (EC-EC-EC-EC)	4.69	1154	[M-H] ⁻	1153	865, 983, 1027, 575, 1135	PC tetramer D ₁
PC tetramer (E)C-(E)C-(E)C-(E)C isomer 1	3.65	1154	[M-H] ⁻	1153	983, 865, 575, 1027, 1135	PC tetramer D ₁
PC tetramer (E)C-(E)C-(E)C-(E)C isomer 2	4.01	1154	[M-H] ⁻	1153	865, 1027, 983, 1135, 575	PC tetramer D ₁
PC pentamer E ₁ (EC-EC-EC-EC-EC)	4.81	1442	[M-2H] ²⁻	720	635, 644, 577, 863, 1151	PC pentamer E ₁
PC pentamer (E)C-(E)C-(E)C-(E)C-(E)C isomer	4.40	1442	[M-2H] ²⁻	720	635, 644, 577, 863, 1151	PC pentamer E ₁

Supplementary Table 2. Information used for semi-quantification of type-B procyanidins (PCs) with a polymerization degree between 3 and 20. Procyanidins were identified as the peaks generated through the selection of a specific m/z from the total ion current (TIC) in a specific range of retention time. MW means molecular weight; m/z means mass to charge ratio; STD means standard used for quantification; $[M-H]^-$, $[M-2H]^{2-}$ and $[M-3H]^{3-}$ indicate single charged, double charged and triple charged ions, respectively.

B-type PCs	MW (Da)	Molecular ion used for quantification	m/z	STD
PCs (E)C-(E)C-(E)C	1154	$[M-H]^-$	1153	PC trimer C ₁
PCs (E)C-(E)C-(E)CG	1018	$[M-H]^-$	1017	PC trimer D ₁
PCs (E)C-(E)CG-(E)CG	1170	$[M-2H]^{2-}$	584	PC trimer D ₁
PCs (E)CG-(E)CG-(E)CG	1322	$[M-2H]^{2-}$	660	PC trimer D ₁
PCs [(E)C] ₄	1154	$[M-H]^-$	1153	PC tetramer D ₁
PCs [(E)C] ₅	1442	$[M-2H]^{2-}$	720	PC pentamer E ₁
PCs [(E)C] ₆	1730	$[M-2H]^{2-}$	864	PC pentamer E ₁
PCs [(E)C] ₇	2018	$[M-2H]^{2-}$	1008	PC pentamer E ₁
PCs [(E)C] ₈	2306	$[M-2H]^{2-}$	1152	PC pentamer E ₁
PCs [(E)C] ₉	2594	$[M-2H]^{2-}$	1296	PC pentamer E ₁
PCs [(E)C] ₁₀	2882	$[M-2H]^{2-}$	1440	PC pentamer E ₁
PCs [(E)C] ₁₁	3170	$[M-2H]^{2-}$	1584	PC pentamer E ₁
PCs [(E)C] ₁₂	3458	$[M-2H]^{2-}$	1728	PC pentamer E ₁
PCs [(E)C] ₁₃	3746	$[M-2H]^{2-}$	1872	PC pentamer E ₁
PCs [(EC)] ₁₄	4034	$[M-3H]^{3-}$	1344	PC pentamer E ₁
PCs [(EC)] ₁₅	4322	$[M-3H]^{3-}$	1440	PC pentamer E ₁
PCs [(E)C] ₁₆	4610	$[M-3H]^{3-}$	1536	PC pentamer E ₁
PCs [(E)C] ₁₇	4898	$[M-3H]^{3-}$	1632	PC pentamer E ₁
PCs [(E)C] ₁₈	5186	$[M-3H]^{3-}$	1728	PC pentamer E ₁
PCs [(EC)] ₁₉	5474	$[M-3H]^{3-}$	1824	PC pentamer E ₁
PCs [(EC)] ₂₀	5762	$[M-3H]^{3-}$	1920	PC pentamer E ₁

Supplementary Table 3. Chromatographic and spectrometric properties of the monitored flavan-3-ol metabolites. Nomenclature of metabolites is reported as proposed by Kay et al [3] and the synonyms and incorrect nomenclature are reported under brackets, as well as abbreviations. RT means retention time; CE means collision energy; STD means standard use for quantification; Ref. is the reference in the literature used for identification of compounds without an available standard. Compounds with an asterisk (*) represent novel metabolites reported here for the first time.

	RT (min)	Parent ion [M-H] ⁻ (m/z)	S-Lens	Quantifier		Qualifier(s)		STD (in bold those reference compounds used to quantify the corresponding metabolite or its coeluting isomers)	Ref.
				Product ion (m/z)	CE (V)	Product ion (m/z)	CE (V)		
Procyanidin B ₂ (PC B ₂)	4.23	577	113	407	25	289,425	27,17	Procyanidin B₂	
(-)-Epigallocatechin (EGC)	3.33	305	95	179	20	221	20	(-)-Epigallocatechin	
(-)-Epigallocatechin-gallate (EGCG)	4.44	457	98	169	20	305	20	(-)-Epigallocatechin-gallate	
(Epi)gallocatechin-sulfate (EGC-S)	-	385	95	305	30	221,179	20,20		
(Epi)gallocatechin-glucuronide (EGC-Glc)	4.12	481	92	305	32	113,221	19,30	4'-Hydroxycinnamic acid-3'-glucuronide	[4,5]
Methoxy-(epi)gallocatechin-sulfate (MeO-EGC-S)	5.75	399	87	319	20	304	26	3'-Methoxycinnamic acid-4'-sulfate	[4,5]
Methoxy-(epi)gallocatechin-glucuronide (MeO-EGC-Glc)	4.31	495	98	319	27	175	18	4'-Methoxycinnamic acid-3'-glucuronide	[4,5]
(Epi)gallocatechin-sulfate-glucuronide (EGC-S-Glc)	-	561	98	385	27	305,481	27,20		[4]
Methoxy-(epi)gallocatechin-sulfate-glucuronide (MeO-EGC-S-Glc)	-	575	98	399	27	319,304	27,27		[4,5]
(+)-Catechin (C)	3.68	289	103	245	18	205,124,151	21,30,30	(+)-Catechin	
(-)-Epicatechin (EC)	4.34	289	103	245	18	205,124,151	21,30,30	(-)-Epicatechin	
(Epi)catechin-sulfate (EC-S)	6.00	369	93	289	20	245	16	4'-Hydroxyphenylpropanoic acid-3'-sulfate	[6,9]

(Epi)catechin-glucuronide isomer 1 (EC-Glc 1)	4.34	465	98	289	27	113	19	4'-Hydroxycinnamic acid-3'-glucuronide	[6,9]
(Epi)catechin-glucuronide isomer 2 (EC-Glc 2)	4.64	465	98	289	27	113	19	4'-Hydroxycinnamic acid-3'-glucuronide	[6,9]
Methoxy-(epi)catechin-sulfate (MeO-EC-S)	6.21	383	92	303	20	288	26	3'-Methoxycinnamic acid-4'-sulfate	[8,9]
Methoxy-(epi)catechin-glucuronide isomer 1 (MeO-EC-Glc 1)	4.28	479	98	303	35	288,245,113	32,16,19	4'-Methoxycinnamic acid-3'-glucuronide	[8,9]
Methoxy-(epi)catechin-glucuronide isomer 2 (MeO-EC-Glc 2)	4.61	479	98	303	35	288,245,113	32,16,19	4'-Methoxycinnamic acid-3'-glucuronide	[8,9]
(Epi)catechin-sulfate-glucuronide (EC-S-Glc)	-	545	98	369	27	289	27		[4,5]
1-(3',5'-Dihydroxyphenyl)-3-(2'',4'',6''-trihydroxyphenyl)-propan-2-ol (3',5'-DiOH-PPOL)	-	291	86	123	30	167	30		[10]
1-(3',4'-Dihydroxyphenyl)-3-(2'',4'',6''-trihydroxyphenyl)-propan-2-ol (3',4'-DiOH-PPOL)	-	291	86	123	30	247,205	16,30		
1-(Hydroxyphenyl)-3-(2'',4'',6''-trihydroxyphenyl)-propan-2-ol (OH-PPOL)	-	275	86	231	16	191	30		[10]
5-(3',4',5'-Trihydroxyphenyl)-γ-valerolactone (3',4',5'-triHPVL)	2.49	223	75	179	21	138	26	5-(3',4',5'-Trihydroxyphenyl)-γ-valerolactone	
5-(Dihydroxyphenyl)-γ-valerolactone-sulfate (3',4',5' isomers) (DiHPVL-S)	5.33	303	84	223	20	179	30	5-(5'-Hydroxyphenyl)- γ -valerolactone-3'-sulfate	[4,11]
5-(Dihydroxyphenyl)-γ-valerolactone-glucuronide (3',4',5') isomer 1 (DiHPVL-Glc iso1)	3.29	399	87	223	30	113	25	5-(5'-Hydroxyphenyl)- γ -valerolactone-3'-glucuronide	[4,11,12]
5-(Dihydroxyphenyl)-γ-valerolactone-glucuronide (3',4',5') isomer 2 (DiHPVL-Glc iso2)	4.5	399	87	223	30	113	25	5-(5'-Hydroxyphenyl)- γ -valerolactone-3'-glucuronide	[4,11,12]

5-(Hydroxyphenyl)- γ -valerolactone-methoxy-sulfate (3',4',5') isomer 1 (HPVL-MeO-S iso1)	5.56	317	97	237	23	222	40	5-(5'-Hydroxyphenyl)- γ -valerolactone-3'-sulfate	[4,11]
5-(Hydroxyphenyl)- γ -valerolactone-methoxy-sulfate (3',4',5') isomer 2 (HPVL-MeO-S iso2)	6.12	317	97	237	23	222	40	5-(5'-Hydroxyphenyl)- γ -valerolactone-3'-sulfate	[4,11]
5-(Hydroxyphenyl)- γ -valerolactone-methoxy-glucuronide (3',4',5') isomer 1 (HPVL-MeO-Glc iso1)	4.62	413	87	237	30	222, 113	30,30	5-(5'-Hydroxyphenyl)- γ -valerolactone-3'-glucuronide	[4,11]
5-(Hydroxyphenyl)- γ -valerolactone-methoxy-glucuronide (3',4',5') isomer 2 (HPVL-MeO-Glc iso2)	3.96	413	87	222	30	237,113	30,30	5-(5'-Hydroxyphenyl)- γ -valerolactone-3'-glucuronide	[4,11]
5-(Hydroxyphenyl)- γ -valerolactone-sulfate-glucuronide (3',4',5' isomers) (HPVL-S-Glc)*	4.08	479	95	303	35	113,223	19,40	5-(5'-Hydroxyphenyl)- γ -valerolactone-3'-glucuronide	
5-(3',5'-Dihydroxyphenyl)- γ -valerolactone (3',5'-diHPVL)	3.98	207	72	122	21	163,121,119	18,22,21	5-(3',5'-Dihydroxyphenyl)- γ -valerolactone	
5-(Hydroxyphenyl)- γ -valerolactone-sulfate (3',5' isomers) (3',5'_HPVL-S)	5.52	287	96	207	23	163	35	5-(5'-Hydroxyphenyl)- γ -valerolactone-3'-sulfate	
5-(Hydroxyphenyl)- γ -valerolactone-glucuronide (3',5' isomers) (3',5'_HPVL-Glc)	3.57	383	92	207	27	113	30	5-(5'-Hydroxyphenyl)- γ -valerolactone-3'-glucuronide	
5-Phenyl- γ -valerolactone-disulfate (3',5' isomers) (3',5'_PVL-S-Glc)	-	367	93	287	20	207	20		[11]
5-(3',4'-Dihydroxyphenyl)- γ -valerolactone (3',4'-diHPVL)	4.30	207	72	163	18	121,122,123	22,21,20	5-(3',4'-Dihydroxyphenyl)- γ -valerolactone	
5-(Hydroxyphenyl)- γ -valerolactone-sulfate (3',4' isomers) (HPVL-S)	6.20	287	96	207	23	163	35	5-(3'-Hydroxyphenyl)- γ -valerolactone-4'-sulfate	

5-(Hydroxyphenyl)-γ-valerolactone-glucuronide (3',4' isomers) (HPVL-Glc)	4.49	383	92	207	27	113	30	5-(5'-Hydroxyphenyl)- γ -valerolactone-3'-glucuronide	[11-14]
5-Phenyl-γ-valerolactone-methoxy-sulfate (3',4' isomers) (PVL-MeO-S)	6.41	301	87	221	23	206	40	5-(5'-Hydroxyphenyl)- γ -valerolactone-3'-sulfate	[6,11,13,14]
5-Phenyl-γ-valerolactone-methoxy-glucuronide (3',4' isomers) (PVL-MeO-Glc)	4.61	397	90	221	30	113	30	5-(5'-Hydroxyphenyl)- γ -valerolactone-3'-glucuronide	[11,13,14]
5-Phenyl- γ -valerolactone-sulfate-glucuronide (3',4' isomers) (PVL-S-Glc)	-	463	87	287	27	207,383,113	40,20,19		[8,11,13,14]
5-Phenyl- γ -valerolactone-disulfate (3',4' isomers) (3',4'_PVL-S-Glc)	-	367	93	287	20	207	20		[11]
5-(4'-Hydroxyphenyl)- γ -valerolactone (4'-HPVL)	4.89	191	67	93	22	147,106,107	16,31,24		
5-(3'-Hydroxyphenyl)-γ-valerolactone (3'-HPVL)	5.04	191	67	106	31	147,107	16,24	5-(3'-Hydroxyphenyl)-γ-valerolactone	
5-Phenyl-γ-valerolactone-sulfate (3'/4' isomers) (PVL-S)	6.47	271	93	191	23	147,106	35,48	5-(Phenyl)-γ-valerolactone-3'-sulfate	
5-Phenyl-γ-valerolactone-4'-glucuronide (PVL-4'-Glc)	4.37	367	93	191	25	113	19	5-(5'-Hydroxyphenyl)- γ -valerolactone-3'-glucuronide	[11,12,14]
5-Phenyl-γ-valerolactone-3'-glucuronide (PVL-3'-Glc)	4.61	367	93	191	25	113	19	5-(5'-Hydroxyphenyl)- γ -valerolactone-3'-glucuronide	[2,8,11-14]
4-Hydroxy-5-(Dihydroxyphenyl)valeric acid-sulfate (3',4',5') (4-OH-MeO-PVA-S)	-	321	75	241	25	197	30		[11]
4-Hydroxy-5-(hydroxyphenyl)valeric acid-sulfate (3',5' isomers) (3',5'_4-OH-HPVA-S)	-	305	95	225	20	207,181	30,20		[11]
4-Hydroxy-5-(3',4'-dihydroxyphenyl)valeric acid (4-OH-3',4'-DiHPVA)	2.80	225	83	101	20	181,179	23,20	5-(3',4'-Dihydroxyphenyl)- γ -valerolactone	[11,12,15]

4-Hydroxy-5-(hydroxyphenyl)valeric acid-sulfate (3',4' isomers) (4-OH-HPVA-S)	4.93	305	95	225	20	207,181	30,20	5-(5'-Hydroxyphenyl)- γ -valerolactone-3'-sulfate	[12-15]
4-Hydroxy-5-(hydroxyphenyl)valeric acid-glucuronide (3',4' isomers) (4-OH-HPVA-Glc)	4.10	401	93	225	30	113	30	5-(5'-Hydroxyphenyl)- γ -valerolactone-3'-glucuronide	[1,4,8,20]
4-Hydroxy-5-phenylvaleric acid-methoxy-sulfate (3',4' isomers) (4-OH-PVA-MeO-S)	-	319	63	239	30	224	30		[13]
4-Hydroxy-5-(hydroxyphenyl)valeric acid (3'/4' isomers) (4-OH-HPVA)	-	209	63	165	12	191,149,151	15,20,18		[11]
4-Hydroxy-5-(phenyl)valeric acid-sulfate (3'/4' isomers) (4-OH-PVA-S)	-	289	103	209	18	147,101	27,32		[11,12,15,16]
4-Hydroxy-5-(phenyl)valeric acid-glucuronide (3'/4' isomers) (4-OH-PVA-Glc)	4.19	385	95	113	30	209	30	5-(5'-Hydroxyphenyl)- γ -valerolactone-3'-glucuronide	[12,15,16]
5-Phenylvaleric acid-methoxy-glucuronide (4,3'/4,4' isomers) (PVA-MeO-Glc)	-	399	87	223	30	208,113	30,25		
5-Phenylvaleric acid-sulfate-glucuronide (4,3'/4,4' isomers) (PVA-S-Glc)	-	465	98	289	27	209,113	19,19		[11]
5-(Phenyl)valeric acid-4-sulfate (PVA-4-S)	-	273	92	193	18	175,149	30,30		[11,16]
5-(Phenyl)valeric acid-4-glucuronide (PVA-4-Glc)	-	369	93	193	20	113	18		[11,16]
4'-Hydroxy-3',5'-dimethoxycinnamic acid (Sinapic acid) (3',5'-DimeO-4'-HCA)	-	223	75	193	31	165,208,179	30,30,21		[17]
3',5'-Dimethoxycinnamic acid-4'-sulfate (Sinapic acid-sulfate) (3',5'-DimeOH-CA-4'-S)	-	303		223					[6]

3',4'-Dihydroxycinnamic acid (Caffeic acid) (3',4'-diHCA)	4.00	179	62	135	19			3',4'-Dihydroxycinnamic acid
3'-Hydroxycinnamic acid-4'-sulfate (Caffeic acid-3-sulfate) (3'-HCA-4'-S)	-	259	62	179	20	135	25	[12]
4'-Hydroxycinnamic acid-3'-sulfate (Caffeic acid-4-sulfate) (4'-HCA-3'-S)	-	259	62	179	20	135	25	[7,18]
3'-Hydroxycinnamic acid-4'-glucuronide (Caffeic acid-4-glucuronide) (3'-HCA-4'-Glc)	3.88	355	98	179	21	113	18	
4'-Hydroxycinnamic acid-3'-glucuronide (Caffeic acid-3-glucuronide) (4'-HCA-3'-Glc)	4.44	355	98	179	21	113	18	4'-Hydroxycinnamic acid-3'-glucuronide
4'-Hydroxy-3'-methoxycinnamic acid (Ferulic acid) (3'-Met-4'-HCA)	4.83	193	71	134	19	178	18	4'-Hydroxy-3'-methoxycinnamic acid
3'-Hydroxy-4'-methoxycinnamic acid (Isoferulic acid) (4'-Met-3'-HCA)	4.92	193	71	134	19	178	18	
Methoxycinnamic acid-4'-sulfate ((Iso)ferulic acid-sulfate) (MeO-CA-S)	6.38	273	92	193	18	178,134,149	28,33,30	3'-Methoxycinnamic acid-4'-sulfate
3'-Methoxycinnamic acid-4'-glucuronide (Ferulic acid-4-glucuronide) (3'-MeO-CA-4'-Glc)	4.22	369	93	193	20	113,134,178	18,39,32	3'-Methoxycinnamic acid-4'-glucuronide
4'-Methoxycinnamic acid-3'-glucuronide (Isoferulic acid-3-glucuronide) (4'-MeO-CA-3'-Glc)	4.64	369	93	113	18	178,193,134	32,20,39	4'-Methoxycinnamic acid-3'-glucuronide
Feruloylglycine (3'-MeO-4'-HCA-Gly)	4.51	250	79	206	14	134,163	22,19	Feruloylglycine
4'-Hydroxycinnamic acid (p-Coumaric acid) (4'-HCA)	4.62	163	62	119	19	93	38	3'-Hydroxycinnamic acid [17]
3'-Hydroxycinnamic acid (m-Coumaric acid) (3'-HCA)	4.89	163	62	119	19	93	38	3'-Hydroxycinnamic acid

Cinnamic acid-4'-sulfate (Coumaric acid-4-sulfate) (CA-4'-S)	-	243	62	163	20	119	35	[7,19]
Cinnamic acid-3'-sulfate (Coumaric acid-3-sulfate) (CA-3'-S)	-	243	62	163	20	119	35	[12]
Cinnamic acid-4'-glucuronide (Coumaric acid-4-glucuronide) (CA-4'-Glc)	-	339	90	163	20	113	16	[19]
Cinnamic acid-3'-glucuronide (Coumaric acid-3-glucuronide) (CA-3'-Glc)	-	339	90	163	20	113	16	[12]
3-(3',4'-Dihydroxyphenyl)propanoic acid (Dihydrocaffeic acid) (3',4'-DiHPPA)	3.32	181	64	137	14	119,121	13,10	
3-(Hydroxyphenyl)propanoic acid-sulfate (3',4' isomers) (Dihydrocaffeic acid-sulfate) (HPPA-S)	6.03	261	96	181	20	137	25	3-(4'-Hydroxyphenyl)propanoic acid-3'-sulfate
3-(3'-Hydroxyphenyl)propanoic acid-4'-glucuronide (Dihydrocaffeic acid-4-glucuronide) (3'-HPPA-4'-Glc)	4.04	357	63	181	21	113	22	3-(4'-Hydroxyphenyl)propanoic acid-3'-glucuronide [12]
3-(4'-Hydroxyphenyl)propanoic acid-3'-glucuronide (Dihydrocaffeic acid-3-glucuronide) (4'-HPPA-3'-Glc)	4.35	357	63	181	21	113	22	3-(4'-Hydroxyphenyl)propanoic acid-3'-glucuronide
3-(4'-Hydroxy-3'-methoxyphenyl)propanoic acid (Dihydroferulic acid) (3'-MeO-4'-HPPA)	4.62	195	73	136	21	151,165	22,18	3-(4'-Hydroxy-3'-methoxyphenyl)propanoic acid
3-(3'-Hydroxy-4'-methoxyphenyl)propanoic acid (Dihydroisoferulic acid) (4'-MeO-3'-HPPA)	4.83	195	73	136	21	151	22	3-(4'-Hydroxy-3'-methoxyphenyl)propanoic acid [12,17]

3-(Methoxy-phenyl)propanoic acid-sulfate (3',4' isomers) (Dihydro(iso)ferulic acid-sulfate) (MeO-PPA-S)	5.91	275	75	195	31	136	17	3-(3'-Methoxy-phenyl)propanoic acid-4'-sulfate	
3-(3'-Methoxy-phenyl)propanoic acid-4'-glucuronide (Dihydroferulic acid-4-glucuronide) (3'-MeO-PPA-4'-Glc)	4.34	371	90	195	26	113	21	3-(4'-Methoxy-phenyl)propanoic acid-3'-glucuronide	[7,12]
3-(4'-Methoxy-phenyl)propanoic acid-3'-glucuronide (Dihydroisoferulic acid-3-glucuronide) (4'-MeO-PPA-3'-Glc)	4.67	371	90	195	26	113	21	3-(4'-Methoxy-phenyl)propanoic acid-3'-glucuronide	
3-(4'-Hydroxyphenyl)propanoic acid (4'-HPPA)	4.38	165	48	121	15	119	20		
3-(3'-Hydroxyphenyl)propanoic acid (3'-HPPA)	4.62	165	48	121	15	119	20	3-(3'-Hydroxyphenyl)propanoic acid	
3-(Phenyl)propanoic acid-sulfate (3'/4' isomers) (PPA-S)	6.20	245	90	165	20	121	15	3-(4'-Hydroxyphenyl)propanoic acid-3'-sulfate	[7,8,12]
3-(Phenyl)propanoic acid-4'-glucuronide (PPA-4'-Glc)	3.90	341	90	165	20	113	18	3-(4'-Hydroxyphenyl)propanoic acid-3'-glucuronide	[7]
3-(Phenyl)propanoic acid-3'-glucuronide (PPA-3'-Glc)	4.40	341	90	113	18	165	20	3-(4'-Hydroxyphenyl)propanoic acid-3'-glucuronide	[7,12]
3',4'-Dihydroxyphenylacetic acid (3',4'-DiHPAA)	-	167	60	123	17				[17]
4'-Hydroxy-3'-methoxyphenylacetic acid (Homovanillic acid) (3'-MeO-4'-HPAA)	4.00	181	64	137	14	121,122,119,166	10,30,13,30	4'-Hydroxy-3'-methoxy-phenylacetic acid	
3'-Hydroxy-4'-methoxyphenylacetic acid	-	181	64	137	14	121,122,119,166	10,30,13,30		[12]

(Isohomovanillic acid) (4'-MeO-3'-HPAA)

3'-Methoxy-phenylacetic acid-4'-sulfate (Homovanillic acid-sulfate) (3'-MeO-PAA-4'-S)	5.30	261	96	137	25	181	20	3-Methoxypropanoic acid-4-sulfate	[12,17,19]
3'-Methoxy-phenylacetic acid-4'-glucuronide (Homovanillic acid-glucuronide) (3'-MeO-PAA-4'-Glc)	3.97	357	63	181	21	113	22	4-Methoxypropanoic acid-3-glucuronide	[12]
3'-Hydroxyphenylacetic acid (3'-HPAA)	3.79	151	40	107	10			3'-Hydroxyphenylacetic acid	
4'-Hydroxyphenylacetic acid (4'-HPAA)	3.17	151	40	107	10			4'-Hydroxyphenylacetic acid	
3,4,5-Trihydroxybenzoic acid (Gallic acid) (3,4,5-TriHBA)	1.03	169	68	125	20			3,4,5-Trihydroxybenzoic acid	
4-Methoxy-3,5-Dihydroxybenzoic acid (4-Methoxygallic acid) (4-MeO-3,5-DiHBA)	2.36	183	70	168	26	139	20	3-Methoxy-4,5-Dihydroxybenzoic acid	[20]
3-Methoxy-4,5-Dihydroxybenzoic acid (3-Methoxygallic acid) (3-MeO-4,5-DiHBA)	2.18	183	70	168	26	139	20	3-Methoxy-4,5-Dihydroxybenzoic acid	
4-Methoxy-dihydroxybenzoic acid (4-Methoxygallic acid-sulfate) (3-MeO-4-HBA-5-S)	-	263	68	183	20	168	26		[17,19]
4-Hydroxy-3,5-dimethoxybenzoic acid (Syringic acid) (3,5-DimeO-4-HBA)	-	197	70	182	20	153	14		[17]
3,5-Dimethoxybenzoic acid-4-sulfate (Syringic acid-sulfate) (3,5-DimeO-BA-4-S)	-	277	70	197	20	182,153	26		[7]
3,4-Dihydroxybenzoic acid (Protocatechuic acid) (3,4-DiHBA)	1.65	153	64	109	20	108	27	3,4-Dihydroxybenzoic acid	
Hydroxybenzoic acid-sulfate (3,4 isomers) (Protocatechuic acid-sulfate) (HBA-S)	5.76	233	85	153	20	109	33		

3-Hydroxybenzoic acid-4-glucuronide (Protocatechuic acid-4-glucuronide) (3-HBA-4-Glc)	2.16	329	81	153	21	113	18	
4-Hydroxybenzoic acid-3-glucuronide (Protocatechuic acid-3-glucuronide) (4-HBA-3-Glc)	3.31	329	81	153	21	113	18	
4-Hydroxy-3-methoxybenzoic acid (Vanillic acid) (3-MeO-4-HBA)	3.85	167	60	152	18	108,123	21,17	4-Hydroxy-3-methoxybenzoic acid
Methoxybenzoic acid-sulfate (3,4 isomers) ((Iso)Vanillic acid-sulfate) (MeO-BA-S)	6.15	247	72	167	17	152,123	27,17	3-Methoxybenzoic acid-4-sulfate
3-Methoxybenzoic acid-4-glucuronide (Vanillic acid-4-glucuronide) (3-MeO-BA-4-Glc)	1.80	343	92	167	23	152,113	40,17	3-Methoxybenzoic acid-4-glucuronide
4-Methoxybenzoic acid-3-glucuronide (Isovanillic acid-3-glucuronide) (4-MeO-BA-3-Glc)	3.07	343	92	113	17	167,152	23,40	
4-Hydroxybenzoic acid (4-HBA)	2.78	137	70	93	16	92	26	4-Hydroxybenzoic acid
3-Hydroxybenzoic acid (3-HBA)	3.67	137	70	93	16	92	26	
Benzoic acid-sulfate (3/4 isomers) (BA-S)	5.86	217	70	137	20	93	16	Benzoic acid-4-sulfate
Benzoic acid-4-glucuronide (BA-4-Glc)	1.97	313	78	113	15	137	20	
Benzoic acid-3-glucuronide (BA-4-Glc)	-	313	78	113	15	137	20	
4'-Hydroxyhippuric acid (4'-HHA)	1.89	194	72	100	11	150	15	4'-Hydroxyhippuric acid
Hippuric acid (HA)	3.87	178	61	134	15	77.2	22	Hippuric acid
4-Hydroxybenzaldehyde (4-HBAL)	3.67	121	89	92	27	91	22	4-Hydroxybenzaldehyde
2,6/2,3-Dihydroxybenzene-1-sulfate (Pyrogallol-sulfate) (Pyr-S)	-	205	83	125	20	97	15	[12,17]

2,6/2,3-Dihydroxybenzene-1-glucuronide (Pyrogallol-glucuronide) (Pyr-Glc)	3.70	301	87	125	27	113	15	Benzoic acid-4-glucuronide	[12]
2-Hydroxy-3/6-methoxybenzene-1-sulfate or 3-Hydroxy-2-methoxybenzene-1-sulfate (Methoxypyrogallol-sulfate) (MeO-Pyr-S)	4.70	219	68	124	24	139	20	3-Methoxybenzoic acid-4-sulfate	[17,20]
2-Hydroxy-benzene-1-sulfate (Catechol-sulfate) (Cat-S)	5.70	189	70	109	20	81	20	Benzoic acid-4-sulfate	[6,7,12,17]
2-Hydroxy-benzene-1-glucuronide (Catechol-glucuronide) (Cat-Glc)	-	285	78	109	27	113	15		[12]
2-Hydroxy-4/5-methylbenzene-1-sulfate (Methylcatechol-sulfate) (Met-Cat-S)	5.17	203	70	123	20	108	26	3-Methoxybenzoic acid-4-sulfate	[6,7,12,17]

Supplementary Table 4. Quantity (g/100g) of flavan-3-ol monomers ((epi)catechins and (epi)gallo catechins), dimers (procyanidin and prodelphinidin dimers), trimers, oligomers and polymers in the plant extracts. DP means degree of polymerization, GT means green tea extract, GSM means grape seed extract rich in monomers, and GSP means grape seed extract rich in procyanidins. HPLC indicates the quantity calculated through HPLC-MSⁿ analysis, and TS indicates the amount indicated by the manufacturer in the technical sheet (TS). NS means that the value is not given by the manufacturer (not specified).

Flavan-3-ols	GT_HPLC	GT_TS	GSM_HPLC	GSM_TS	GSP_HPLC	GSP_TS
Monomers	62.6	73.1	31.0	37.1	7.9	6.4
(Epi)catechins	17.3	22.2	31.0	37.1	7.9	6.4
(Epi)gallo catechins	45.3	50.9	-	-	-	-
Dimers	5.4	-	19.8	17.8	6.4	7.4
Procyanidin dimers	2.1	-	19.8	17.8	6.4	7.4
Prodelphinidin dimers	3.3	-	-	-	-	-
Procyanidin trimers	-	-	10.2	NS	10.0	NS
Procyanidins with DP$\geq$2	5.4	-	34.3	47.8	20.7	86.2
Procyanidins with DP>2	-	-	14.5	30.0	14.3	78.8
Procyanidins with DP>3	-	-	4.3	NS	7.3	NS
Procyanidin oligomers (2 $\leq$ DP $\leq$ 10)	5.4	-	34.3	NS	19.5	NS
Procyanidin polymers (10<DP $\leq$ 20)	-	-	<0.1	NS	1.2	NS
Total flavan-3-ols	68.0	73.1	65.3	84.9	28.6	92.6

Supplementary Table 5. Correlation analysis between values of urinary excretion of colonic classes obtained using the volume of 24 h urine (μmol) and values obtained using the urinary creatinine concentration ($\mu\text{mol/mol}$ creatinine). Correlation coefficients are indicated, and asterisks are used to indicate their statistical significance (one asterisk for $p < 0.05$, two for $p < 0.01$).

Colonic class of flavan-3-ol metabolites	Correlation coefficient
(Epi)(gallo)catechins ((E)GCs)	0.99**
(Epi)catechins ((E)Cs)	0.97**
3',4',5'- Trihydroxyphenyl- γ -valerolactones (3',4',5'-TriHPVLs)	1.00**
3',5'-Dihydroxyphenyl- γ -valerolactones (3',5'-DiHPVLs)	1.00**
3',4'-Dihydroxyphenyl- γ -valerolactones (3',4'-DiHPVLs)	0.89**
Hydroxyphenyl- γ -valerolactones (HPVLs)	0.99**
4-Hydroxy-3',4'-dihydroxyphenylvaleric acids (4-OH-3',4'-DiHPVAs)	0.85**
4-Hydroxy-hydroxyphenylvaleric acids (4-OH-HPVAs)	1.00**
3',4'-Hydroxycinnamic acids (3',4'-DiHCAs)	0.88**
4'-Hydroxycinnamic acids (4'-HCAs)	0.97**
3'-Hydroxycinnamic acids (3'-HCAs)	0.90**
3',4'-Dihydroxyphenylpropanoic acids (3',4'-DiHPPAs)	0.83**
Hydroxyphenylpropanoic acids (HPPAs)	0.93**
3',4'-Hydroxyphenylacetic acids (3',4'-DiHPAAs)	0.78**
4'-Hydroxyphenylacetic acids (4'-HPAAs)	0.87**
3'-Hydroxyphenylacetic acids (3'-HPAAs)	0.95**
3,4,5-Dihydroxybenzoic acids (3,4,5-TriHBAs)	0.96**
3,4-Dihydroxybenzoic acids (3,4-DiHBAs)	0.82**
Hydroxybenzoic acids (HBAs)	0.92**
Hippuric acids (HAs)	0.88**
Hydroxybenzaldehydes (OH-BALs)	0.93**
1,2,3-Trihydroxybenzenes (1,2,3-TriOH-BZs)	0.96**
1,2-Dihydroxybenzenes (1,2-DiOH-BZs)	0.91**
Total flavan-3-ol metabolites	0.84**
Total flavan-3-ol metabolites without hippuric acid derivatives	0.86**

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4.3. A chronic human intervention study

Unravelling phenolic metabotypes in the frame of the COMBAT study, a randomized, controlled trial with cranberry supplementation

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Abstract

Cranberry (poly)phenols may have potential health benefits. Circulating (poly)phenol metabolites can act as mediators of these effects, but they are subjected to an extensive inter-individual variability. This study aimed to quantify both plasma and urine (poly)phenol metabolites following a 12-week intake of a cranberry powder in healthy older adults, and to investigate inter-individual differences by considering the existence of urinary metabotypes related to dietary (poly)phenols. Up to 13 and 67 metabolites were quantified in plasma and urine respectively. Cranberry consumption led to changes in plasma metabolites, mainly hydroxycinnamates and hippuric acid. Individual variability in urinary metabolites was assessed using different data sets and a combination of statistical models. Three phenolic metabotypes were identified, colonic metabolism being the main driver for subject clustering. Metabotypes were characterized by quali-quantitative differences in the excretion of some metabolites such as phenyl- γ -valerolactones, hydroxycinnamic acids, and phenylpropanoic acids. Metabotypes were further confirmed when applying a model only focused on flavan-3-ol colonic metabolites. 5-(3',4'-dihydroxyphenyl)- γ -valerolactone derivatives were the most relevant metabolites for metabotyping. Metabotype allocation was well preserved after 12-week intervention. This metabotyping approach for cranberry metabolites represents an innovative step to handle the complexity of (poly)phenol metabolism in free-living conditions, deciphering the existence of metabotypes derived from the simultaneous consumption of different classes of (poly)phenols. These results will help contribute to studying the health effects of cranberries and other (poly)phenol-rich foods, mainly considering gut microbiota-driven individual differences.

Keywords

Cranberry; phenolic metabolites; inter-individual variability; metabolic phenotypes; phenyl- γ -valerolactones

4.3.1 Introduction

Increasing evidence from cohort studies and intervention trials suggests that modest long-term intake of (poly)phenols can reduce the risk of chronic diseases, including cardiometabolic diseases, cognitive decline, and neurodegenerative diseases [1,2]. In particular, American cranberry (*Vaccinium macrocarpon*) (poly)phenols have shown beneficial effects in the prevention of urinary tract infections, vascular diseases and cognitive disfunctions [2–5]. Cranberry is a rich source of different classes of (poly)phenols, in particular type-A oligomeric proanthocyanidins and flavan-3-ol monomers, flavonols, anthocyanidins, and phenolic acids. Following ingestion, (poly)phenols are poorly absorbed in the upper intestinal tract and reach the colon, where they undergo modifications by the gut microbiota, being converted to smaller catabolites that are easily absorbed and then conjugated by human phase II enzymes [1]. Indeed, many circulating (poly)phenol metabolites, including benzaldehydes, benzene derivatives (catechols and pyrogallols), and phenolic acids (benzoic, phenylacetic, phenylpropanoic, cinnamic and hippuric acids), derive from the metabolism of these (poly)phenols classes [2,6,7]. Interestingly, the two main classes of cranberry (poly)phenols (hydroxycinnamic acids and flavan-3-ols) share common phenolic metabolites in their degradation pathways, as well as high inter-individual variability in their production [8–10]. Flavan-3-ol catabolism also generates specific metabolites such as phenylvaleric acids, 4-hydroxyphenylvaleric acids, and phenyl- γ -valerolactones (PVLs), which have shown protective activities in in vitro and in vivo studies [11]. To date, few studies have evaluated the metabolism of cranberry (poly)phenols in humans, mainly focusing on the analysis of structurally-related anthocyanin metabolites [12] or PVLs, which are considered biomarkers of flavan-3-ol intake [13]. Only a few comprehensive investigations have been conducted on several classes of cranberry (poly)phenols [14–17] or on the impact of chronic cranberry consumption on the quali-quantitative profile of (poly)phenolic metabolites [18]. In addition, the inter-individual variation in the plasma and urinary profile has been scarcely addressed despite its potential contribution to cranberry health effects. In this sense, a recent work has investigated the existence of metabolic phenotypes (aka metabotypes) for cranberry flavan-3-ols, defining three clusters of individuals based on differences in excreted amount and profile of dihydroxyPVLs, monohydroxyPVLs and 3-(hydroxyphenyl)propanoic acids (HPPAs) [19]. A methodological workflow for the definition of urinary phenolic metabotypes has also been proposed, defining a strategy to specifically search for metabolic phenotypes not characterized by the dichotomic excretion/non-excretion of specific phenolic metabolites [19]. This

metabotyping approach may be a strategy to handle the inter-individual variability in (poly)phenol bioavailability and to investigate its influence on the monitored health effects [20]. Metabotyping could be, therefore, a key success factor in personalized approaches, supporting tailored dietary advice to decrease disease risks and promote health at a population level [21]. However, rather than focusing on individual classes of (poly)phenols, comprehensive translatable approaches are needed.

The present study aimed to identify and quantify (poly)phenol metabolites in human biological samples (plasma and urine) after chronic intake of cranberry in healthy older adults, investigating the inter-individual differences in the profile of phenolic metabolites in fasted spot urine samples. The pipeline proposed by Mena et al [19] for cranberry flavan-3-ols was used here to investigate the existence of (poly)phenol metabotypes for the first time, in the context of a randomized, controlled clinical trial with no major dietary restrictions.

4.3.2. Materials and methods

Materials

All chemicals and solvents used in this study were of analytical grade, and are indicated in Supplementary Information S1.1.

Intervention study

Plasma and urine samples were obtained from the COMBAT study (The impact of Cranberries On the Microbiome and Brain in healthy Ageing sTudy), which was fully reported in [4]. The study was registered at clinicaltrials.gov as NCT03679533 and at [ISRCTN](https://www.isrctn.com) as ISRCTN76069316, and was conducted according to the guidelines laid down in the current revision of the Declaration of Helsinki. Informed consent was obtained for all participants, and all procedures involving human subjects were approved by the University of East Anglia's Faculty of Medicine and Health Sciences Ethical Review Committee (Reference: 201819 – 039) and the Health Research Authority (IRAS number: 237251; HRA number: 18/HRA/1339). The COMBAT study was a single-centre, 12-week randomized, double-blind, placebo-controlled parallel intervention trial designed to investigate the impact of cranberry consumption on the gut microbiome, cognition, and brain physiology. It involved the recruitment of 60 healthy older adults, both male and female, aged between 50 and 80 years old. 31 subjects were assigned to the control group, and the remaining 29 were assigned to the treatment group. The intervention consisted of the intake of freeze-dried cranberry powder

(Cranberry Institute, USA) in a dose providing 375 mg proanthocyanidins, 85 mg flavonols, 59 mg anthocyanins, and 70 mg of phenolic acids per day. Details are reported in Table S1 in Supplementary Information and in the study by Flanagan and colleagues [4]. The intervention was provided in sachets (4.5 g each) of cranberry powder designed to be incorporated into food and beverages. Participants took two sachets daily, one in the morning and one in the evening, to maximize the physiological impact according to the current knowledge of bioavailability [2]. The control group was supplemented with a placebo powder. Apart from adding the powder, participants were asked not to change their dietary or caloric intake. However, they were asked to reduce their consumption of certain vitamin supplements and to avoid the intake of supplements that could significantly influence the outcome measures, or supplements having a high flavonoid intake, defined as more than 15 portions of flavonoid-rich foods per day [22]. In particular, participants were screened and were eligible if they consumed <15 portions of high polyphenol foods per day. After the participants were randomized into the study, they were asked to keep the same diet as usual except for limiting certain high-polyphenol foods. Spot plasma and urine samples were collected before the treatment (baseline visit V1, 0 weeks) and after the intervention (follow-up visit V2, 12 weeks). Plasma samples were not collected from 15 subjects (8 in the cranberry group and 7 in the control group) after the intervention because of the outbreak of the COVID-19 pandemic. For the study purpose, plasma and urine samples were analyzed to obtain data on metabolite concentration for a total of 210 observations (90 for plasma and 120 for urine). These observations were used for statistical analyses.

Sample analysis

Urinary extraction of phenolic metabolites was performed using microelution solid phase extraction (μ SPE) according to validated protocols, with small modifications [14]. Briefly, plasma (350 μ L) and urine (250 μ L) were diluted (1:1) with phosphoric acid 4% to limit phenolic-protein interactions. Each sample (600 μ L for plasma and μ L 400 for urine) was loaded on a 96 well μ SPE plate, washed with water (200 μ L) and 0.2% acetic acid (200 μ L), and finally eluted with methanol (60 μ L for plasma and 200 μ L for urine). Samples were analyzed through high-performance liquid chromatography coupled with tandem mass spectrometry (uHPLC-MS/MS) as described by Castello et al [23]. More details are indicated in Supplementary Information S1.2. Up to 74 compounds were simultaneously monitored in selective reaction monitoring (SRM) mode, and 67 metabolites were detected and quantified (Table S2 in Supplementary Information). Chromatograms, mass spectral data and data processing were performed using Xcalibur software 2.1 (Thermo Fisher Scientific Inc.). The

nomenclature used follows the current recommendations for (poly)phenol catabolites [24]. Plasma metabolite data were expressed as nmol/L, while urinary concentrations were normalized using creatinine concentration and expressed as $\mu\text{mol/mol}$ creatinine. Urinary creatinine analysis was performed by uHPLC-MS/MS after dilution of samples with water 0.1% formic acid (1:1000) (details are described in Supplementary Information S1.3). Sums of urinary metabolites belonging to the same aglycone moiety were calculated, defining 20 different phenolic classes. Hippuric acid derivatives (namely, hippuric acid and 4'-hydroxyhippuric acid) were not taken into consideration because they have a heterogeneous origin (both exogenous and endogenous) and their inclusion did not modify the models presented herein. This way, two different data sets were considered, one (DS1) consisting of 120 observations (samples) and 65 variables corresponding to individual metabolites, and one (DS2) consisting of 120 observations (samples) and 19 variables corresponding to phenolic classes. Moreover, one additional data set (DS3) considering individual metabolites belonging to PVLs and HPPAs was considered according to previous evidence about flavan-3-ol metabolites [6,19]. DS3 consisted of 120 observations (samples) and 17 variables, including metabolites belonging to HPPAs (isomers 3' and 4'), monohydroxyPVLs (isomers 3' and 4'), and dihydroxyPVLs (3',4').

Statistical analysis

All data were expressed as mean values $\pm$ standard error of the mean (SEM). Tests for assessing data normality and homoscedasticity were performed using the Kolmogorov–Smirnov and Levene's tests, respectively. Comparisons between the control and the treatment groups were made by independent sample Student *t* test for normally distributed variables or Mann-Whitney U test for non-normally distributed variables. Comparisons between concentrations at the baseline and follow-up visits were made by dependent sample Student *t* test for normally distributed variables and Wilcoxon test for non-normally distributed variables. Differences were considered significant at *p* value < 0.05. These statistical analyses were performed using IBM SPSS Statistics version 26 (IBM, Chicago, IL, USA).

Principal component analysis (PCA) was carried out to investigate inter-individual differences in the urinary excretion of (poly)phenol metabolites. PCA was performed using SIMCA 16.0.1 software (Sartorius Stedim Data Analytics, Umea, Sweden). Data sets were not subjected to any data transformation, since Mena et al [19] suggested that this strategy results in better models compared to logarithmic and power transformation. Data sets were subjected to three different mean

centering plus scaling methods: only mean centering, unit variance (UV) scaling or autoscaling, and Pareto scaling. Centering converted the data to variations around zero instead of around the mean; UV scaling used standard deviation as scaling factor, while Pareto scaling used the square root of standard deviation [25]. All models were set by default with two principal components (PCs). The parameters used to evaluate the quality of each model and data interpretability were R^2X and Q^2 , which measure the model fit (or explained variation) and the predictive ability, respectively. The models obtained pre-treating data with mean centering plus UV scaling were selected, since the results were in accordance with those obtained by Mena et al [19]. Results obtained pre-treating data with the other two methods (mean centering, and mean centering plus Pareto scaling) are not shown.

To investigate the causes behind the observed inter-individual variation in the excretion of (poly)phenol metabolites, partial least squares discriminant analysis (PLS-DA) was applied using SIMCA 16.0.1 software. Different explorative PLS-DA models were carried out to define the optimal number of clusters and the observations in each cluster. The model that maximized the validation parameters was selected; this final model considered 3 clusters. Observations were assigned to clusters based on their PC scores. Variables were autoscaled applying centering plus unit variance scaling. Model validity was assessed by R^2X (variation in the data), R^2Y (variation in the cluster), Q^2 (goodness of fit of the validation), the random permutation test, and CV-ANOVA within the SIMCA package. The most relevant metabolites from the whole set of metabolites (selection of variables) were identified using the Variable Importance in Projection (VIP) scores, which allows to measure the importance of each variable in the projection used in a PLS model [26]. Variables with VIP scores greater than 1 were considered relevant for the model. The ROC curve was built to assess the classification performance of the PLS-DA models.

The normality of phenolic classes distribution within the clusters identified with PCA using DS1 was checked through the Kolmogorov–Smirnov test. For normally distributed variables, one-way repeated measures analysis of variance (ANOVA) was performed to compare the mean concentrations among the clusters. *Post-hoc* analysis was conducted using Dunnett's T3 test with Bonferroni correction, since variances in clusters were non equal (heteroscedastic variables). For non-normally distributed variables, non-parametric Kruskal–Wallis test with *post hoc* pairwise multiple comparison was used. Differences were considered significant at p value < 0.05. All these univariate statistical analyses were performed using IBM SPSS Statistics version 26.

4.3.3. Results

Plasma phenolic metabolites

A comprehensive panel of (poly)phenol metabolites was targeted in both plasma and urine samples. A total of 13 metabolites were identified and quantified in plasma (Table 1). These metabolites belong to several phenolic classes, including flavonols, PVLs, cinnamic acids, phenylpropanoic acids, phenylacetic acids, benzoic acids, benzaldehydes, hippuric acids, and catechols. Concentrations in fasting conditions ranged from 10 to 90 nmol/L for all the metabolites without hippuric acid.

When comparing the treatment and placebo groups at baseline (Table 1), there were no statistically significant differences for any metabolite in plasma except for kaempferol-3-glucuronide, which was lower in the treatment group ($p=0.026$). At the follow-up visit, plasma concentrations of 4 metabolites were statistically higher in the treatment group: 3',4'-dihydroxycinnamic acid (increased around 68%, $p=0.001$), 3'-methoxycinnamic acid-4'-glucuronide (108%, $p=0.005$), hippuric acid (69%, $p=0.001$), and 2-hydroxy-4/5-methylbenzene-1-sulfate (75%, $p<0.001$). The plasma concentration for total metabolites was also significantly higher in the treatment group than in the placebo (increased by 63%, $p=0.001$) and remained higher even when hippuric acid derivatives were not considered (increased by 20%, $p=0.033$).

When comparing the baseline and follow-up plasma concentrations within the placebo group, the level of 3-(3'-hydroxyphenyl)propanoic acid significantly decreased its level ($p=0.026$) while that of 4-hydroxybenzaldehyde increased it ($p=0.021$). Total metabolites without hippuric acid derivatives showed a small (17%), but significant decrease ($p=0.045$). Comparisons within the cranberry group accounted for significant increases in 3 metabolites: 3',4'-dihydroxycinnamic acid ($p=0.002$), 4-hydroxybenzaldehyde ($p=0.003$), and hippuric acid ($p=0.002$). Total metabolites also increased by 1502 ± 84 nM (about 47%) for the treatment group in a statistically significant way when considering hippuric acid derivatives ($p=0.002$).

Table 1. Plasma (poly)phenol metabolite concentration at baseline and at follow-up for placebo and cranberry groups. Data are indicated as mean $\pm$ SEM and are expressed as nmol/L. Compounds presenting different uppercase letters indicate statistically significant differences between placebo and cranberry groups at baseline or follow-up. Compounds changing from baseline to follow-up are marked with an asterisk (*). Nomenclature of metabolites is reported as proposed by Kay et al [24].

Metabolite	Placebo group, baseline (nmol/L)	Cranberry group, baseline (nmol/L)	Placebo group, follow-up (nmol/L)	Cranberry group, follow-up (nmol/L)
Kaempferol-3-glucuronide	33 $\pm$ 7 ^A	17 $\pm$ 4 ^B	28 $\pm$ 5	19 $\pm$ 4
5-(Phenyl)- γ -valerolactone-methoxy-glucuronide (3',4') isomer 1	17 $\pm$ 2	18 $\pm$ 2	13 $\pm$ 2	18 $\pm$ 2
3',4'-Dihydroxycinnamic acid	20 $\pm$ 2	21 $\pm$ 3	19 $\pm$ 3	32 $\pm$ 3 ^{*A}

3'-Methoxycinnamic acid-4'-glucuronide	16±3	18±4	12±2 ^B	25±3 ^A
3-(3'-Hydroxyphenyl)propanoic acid	77±11	51±9	41±10 *	39±8
3'-Hydroxyphenylacetic acid	68±7	78±9	74±6	87±9
4-Hydroxybenzoic acid	43±16	24±5	38±11	27±3 *
Benzoic acid-4-sulfate	15±2	23±5	14±2	28±5
Benzoic acid-3-sulfate	15±3	26±8	12±2	20±5
4'-Hydroxyhippuric acid	43±5	39±4	41±5	43±5
Hippuric acid	2756±234	2824±336	2532±371 ^B	4287±413 ^{*A}
4-Hydroxybenzaldehyde	13±1	11±1	18±2 *	19±3 *
2-Hydroxy-4/5-methylbenzene-1-sulfate	32±3	33±3	24±2 ^B	42±3 ^A
Total metabolites	3150±250	3184±358	2869±384 ^B	4686±430 ^{*A}
Total metabolites without hippuric acid derivatives	351±23	321±28	296±22 ^B	356±23 ^A

Urinary phenolic profile

In spot urine samples, up to 67 metabolites were quantified (Table S3 in Supplementary Information). They belonged to the following classes: flavonols, flavan-3-ols, PVLs, phenylvaleric acids, cinnamic acids, phenylpropanoic acids, phenylacetic acids, benzoic acids, benzaldehydes, hippuric acids, and benzene derivatives (catechols). Without considering hippuric acid derivatives (hippuric acid and 4'-hydroxyhippuric acid), which were the most abundant metabolites (74.3% of total metabolites), PVLs and phenylacetic acids were the most represented classes in all samples (on average, 36.4% and 33.6%, respectively), followed by benzoic acids (9.2%), phenylpropanoic acids (8.8%), and cinnamic acids (8.1%). The less represented classes were flavonols (1.2%), flavan-3-ols (0.8%), catechols (0.4%) and benzaldehydes (0.1%) (Figure 1).

No statistically significant differences in urinary metabolite concentrations were seen between placebo and treatment groups at baseline (Table S3). At follow-up, 5 metabolites were excreted at significantly higher concentrations in the placebo than in the treatment group: 5-phenyl- γ -valerolactone-methoxy-glucuronide isomer 2 ($p=0.034$), 5-phenyl- γ -valerolactone-sulfate-glucuronide ($p=0.046$), 4-methoxyphenylacetic acid-3-sulfate ($p=0.007$), benzoic acid-4-sulfate ($p=0.036$), and 4'-hydroxyhippuric acid ($p=0.014$). However, no statistically significant differences were seen in total metabolites. Comparing samples at baseline and at follow-up, 28 and 44 metabolites increased significantly in the treatment and control groups, respectively. Also, total metabolites were higher at follow-up in both groups, although no significance was reached due to the high data variance (Table S3).

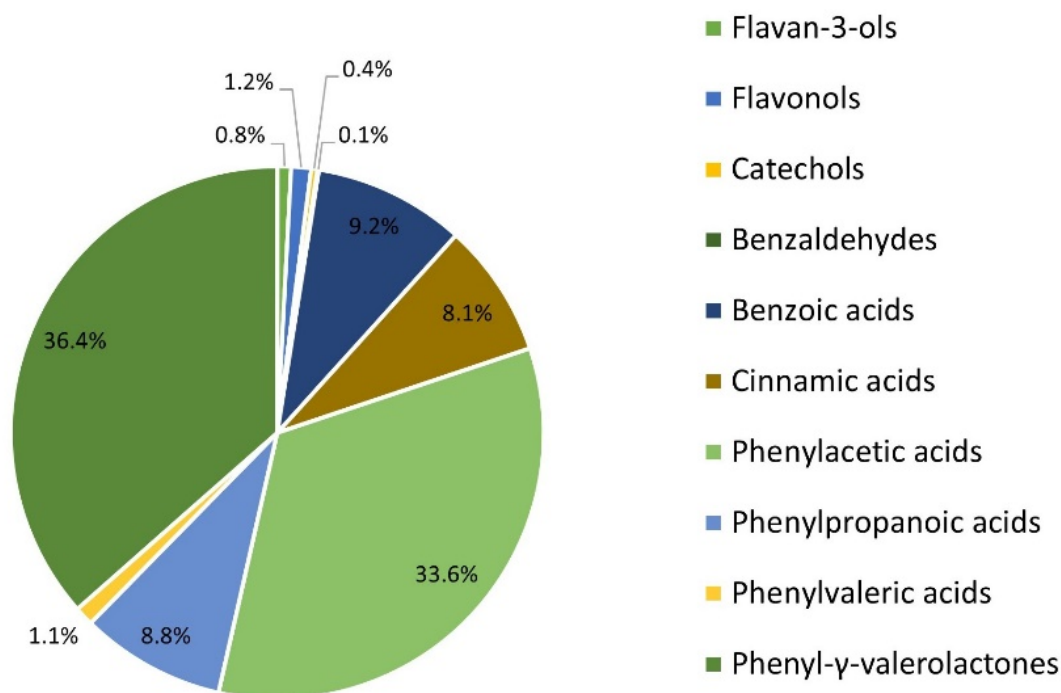


Figure 1. Phenolic metabolite profile of urine spot samples. The distribution of (poly)phenol metabolite classes does not consider hippuric acid derivatives.

Unsupervised multivariate analysis shows inter-individual variability in the urinary excretion of phenolic metabolites

Unsupervised PCA was carried out to explore the inter-individual and inter-group variability in the urinary excretion of (poly)phenol metabolites. First, all metabolites were considered as variables (data set DS1) except for hippuric acid derivatives (hippuric acid and 4'-hydroxyhippuric acid), which were excluded because they derive from different dietary sources -not only phenolics- and endogenous metabolites. Two principal components (PCs) explained 43.1% of the total variability. PC1 and PC2 explained 33.4% and 9.7% of the observed variation, respectively. It was possible to identify a pattern of metabolite (variable) distribution in the loading plots (Figure 2A), reflecting differences in colonic metabolism according to the aglycone moieties: PVLs grouped together and separated from low molecular weight phenolic acids such as cinnamic, phenylpropanoic, phenylacetic, and benzoic acids. This colonic metabolism-based pattern accounted for the potential existence of phenolic metabolotypes and was not associated with the treatment type (placebo or cranberry) or time (baseline or follow-up). This likely indicated that inter-individual differences were greater than study-related differences regarding the spot urinary phenolic profile. Clustering was carried out based on the scores of each observation in the PCA model. Three clusters were defined: observations with a positive score for both PC1 and PC2 were assigned to cluster 1, observations

with a positive score for PC1 and a negative score for PC2 were assigned to cluster 2, and observations with a negative score for PC1 and a positive or negative score for PC2 were allocated in cluster 3. Samples in the top right quadrant (cluster 1) were characterized by a higher excretion of 5-(3',4'-dihydroxyphenyl)- γ -valerolactone, 5-(hydroxyphenyl)- γ -valerolactone-sulfate (3',4' isomers), 5-(4'-hydroxyphenyl)- γ -valerolactone-3'-glucuronide, 5-(3'-hydroxyphenyl)- γ -valerolactone-4'-glucuronide, 5-phenyl- γ -valerolactone-sulfate-glucuronide, 5-phenyl- γ -valerolactone-methoxy-sulfate (isomer 1), 5-phenyl- γ -valerolactone-methoxy-glucuronide (isomer 1), 5-phenyl- γ -valerolactone-3'-sulfate, 5-phenyl- γ -valerolactone-3'-glucuronide, 4-hydroxy-5-(hydroxyphenyl)valeric acid-sulfate, and epicatechin-glucuronide (isomer 1). Samples in the bottom right quadrant (cluster 2) were characterized by a more abundant excretion of several low molecular weight phenolic metabolites belonging to cinnamic, phenylpropanoic, phenylacetic, and benzoic acids, benzaldehydes and catechols. Samples in the left quadrants (cluster 3) were characterized by a low excretion of all these metabolites.

Then, phenolic classes instead of individual phenolics were considered variables (data set DS2). Classes were defined grouping metabolites according to their aglycon moiety, obtaining 19 groups: flavonols, flavan-3-ols, 5-(3',4'-dihydroxyphenyl)- γ -valerolactones (3',4'-diHPVLs), 5-(3'-hydroxyphenyl)- γ -valerolactones (3'-HPVLs), 5-(4'-hydroxyphenyl)- γ -valerolactones (4'-HPVLs), 4-hydroxy-5-(hydroxyphenyl)valeric acids (4-OH-HPVAs), 3',4'-dihydroxycinnamic acids (3',4'-diHCAs), 3'-hydroxycinnamic acids (3'-HCAs), 4'-hydroxycinnamic acids (4'-HCAs), (3',4'-dihydroxyphenyl)propanoic acids (3',4'-diHPPAs), 3-(hydroxyphenyl)propanoic acids (HPPAs), 3',4'-dihydroxyphenylacetic acids (3',4'-diHPAAs), 3'-hydroxyphenylacetic acids (3'-HPAAs), 4'-hydroxyphenylacetic acids (4'-HPAAs), 3,4-dihydroxybenzoic acids (3,4-diHBAs), 3-hydroxybenzoic acids (3-HBAs), 4-hydroxybenzoic acids (4-HBAs), 4-hydroxybenzaldehydes (4-HBALs), and catechols. This strategy allowed considering only differences arising from the colonic microbial catabolism of (poly)phenols, excluding the inter-individual variation in phase II metabolism. Two PCs accounted for 52.1% of the total variability, PC1 and PC2 explaining 43.2% and 8.9% of the observed variation, respectively. The distribution observed was similar to that already described when considering all the metabolites (DS1), confirming the inter-individual differences due to colonic metabolism (Figure 2B). As explained above, three clusters were identified, and observations were assigned to each class according to their PC scores. Samples in the top right quadrant (cluster 1) were characterized by a higher excretion of 3',4'-diHPVLs, 3'-HPVLs, and 4-OH-HPVAs, while samples in the bottom right quadrant (cluster 2) were characterized by a more abundant excretion of 3'-

HCAs, HPPAs, and 3-HBAs. Samples in the left quadrants (cluster 3) were characterized by a low excretion of all metabolite classes.

The third data set (DS3) considered was composed only of flavan-3-ol metabolites belonging to diHPVLs, HPVLs, and HPPAs. Two PCs explained 62.5% of the total variability (38.3% and 21.9% for PC1 and PC2, respectively). As observed for all metabolites (Figure 2C), the treatment type (placebo or cranberry) or time (baseline or follow-up) did not influence the distribution of the observations. Three clusters were set by allocating the observations as previously indicated for DS1: samples in the bottom right quadrant (cluster 2) were mainly characterized by a higher excretion of diHPVLs like 5-(3',4'-dihydroxyphenyl)- γ -valerolactone, 5-(hydroxyphenyl)- γ -valerolactone-sulfate (3',4' isomers), 5-(4'-hydroxyphenyl)- γ -valerolactone-3'-glucuronide, its isomer 5-(3'-hydroxyphenyl)- γ -valerolactone-4'-glucuronide, 5-phenyl- γ -valerolactone-sulfate-glucuronide, 5-phenyl- γ -valerolactone-methoxy-sulfate (isomer 1), and 5-phenyl- γ -valerolactone-methoxy-glucuronide (isomer 1), as well as by 5-phenyl- γ -valerolactone-3'-sulfate; samples in the top right quadrant (cluster 1) were characterized by a more abundant excretion of 5-phenyl- γ -valerolactone-methoxy-sulfate (isomer 2), 5-phenyl- γ -valerolactone-methoxy-glucuronide (isomer 2), HPVLs like 5-phenyl- γ -valerolactone-3'-glucuronide, 5-phenyl- γ -valerolactone-4'-sulfate, and 5-phenyl- γ -valerolactone-4'-glucuronide, and HPPAs like 3-(hydroxyphenyl)propanoic acid, 3-(phenyl)propanoic acid-sulfate (3'/4' isomers), 3-(phenyl)propanoic acid-3'-glucuronide and 3-(phenyl)propanoic acid-4'-glucuronide. Samples in the left quadrants (cluster 3) were characterized by a low excretion of all the metabolites considered.

For all data sets, PC1 and PC2 resulted in discriminating samples on a quantitative and qualitative basis, respectively. Although PC1 discriminated more than PC2, it was less useful in characterizing specific metabolite patterns. On the contrary, PC2 was very helpful in characterizing metabolic profiles.

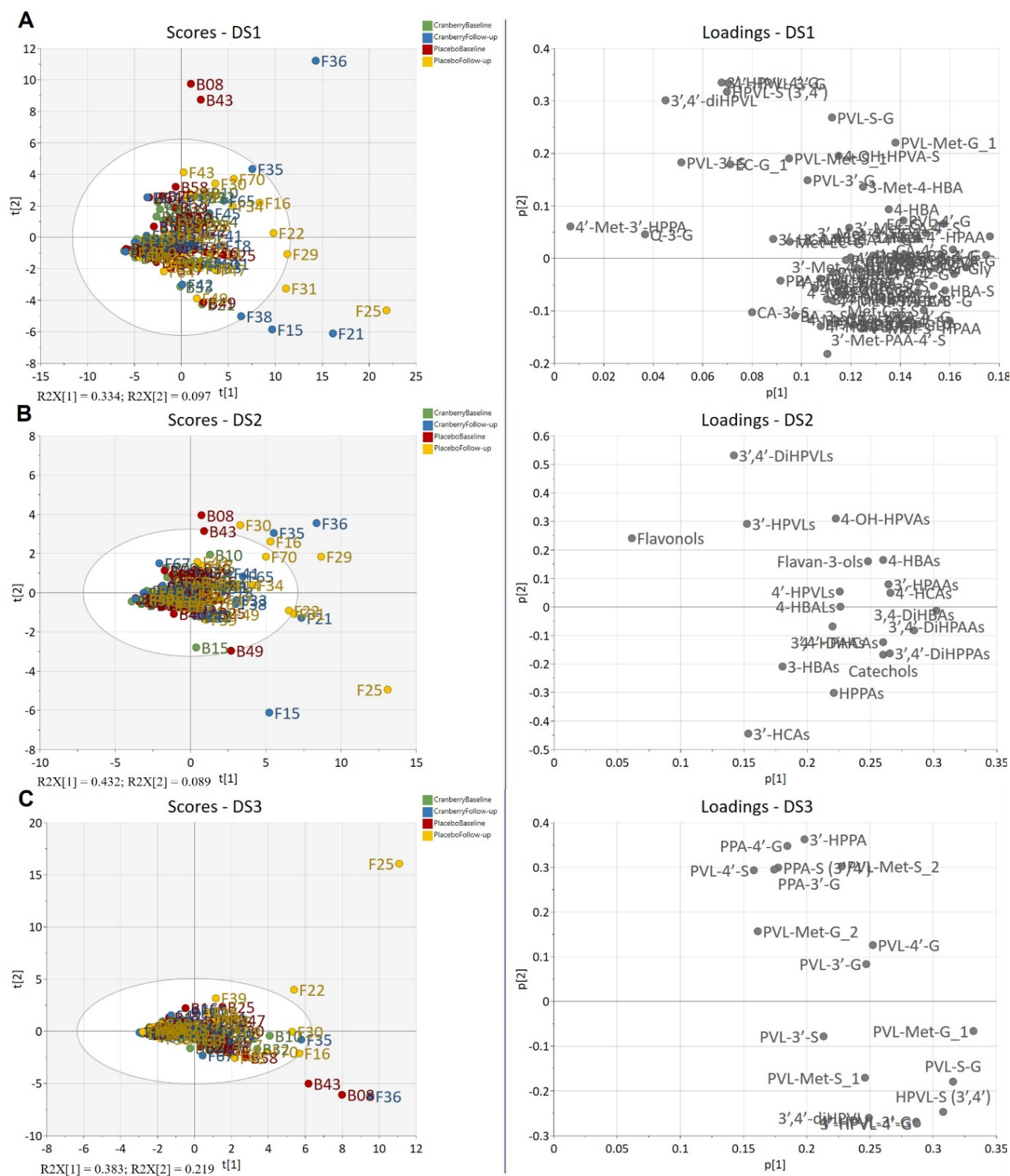


Figure 2. Score and loadings plots resulting after PCA on autoscaled data for individual metabolites (DS1, **A**), phenolic classes (DS2, **B**), and metabolites belonging to HPPAs (isomers 3' and 4'), monohydroxyPVLs (isomers 3' and 4'), and dihydroxyPVLs (3',4') (DS3, **C**). Metabolite abbreviations in the loading plot are indicated in Table S2 in Supplementary Information. Letters B and F for the observations in the score plot indicate subjects at baseline and follow-up visits, respectively.

Supervised multivariate analysis: metabotypes in the excretion of phenolic metabolites

Supervised PLS-DA was carried out to predict which metabolite(s) could explain the inter-individual variability obtained in the PCA outcomes, and to stratify subjects into groups sharing a similar excretion profile (Figure 3 A, B, C). Clustering according to PC scores was used to classify individuals into 3 groups characterized by different metabolite profiles or a low excretion of all metabolites. All the PLS-DA models passed cross-validation by CV-ANOVA ($p=1.1e^{-31}$ for DS1, $p=4.3e^{-32}$ for DS2, $p=1.3e^{-33}$ for DS3) and by random permutation. The model with the highest explained variance was obtained considering DS3 ($R^2X=0.62$), followed by DS2 ($R^2X=0.52$). The DS1 model had the lowest explained variance ($R^2X=0.43$), but it was acceptable and presented the highest predictive ability ($Q^2=0.50$). A similar predictive ability was obtained using DS2 ($Q^2=0.49$), while this value is lower for DS3 ($Q^2=0.43$). R^2Y values were greater than 0.50 for all models (0.57, 0.55 and 0.52 for DS1, DS2, DS3, respectively). These results confirmed model validation and excluded data overfitting. In addition, the ROC curves confirmed the good classification performance of each model (Figure S1 in Supplementary Information).

For DS1, the most relevant metabolites (VIP score >1.25) were mainly diHPVL derivatives, epicatechin-glucuronide (isomer 1), 3'-methoxy-phenylacetic acid-4'-sulfate, and 4-hydroxy-5-(hydroxyphenyl)valeric acid-sulfate (Figure S2A in Supplementary Information). This was also observed in the DS2 model, where the highest VIP was recorded for diHPVLs. Other relevant classes were 4-OH-HPVAs, catechols, HPPAs, 3'-HCAs, 3',4'-diHPAAs, 3-HBAs and 3,4-diHBAs (Figure S2B in Supplementary Information). For DS3, the highest VIP was for 5-(hydroxyphenyl)- γ -valerolactone-sulfate (3',4'), followed by other diHPVLs, phenylpropanoic acid-3'-glucuronide, phenylpropanoic acid-4'-glucuronide, and 5-(phenyl)- γ -valerolactone-4'-glucuronide (Figure S2C in Supplementary Information).



Figure 3. Score and loadings plots resulting after PLS-DA with clusters obtained using PC score-based models for individual metabolites (DS1, **A**), phenolic classes (DS2, **B**), and metabolites belonging to HPPAs (isomers 3' and 4'), monohydroxyPVLs (isomers 3' and 4'), and dihydroxyPVLs (3',4') (DS3, **C**). Metabolite abbreviations in the loading plot are indicated in Table S2 in Supplementary Information. Letters B and F for the observations in the score plot indicate subjects at baseline and follow-up visits, respectively.

In general, the PLS-DA models were representative of metabolotypes reflecting a different microbial metabolism of (poly)phenols. Observations belonging to cluster 1 for DS1/2 and cluster 2 for DS3, which were characterized by a higher excretion of 3',4'-diHPVLs and 3'-HPVLs, were assigned to metabolotype 1. On the contrary, observations belonging to cluster 2 for DS1/2 and cluster 1 for DS3, characterized by a more abundant excretion of 3'-HCAs, HPPAs, and 3-HBAs, were assigned to metabolotype 2. The remaining observations belonging to cluster 3 for all models were assigned to metabolotype 3, characterized by a low metabolite excretion. Agreement among models, calculated as a percentage of observations with the same metabolotype for the models considered, accounted for well-preserved metabolotypes regardless of the data set used. A high accordance between the models was found: 87% between models using DS1 and DS2, 73% between models using DS3 and DS1/DS2, and 68% using all models.

Considering the individual metabolotype shifts from baseline to follow-up for models DS1 and DS3 (Table 2), half of the subjects (55%) maintained the same metabolotype. Most of the metabolotype shifts involved metabolotype 3 (35% for DS1 and 22% for DS3), which changed into metabolotype 2 (18% for DS1 and 8% for DS3) or into metabolotype 1 (17% for DS1 and 13% for DS2). The other metabolotype shifts involved metabolotype 1 (8% for DS1 and 17% for DS3), which changed mainly into metabolotype 3 (7% for DS1 and 12% for DS3), and metabolotype 2, which was the most stable one, with only 1 and 4 subjects undergoing a change for DS1 and DS3, respectively. Qualitative changes of metabolotype, namely shifts from metabolotype 2 to 1 or vice versa, were very limited (2% for DS1 and 8% for DS3) and, for DS1, involved only metabolotype 1, confirming the stability of metabolotype 2. As expected, metabolotype shifts obtained with DS2 were similar to the ones obtained with DS1, with the exception of the shift from metabolotype 3 to metabolotype 2, which was less frequent for DS2. No differences in metabolotype shifts were observed between control and cranberry groups.

Table 2. Number and frequency of observations involved in the metabolotype shifts observed using models built with data set 1 (DS1), data set 2 (DS2) and data set 3 (DS3).

Shifts	Number of observations			Percentage of observations		
	DS1	DS2	DS3	DS1	DS2	DS3
1 to 2	1	1	3	2%	2%	5%
1 to 3	4	4	7	7%	7%	12%
2 to 1	0	0	2	0%	0%	3%
2 to 3	1	0	2	2%	0%	3%
3 to 1	10	11	8	17%	18%	13%
3 to 2	11	6	5	18%	10%	8%
No shifts	33	38	33	55%	63%	55%

Univariate statistics account for differences in metabolite excretion among metabolotypes

Results from PLS-DA outcomes and discriminating classes were confirmed by univariate statistics. Considering phenolic classes and PC score-based clustering using DS1 (Table 3), the small groups of high excretors (metabotypes 1 and 2) showed statistically significant differences in the urinary excretion of most metabolite classes with respect to metabotype 3. Metabotypes 1 and 2 differed statistically only in the excretion of 3'-HPVLs ($p=0.001$) and 3',4'-DiHPVLs ($p=0.0005$), which were higher for metabotype 1. Metabotype 2 presented higher levels of many phenolic acids like 3'-HCAs, HPPAs, and 3-HBAs, although these differences did not reach statistical significance. Metabotype 1 was also characterized by the highest excretion of total metabolites (without considering hippuric acid derivatives) ($p=0.010$), while metabotype 3 presented the lowest (Table 3).

Table 3. Urinary excretion of phenolic classes, total metabolites (without considering hippuric acid derivatives) and metabolites belonging to 3'-(hydroxyphenyl)- γ -valerolactones and 3',4'-(dihydroxyphenyl)- γ -valerolactones per each metabotype defined after applying PCA-based clustering to DS1. Data are indicated as mean $\pm$ SEM and are expressed as μ mol/mol creatinine. Different lowercase letters indicate significant mean differences between clusters ($p<0.05$).

Phenolic metabolite class	Metabotype 1	Metabotype 2	Metabotype 3
Flavonols	96.5 $\pm$ 41.2 ^a	77.3 $\pm$ 33.4 ^a	29.8 $\pm$ 7.2 ^b
Flavan-3-ols	62.0 $\pm$ 6.7 ^a	43.7 $\pm$ 4.8 ^a	25.5 $\pm$ 1.4 ^b
3',4'-DiHPVLs	3476.6 $\pm$ 417.1 ^a	1009.7 $\pm$ 133.6 ^b	871.8 $\pm$ 110.5 ^b
3'-HPVLs	159.0 $\pm$ 26.2 ^a	61.4 $\pm$ 12.1 ^b	48.3 $\pm$ 5.2 ^b
4'-HPVLs	78.3 $\pm$ 10.4 ^a	73.0 $\pm$ 16.0 ^a	32.6 $\pm$ 3.4 ^b
4-OH-HPVAs	114.2 $\pm$ 17.3 ^a	72.3 $\pm$ 14.8 ^a	23.0 $\pm$ 2.9 ^b
3',4'-DiHCAs	452.3 $\pm$ 57.0 ^a	527.6 $\pm$ 69.7 ^a	179.2 $\pm$ 15.6 ^b
3'-HCAs	31.6 $\pm$ 3.4 ^a	70.5 $\pm$ 18.4 ^a	20.1 $\pm$ 2.0 ^b
4'-HCAs	13.2 $\pm$ 2.1 ^a	15.3 $\pm$ 3.2 ^a	6.0 $\pm$ 0.5 ^b
3',4'-DiHPPAs	347.2 $\pm$ 36.2 ^a	474.7 $\pm$ 78.5 ^a	130.4 $\pm$ 11.0 ^b
HPPAs	221.5 $\pm$ 57.2 ^a	317.6 $\pm$ 105.6 ^a	61.9 $\pm$ 6.7 ^b
3',4'-DiHPAAs	287.5 $\pm$ 29.8 ^a	344.9 $\pm$ 37.1 ^a	120.8 $\pm$ 7.5 ^b
3'-HPAAs	784.3 $\pm$ 97.9 ^a	807.7 $\pm$ 115.3 ^a	355.1 $\pm$ 25.3 ^b
4'-HPAAs	1140.3 $\pm$ 133.0 ^a	1095.3 $\pm$ 115.1 ^a	479.7 $\pm$ 39.7 ^b
3,4-DiHBAs	77.2 $\pm$ 8.0 ^a	100.7 $\pm$ 13.0 ^a	28.3 $\pm$ 1.9 ^b
3-HBAs	91.1 $\pm$ 24.5 ^a	152.2 $\pm$ 35.1 ^a	40.8 $\pm$ 5.3 ^b
4-HBAs	432.0 $\pm$ 43.4 ^a	426.8 $\pm$ 65.7 ^a	176.6 $\pm$ 12.0 ^b
4-HBALs	6.0 $\pm$ 1.0 ^a	7.1 $\pm$ 1.1 ^a	2.7 $\pm$ 0.2 ^b
Catechols	21.1 $\pm$ 3.9 ^a	36.2 $\pm$ 5.1 ^a	8.3 $\pm$ 0.9 ^b
Total	7892.0$\pm$523.1^a	5713.9$\pm$467.7^b	2640.9$\pm$164.0^c
3',4'-DiHPVL	55.0 $\pm$ 9.4 ^a	8.9 $\pm$ 1.9 ^b	14.0 $\pm$ 2.4 ^b
HPVL-S (3',4')	1944.8 $\pm$ 197.2 ^a	583.5 $\pm$ 105.9 ^b	531.5 $\pm$ 72.6 ^b
3'-HPVL-4'-G	206.6 $\pm$ 40.3 ^a	33.8 $\pm$ 4.6 ^b	47.2 $\pm$ 8.3 ^b
4'-HPVL-3'-G	740.2 $\pm$ 149.1 ^a	98.7 $\pm$ 13.7 ^b	136.2 $\pm$ 22.5 ^b
PVL-Met-S 1	8.1 $\pm$ 0.9 ^a	4.4 $\pm$ 0.6 ^b	3.1 $\pm$ 0.3 ^b
PVL-Met-S 2	18.8 $\pm$ 2.8 ^a	25.5 $\pm$ 14.7 ^a	4.6 $\pm$ 0.6 ^b
PVL-Met-G 1	167.6 $\pm$ 15.0 ^a	98.2 $\pm$ 10.3 ^b	53.9 $\pm$ 4.0 ^b

PVL-Met-G 2	39.0±7.8 ^a	50.3±6.4 ^a	18.4±2.7 ^b
PVL-S-G	296.6±37.1 ^a	106.4±13.0	62.9±8.1 ^c
PVL-3'-S	59.5±14.3 ^a	14.6±2.8 ^b	15.6±2.6 ^b
PVL-3'-G	99.6±13.6 ^a	46.8±10.6 ^b	32.7±3.8 ^b

Exploring the differences in the excretion of single metabolites belonging to 3'-HPVL and 3',4'-DiHPVL classes among metabotypes revealed that all the individual metabolites were significantly higher in metabotype 1, except for the isomers 2 of 5-phenyl-γ-valerolactone-methoxy-sulfate (3',4') and 5-phenyl-γ-valerolactone-methoxy-glucuronide (3',4') (Table 3). These two metabolites were lower in metabotype 3 and did not contribute to differentiating metabotype 1 and 2. So, most 3'-HPVLs and 3',4'-DiHPVLs may help discriminate cranberry phenolic metabotypes 1, 2, and 3 on the basis of quali-quantitative differences.

4.3.4. Discussion

Cranberry supplementation in older adults for 12 weeks has been related to improved episodic memory, through improved regional brain perfusion [4]. A recent study also accounted for the role of cranberry consumption in the vascular system via increased flow-mediated dilation, this positive effect on the endothelial function being associated with (poly)phenol metabolites [18]. Our work addressed the (poly)phenolic profile of two biofluids, plasma and urine, after chronic intake of cranberry in healthy older adults, also considering the differential individual excretion of (poly)phenolic metabolites. A total of 13 metabolites were quantified in plasma samples after overnight fasting, in line with other studies reporting acute or chronic consumption of cranberry [3,14,18]. Some (poly)phenolic metabolites increased upon chronic intake of cranberry, which was used as a measure of compliance with the cranberry treatment [4]. Main increases in plasma phenolic metabolites derived from hydroxycinnamic acid derivatives, such as 3',4'-dihydroxycinnamic acid and 3'-methoxycinnamic acid-4'-glucuronide, in good agreement with previous studies [14,18].

A total of 67 metabolites were quantified in spot urine samples, PVLs and phenylacetic acids being the most represented classes. PVLs are specific flavan-3-ol metabolites, while phenylacetic acids can derive from hydroxycinnamic acids and other flavonoids, including flavan-3-ols [1,2,11]. Interestingly, the cranberry treatment did not influence the urinary phenolic profile. This could be due to the sample type and collection time since spot samples were collected, and there was no control on the time of consumption of the 2 cranberry doses during the day. Moreover, the participants' diet was not controlled, and their (poly)phenol intake was not estimated during the

intervention, adding a possible confounding factor due to the realistic nature of the intervention. On the other hand, the increase in many phenolic metabolites after the placebo treatment may indicate a spontaneous adjustment of the participants' diet toward healthier dietary patterns during the study, or at least a change in their diet a few days before the follow-up sampling. Indeed, these results were in line with the intervention carried out by Heiss and colleagues [18], where shifts for only some metabolites were recorded in 24-h urines upon 1 month of cranberry powder consumption, and with the conclusions of Feliciano and colleagues [15], who suggested that only 6.2% of the total ingested (poly)phenols were recovered in urine after acute consumption of cranberry juice.

Applying PCA to urinary data reflected considerable differences in the metabolism of (poly)phenols, mainly attributed to microbial catabolism, and was not associated with the treatment type or time. This indicated that inter-individual differences were somehow greater than study-related differences according to the samples analyzed. Similar results were obtained by Mena et al [19] for flavan-3-ol catabolites upon cranberry consumption, where no pattern of metabolite distribution was associated with the intervention study or visit type. Indeed, despite the significant results found in intervention trials with cranberries [4,18], the beneficial role of (poly)phenols has not been demonstrated consistently yet because of the heterogeneity in the individual response to their consumption [27,28]. Subjects' heterogeneity is likely related to differences in the microbial metabolism of (poly)phenols, but it could also be influenced by the variability in the consumption of flavan-3-ols and other (poly)phenol classes during the experiment. This second source of variability could not be excluded due to the lack of control of the (poly)phenol intake along the study.

The inter-individual variability in the metabolism and physiological response to (poly)phenols is influenced by several factors, including gut microbiota, genetic background, sex, ethnicity, age, lifestyle (smoking, diet, physical activity), (patho)physiological status and medication [29–31]. Inter-individual differences in the colonic metabolism of (poly)phenols have been reported for different classes of these compounds and result in an extensive quali-quantitative variation in the circulating phenolic metabolites [20]. Handling these individual differences through metabotyping approaches may contribute to understanding the drivers of the existing variability and investigating its impact on the health effects of (poly)phenol-containing foods. A quali-quantitative approach for metabotyping phenolic metabolites has been proposed by our research group, using cranberry flavan-3-ols as an example [19]. This strategy is based on clustering

subgroups of individuals sharing similar metabolic profiles when all subjects produce most of the (poly)phenol metabolites of a specific catabolic pathway but in different proportions, as occurring for flavan-3-ols and other main dietary (poly)phenols. This approach has never been applied before to complex food matrices presenting a wide set of (poly)phenols and for metabolites belonging to different metabolic pathways, as is the case of cranberry. Here, the lack of major differences between control and cranberry treatments may lead to hypothesize that consumption of (poly)phenols from the daily diet mask the cranberry treatment effect, at least regarding the urinary phenolic profile. Therefore, using this metabotyping approach for (poly)phenol metabolites, as done here, represents a new step in handling the complexity of (poly)phenol metabolism in free-living conditions, deciphering the existence of metabotypes derived from the simultaneous consumption of different classes of (poly)phenols. To analyze the inter-individual variability registered in the data and provide methodological insights for future research, PCA was applied considering three different data sets: all individual metabolites (DS1), which may account for differences in phase II conjugation and colonic metabolism; phenolic classes obtained grouping metabolites according to their aglycone moiety (DS2), accounting only for differences in the colonic metabolism; and metabolites belonging to HPPAs, HPVLs and diHPVLs (DS3), related to the metabolism of flavan-3-ols, in line with previous works [6,19]. Since no major increases in urinary metabolites were found following treatment with cranberry compared to placebo, all subjects were included as observations, both at baseline and at follow-up. This approach was also used because flavan-3-ols, along with other cranberry (poly)phenols such as hydroxycinnamic acids, are the most consumed (poly)phenols in Western diets [32,33]. All models highlighted a colonic metabolism-based pattern in the metabolite excretion, likely indicating that gut microbiota was the main determinant of the inter-individual variability observed and the main driver of subject clustering into putative metabotypes. However, it is necessary to highlight that clustering could also be influenced by quali-quantitative differences in the consumption of (poly)phenols during the experiment. Subjects clustering in DS1 and DS2 was driven by the excretion of 5-carbon side chain ring fission metabolites, in particular 3',4'-diHPVLs, 3'-HPVLs and 4-OH-HPVAs, and 3-carbon side chain ring fission metabolites, namely 3'-HPPAs and 3'-HCAs. The former metabolites characterized metabotype 1 and the latter metabotype 2. Metabotype 3 was the most prevalent one and was defined by a low excretion of all metabolites, differing from the other two on a quantitative basis. Similar results were obtained by Mena et al [19] investigating the existence of colonic metabotypes in the excretion of cranberry flavan-3-ol metabolites: one

group was characterized by a high excretion of 5-(4'-hydroxyphenyl)- γ -valerolactone and 3-(hydroxyphenyl)propanoic acid derivatives, a second group was associated to a relevant excretion of 5-(3',4'-dihydroxyphenyl)- γ -valerolactones and 5-(3'-hydroxyphenyl)- γ -valerolactones, and a third larger group showed a low metabolite excretion profile.

The DS3 model focused on flavan-3-ols fitted well DS1 insights and previous data [6,19,34]. First, this outcome further confirms the existence of urinary metabotypes in the excretion of flavan-3-ol catabolites proposed for tea and in another cranberry study [6,19]. Second, flavan-3-ol metabolites may be key in determining (poly)phenol metabotypes, and flavan-3-ol metabotypes may be highly predictive of (poly)phenol metabotypes. This was confirmed by the relevance of 3',4'-diHPVLs (or its sulfate derivative) as a discriminating class (or metabolite) and the good classification performance of all the models. PVLs, in particular the sulfate and glucuronide derivatives of 5-(3',4'-dihydroxyphenyl)- γ -valerolactone, are the main circulating metabolites of flavan-3-ols [11,35], which are in turn the most important dietary class of (poly)phenols together with hydroxycinnamic acids [36]. The role of some PVLs as key phenolic metabolites and their intrinsic microbiota-driven individual variability might be of help to classify individuals and conduct further metabotyping or personalized nutrition studies. Nevertheless, more efforts are needed to understand better the relationships between PVLs, the gut microbiota consortium in charge of their metabolism, and the rest of main dietary classes of (poly)phenols. In addition, results suggested that cranberry (poly)phenols, in particular the A-type oligomeric proanthocyanidins characterizing this fruit, are likely stable in the gut and poorly catabolized, not contributing significantly to the pool of circulating flavan-3-ols or their downstream gut microbiota metabolites. These considerations are in line with the existing evidence from *in vitro* fermentation studies and human trials, showing that proanthocyanidins are less converted to microbial catabolites than monomers, with type-A oligomers being more resistant than B-type [37–40].

Last, metabotypes 1 and 2, but not metabotype 3, remained stable over time. This is interesting as it may confirm the stability in the production profile of phenolic metabolites for 12 weeks. The fact that metabotype 3 changed after the intervention period towards metabotypes characterized by higher production of phenolic catabolites should be a positive result if these metabolites are bioactive. This change in the metabotype may account for a shift of the microbiota profile due to increases in specific genera, as already seen for urolithins with non-producers [41] or in the alpha-diversity, since the microbial species behind the catabolic pathways

of many (poly)phenols classes are poorly understood. Shifts involving metabotype 3 could also be influenced by changes in the amount of flavan-3-ols ingested with the daily diet, which could be a confounding factor. However, these hypotheses require further research, something warranted.

4.3.5. Conclusions

Chronic treatment with cranberry powder for 12 weeks led to changes in the plasma concentrations of circulating phenolic metabolites, in particular hydroxycinnamic acids and hippuric acid. Inter-individual differences in the excretion of phenolic metabolites were assessed using a comprehensive pipeline to unravel phenolic metabotypes, and three metabotypes were identified. They were characterized mainly by quali-quantitative differences in the production of phenolic metabolites of colonic origin, belonging to the catabolic pathway of different classes of dietary (poly)phenols. The most relevant class of metabolites for subjects' metabotyping was 3',4'-diHPVLs. The identification of metabotypes in the excretion of (poly)phenol metabolites could be helpful in unveiling the health-promoting activity of phenolic metabolites associated with cranberry consumption. Investigating these individual differences considering gut microbiota profiles and functional measures deserves further efforts. Future intervention trials investigating the beneficial effects of (poly)phenol intake while considering inter-individual differences should (1) control volunteers' diet using dietary records/recalls during the sampling period and the day(s) before, and (2) optimize the time and type of sampling, for example collecting 24 h urine or providing harmonized recommendations on food consumption timing when spot urine samples are collected.

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Authors' Contributions

AN, MH, and DV contributed to the conception and design of the study; EF contributed to the day-to-day management of the clinical study and sample acquisition; NT, CF, DDR, and PM contributed to the methodology; AN, , MH, DV, DDR, and PM acquired funding; NT performed sample and data analysis and visualized the data; CF, LB, and PM supervised analysis; NT and PM wrote the manuscript. All the authors performed reviewing and editing of the article and consented to the final article.

Data Availability Statement

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

Declaration of interest statement

DV, MH, and AN received funding from the Cranberry Institute. DDR and PM received a research grant from the Cranberry Institute in the past. The rest of the authors declare no conflict of interest.

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Electronic Supplementary Information (ESI)

S1.1 Materials

4-Hydroxybenzaldehyde, 3,4-dihydroxybenzaldehyde, 4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 3-(4'-hydroxyphenyl)propanoic acid, 3-(3'-hydroxyphenyl)propanoic acid, 3-(3',4'-dihydroxyphenyl)propanoic acid, 3-(4'-hydroxy-3'-methoxyphenyl)propanoic acid, 4'-hydroxy-3'-methoxycinnamic acid, 4'-hydroxyphenylacetic acid, 3'-hydroxyphenylacetic acid, 3',4'-dihydroxyphenylacetic acid, hippuric acid, and quercetin 3-glucuronide were purchased from Sigma-Aldrich (St. Louis, MO, USA). 3'-Methoxycinnamic acid-4'-sulfate, 3'-methoxycinnamic acid-4'-glucuronide, 4'-methoxycinnamic acid-3'-sulfate, 4'-methoxycinnamic acid-3'-glucuronide, 3-(3'-methoxyphenyl)propanoic acid-4'-sulfate, 3-(4'-methoxyphenyl)propanoic acid-3'-glucuronide, 3-(4'-hydroxyphenyl)propanoic acid-3'-sulfate, 3-(4'-hydroxyphenyl)propanoic acid-3'-glucuronide, 3'-hydroxycinnamic acid-4'-glucuronide and 4'-hydroxycinnamic acid-3'-glucuronide were purchased from Toronto Research Chemicals (Toronto, ON, Canada). 4-Hydroxy-3-methoxybenzoic acid and 4'-hydroxy-3'-phenylacetic acid were purchased from Alfa Aesar (Thermo Fisher (Kandel) GmbH, Postfach, Karlsruhe, Germany) and from Extrasynthese (Genay Cedex, France), respectively. 4'-Hydroxyhippuric acid was purchased from Bachem Ltd. (St Helens, UK). Prof. Alan Crozier (University of Glasgow) kindly supplied feruloylglycine. 3-Methoxybenzoic acid-4-glucuronide, 3-methoxybenzoic acid-4-sulfate, 4-methoxybenzoic acid-3-glucuronide, 4-hydroxybenzoic acid-3-sulfate, 4-hydroxybenzoic acid-3-glucuronide, benzoic acid-4-sulfate, benzoic acid-4-glucuronide were provided by Dr. Colin Kay (N.C. State University's Plants for Human Health Institute). 5-(3',4'-Dihydroxyphenyl)- γ -valerolactone, 5-phenyl- γ -valerolactone-3'-sulfate, 5-(3'-hydroxyphenyl)- γ -valerolactone-4'-sulfate, 5-(5'-hydroxyphenyl)- γ -valerolactone-3'-glucuronide were synthesized in house using the synthetic strategy previously outlined by Curti and colleagues for aglycones [1], and by Brindani and colleagues for phase II conjugates [2].

S1.2 Sample analysis

Samples were analyzed through a UHPLC DIONEX Ultimate 3000 fitted with a TSQ Vantage Triple Quadrupole Mass Spectrometer (Thermo Fisher Scientific Inc., San José, CA, USA) equipped with a heated-electrospray ionization source (H-ESI-II; Thermo Fisher Scientific Inc.). Separations were performed with a Kinetex EVO C18 (100 × 2.1 mm), 2.6 μ m particle size (Phenomenex). For UHPLC, mobile phase A was water containing 0.01% formic acid, and mobile phase B was acetonitrile

containing 0.01% formic acid. The gradient started with 5% B, keeping isocratic conditions for 0.5 min, reaching 95% B at 7 min, followed by 1 min at 95% B and then 4 min at the start conditions to re-equilibrate the column. The flow rate was set at 0.4 mL/min, the injection volume was 5 μ L, and the column was thermostatted at 40°C. The MS worked in negative ionization mode with capillary temperature at 270°C, while the source was at 300°C. The sheath gas flow was 60 units, while auxiliary gas pressure was set to 10 units. The source voltage was 3 kV. Ultra-high-purity argon gas was used for collision-induced dissociation (CID). Metabolite identification was carried out by comparison of the retention time with commercial or in-house synthesized standards and/or MS/MS fragmentation patterns and retention times. Quantification was performed with calibration curves of standards, when available; when not available, metabolites were quantified with the most structurally similar compound.

S1.3 Creatinine analysis

Samples were analyzed through Accela uHPLC 1250 apparatus equipped with a Linear Ion Trap Mass Spectrometer (LIT-MS) (LTQ XL, Thermo Fisher Scientific Inc.), fitted with a heated-electrospray ionization source (H-ESI-II). Separation was carried out by means of a XSelect HSS T3 column (50x 2.1 mm; 2.5 μ m particle size; Waters, Ireland) installed with a precolumn cartridge (Phenomenex, CA, USA). For uHPLC, mobile phase A was water containing 0.1% formic acid and mobile phase B was acetonitrile containing 0.1% formic acid. Chromatographic separation was performed in an isocratic mode keeping A and B at 50% for 2 min (duration of a single run). The flow rate was set at 0.2 mL/min, the injection volume was 5 μ L, and the column was thermostatted at 40°C. The H-ESI-II interface was set to a capillary temperature of 275 °C and the source heater temperature was 300 °C. The sheath gas (N₂) flow rate was set at 40 (arbitrary units) and the auxiliary gas (N₂) flow rate at 5. The source voltage was 4.5 kV, and the capillary voltage and tube lens voltage were +20 and +95 V, respectively. Ultra-high purity helium gas and a CID equal to 55 was used to obtain MS² fragmentation. Creatinine was analyzed with a full-scan MS² analysis in a positive ionisation mode, monitoring all the ions with a m/z value between 35 and 200 Da. For identification and quantification, the molecular ion with m/z =114 and the fragment ion with m/z =86 were considered. Identification and quantification was performed by comparison with the standard (concentration range for the calibration curves: 0.1-100 μ mol/L). Chromatograms, mass spectral data and data processing were performed using Xcalibur software 2.1. Creatinine concentration in urine samples was expressed as mol/L.

Supplementary Table 1. Amount of (poly)phenols contained in the daily dose of cranberry powder provided to subjects (9 grams, 2 sachets of 4.5 grams).

(Poly)phenols	Quantity (mg)
Total polyphenols	587.5
Proanthocyanidins (PACs)	374.6
Soluble PACs	280.8
Insoluble PACs	93.4
(–)-Epicatechin	0.387
(+)-Catechin	0.072
Flavonols	84.6
Quercetin-3- <i>O</i> -rhamnoside equivalents	81
Quercetin	2.3
Kaempferol	0.045
Isorhamnetin	0.34
Myricetin	0.64
Laricitrin	0.08
Syringetin	0.23
Anthocyanins	58.5
Cyanidin-3- <i>O</i> -galactoside	6.8
Cyanidin-3- <i>O</i> -arabinoside	3.8
Peonidin-3- <i>O</i> -galactoside	35.0
Peonidin-3- <i>O</i> -arabinoside	12.9
Phenolic acids	69.8
Benzoic acid	2.3
3,4-Dihydroxybenzoic acid (Protocatechuic acid)	6.9
Cinnamic acid	2.0
4'-Hydroxycinnamic acid (<i>p</i> -Coumaric acid)	1.9
3',4'-Dihydroxycinnamic acid (Caffeic acid)	0.8
5-Caffeoylquinic acid	4.7
Coumaroyl-glucose	24.3
Feruloyl-glucose	8.9
Feruloylquinic acid	0.4
Sinapoyl-glucose	3.6
Caffeoyl-glucose I	2.4
Caffeoyl-glucose II	4.8
Glucosyl-caffeoyl-glucose	6.8

Supplementary Table 2. Chromatographic and spectrometric properties of the monitored (poly)phenol metabolites. Nomenclature of metabolites is reported as proposed by Kay et al [3] and the synonyms are reported under brackets, as well as abbreviations. RT means retention time; CE means collision energy; STD means standard use for the quantification; Ref. is the reference in the literature used for the identification of compounds without an available standard.

Compound (in bold the compounds detected and quantified in urine; compounds quantified in plasma are underlined)	RT (min)	Parent ion [M-H] ⁻ (m/z)	S-Lens	Quantifier		Qualifier(s)		STD (in bold those reference compounds used to quantify the corresponding metabolite)	Ref.
				Product ion (m/z)	CE (V)	Product ion (m/z)	CE (V)		
<u>Kaempferol-3-glucuronide (K-3-G)</u>	5.71	461	90	285	27	113	30	Quercetin-3-glucuronide	[4]
Quercetin-3-glucuronide (Q-3-Glc)	5.12	477	91	301	28	113	30	Quercetin-3-glucuronide	
(Epi)catechin-glucuronide isomer 1 (EC-Glc 1)	4.48	465	98	289	27	113	19	4'-Hydroxycinnamic acid-3'-glucuronide	[5-7]
(Epi)catechin-glucuronide isomer 2 (EC-Glc 2)	5.00	465	98	289	27	113	19	4'-Hydroxycinnamic acid-3'-glucuronide	[5-7]
Methoxy-(epi)catechin-glucuronide (Met-EC-Glc)	5.90	479	98	303	35	288, 245, 113	32,16,19	4'-Methoxycinnamic acid-3'-glucuronide	[7]
5-(3',4'-Dihydroxyphenyl)-γ-valerolactone (3',4'-diHPVL)	4.35	207	81	122	21	163, 121	18,22	5-(3',4'-Dihydroxyphenyl)-γ-valerolactone	
5-(Hydroxyphenyl)-γ-valerolactone-sulfate (3',4' isomers) (3'-HPVL-4'-S and 4'-HPVL-3'-S)	5.70	287	96	207	23	163	35	5-(3'-Hydroxyphenyl)-γ-valerolactone-4'-sulfate	
5-(3'-Hydroxyphenyl)-γ-valerolactone-4'-glucuronide (3'-HPVL-4'-Glc)	4.18	383	92	207	27	113	30	5-(5'-Hydroxyphenyl)-γ-valerolactone-3'-glucuronide	[8-10]
5-(4'-Hydroxyphenyl)-γ-valerolactone-3'-glucuronide (4'-HPVL-3'-Glc)	4.38	383	92	207	27	113	30	5-(5'-Hydroxyphenyl)-γ-valerolactone-3'-glucuronide	
5-Phenyl-γ-valerolactone-methoxy-sulfate (3',4') isomer 1 (PVL-Met-S 1)	5.67	301	87	221	23	206	40	5-(3'-Hydroxyphenyl)-γ-valerolactone-4'-sulfate	[5,9,10]

5-Phenyl-γ-valerolactone-methoxy-sulfate (3',4') isomer 2 (PVL-Met-S 2)	5.90	301	87	221	23	206	40	5-(3'-Hydroxyphenyl)-γ-valerolactone-4'-sulfate	[5,9,10]
5-Phenyl-γ-valerolactone-methoxy-glucuronide (3',4') isomer 1 (PVL-Met-Glc 1)	4.55	397	90	221	30	113	30	5-(5'-Hydroxyphenyl)-γ-valerolactone-3'-glucuronide	[9,10]
5-Phenyl-γ-valerolactone-methoxy-glucuronide (3',4') isomer 2 (PVL-Met-Glc 2)	5.05	397	90	221	30	113	30	5-(5'-Hydroxyphenyl)-γ-valerolactone-3'-glucuronide	[9,10]
5-Phenyl-γ-valerolactone-sulfate-glucuronide (3',4') (PVL-S-Glc)	4.83	463	87	287	27	207, 383, 113	40,20,19	5-(5'-Hydroxyphenyl)-γ-valerolactone-3'-glucuronide	[7,9,10]
5-Phenyl-γ-valerolactone-4'-sulfate (PVL-4'-S)	5.54	271	93	191	23	147	35	5-(Phenyl)-γ-valerolactone-3'-sulfate	[2,10]
5-Phenyl-γ-valerolactone-3'-sulfate (PVL-3'-S)	5.85	271	93	191	23	147, 106	35,48	5-(Phenyl)-γ-valerolactone-3'-sulfate	
5-Phenyl-γ-valerolactone-4'-glucuronide (PVL-4'-Glc)	4.26	367	93	191	25	113	19	5-(5'-Hydroxyphenyl)-γ-valerolactone-3'-glucuronide	[8,10]
5-Phenyl-γ-valerolactone-3'-glucuronide (PVL-3'-Glc)	4.64	367	93	191	25	113	19	5-(5'-Hydroxyphenyl)-γ-valerolactone-3'-glucuronide	[2,9,10]
4-Hydroxy-5-(hydroxyphenyl)valeric acid-sulfate (3',4') (4-OH-HPVA-S)	4.83	305	95	225	20	207, 181	30,20	5-(5'-Hydroxyphenyl)-γ-valerolactone-3'-sulfate	[10]
4-Hydroxy-5-(hydroxyphenyl)valeric acid-glucuronide (3',4') (4-OH-HPVA-Glc)	4.50	401	93	225	30	113	30	5-(5'-Hydroxyphenyl)-γ-valerolactone-3'-glucuronide	[9,10]
3',4'-Dihydroxycinnamic acid (Caffeic acid) (3',4'-diHCA)	4.00	179	62	135	19			3',4'-Dihydroxycinnamic acid	
3'-Hydroxycinnamic acid-4'-sulfate (Caffeic acid-3-sulfate) (3'-HCA-4'-S)	5.14	259	62	179	20	135	25	3-(4'-Hydroxyphenyl)propanoic acid-3'-sulfate	[6,10]
4'-Hydroxycinnamic acid-3'-sulfate (Caffeic acid-4-sulfate) (4'-HCA-3'-S)	4.80	259	62	179	20	135	25	3-(4'-Hydroxyphenyl)propanoic acid-3'-sulfate	[6,10]

3'-Hydroxycinnamic acid-4'-glucuronide (Caffeic acid-4-glucuronide) (3'-HCA-4'-Glc)	3.00	355	98	179	21	113	18	
4'-Hydroxycinnamic acid-3'-glucuronide (Caffeic acid-3-glucuronide) (4'-HCA-3'-Glc)	4.12	355	98	179	21	113	18	4'-Hydroxycinnamic acid-3'-glucuronide
4'-Hydroxy-3'-methoxycinnamic acid (Ferulic acid) (3'-Met-4'-HCA)	4.88	193	71	134	19	178	18	4'-Hydroxy-3'-methoxycinnamic acid
3'-Hydroxy-4'-methoxycinnamic acid (Isoferulic acid) (4'-Met-3'-HCA)	4.95	193	71	134	19	178	18	
3'-Methoxycinnamic acid-4'-sulfate (Ferulic acid-4-sulfate) (3'-Met-CA-4'-S)	5.30	273	92	193	18	178, 134, 149	28,33,30	3'-Methoxycinnamic acid-4'-sulfate
4'-Methoxycinnamic acid-3'-sulfate (Isoferulic acid-3-sulfate) (4'-Met-CA-3'-S)	5.60	273	92	193	18	178, 134	28,33	4'-Methoxycinnamic acid-3'-sulfate
<u>3'-Methoxycinnamic acid-4'-glucuronide (Ferulic acid-4-glucuronide) (3'-Met-CA-4'-Glc)</u>	3.88	369	93	193	20	113, 134, 178	18,39,32	3'-Methoxycinnamic acid-4'-glucuronide
4'-Methoxycinnamic acid-3'-glucuronide (Isoferulic acid-3-glucuronide) (4'-Met-CA-3'-Glc)	4.49	369	93	113	18	178, 193, 134	32,20,39	4'-Methoxycinnamic acid-3'-glucuronide
Feruloylglycine (Fer-Gly)	4.50	250	79	206	14	134, 163	22,19	Feruloylglycine
3'-Hydroxycinnamic acid (<i>m</i>-Coumaric acid) (3'-HCA)	4.90	163	62	119	19	93	38	3'-Hydroxycinnamic acid
Cinnamic acid-4'-sulfate (Coumaric acid-4-sulfate) (CA-4'-S)	5.21	243	62	163	20	119	35	3-(4'-Hydroxyphenyl)propanoic acid-3'-sulfate [8]
Cinnamic acid-3'-sulfate (Coumaric acid-3-sulfate) (CA-3'-S)	5.47	243	62	163	20	119	35	3-(4'-Hydroxyphenyl)propanoic acid-3'-sulfate [8]
3-(3',4'-Dihydroxyphenyl)propanoic acid (Dihydrocaffeic acid) (3',4'-diHPPA)	3.34	181	64	137	14	119, 121	13,10	

3-(4'-Hydroxyphenyl)propanoic acid-3'-sulfate (Dihydrocaffeic acid-3-sulfate) (4'-HPPA-3'-S)	5.09	261	96	181	20	137	25	3-(4'-Hydroxyphenyl)propanoic acid-3'-sulfate	[8]
3-(3'-Hydroxyphenyl)propanoic acid-4'-glucuronide (Dihydrocaffeic acid-4-glucuronide) (3'-HPPA-4'-Glc)	3.75	357	63	181	21	113	22	3-(4'-Hydroxyphenyl)propanoic acid-3'-glucuronide	
3-(4'-Hydroxyphenyl)propanoic acid-3'-glucuronide (Dihydrocaffeic acid-3-glucuronide) (4'-HPPA-3'-Glc)	4.25	357	63	181	21	113	22	3-(4'-Hydroxyphenyl)propanoic acid-3'-glucuronide	
3-(4'-Hydroxy-3'-methoxyphenyl)propanoic acid (Dihydroferulic acid) (3'-Met-4'-HPPA)	4.65	195	73	136	21	151	22	3-(4'-Hydroxy-3'-methoxyphenyl)propanoic acid	
3-(3'-Hydroxy-4'-methoxyphenyl)propanoic acid (Dihydroisoferulic acid) (4'-Met-3'-HPPA)	4.85	195	73	136	21	151	22	3-(4'-Hydroxy-3'-methoxyphenyl)propanoic acid	[8]
3-(3'-Methoxy-phenyl)propanoic acid-4'-sulfate (Dihydroferulic acid-4-sulfate) (3'-Met-PPA-4'-S)	5.18	275	75	195	31	136	17	3-(3'-Methoxy-phenyl)propanoic acid-4'-sulfate	
3-(4'-Methoxy-phenyl)propanoic acid-3'-sulfate (Dihydroisoferulic acid-3-sulfate) (4'-Met-PPA-3'-S)	5.35	275	75	195	31	136	17	3-(3'-Methoxy-phenyl)propanoic acid-4'-sulfate	[6,8]
3-(3'-Methoxy-phenyl)propanoic acid-4'-glucuronide (Dihydroferulic acid-4-glucuronide) (3'-Met-PPA-4'-Glc)	4.14	371	90	195	26	113	21	3-(4'-Methoxy-phenyl)propanoic acid-3'-glucuronide	[6,8]
3-(4'-Methoxy-phenyl)propanoic acid-3'-glucuronide (Dihydroisoferulic acid-3-glucuronide) (4'-Met-PPA-3'-Glc)	4.52	371	90	195	26	113	21	3-(4'-Methoxy-phenyl)propanoic acid-3'-glucuronide	
<u>3-(3'-Hydroxyphenyl)propanoic acid (3'-HPPA)</u>	4.65	165	48	121	15	119	20	3-(3'-Hydroxyphenyl)propanoic acid	

3-(Phenyl)propanoic acid-sulfate (PPA-S)	5.30	245	90	165	20	121	15	3-(4'-Hydroxyphenyl)propanoic acid-3'-sulfate	[6,7]
3-(Phenyl)propanoic acid-4'-glucuronide (PPA-4'-Glc)	3.90	341	90	165	20	113	18	3-(4'-Hydroxyphenyl)propanoic acid-3'-glucuronide	[6]
3-(Phenyl)propanoic acid-3'-glucuronide (PPA-3'-Glc)	4.40	341	90	165	20	113	18	3-(4'-Hydroxyphenyl)propanoic acid-3'-glucuronide	[6,8]
3',4'-Dihydroxyphenylacetic acid (3',4'-DiHPAA)	2.14	167	60	123	17				
4'-Hydroxy-3-methoxy-phenylacetic acid (Homovanillic acid) (3'-Met-4'-HPAA)	4.06	181	64	137	14	121, 122, 119, 166	10,30,13,30	4'-Hydroxy-3'-methoxy-phenylacetic acid	
3'-Methoxy-phenylacetic acid-4'-sulfate (Homovanillic acid-sulfate) (3'-Met-PA-4'-S)	4.40	261	96	137	25	181	20	3-Methoxybenzoic acid-4-sulfate	[4,8]
4'-Methoxy-phenylacetic acid-3'-sulfate (Isohomovanillic acid-sulfate) (4'-Met-3'-HPAA)	4.60	261	96	137	25	181	20	4-Methoxybenzoic acid-3-sulfate	[4,8]
<u>3'-Hydroxyphenylacetic acid (3'-HPAA)</u>	3.82	151	40	107	10			3'-Hydroxyphenylacetic acid	
4'-Hydroxyphenylacetic acid (4'-HPAA)	3.21	151	40	107	10			4'-Hydroxyphenylacetic acid	
3,4-Dihydroxybenzoic acid (Protocatechuic acid) (3,4-DiHBA)	1.65	153	64	109	20	108	27	3,4-Dihydroxybenzoic acid	
Hydroxybenzoic acid-sulfate (Protocatechuic acid-sulfate) (HBA-S)	4.21	233	85	153	20	109	33	3-Hydroxybenzoic acid-4-sulfate	
3-Hydroxybenzoic acid-4-glucuronide (Protocatechuic acid-4-glucuronide) (3-HBA-4-GlcUA)	1.24	329	81	153	21	113	18	3-Hydroxybenzoic acid-4-glucuronide	
4-Hydroxybenzoic acid-3-glucuronide (Protocatechuic acid-3-glucuronide) (4-HBA-3-Glc)	1.92	329	81	153	21	113	18	4-Hydroxybenzoic acid-3-glucuronide	
4-Hydroxy-3-methoxybenzoic acid (Vanillic acid) (3-Met-4-HBA)	3.85	167	60	152	18	108, 123	21,17	4-Hydroxy-3-methoxybenzoic acid	

3-Methoxybenzoic acid-4-sulfate (Vanillic acid-4-sulfate) (3-Met-BA-4-S)	4.51	247	72	167	17	152, 123	27,17	3-Methoxybenzoic acid-4-sulfate	
4-Methoxybenzoic acid-3-sulfate (Isovanillic acid-3-sulfate) (4-Met-BA-3-S)	4.75	247	72	167	17	152	27	4-Methoxybenzoic acid-3-sulfate	
3-Methoxybenzoic acid-4-glucuronide (Vanillic acid-4-glucuronide) (3-Met-BA-4-Glc)	1.80	343	92	167	23	152, 113	40,17	3-Methoxybenzoic acid-4-glucuronide	
4-Methoxybenzoic acid-3-glucuronide (Isovanillic acid-3-glucuronide) (4-Met-BA-3-Glc)	3.07	343	92	113	17	167, 152	23,40	4-Methoxybenzoic acid-3-glucuronide	
3-Hydroxybenzoic acid (3-HBA)	3.68	137	70	93	16	92	26		
<u>4-Hydroxybenzoic acid (4-HBA)</u>	2.75	137	70	93	16	92	26	4-Hydroxybenzoic acid	
<u>Benzoic acid-4-sulfate (BA-4-S)</u>	4.20	217	70	137	20	93	16	Benzoic acid-4-sulfate	
<u>Benzoic acid-3-sulfate (BA-3-S)</u>	4.63	217	70	137	20	93	16	Benzoic acid-4-sulfate	[5,6,8]
Benzoic acid-4-glucuronide (BA-4-Glc)	1.26	313	78	113	15	137	20	Benzoic acid-4-glucuronide	
Benzoic acid-3-glucuronide (BA-3-Glc)	1.79	313	78	113	15	137	20	Benzoic acid-4-glucuronide	[5,6]
<u>4'-Hydroxyhippuric acid (4'-HHA)</u>	1.74	194	72	100	11	150	15	4'-Hydroxyhippuric acid	
<u>Hippuric acid (HA)</u>	3.57	178	61	134	15	77.2	22	Hippuric acid	
<u>4-Hydroxybenzaldehyde (4-HBAL)</u>	3.74	121	89	92	27	91	22	4-Hydroxybenzaldehyde	
<u>2-Hydroxy-4/5-methylbenzene-1-sulfate (4-Methylcatechol-sulfate) (Met-Cat-S)</u>	5.17	203	70	123	20	108	26	3-Methoxybenzoic acid-4-sulfate	[4-6]

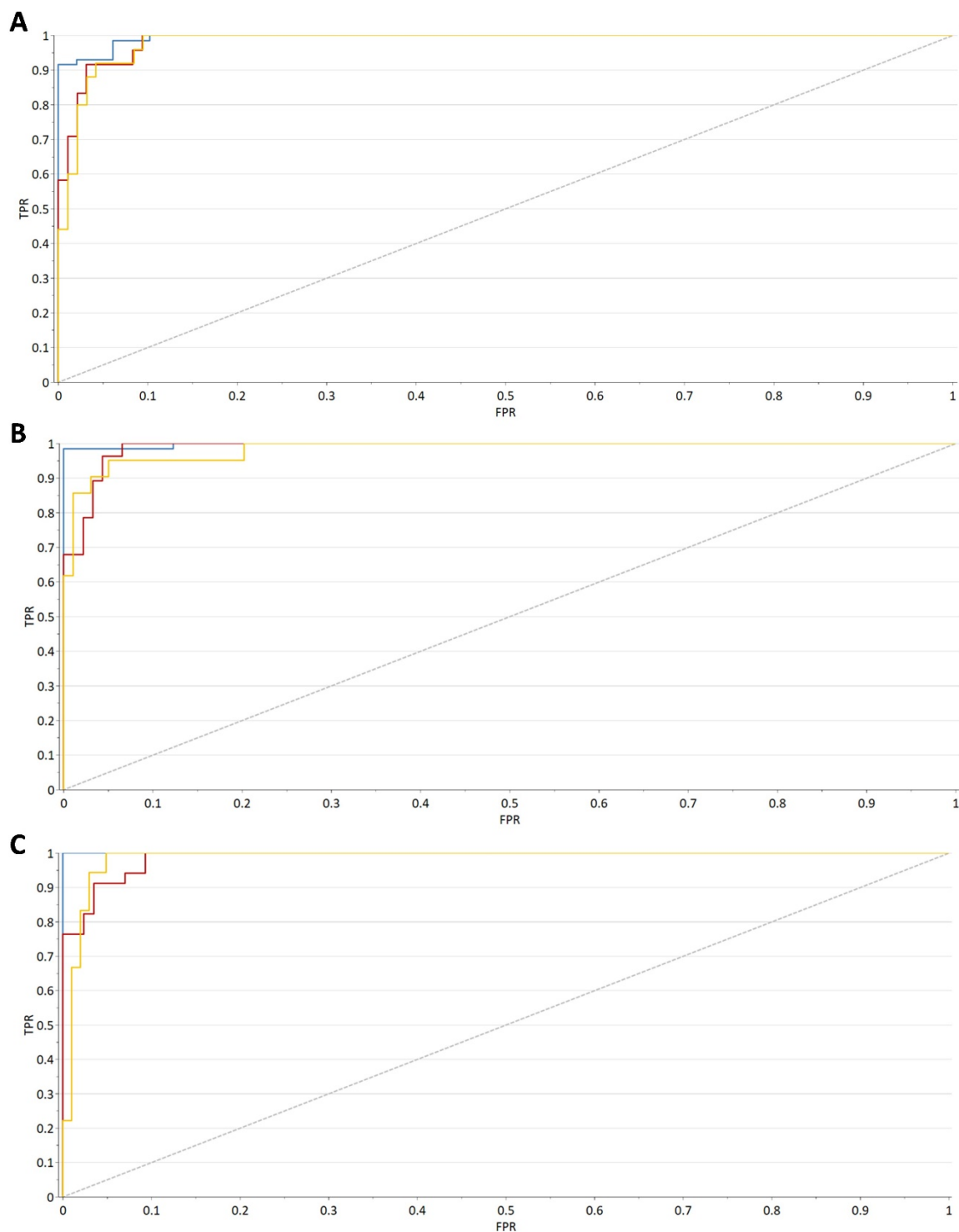
Supplementary Table 3. Urine (poly)phenol metabolite concentration at baseline and at follow-up for placebo and cranberry groups. Data are indicated as mean±SEM and are expressed as µmol/mol creatinine. Compounds presenting different uppercase letters indicate statistically significant differences between placebo and cranberry groups at baseline or follow-up. Compounds changing from baseline to follow-up are marked with an asterisk (*). Nomenclature of metabolites is reported as proposed by Kay et al [3].

Urinary metabolite	Placebo group, baseline (µmol/mol creatinine)	Cranberry group, baseline (µmol/mol creatinine)	Placebo group, follow-up (µmol/mol creatinine)	Cranberry group, follow-up (µmol/mol creatinine)
Quercetin-3-glucuronide (Q-3-G)	62.5±27.5	32.9±10.2	71.2±32.1	43.5±15.1
(Epi)catechin-glucuronide isomer 1 (EC-G 1)	2.8±0.7	2.7±0.6	2.7±0.7	3.8±1.0
(Epi)catechin-glucuronide isomer 2 (EC-G 2)	7.6±0.8	9.0±1.1	18.3±2.4 *	15.0±1.6 *
Methoxy-(epi)catechin-glucuronide (Met-EC-G)	14.1±1.1	16.2±2.3	26.7±2.6 *	27.6±4.7 *
5-(3',4'-Dihydroxyphenyl)-γ-valerolactone (3',4'-diHPVL)	24.1±7.2	19.5±5.8	21.2±4.3	19.5±5.1
5-(Hydroxyphenyl)-γ-valerolactone-sulfate (3',4' isomers) (3'-HPVL-4'-S and 4'-HPVL-3'-S)	945.3±193.4	751.0±130.5	900.5±156.3	689.6±154.5
5-(3'-Hydroxyphenyl)-γ-valerolactone-4'-glucuronide (3'-HPVL-4'-G)	311.4±101.8	211.7±43.5	230.7±50.4	240.0±102.1
5-(4'-Hydroxyphenyl)-γ-valerolactone-3'-glucuronide (4'-HPVL-3'-G)	96.9±26.4	68.3±14.2	65.1±13.2	74.1±30.7 *
5-Phenyl-γ-valerolactone-methoxy-sulfate (3',4') isomer 1 (PVL-Met-S 1)	4.6±0.7	3.5±0.6	5.0±0.7	4.3±0.7
5-Phenyl-γ-valerolactone-methoxy-sulfate (3',4') isomer 2 (PVL-Met-S 2)	6.9±1.8	4.7±0.7	25.2±11.8	9.8±2.2
5-Phenyl-γ-valerolactone-methoxy-glucuronide (3',4') isomer 1 (PVL-Met-G 1)	82.8±11.5	70.6±8.0	97.5±13.3 *	92.0±13.7 *
5-Phenyl-γ-valerolactone-methoxy-glucuronide (3',4') isomer 2 (PVL-Met-G 2)	24.1±5.5	17.0±2.8	47.3±7.3 ^A	27.2±4.6 ^B
5-Phenyl-γ-valerolactone-sulfate-glucuronide (3',4') (PVL-S-G)	96.7±20.4	87.5±16.5	166.4±27.3 ^A	122.5±30.5 ^B
5-Phenyl-γ-valerolactone-4'-sulfate (PVL-4'-S)	10.4±2.9	9.0±1.7	11.6±3.2	10.9±1.6
5-Phenyl-γ-valerolactone-3'-sulfate (PVL-3'-S)	28.4±6.7	23.8±9.5	24.1±5.3	20.2±7.6
5-Phenyl-γ-valerolactone-4'-glucuronide (PVL-4'-G)	35.7±6.2	32.0±5.1	44.6±9.7	46.5±10.5
5-Phenyl-γ-valerolactone-3'-glucuronide (PVL-3'-G)	61.6±19.9	49.3±11.1	50.1±10.6	57.5±9.6
4-Hydroxy-5-(hydroxyphenyl)valeric acid-sulfate (3',4') (4-OH-HVA-S)	28.9±6.7	25.3±5.5	53.9±12.0 *	36.5±9.8
4-Hydroxy-5-(hydroxyphenyl)valeric acid-glucuronide (3',4') (4-OH-HVA-G)	10.0±1.5	7.5±1.8	23.7±6.4 *	19.2±3.4 *
3',4'-Dihydroxycinnamic acid (Caffeic acid) (3',4'-diHCA)	0.6±0.04	0.6±0.04	0.9±0.08 *	1.1±0.10
3'-Hydroxycinnamic acid-4'-sulfate (Caffeic acid-3-sulfate) (3'-HCA-4'-S)	7.1±1.8	8.2±1.3	5.9±1.2	7.8±1.4
4'-Hydroxycinnamic acid-3'-sulfate (Caffeic acid-4-sulfate) (4'-HCA-3'-S)	15.1±2.2	18.5±3.4	19.7±2.6	22.2±5.1

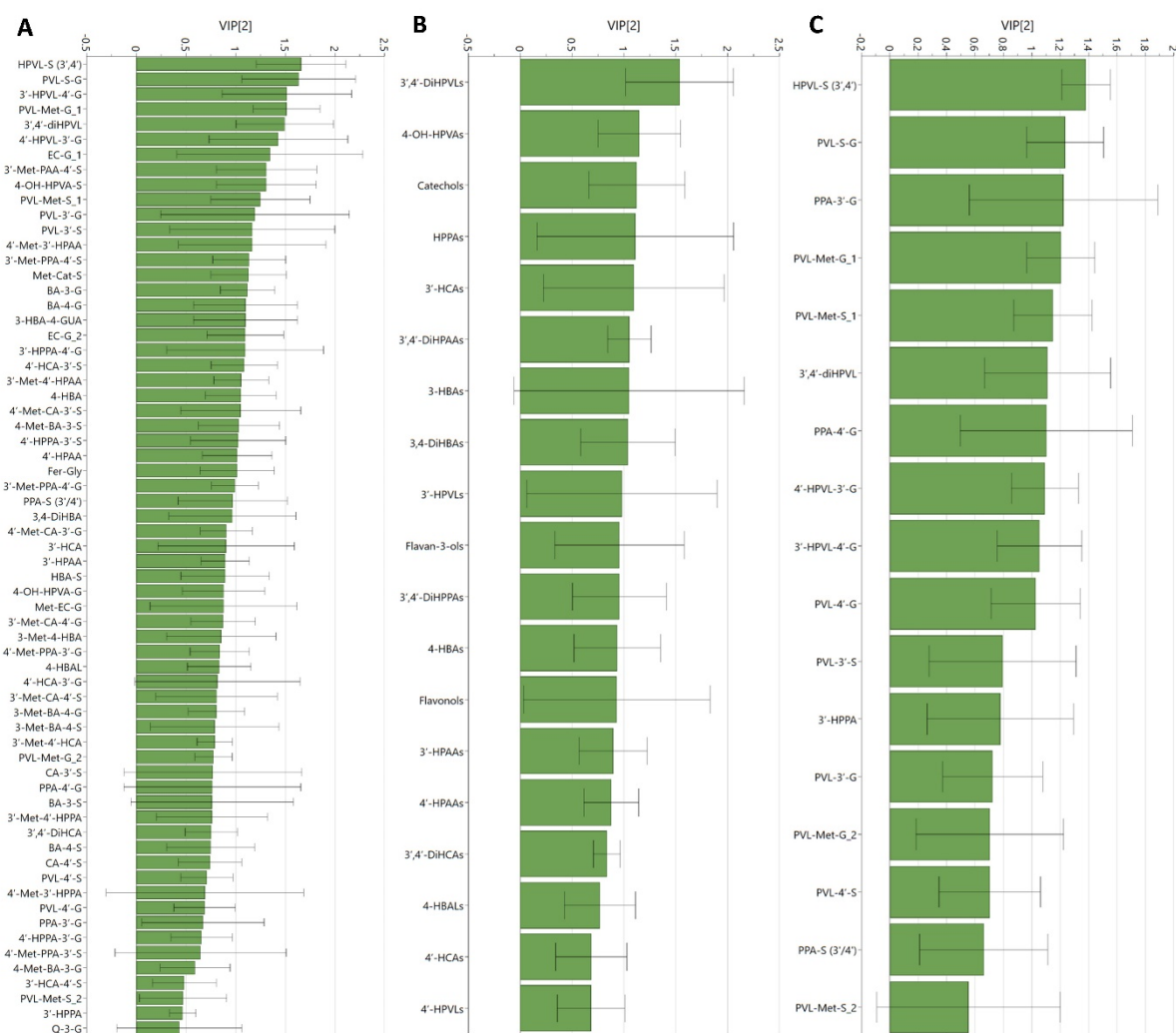
4'-Hydroxycinnamic acid-3'-glucuronide (Caffeic acid-3-glucuronide) (4'-HCA-3'-G)	0.5±0.2	0.7±0.3	5.3±1.6 *	7.1±2.2 *
4'-Hydroxy-3'-methoxycinnamic acid (Ferulic acid) (3'-Met-4'-HCA)	3.1±0.5	3.1±0.5	5.7±1.0 *	7.9±1.7 *
3'-Methoxycinnamic acid-4'-sulfate (Ferulic acid-4-sulfate) (3'-Met-CA-4'-S)	29.5±4.9	41.5±11.2	43.7±11.1	47.2±8.9
4'-Methoxycinnamic acid-3'-sulfate (Isoferulic acid-3-sulfate) (4'-Met-CA-3'-S)	1.8±0.3	1.5±0.4	2.4±0.5	3.5±0.6 *
3'-Methoxycinnamic acid-4'-glucuronide (Ferulic acid-4-glucuronide) (3'-Met-CA-4'-G)	58.5±10.0	96.7±19.6	89.7±15.8 *	128.1±19.5
4'-Methoxycinnamic acid-3'-glucuronide (Isoferulic acid-3-glucuronide) (4'-Met-CA-3'-G)	18.3±2.9	22.0±3.4	29.5±5.9 *	42.4±29.5 *
Feruloylglycine (Fer-Gly)	78.4±11.7	86.1±14.6	118.1±22.5 *	150.9±27.4 *
3'-Hydroxycinnamic acid (<i>m</i> -Coumaric acid) (3'-HCA)	4.7±0.7	3.4±0.6	9.6±2.6 *	5.7±1.0 *
Cinnamic acid-4'-sulfate (Coumaric acid-4-sulfate) (CA-4'-S)	7.6±0.8	8.0±1.0	11.2±2.6	10.9±2.2
Cinnamic acid-3'-sulfate (Coumaric acid-3-sulfate) (CA-3'-S)	25.7±3.4	25.1±8.2	25.8±4.6	31.7±14.1
3-(4'-Hydroxyphenyl)propanoic acid-3'-sulfate (Dihydrocaffeic acid-3-sulfate) (4'-HPPA-3'-S)	75.1±13.1	69.3±11.4	101.1±23.0	90.0±13.1
3-(3'-Hydroxyphenyl)propanoic acid-4'-glucuronide (Dihydrocaffeic acid-4-glucuronide) (3'-HPPA-4'-G)	4.7±1.2	5.3±1.3	6.3±0.9 *	5.4±1.5
3-(4'-Hydroxyphenyl)propanoic acid-3'-glucuronide (Dihydrocaffeic acid-3-glucuronide) (4'-HPPA-3'-G)	3.7±0.8	4.0±0.7	4.2±1.1	3.5±0.7
3-(4'-Hydroxy-3'-methoxyphenyl)propanoic acid (Dihydroferulic acid) (3'-Met-4'-HPPA)	34.5±10.6	23.7±4.9	57.1±21.3 *	42.6±9.4
3-(3'-Hydroxy-4'-methoxyphenyl)propanoic acid (Dihydroisoferulic acid) (4'-Met-3'-HPPA)	10.1±4.9	8.4±2.8	30.6±15.7 *	7.1±1.2
3-(3'-Methoxy-phenyl)propanoic acid-4'-sulfate (Dihydroferulic acid-4-sulfate) (3'-Met-PPA-4'-S)	7.4±1.2	7.1±1.2	15.2±3.4 *	14.9±4.1 *
3-(4'-Methoxy-phenyl)propanoic acid-3'-sulfate (Dihydroisoferulic acid-3-sulfate) (4'-Met-PPA-3'-S)	2.9±0.5	3.8±1.3	5.4±1.2	10.7±4.0 *
3-(3'-Methoxy-phenyl)propanoic acid-4'-glucuronide (Dihydroferulic acid-4-glucuronide) (3'-Met-PPA-4'-G)	15.1±3.1	14.4±2.4	21.8±4.5 *	25.1±4.3
3-(4'-Methoxy-phenyl)propanoic acid-3'-glucuronide (Dihydroisoferulic acid-3-glucuronide) (4'-Met-PPA-3'-G)	41.7±6.3	41.2±6.8	69.1±14.0 *	98.7±23.7 *
3-(3'-Hydroxyphenyl)propanoic acid (3'-HPPA)	43.2±13.6	24.8±5.7	142.8±69.4 *	27.0±5.3
3-(Phenyl)propanoic acid-sulfate (PPA-S)	74.5±15.8	48.4±8.1	125.4±28.7 *	71.4±14.5
3-(Phenyl)propanoic acid-4'-glucuronide (PPA-4'-G)	3.9±0.8	3.2±0.8	6.4±2.1	4.5±1.2
3-(Phenyl)propanoic acid-3'-glucuronide (PPA-3'-G)	1.5±0.4	1.3±0.4	1.8±0.6	0.9±0.2
4'-Hydroxy-3'-methoxy-phenylacetic acid (Homovanillic acid) (3'-Met-4'-HPAA)	150.2±12.8	132.7±13.4	233.0±33.7 *	204.2±30.5 *
3'-Methoxy-phenylacetic acid-4'-sulfate (Homovanillic acid-sulfate) (3'-Met-PAA-4'-S)	10.9±2.5	14.8±4.4	23.0±4.7 *	19.6±5.2
4'-Methoxy-phenylacetic acid-3'-sulfate (Isohomovanillic acid-sulfate) (4'-Met-3'-HPAA)	2.3±0.6	1.7±0.4	6.1±1.3 ^{*A}	3.2±0.7 ^{*B}
3'-Hydroxyphenylacetic acid (3'-HPAA)	409.3±38.5	409.5±47.1	712.3±99.6 *	606.3±97.0 *

4'-Hydroxyphenylacetic acid (4'-HPAA)	489.7±67.4	640.8±71.7	1047.4±137.2 *	778.4±87.6
3,4-Dihydroxybenzoic acid (Protocatechuic acid) (3,4-DiHBA)	10.5±1.2	12.4±1.7	18.1±1.8 *	23.1±4.2 *
Hydroxybenzoic acid-sulfate (Protocatechuic acid-sulfate) (HBA-S)	4.1±0.7	4.0±0.7	8.9±2.3 *	6.8±1.3 *
3-Hydroxybenzoic acid-4-glucuronide (Protocatechuic acid-4-glucuronide) (3-HBA-4-GUA)	2.8±0.6	3.4±0.6	5.3±0.8 *	5.7±1.4
4-Hydroxy-3-methoxybenzoic acid (Vanillic acid) (3-Met-4-HBA)	2.2±0.4	2.3±0.3	3.4±0.6 *	4.0±0.9 *
3-Methoxybenzoic acid-4-sulfate (Vanillic acid-4-sulfate) (3-Met-BA-4-S)	5.6±0.9	6.5±1.8	13.3±4.6 *	9.2±2.2
4-Methoxybenzoic acid-3-sulfate (Isovanillic acid-3-sulfate) (4-Met-BA-3-S)	1.0±0.3	0.9±0.3	2.2±0.5 *	2.0±0.5 *
3-Methoxybenzoic acid-4-glucuronide (Vanillic acid-4-glucuronide) (3-Met-BA-4-G)	7.0±1.2	7.3±1.1	16.2±4.1 *	14.6±3.3 *
4-Methoxybenzoic acid-3-glucuronide (Isovanillic acid-3-glucuronide) (4-Met-BA-3-G)	1.1±0.3	1.6±0.4	2.9±0.9 *	4.3±1.4 *
4-Hydroxybenzoic acid (4-HBA)	124.1±19.8	106.8±9.5	195.0±19.7 *	161.6±19.5 *
Benzoic acid-4-sulfate (BA-4-S)	99.3±15.3	85.7±12.0	209.8±46.6 * ^A	114.3±18.5 * ^B
Benzoic acid-3-sulfate (BA-3-S)	72.1±25.0	44.0±9.2	97.4±22.4 *	69.8±17.8
Benzoic acid-4-glucuronide (BA-4-G)	2.8±0.6	3.4±0.6	5.3±0.8 *	5.7±1.4
Benzoic acid-3-glucuronide (BA-3-G)	1.9±0.3	1.8±0.2	3.8±0.6 *	3.5±0.6 *
4'-Hydroxyhippuric acid (4'-HHA)	243.3±59.5	136.8±16.9	409.6±58.7 * ^A	243.1±44.3 * ^B
Hippuric acid (HA)	8610.9±890.6	8506.9±929.3	18062.2±2716.1 *	18708.3±243.1 *
4-Hydroxybenzaldehyde (4-HBAL)	2.1±0.3	2.7±0.4	6.8±1.0 *	5.5±0.737 *
2-Hydroxy-4/5-methylbenzene-1-sulfate (4-Methylcatechol-sulfate) (Met-Cat-S)	12.8±3.1	10.5±2.0	26.4±24.9 *	16.6±2.9
Total metabolites	12659.0±1234.7	12148.1±1201.2	23948.7±3201.4 *	23429.2±3568.4
Total metabolites without hippuric acid derivatives	3813.8±442.3	3504.5±338.0	5476.9±624.4	4486.8±570.2

Supplementary Figure S1. ROC plots obtained from PLS-DA models for individual metabolites (DS1, A), phenolic classes (DS2, B), and metabolites belonging to HPPAs (isomers 3' and 4'), monohydroxyPVLs (isomers 3' and 4'), and dihydroxyPVLs (3',4') (DS3, C). TPR and FPR indicate true positive and false positive rates, respectively. Yellow color is used for metabotype 1, red for metabotype 2 and blue for metabotype 3.



Supplementary Figure S2. Variable Importance in Projection (VIP) scores obtained from PLS-DA models for individual metabolites (DS1, A), phenolic classes (DS2, B), and metabolites belonging to HPPAs (isomers 3' and 4'), monohydroxyPVLs (isomers 3' and 4'), and dihydroxyPVLs (3',4') (DS3, C). Metabolite abbreviations in the loading plot are indicated in Table S2. Letters B and F for the observations in the score plot indicate subjects at baseline and follow-up visits, respectively.



Supplementary References

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4.4. A systematic review of human studies

The impact of genetic variations on the absorption, distribution, metabolism, and excretion of dietary (poly)phenols: a critical systematic review

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Abstract

Response to (poly)phenol intake is highly variable among subjects, and genetic variants may contribute to such variability. The present study was undertaken to fill in this gap of knowledge and was designed as a systematic review of the literature. Twelve studies were selected, all dealing with the association between single nucleotide polymorphisms (SNPs) in genes involved in (poly)phenol ADME (absorption, distribution, metabolism, and excretion) and the interindividual variability in (poly)phenol bioavailability and metabolism. Seven studies investigated the effects of genetic variations on the metabolism of flavan-3-ols, of which six focused on green tea catechins and one from flavan-3-ols deriving from free diet. Two studies focused on isoflavone metabolites deriving from soy or free diet, one study dealt with flavanones contained in orange juice, another study assessed the effects of SNPs on the metabolism of flaxseed-derived lignans, and the last study explored the impact of genetic variations on the metabolism of (poly)phenols from free diet. In total, 88 SNPs in 33 genes were studied. Of these 33 genes, slightly more than a half ($n=18$, 55%) of them were related to drug/xenobiotic metabolism. More specifically, two (11%) were involved in absorption, seven (39%) in phase I metabolism, four (22%) in phase II metabolism, and five (28%) in excretion. The remaining 15 genes were related to steroid hormone metabolism. The SNPs analyzed could refer to non-coding regions (intron, untranslated regions, upstream transcription control elements) or to coding sequences. In some cases, statistical significant impacts could be detected, even though the p value was nominal and the significance disappeared when correcting for multiple testing. Overall, the quality of the studies here overviewed appears to be low-medium, showing a weak methodological strength, statistical robustness, reproducibility, and generalizability. Only a few studies applied stringent statistical criteria, and recruited sufficiently large and diverse samples to enable reaching solid and reliable conclusions. Most studies included in the present systematic review show methodological weaknesses and deficiencies, including small samples, biases and confounding, and nominal effect sizes, among others. Studies employing larger samples, leveraging integrative bioinformatics approaches and genome-wide linkage are warranted.

Keywords

(Poly)phenols, interindividual variability, genome, single nucleotide polymorphisms, metabolism, systematic review

4.4.1. Introduction

(Poly)phenols are a large family of phytochemicals comprising over 8,000 heterogeneous compounds [1,2], that can be found in a variety of beverages and foods of plant origin, including coffee, tea, cocoa, red wine, fruits, vegetables, seeds, legumes, and cereals [2,3]. These phytochemicals are derived *via* the shikimate/phenylpropanoid and the polyketide acetate/malonate pathways [1,4], both present in plants, and are characterized by the presence of at least one phenolic group [4]. Dietary intake of food (poly)phenols often exceeds 1 g/day [5]. Regarding (poly)phenol subclasses, the principal contributors to the intake in the European diet are hydroxycinnamic acids and flavan-3-ols [5]; other important classes of polyphenols include anthocyanins, flavonols, flavanones, hydroxybenzoic acids, tannins, lignans, alkylresorcinols, and tyrosols. Minor contributors are stilbenes, furanocoumarins, flavones, and isoflavones, among others [5].

A growing body of scholarly research suggests that high (poly)phenol intake and supplementation may protect against non-communicable chronic diseases, including cardiovascular, neurodegenerative, and dysmetabolic diseases [6-9]. However, evidence is not compelling yet, due to the high heterogeneity among studies [10]. Various factors can influence the effects of (poly)phenols on human health, thus determining a high interindividual variability and contributing to explaining the contrasting findings reported in the literature [10].

These factors can be exogenous, including the variability of (poly)phenol content in foods [11], and the high degree of diversification of chemical structures (modifications and substitutions at various positions of the backbone structure, and polymerization of monomeric units) [12]. The content and quality of (poly)phenols in foods can depend on the plant variety (*cultivar*), its cultivation technique [13,14], and the climate [15]. Further sources of variability are food matrices and the complex interplay of (poly)phenols with other components of the diet (fiber, and micro- and macro-nutrients) [16].

However, endogenous factors are considered to be the principal source of heterogeneity in (poly)phenol effectiveness and physiological response. In particular, this variability mainly originates from differences in the absorption, distribution, metabolism, and excretion (ADME) process, and such differences derive from a number of individual factors including personal characteristics (age, sex/gender, and ethnicity), lifestyle (diet habits, smoking, physical activity,

medication), pathophysiological status, gut microbiota, and genetic background [17-21]. These various levels of diversity - spanning from bio- and chemo-diversity to nutritional diversity, can influence (poly)phenol bioaccessibility (i.e., the amount available for absorption in the intestine after digestion), its bioavailability (i.e., the proportion of the molecule available after digestion, absorption, and metabolism), and its metabolism [22,23].

In the case of the bioavailability and metabolism of (poly)phenols, genetic variations and microbiota composition/activity are the major contributors to variability. Genetic variations, also known as “Single Nucleotide Polymorphisms” (SNPs) can, at least partially, explain differences in the response to dietary (poly)phenol exposure [24]. A further layer of variability is given by gut microbiota [18], which can be considered a key interplayer in a variety of individual physiological processes and metabolic phenotypes (also known as metatypes). The human microbiome has been linked with several diseases too, as well as with host genetic variants. As such, human genetic variants are expected to play a major role in causally explaining various complex and overlapping physiological and pathophysiological phenomena, ranging from food intake to the composition of the microbiome and their impacts on food metabolism and human health [18]. However, to the best of our knowledge, there is a dearth of information concerning the impact of the human genome on the response to dietary (poly)phenol exposure in terms of inter-individual variability in the bioavailability and metabolism of (poly)phenols [24].

Following ingestion, dietary (poly)phenols, present usually in a glycosylated form, can be hydrolyzed by human enzymes present in the upper gastrointestinal tract, and the released aglycones can be absorbed becoming the substrate for phase II enzymes present in enterocytes and hepatocytes [2,3]. However, the majority of (poly)phenols are not absorbed and reach intact the colon, where they undergo modifications by the gut microbiota, being converted to smaller catabolites that are easily absorbed and conjugated by phase II enzymes in colonocytes and hepatocytes [2,3]. So, the molecules present in the circulation and potentially available for target tissues are phase II metabolites, both of human and microbial-human origin, which are finally excreted through urine. SNPs in genes involved in (poly)phenol ADME, including transporters, glycosidases, and phase II enzymes (sulfotransferases or SULTs, UDP-glucuronosyltransferases or UGTs, and catechol-*O*-methyltransferase or COMT), could have an impact on the amount and type of metabolites excreted [17], as pictorially shown in Figure 1.

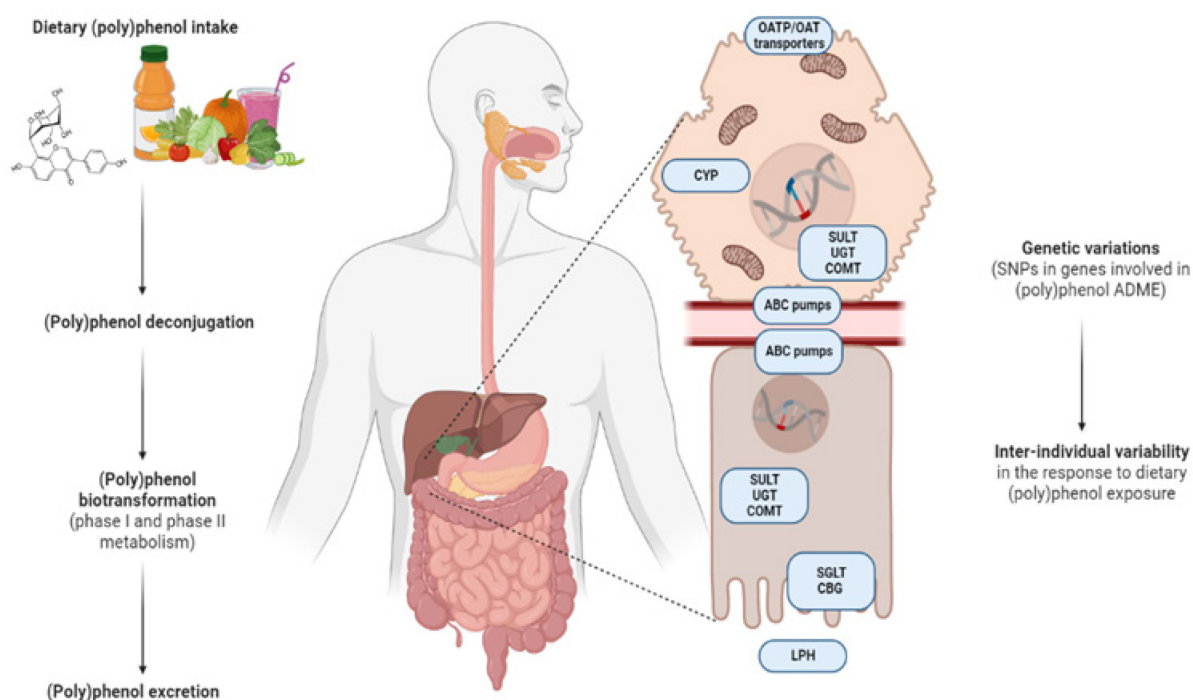


Figure 1. Representation of the process of (poly)phenol absorption, metabolism and excretion, and indication of genes carrying genetic variations that can determine the interindividual variability in the response to dietary (poly)phenol intake.

A better understanding of the genetic component would enable and enhance the translation of our current knowledge about these compounds into more effective and personalized dietary advice. A recently published review found that more research on the factors that underlie the differences in the response to (poly)phenol exposure is urgently needed [10]. Therefore, the present study was undertaken to fill in this gap of knowledge, with the aims of systematically identifying and appraising genetic factors associated with inter-individual variability in (poly)phenol ADME after their dietary intake, and assessing their causal relationships with (poly)phenol bioavailability and metabolism, by conducting a systematic review of the literature.

4.4.2. Materials and methods

Study protocol

An *a priori* study protocol was devised and registered within the “International Prospective Register of Systematic Reviews” (PROSPERO, Centre for Reviews and Dissemination (CRD), University of York, York, UK) with the registration code CRD42023416767 (accessible at https://www.crd.york.ac.uk/prospero/display_record.php?RecordID=416767).

Literature search

A comprehensive literature search was performed with a string of keywords consisting of four broad components ((poly)phenols, dietary exposure/intake, human metabolism, and SNPs), on five major scholarly databases: namely, MEDLINE (via its freely accessible PubMed interface), Scopus, Web of Science (WoS), EMBASE, and “Chemical Abstracts Service” (CAS), a database of the “American Chemical Society” (ACS).

The “Medical Subject Headings” (MeSH) thesaurus, a controlled, hierarchically-organized vocabulary produced by the National Library of Medicine (NLM), was leveraged. When appropriate, the wild-card option (i.e., truncated words) was used. (Poly)phenol compound names were obtained from Phenol-Explorer, which is the most comprehensive database on (poly)phenols [25]. (Poly)phenol metabolite names were obtained from PhytoHub, a freely available electronic database containing detailed information about dietary phytochemicals and their human and animal metabolites. The full string, which was modified and adapted according to each database, is reported in Supplementary Table 1. Moreover, the “Human Genome Epidemiology Literature Finder” (HuGE Navigator Database) based at the “Public Health Genomics and Precision Health Knowledge Base” (PHGKB v8.5) was mined. Extensive cross-referencing was applied. Finally, target journals were hand-searched. The literature search was conducted by three researchers independently. Further details are reported in Supplementary Table 1 and Table 1.

Table 1. Overview of the search strategy adopted in the present review.

Search items	Details
Search string components	(Poly)phenols Dietary exposure/intake Human metabolism SNPs
Databases/registries/repositories mined/searched	PubMed/MEDLINE, Scopus, WoS, EMBASE, CAS/ACS, HuGE Navigator Database (PHGKB v8.5)
Inclusion criteria	P (humans) I/E (exposed to dietary (poly)phenols) C (stratified according to their genotype status; age) O (quantification of (poly)phenol-related

	plasma and/or urinary metabolites by any separation methodology/technique of analytical chemistry) S (any original study, observational/interventional, retrospective/prospective, cross-sectional/longitudinal, randomized/non-randomized, parallel/cross-over, placebo/non-placebo-controlled, pilot, etc.)
Exclusion criteria	P (<i>in vitro</i> studies - cellular -, in silico/computational studies) I/E (exposed to nutrients other than (poly)phenols) C (not providing information about genotype status) O (not quantifying (poly)phenol-related metabolites) S (reviews of any type)
Time filter	None applied
Language filter	None applied
Target journals hand-searched	Biochem Pharmacol; Br J Nutr; Cancer Epidemiol Biomarkers Prev; Eur J Pharmacol; Genes Nutr; Int J Mol Epidemiol Genet; Mol Nutr Food Res; Nutrients; PLoS One

Inclusion and exclusion criteria

Inclusion and exclusion criteria were pre-defined and devised according to the population - intervention/exposure - comparators/comparisons - outcomes - study design (PICOS/PECOS) criteria. Original studies (S) investigating populations (P) consisting of humans, undergoing dietary interventions with (poly)phenols (I) or exposed (E) to dietary (poly)phenols, stratified according to their genotype status (C, comparators/comparisons), and for whom (poly)phenol-related plasma and/or urinary metabolites (O, outcomes) were quantified (i.e., characterized and measured by means of any methodology/technique of analytical chemistry), and reported, were deemed eligible for inclusion.

Studies were excluded if studying non-human populations (P), i.e., if designed as *in vitro* models (like cellular models), animal models, or *in silico*/computational models. Also, articles were not retained in the present review if study subjects were exposed (E) to dietary

compounds other than (poly)phenols or, if, in the case of exposure to dietary (poly)phenols, (poly)phenol-related metabolites were not analytically characterized/measured (O). Another cause of exclusion was given by other studied outcomes, including changes in dietary habits and eating patterns (like energy intake), anthropometric characteristics (body weight, body mass index/BMI, waist circumference), or metabolic responses (for instance, glucose and insulin levels, or circulating leptin, ghrelin, or adiponectin). Finally, studies were excluded if designed as technical notes reporting new methodologies or advancements in techniques of analytical chemistry but narrowly focused on the technical aspects, letters to the editor, editorials, commentaries, and expert opinions, or not containing sufficient quantitative details (S). Reviews (of any type, narrative, systematic, or meta-analysis) were excluded too but were scanned to increase the chance of not missing content relevant to the present review.

Data extraction

The following data were extracted: study authors (in particular, the surname of the first author), study year, study country, study design, sample size, sample characteristics (mean age, sex/gender distribution, BMI, and ancestry, level of physical activity practiced, smoking status, alcohol intake, use of drugs, contraceptives, and hormone therapy, dietary habits and how the dietary assessment was performed), SNPs and related genes, information related to genotyping technique, genotype frequencies, and Hardy-Weinberg equilibrium, type of genetic modeling conducted, statistical analysis carried out and statistical threshold adopted, goodness-of-fit evaluation and validation, type and source of dietary (poly)phenol intervention/exposure, intervention duration and timepoints, assessment of adherence to the intervention protocol, metabolites searched in biological samples, technique of analytical chemistry utilized for metabolite characterization and quantification, and impact of the SNP on dietary (poly)phenol ADME. Finally, study strengths and limitations were also recorded, along with trial registration (only if the study was designed as a randomized trial), funding/sponsoring and its role (if any), and whether a checklist for findings reporting was followed.

A specifically designed Excel spreadsheet was utilized. Three researchers independently extracted data. Further details about the search strategy adopted in the present systematic review are reported in Table 1 and in Supplementary Table 1.

Methodological assessment

As foreseen by the PRISMA methodology [26], study quality appraisal was evaluated by applying the framework for evaluating scientific validity in nutritional genomics developed by Keathley et al. [27]. This framework consists of several items related to various dimensions (namely, study design and quality, generalizability, directness, consistency, precision, confounding, effect size, biological plausibility, publication/funding bias, allele and nutrient dose-response, and summary levels of evidence).

Bioinformatics analysis

The retrieved lists of genes and related SNPs associated with (poly)phenol metabolism were extensively annotated using a wide range of bioinformatics tools. Genes were functionally annotated by using Gene Ontology (GO), along its three major domains (biological processes, molecular functions, and cellular components). The web browser located at the Lewis-Sigler Institute for Integrative Genomics, Princeton University, Princeton, New Jersey, USA, accessible at <https://go.princeton.edu/cgi-bin/GOTermFinder>, was utilized. GO terms were visualized by means of the “Reduce plus visualize Gene Ontology” (REVIGO) tool (freely available at <http://revigo.irb.hr/>). Briefly, the list of GO terms associated in a statistically significant fashion with the genes involved in (poly)phenol metabolism was transformed into a matrix of the GO term pairwise semantic similarities. Then, Multidimensional Scaling (MDS) was applied to reduce the dimensionality of the matrix. Semantically similar GO terms are displayed together in the plot, along the axes of the semantic space. The SNPs were annotated using the SNPinfo Web Server, accessible at <https://snpinfo.niehs.nih.gov/snpinfo/snpfunc.html>. Locations of SNPs along the human genome were displayed using the PhenoGram plot (accessible at <http://visualization.ritchielab.org/phenograms/plot>). Finally, the overlapping genes and SNPs involved in (poly)phenol metabolism were visualized by means of Venn’s networks generated using an online generator (accessible at <http://www.ehbio.com/test/venn/#/>).

4.4.3. Results

Literature search

The initial literature search (updated as of September 24, 2023) yielded a pool of 23,513 items, which remained 5,310 after removing the duplicates. A total of 5,288 items were discarded based on title and/or abstract. Twenty-two studies [28-49] were scrutinized in full text. Based on the above-mentioned inclusion and exclusion criteria, ten studies [28-37] were “excluded

with reason” (Supplementary Table 2). Finally, twelve studies [38-49] were retained in the present systematic review, overviewed in Table 2 and detailed in Supplementary Tables 3 and 4. The flowchart adopted to manage the study retrieval and inclusion/exclusion is pictorially depicted in Figure 2.

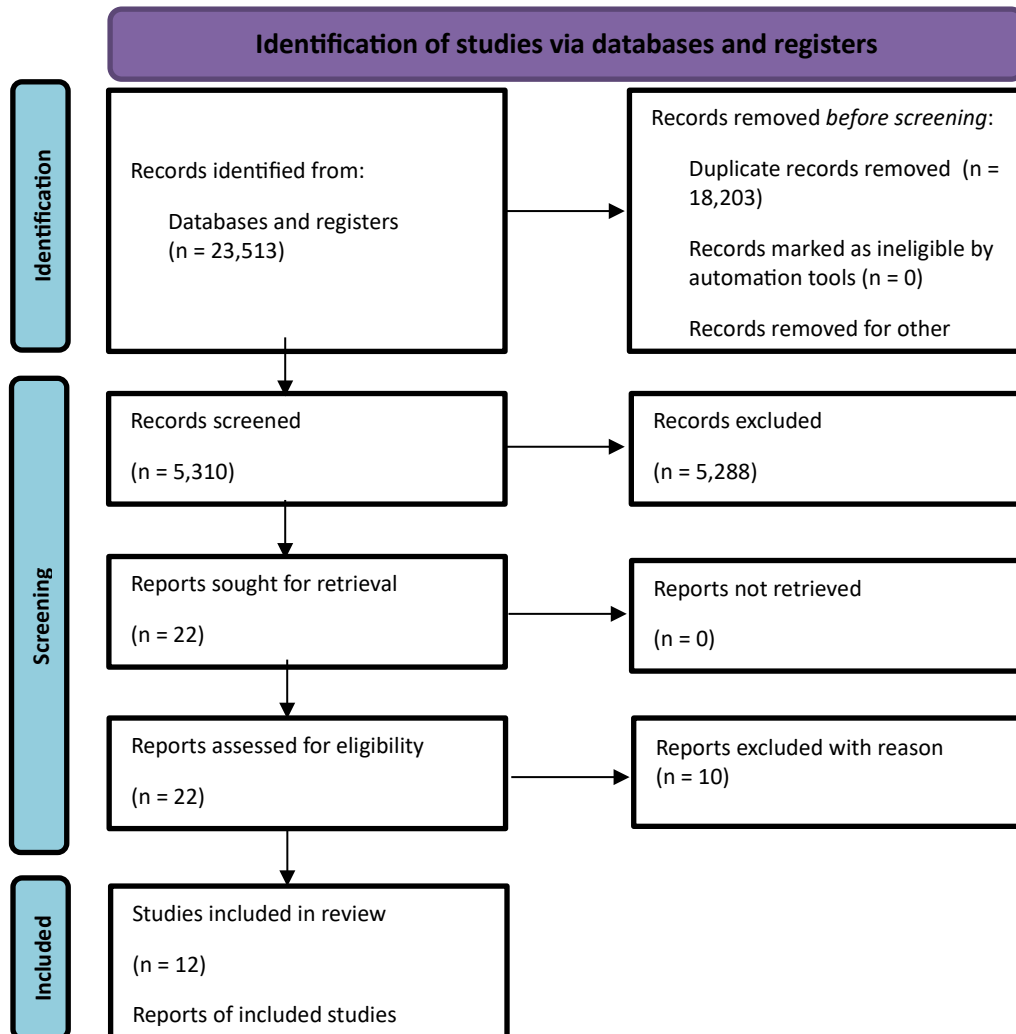


Figure 2. Pictorial flow-chart of the search strategy adopted in the present systematic review.

Table 2. Overview of the studies included in the present systematic review.

Study	Study design	Country	Sample size	SNPs	Dietary (poly)phenols	(Poly)phenol source	Intervention duration	Impact of SNP
Brown et al., 2011 (38)	Single-centre, placebo-controlled, double-blinded, cross-over study with age- and BMI-matched groups	UK	64 subjects aged 40-69y, 100%M, overweight or obese	COMT (rs4680)	Flavan-3-ols (mainly catechins and gallic catechins)	Green tea	6 weeks	Lower concentrations in homozygous G individuals
Chang et al., 2019 (39)	Randomized cross-over study	USA	252 subjects aged 59.4±6.2y, 100%F, BMI 30.5±7.6	60 functional SNPs in 29 genes (ARPC1A, BAIAP2L1, BMF, COMT, CYP17A1, CYP19A1, CYP1A1, CYP1B1, CYP3A4, CYP3A5, ESR1, GCKR, HHEX, HSD17B1, JMJD1C, LHCGR, NR2F2, PRMT6, SHBG, SLCO1B1, SULT1A1, TDGF3, TSPYL5, UGT1A1, ZBTB10, ZKSCAN5, ZNF652), and 60 SNPs selected as AIMS	Lignans	Ground brown flaxseed	6 weeks	Impact of SNPs on ENL excretion
Fraga et al., 2022 (40)	Non-randomized, pre-post design study	Brazil	46 subjects aged 26.26±4.50y [19-38y], 20M (43.5%) and 26F (56.5%), BMI 23.23±2.54 [18.50-29.9]	SULT1A1 (rs3760091, rs4788068), SULT1C4 (rs1402467), ABCC2 (rs8187710)	Flavanones (mainly hesperetin and naringenin)	Pasteurized orange juice; a single dose of 500 mL; 25.46 mg/100 mL of hesperidin and 1.95 mg/100 mL of narirutin)	24 hours	Significant ($p < 0.05$) relationship between SNPs and high excretion of phase II flavanone metabolites
Hong et al., 2012 (41)	Cross-sectional, ancillary sub-analysis of a population-based study	South Korea	1,391 healthy subjects aged 52.0y, 758M (54.5%), 633F (45.5%), BMI 24.9	HACE1 (rs6927608, rs4946645, rs11759010, rs17065302, and rs4245525)	Equol	Soy	Not applicable (observational)	Significant effects of 5 SNPs clustered in the 6q21 region, the most significant of which was rs6927608 (A>C)

Inoue- Choi et al., 2010 (42)	Cross-sectional sub- analysis nested within the Shanghai Cohort Study	China	660 subjects aged 56.7±5.0y, 100%M	COMT (rs4680)	Flavan-3-ols (mainly catechins and gallocatechins)	Green tea	Not applicable (observation ale)	Urinary tea catechins were lower by 35-45% among men carrying the LL genotype ($p=0.007$). When consuming greater amount of green tea (≥ 5 g/day), urinary tea catechins did not differ according to COMT genotype
Lorenz et al., 2014 (43)	Non-randomized, pre-post design study	Germany	24 subjects (10M, 41.7%, and 14F, 58.3%)	COMT (rs6269, rs4633, rs4680, rs4818)	EGCG	Pure compound	2 hours	None
Miller et al., 2012a (44)	Pilot, non-placebo- controlled trial	UK	20 overweight and obese individuals, aged 54.6±3.2y [18-70y], 14M (70.0%) and 6F (30.0%)	COMT (rs4680)	Flavan-3-ols (mainly catechins and gallocatechins): EGCG 448, EGC 178, E 96, EG 66 mg	Green tea	8 hours	None
Miller et al., 2012b (45)	Randomized, double-blinded, placebo-controlled, crossover trial, with age- and BMI- matched groups	UK	47 overweight/obe se adults, 100%M	COMT (rs4680)	Flavan-3-ols (mainly catechins and gallocatechins)	Green tea	24 hours	None
Momma et al., 2023 (46)	Cross-sectional, ancillary sub- analysis of a	UK	16,672 subjects, 9020F (54.1%), 7652M (45.9%)	PON1 (rs662, rs854560, rs705379, rs705381, and rs854572)	Flavan-3-ols	Diet (not better characterized)	Not applicable (observation al)	No differences in the sum of 5- (4'- hydroxyphenyl)-

	population-based study							γ-valerolactone-3'-sulfate and 5-(4'-hydroxyphenyl)-γ-valerolactone-3'-glucuronide metabolites in men, and only small differences among rs662 and rs705381 alleles in women with no clinical and physiological impact
Muhrez, et al., 2017 (47)	Metabolomics study	France	53 aged 23y [20-59y], 24M (45.3%) and 29F (54.7%)	ABCC2/MRP2 (rs717620, rs3740066, rs2273697)	NA	Free diet	Not applicable (observational)	SNPs associated with some (poly)phenol urinary metabolites
Scholl et al., 2018 (48)	Population-based, open, one-arm nutrkinetic study	Germany	84 aged 24.5±3.9y [19-49y], 30M (35.7%) and 54F (64.3%), BMI 22.7±2.6	MRP2/ABCC2 (rs2273697, rs3740066, rs717620, rs8187710), OATP1B1/SLCO1B1 (rs4149056, rs2306283, rs11045819), Pgp/ABCB1 (rs1045642, rs1128503, rs2032582), COMT (rs4680), SULT1A1 (rs750155), UGT1A128 (rs8175347)	Flavan-3-ols (mainly catechins and gallocatechins)	Green tea	5 days	Impact of SNPs of MRP2 and OATP1B1
Wakeling and Ford, 2012 (49)	Non-randomized, pre-post design study	UK	100 aged 33.0±9.3y ([18-50y], median 31.5y), 100%F, BMI 23.4±3.4	UGT1A1*28, CBG (rs358231), ABCG2 (rs2231142), and ABCC2 (rs2273697), LPH (rs3754689)	Isoflavones (mainly glycitein, daidzein and genistein)	Soy	24 hours	Impact of SNPs on isoflavone excretion

Overview of the studies included: sample features

Articles were published between 2010 [42] and 2023 [46]. Sample sizes ranged from 20 [44] to 16,672 [46] subjects (median 74), totaling 19,413 participants. The age of the participants varied from 18 [44,49] to 75 years [39]. Five studies [38,39,42,45,49] recruited populations consisting of males [38,42,45] or females [39,49] only. In the remaining seven studies [40,41,43,44,46,47,48], the percentage of male subjects ranged from 35.7% [48] to 70.0% [44]. Of note, only two studies [46,48] reported and analyzed data stratified according to sex/gender.

Most studies (67%) were conducted in Europe - five in the UK [38,44,45,46,49], two in Germany [43,48], and one in France [47]. Two studies were carried out in the Americas – one in the USA [39], and one in Brazil [40] – and two in Asia, one in China [42], and one in South Korea [41]. As such, information was provided mostly for European/Caucasian ancestry, with only one study investigating African ancestry [39] along with Caucasian ancestry, and other two focusing exclusively on Asiatic ancestry [41,42].

Concerning BMI, no information was provided in four studies [42,43,46,47]. When reported, BMI ranged from 18.5 to 38.0 kg/m², that is to say, a range of populations - normal weight, overweight, and obese - have been investigated in the studies included in the present systematic review, with a higher representation of the latter.

Overview of the studies included: study design

Most studies were interventional (67%), with the exception of four studies [41,42,46,47], which were designed as observational - three [41,42,46] were ancillary, cross-sectional sub-analyses nested within population-based, prospective investigations, and one [47] was a “real-life” metabolomics study. Of the interventional studies, only three [38,39,45] were randomized, double-blinded, cross-over investigations. Furthermore, four studies [40,43,44,49] were single acute intake, one-arm, non-placebo-controlled investigations. Another study [48] was designed as a population-based, open, one-arm nutrikinetic study. Finally, the duration of the dietary intervention varied from a few hours/one day [40,49] to six weeks [38,39], thus including both acute and chronic trials.

Overview of the studies included: confounders and mediators/moderators of dietary (poly)phenol metabolism

Several variables, including behavioral lifestyles (smoking, drugs, alcohol intake, and exercise/physical activity), are known to confound or interact with - mediate/moderate - dietary (poly)phenol metabolism. Despite the importance of these variables, high heterogeneity in their reporting was found.

Concerning smoking status, no information was provided for three studies [43,46,47]. Four studies [39,41,42,48] included smokers, whereas all the other studies excluded smokers from enrollment. When reported, the rate of current smokers varied considerably from 19.8% [39] to 60% [42]. Regarding alcohol intake, no information was provided for five studies [39,43,46,47,49]. When reported, the rate of habitual alcohol consumers ranged from 5.9% [41] to 89.3% [48]. All the other studies excluded alcohol consumers or asked participants to refrain from alcohol consumption from 24 [44] to 48 hours [45] prior to the investigation. Concerning exercise and physical activity, no information was provided for eight studies [39,41-44,46,47,49].

Finally, there was a considerable degree of heterogeneity also in the reporting of drugs and contraceptives/hormone therapy. Information about the former variable was not disclosed in five studies [41-43,46,47], whereas all the other studies excluded patients on drugs from enrollment. Concerning the latter variable, only one study [48] considered this parameter, with 44.0% and 56.0% of the sample recruited ever and never using contraceptives, respectively. Of note, the use of contraceptives was found to impact dietary (poly)phenol metabolism.

Sources and classes of dietary (poly)phenols

Six studies [38,42-45,48] studied the effects of genetic variations on the metabolism of green tea flavan-3-ols. Another study focused on flavan-3-ol metabolites deriving from free diet [46]. Two studies [41,49] focused on isoflavone metabolites; another study [40] explored the association between genetic variations and phase II metabolites of flavanones contained in orange juice. One study [39] assessed the effects of SNPs on the metabolism of flaxseed-derived lignans. Finally, one study [47] explored the impact of genetic variations on the levels of some (poly)phenol metabolites derived from free diet, including 3',4'-dihydroxycinnamic acid, 2-(3',4'-dihydroxyphenyl)acetic acid, benzene-1,3-diol, and 4'-hydroxyhippuric acid. Sources and classes of dietary (poly)phenols are pictorially shown in Figure 3.

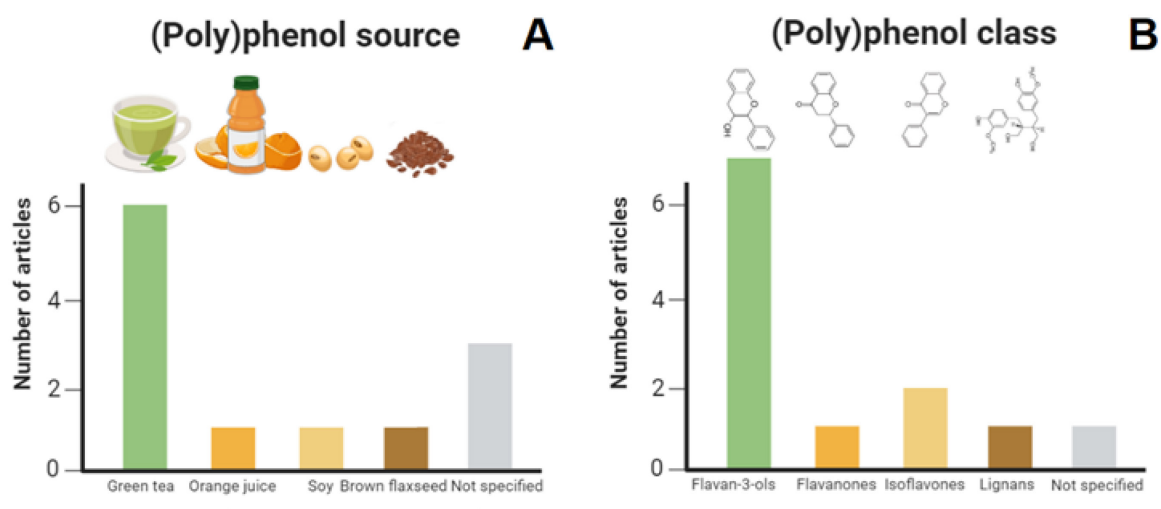


Figure 3. Number of studies investigating a certain (A) (poly)phenol source or (B) (poly)phenol class.

Adherence to the study protocol

Only a few studies explicitly reported whether and how they monitored compliance with the study protocol: by pill count [38], asking the participant to return unconsumed food/beverage (i.e., uneaten flaxseed) [39], or *via* an investigator-led 24-hour dietary recall to monitor compliance with low-flavonoid diet [45].

Dietary assessment and dietary habits

Only two studies [42,48] explored the dietary habits of participants, especially in terms of (poly)phenol intake. These studies included a variety of green tea drinkers, categorized according to their green tea drinking habits, in terms of the range in the amount consumed *per* day or simply dichotomized in habitual/non-habitual green tea consumers. On the contrary, all the other studies did not report any information concerning dietary habits and usual (poly)phenol exposure/intake. They excluded *per* protocol habitual consumers of (poly)phenol-rich foods and beverages, and included populations naive to dietary (poly)phenols and/or on low/very-low (poly)phenol diets.

Dietary assessment was mostly done by carrying out a 24-hour dietary recall. In one study [39], a series of 12 telephone-administered 24-hour dietary recalls were assigned randomly throughout the 5-month active intervention period. Only one study [42], which was observational and not interventional in terms of design, utilized the “Food Frequency Questionnaire” (FFQ) that included 45 food groups or items representing commonly consumed local foods, to assess current eating habits in terms of dietary patterns. Of note, only in one

study [38], participants were prospectively required to record dietary and daily activity information.

Biological samples and chemical characterization analysis

Four studies [41,43,44,48] and one study [46] collected only blood and urine samples, respectively, whilst all the other studies collected both blood and urine. Samples were analyzed using chromatographic separation techniques coupled with detection methods based on mass spectrometry (MS) or ultraviolet/visible spectroscopy. High-performance liquid chromatography, HPLC, was employed in seven studies [38,40,42-44,48,49], coupled with mass spectrometry [38,40,48], ultraviolet/visible (UV/VIS) [49] or electrochemical detectors [42,43,44]. One study [41] and three studies [44,45,46] employed liquid chromatography and ultra-performance liquid chromatography, UPLC, respectively, both coupled with mass spectrometry. Further two studies [39,47] analyzed samples by gas chromatography (GC) coupled with mass spectrometry.

Genetic variations, genotyping, and features of the genes and related SNPs studied

The SNPs retrieved in the present systematic review and related genes are reported in Supplementary Tables 5-8. Overall, 88 SNPs in 33 genes were studied.

A median value of 1 gene *per* study was investigated: the range of genes under study went from one [38,42-47] to 25 [39]. Seven studies [38,42-47] explored one single gene, one study [40] 3 genes, another study [49] five genes, and a further study [48] six genes. Finally, one study [39] appraised 25 genes. Of note, a single study [39] was responsible for most (76%) of the genes investigated and, despite some degree of overlapping, no gene was studied by all the articles included in the present systematic review (Figure 4). It is worthy stressing that a study [41] did not adopt the candidate gene approach and did not explore any preferential gene, as the whole genome was explored.

Of the 33 genes studied, slightly more than a half (n=18, 55%) of them were related to drug/xenobiotic metabolism. More specifically, two (11%; 6% of the entire set of genes) were involved in absorption, seven (39%; 21% of the overall set of genes) in phase I metabolism, four (22%; 12% of all genes) in phase II metabolism, and five (28%; 15% of the entire list of genes) in excretion. The remaining 15 genes (45%) were related, to varying degrees, to steroid hormone metabolism (Figure 5).

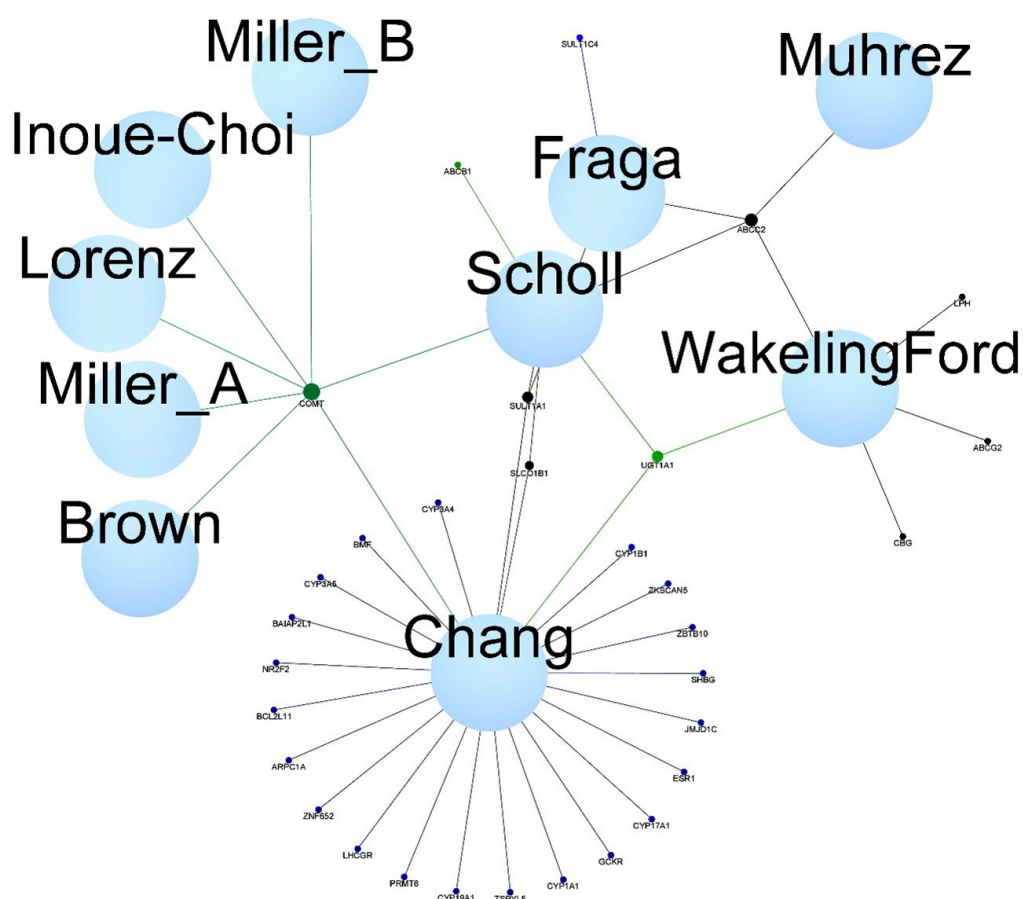


Figure 4. Venn's network showing the overlapping genes involved in (poly)phenol metabolism among studies included in the present systematic review. Only interconnected genes are shown.

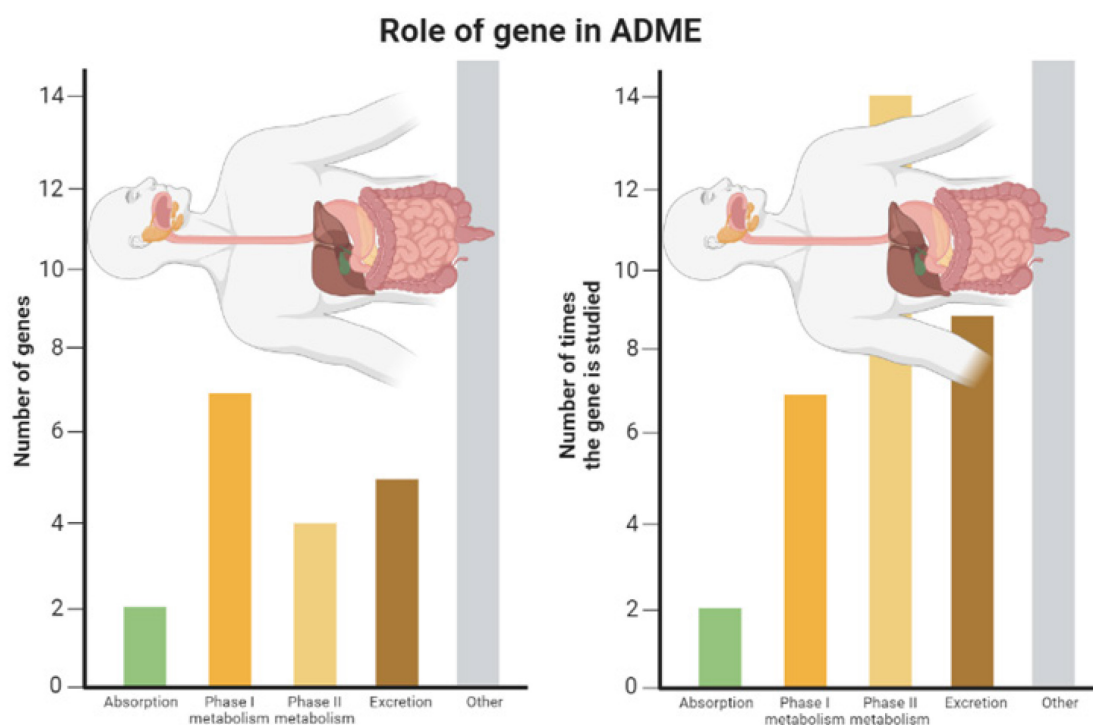


Figure 5. Number of (A) genes with a certain role in (poly)phenol ADME, and (B) times that genes with a certain role were studied.

A median value of 5 SNPs *per* study was explored: the range of SNPs went from one SNP [38,42,44] to sixty [39]. Three studies [38,42,44] studied the impact of a single SNP, one study [47] the effects of three SNPs, two studies [40,43] of four SNPs, and four studies [41,45,46,49] of five SNPs. Finally, one study [48] and a further study [39] explored the effects of panels of 13 and 60 SNPs, respectively. Of note, a single study [39] was responsible for the majority (68%) of the SNPs investigated (Figure 6).

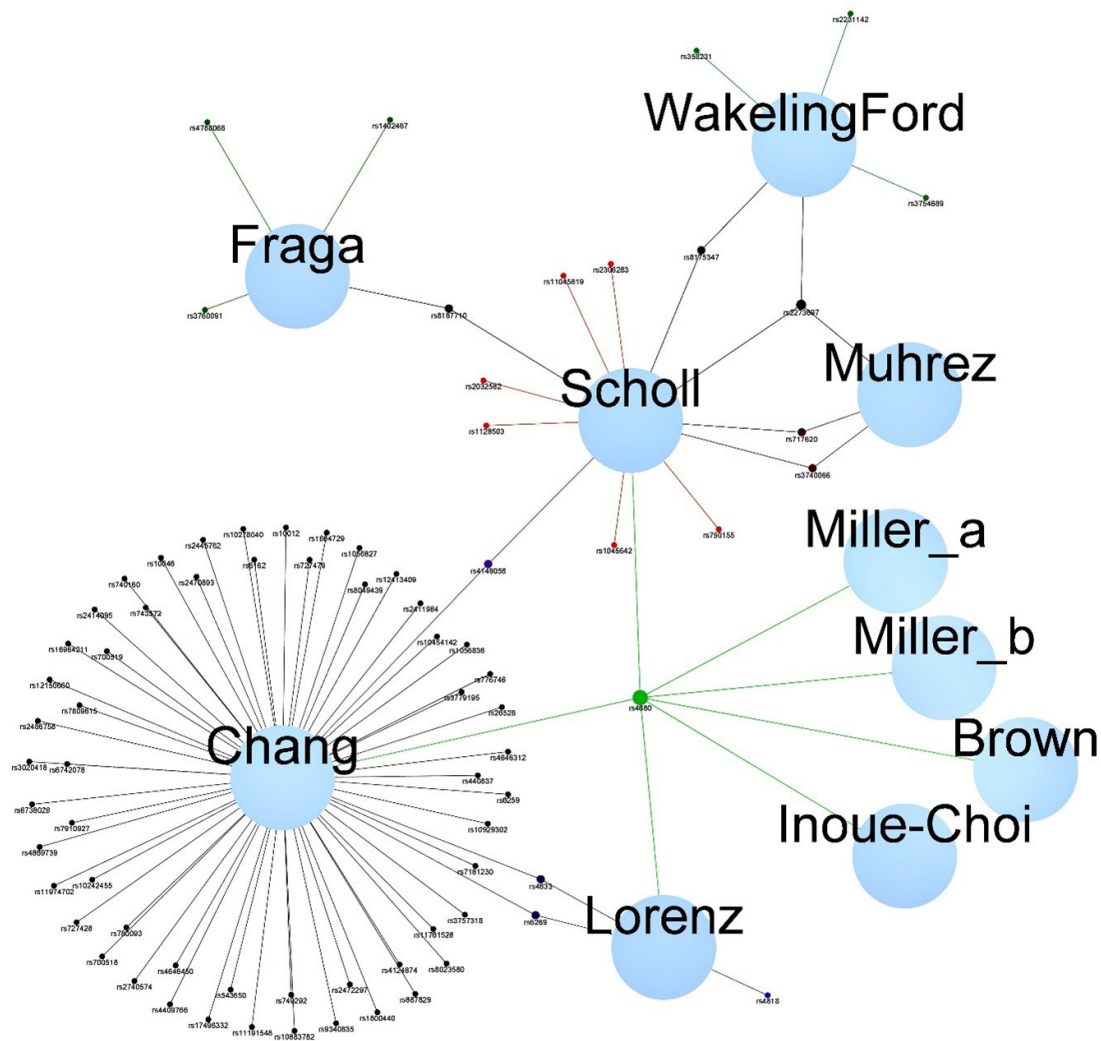


Figure 6. Venn's network showing the overlapping SNPs involved in (poly)phenol metabolism among studies included in the present systematic review. Only interconnected SNPs are shown.

The functional annotation of SNPs and related genes in terms of biological processes, molecular functions, and cellular components is pictorially shown in Supplementary Figures 1 and 2. Forty-five biological processes (Supplementary Table 5, Supplementary Figure 1), 19 molecular functions (Supplementary Table 6, Supplementary Figure 2), and one cellular component (Supplementary Table 7) could be identified. Locations of SNP along human genome are displayed in Figure 7 and in Supplementary Table 8.

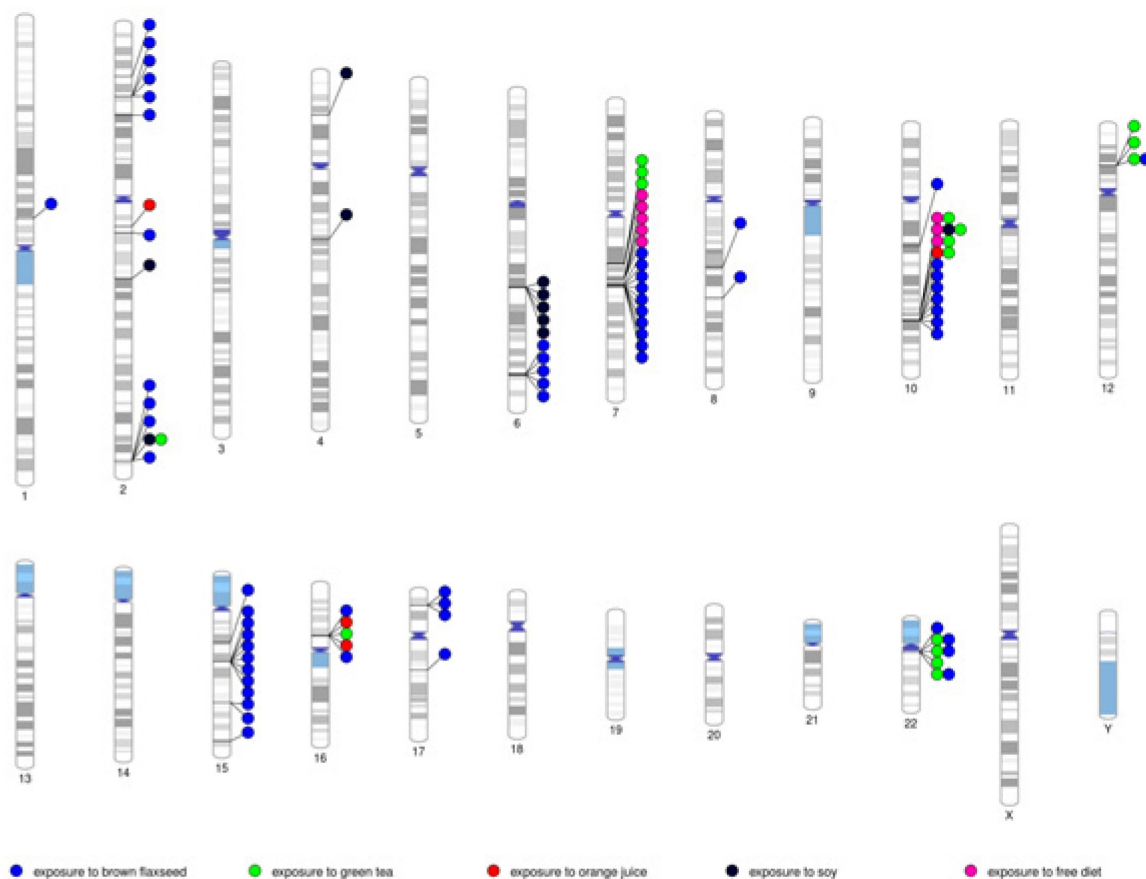


Figure 7. Locations of SNPs involved in (poly)phenol metabolism along the human genome.

In most cases, SNPs had been chosen and tested by the authors, based on knowledge derived from previous *in vitro*, animal or *in silico*/computational studies, or from clinical/epidemiological studies. Only one study [39] devised a systematic methodology for selecting SNPs, integrating data-mining (by surveying the HuGE Navigator Database) and text mining (by comprehensively looking for all published genome-wide association studies, GWAS, of genes coding proteins involved in the metabolism of endo- and xenobiotics, across any phenotype). In most studies [42-45,47-49], genotyping was carried out by TaqMan assay, which utilizes the 5' nuclease activity of Taq polymerase to generate a fluorescent signal during polymerase chain reaction (PCR), or similar techniques. In one study [40], “Kompetitive Allele Specific” PCR (KASP) and agarose gel electrophoresis were utilized, which enable a fast, cheap, but high-quality and highly reliable bi-allelic SNP characterization. In another study [38], genotyping was conducted by direct sequencing. Finally, one study [39] conducted genotyping by multiplexing (utilizing the MassARRAY technology and iPLEX Gold assay). Of note, only one study [41] performed a GWAS. Only one study [46] did not provide any detail about the genotyping technique.

A priori sample size and power analysis

Formal *a priori* sample size and power analyses were conducted only in one study, even if this study [38] conducted such analyses but for their primary (and not secondary) outcome (changes in blood pressure, and not the impact of the genetic variant).

Data quality check, dealing with missing data, and data pre-processing/transformation

Only a few studies explicitly reported the procedures followed to ensure data quality, deal with missing data, and perform data pre-processing/transformation. For instance, one study [38] log-transformed post-intervention analytical values. Similarly, other two studies [42,46] log-transformed the values of all urinary biomarkers, the distributions of which exhibited a markedly skewed trend toward high values. As such, all formal statistical tests were carried out on logarithmically transformed values, presenting geometric (and not arithmetic) means of all urinary (poly)phenol biomarkers. Furthermore, in one of these two studies [42], the authors computed within-batch coefficients of variations on duplicate samples, which were found to vary from 5.8% to 24.6%. Another study [49] checked for normality using the Kolmogorov–Smirnov test, and non-normally distributed data were transformed by ranking according to Blom. Also, the authors reported mean intra-assay (9.1%) and inter-assay (13.4%) CVs and, after quality checks, removed three subjects from their analyses. In the “real-life” metabolomic study [47], 217 metabolites could be initially identified and, after quality checks, 108 metabolites entered the multivariate analyses (Principal Component Analysis, PCA) to check for homogeneity and reproducibility of the dataset and identify eventual outliers. “Orthogonal projections to latent structure discriminant analysis” (OPLS-DA) was carried out in order to maximize the separation between genotypic classes.

Genetic modeling

In most studies, genotypes were simply grouped based on the presence of the minor allele. Only a few studies [38,40] performed extensive and comprehensive genetic modeling. One study [38] conducted four genetic analyses, comparing dominant, recessive, and additive models and model-free computation. Another study [40] tested five genetic models (namely, dominant, co-dominant, over-dominant, recessive, and log-additive). Of note, only one study [40] performed a haplotype analysis.

Furthermore, two studies [44,45] included only homozygous variant individuals, excluding those heterozygous. Finally, two studies [40,43] found evidence of linkage disequilibrium and violation of the Hardy-Weinberg equilibrium, at least for a SNP and in a sub-group of the population recruited.

Analysis of the SNP impact on dietary (poly)phenol metabolism: model building

Only a few studies explicitly reported the statistical procedure that was followed to build their model. A study [44] performed a three-way interaction analysis (SNP genotype, treatment, and time). One study [47] leveraged metabolomics, whilst two studies [44,48] utilized pharmacokinetics/nutrikinetics modeling. In the metabolomic study [47], subsets of metabolites significantly contributing to the discrimination between genetic groups were identified by iteratively performing a stepwise removal of the less contributing metabolites, according to coefficient plots and values of the variable importance on projection. In one of the two nutrikinetics studies [44], mean catechin concentrations at each time point stratified by SNP (more specifically, COMT genotype) were modelled using cubic spline interpolation, and pharmacokinetic parameters were estimated. In the other nutrikinetics study [48], catechin concentrations were modelled using non-compartmental analyses and population pharmacokinetic methods (including linear trapezoidal-linear interpolation to estimate the area under the ROC curve, AUC, correlational analyses, and non-linear mixed-effects modeling techniques).

Statistical analysis, threshold for statistical significance, goodness-of-fit statistics, and model evaluation and validation

The majority of studies carried out multivariate statistical analyses, with the exception of a single study [43]. Most studies did not utilize stringent statistical criteria. Of note, only two studies [39,47] performed corrections for multiple testing, specifically applying Bonferroni correction. Interestingly, the only performed GWAS [41] adopted a statistically significant p-value $<1 \times 10^{-5}$, instead of the genome-wide significance threshold, to avoid type II errors and screen genetic variants with small effects on the trait of interest (i.e., the equal-producing phenotype).

Goodness-of-fit statistics were rarely reported [47,48]. In one study [47], the goodness of fit of the models was estimated by computing R^2 and Q^2 by 7-fold internal cross-validation, and

the analysis of variance (ANOVA) of the cross-validated residuals (or CV-ANOVA). In a pharmacokinetics/nutrikinetics study [48], model selection was based on the objective function value, several goodness-of-fit indicators and plots, and a visual predictive check. A stepwise covariate model-building strategy was leveraged by utilizing forward inclusion of covariates followed by backward elimination.

Major findings

In the next sections, the major findings of the studies retained in the present systematic review are overviewed and compared (see also Table 2). Full details of the outcomes are reported in Supplementary Table 3.

Impact of genetic variations on green tea catechin metabolism

Green tea is one of the most popular and widely consumed beverages worldwide. It is brewed from dried leaves of the plant *Camellia sinensis*. Green tea intake has been associated with anti-carcinogenic, anti-diabetic, anti-atherosclerotic, hypolipidaemic, anti-inflammatory, and anti-obesogenic effects, among others [50]. These protective effects could derive from the modulation of different cellular pathways, including the PI3K/AKT pathway, the NF- κ B pathway, the MAPK pathway, the cAMP/PKA pathway and the 67LR/PP2A pathway [51-53]. Green tea is a source of flavan-3-ols, including epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin (EC), and epicatechin gallate (ECG), collectively known also as (gallo)catechins. The proportion of (gallo)catechins that is absorbed in the small intestine can undergo sulphatation, glucuronidation, and/or methylation by phase II enzymes in enterocytes and hepatocytes, before entering the systemic circulation. In particular, monomers with a 3-*O*-galloyl moiety are quite resistant to sulfation and glucuronidation, and are found in plasma mainly in a free form, being excreted mainly through the biliary system [54,55]. EGCG can be metabolized through methylation producing 4'-*O*-methyl EGCG, 4''-*O*-methyl EGCG and 4',4''-*O*-dimethyl-EGCG [56]. Also, EGC can be methylated in position 4', giving origin to 4'-methyl-EGC, which can be found in plasma and urine. The fraction of (gallo)catechins that are not absorbed in the smaller intestine (70% of catechins and 90% of gallo catechins) reach the colon and are catabolized to phenyl- γ -valerolactones (PVLs) and smaller phenolic acids, which are absorbed through passive diffusion, conjugated by phase II enzymes, and excreted through urine [2,57].

The enzyme catechol-*O*-methyltransferase (COMT), encoded by a gene located at 22q11.21, is responsible for catalyzing a major metabolic pathway of flavonoids and their gut microbiota metabolites, namely their *O*-methylation, which is the transfer of a methyl group from *S*-adenosylmethionine (SAM) to one of the hydroxyl groups of catechols, to produce *O*-methylated catechols and *S*-adenosyl-L-homocysteine (SAH). This biotransformation mainly occurs in the intestinal tract, liver, and kidney [58]. Two isoforms of COMT have been demonstrated to exist: a soluble form of the protein, which is the predominant one in most tissues, and a membrane-bound form. The genetic polymorphism rs4680 is a quite common missense variant (G to A base change) resulting in a valine-to-methionine amino acid substitution at position 108 in the soluble protein and at position 158 in the membrane-bound form. This mutation, which can be found in insulin-resistant and type 2 diabetics, seems to alter the function of the COMT enzyme by decreasing its thermostability and resulting in a 30-40% reduction of its activity [58].

Six studies [38,42-45,48] explored the impact of COMT-related genetic variations on the metabolism of green tea (gallo)catechins. In the study by Brown et al. [38], the authors recruited a sample of 83 subjects, who consumed decaffeinated green tea extract (about six-to-eight cups of moderate-strength green tea daily). Plasma concentrations of EGCG were undetectable at baseline and remained undetectable after dietary supplementation in the placebo group, but increased in the experimental group. Urinary excretion over 24 h of EGC and 4'-*O*-methyl EGC was similar for the two groups at baseline, but post-intervention increased in the experimental group by 28-fold and 34-fold, respectively. Mean urinary concentrations of EGC and 4'-*O*-methyl-EGC were significantly lower ($p < 0.01$) in individuals homozygous for the high-activity COMT G-allele, potentially reflecting increased metabolic flux and a more rapid turnover and conversion to downstream metabolites, with respect to individuals carrying at least one copy of the low-activity COMT A-allele.

In the study by Inoue-Choi et al. [42], 660 subjects, self-identified as daily green tea drinkers, were recruited from four small, geographically defined communities from the Greater Shanghai metropolitan area, and stratified according to COMT genotype: 343 carried the homozygous wild-type genotype, 49 the homozygous variant genotype, and 268 the heterozygous genotype. Comparing the three COMT genotype groups, statistically significant differences, even though nominal or borderline, could be found for the five (poly)phenol metabolites under study, when considered each one individually (EGC, EC, 4'-*O*-methyl-EGC,

3',4'-diHPVL, 3',4',5'-triHPVL). Considering all the metabolites altogether, the difference was highly significant ($p=0.007$). More specifically, the authors found that those carrying the homozygous low-activity genotype had lower urinary levels of five tea (poly)phenol metabolites by 35-45%, compared to those with the wild type high-activity genotype or the heterozygous variant genotype (who had, on the contrary, comparable levels of urinary metabolites). The authors speculated that they were able to retain more green tea (poly)phenols in their bodies and benefit more from green tea intake. Of note, when stratifying according to daily green tea consumption, low green tea consumers (<5g/day) had lower urinary metabolite concentrations in both homozygous low-activity and heterozygous genotype groups, compared with the high-activity homozygous wild-type genotype, whilst the difference between high- and intermediate-activity COMT genotype tended to disappear with increasing green tea consumption ($p=0.18$).

Conversely, the study by Lorenz et al. [43] found no impact of COMT genotype. The authors explored the enzymatic activities of four functional COMT SNPs (rs4680, rs6269, rs4633, and rs4818) determined in red blood cells in a sample of 24 healthy human volunteers supplemented with pure EGCG. EGCG plasma levels and COMT enzyme activities in erythrocytes were measured before and two hours after intervention. While enzymatic COMT activities were affected by the COMT SNPs ($p<0.001$), EGCG plasma levels significantly increased after the intervention and were not influenced by any of the COMT SNPs and different enzyme activities. Furthermore, EGCG ingestion did not result in impairment of COMT activity, which significantly increased by 24% after EGCG consumption.

Similarly, the study by Miller et al. [44] found no influence of COMT genotype. The authors recruited twenty participants, who were exposed to decaffeinated green tea extract. Maximum plasma concentrations of 1.09 μ M, 405, 331, 160, and 77.1 nM were reached for EGCG, EGC, EC, ECG, and 4'-O-methyl EGCG at 81.5, 98.5, 99.0, 85.5 and 96.5 min, respectively. Bimodal and single-peaked curves could be observed for the non-gallated and gallated green tea catechins, respectively, probably reflecting a meal effect from the high-carbohydrate breakfast administered 1 hour after the capsule consumption. It is hypothesized that gallated species, different from non-gallated ones, may form complexes with the food components, resulting in a single peak being observed. Other possible explanations could be a depletion in glucuronidation precursors in the fasted status, or enterohepatic circulation of absorbed EGC and EC. No statistically significant differences based on COMT genotype could be found in

terms of maximum concentration, time to reach maximum concentration, area under the time-concentration curve (AUC), EGCG half-life absorption, and EGCG half-life elimination. However, a trend towards a greater maximum concentration and AUC response in the plasma concentration curve for 4'-O-methyl-EGCG in the COMT AA genotype group could be noted. Also, a tendency towards a higher maximum plasma concentration for the AA genotype for EC, EGC and ECG was detected, along with higher median concentrations of 4''-O-methyl-EGCG, which was detected and quantified only in few samples.

The authors were able to replicate their findings in another, larger study [45], with 47 participants. A genotypic effect was observed for urinary 4'-O-methyl EGC during the first 5.5 h, with the COMT GG group exhibiting a greater concentration ($p=0.049$). No differences could be found for the remaining 18.5 h collection or for total EGC during the 5.5 h and 18.5 h collections. Finally, no difference in concentrations of plasma metabolites (C, EC, EGC, ECG, EGCG, 4'-O-methyl-EGCG, 4''-O-methyl EGCG, 4',4''-O-dimethyl-EGCG) could be detected. According to the authors, these findings are coherent with the hypothesis of a slower methylation enzymatic function in the COMT AA genotype individuals, as well as with a differential preference for methylation position ("regioselectivity") induced by the genetic variation, which could explain why, in the COMT GG group, secondary and tertiary methylation products, such as 4',4''-O-dimethyl EGCG and 3',4',4''-O-trimethyl EGCG, are more likely to be detected with respect to primary methylation products [44,45]. On the other hand, all these differences are, overall, slight and not statistically significant, suggesting that green tea (gallo)catechins, having low bioavailability and being poorly absorbed in the small intestine and quickly eliminated, may have better enzyme-substrate interactions and binding affinity than endogenous substrates, which could counteract, at least partly, the effects of the polymorphism.

Whilst COMT genotype as potential genetic factor mediating green tea exposure/intake and metabolic response has been the most investigated, other studied SNPs are related to genes coding members of the family of drug transporters, which include uptake (Organic Anion Transporting Polypeptide type 1B1, OATP1B1/SLCO1B1) and efflux (Multi-drug Resistance-associated Protein type 2, MRP2/ABCC2) transporters [59]. The former is specifically expressed in the liver, on the basolateral membrane of hepatocytes, whereas the latter is an ATP-binding cassette protein and is expressed in several human tissues, being of particular importance for intestinal drug absorption and hepatic drug elimination.

In the nutrkinetics study by Scholl et al. [48], 84 healthy participants took green tea extract capsules for 5 days. On day 5, plasma profiles for EGCG, EGC and ECG were obtained by collecting venous blood samples 0.5, 1, 2, 3, 4, 5, 7, and 9 hours after the last green tea extract capsule consumption. A strong between-subject variability in pharmacokinetics was found, with maximum plasma concentrations varying more than 6-fold (6.1, 7.7 and 6.6-fold for EGCG, EGC and ECG, respectively). For EGCG and ECG, the highest interindividual variabilities concerned the zero-and first-order absorption processes, whilst for EGC, the highest interindividual variability regarded the central volume of distribution and the intercompartmental distribution. The AUCs of EGCG, EGC and ECG were 877.9 (360.8-1576.5), 35.1 (8.0-87.4), and 183.6 (55.5-364.6) h x µg/L respectively, and the elimination half-lives were 2.6 (1.8-3.8), 3.9 (0.9-10.7) and 1.8 (0.8-2.9) hours, respectively. Metabolite concentrations were found to decline in a biexponential fashion. Genetic polymorphisms in genes of the drug transporters ABCC2/MRP2 and OATP1B1 were found to explain, at least partly, the high variability in pharmacokinetic parameters. More specifically, wildtype carriers of ABCC2 rs717620 exhibited 26% less EGCG relative bioavailability than carriers of the variant allele ($p=0.00721$). Additionally, total body clearance of EGCG was lower in carriers of the C allele of rs4149056 ($p=0.01212$). Concerning EGC, its pharmacokinetics was affected by MRP2 rs3740066 ($p=0.00153$, a higher intercompartmental clearance in wildtype carriers), OATP1B1 rs2306283 ($p=0.00163$, a 35% reduction in total body clearance in wildtype carriers), ACBC2 rs717620 ($p=0.01654$, a reduction in central volume of distribution in carriers of the T allele), UGT1A128 ($p=0.01795$, a 26% reduction in total body clearance in wildtype carriers), and COMT rs4680 ($p=0.02350$, a 24% reduction in relative bioavailability in carriers of the low-activity AA genotype). Of note, habitual tea drinking was not found to have an impact on the kinetic of any of the metabolites measured.

Impact of genetic variations on flaxseed lignan metabolism

Flaxseed is a source particularly rich in lignans, especially secoisolariciresinol diglucoside, which is poorly absorbed in the smaller intestine and reaches the lower part of the gastrointestinal tract, where it is deglycosylated to secoisolariciresinol and converted by the intestinal microbiota to the enterolignans enterodiols and enterolactone [60-61]. Enterodiols, in turn, rapidly converted to enterolactone, which is therefore the predominant circulating lignan. The effects of enterolignans are complex, in that they can have both estrogenic and

anti-estrogenic actions, depending on their concentration and on individual factors, including age and the reproductive/menopausal status of the subject [62-65]. Moreover, they can exert other protecting activities, including anti-tumor and anti-oxidant effects, through the modulation of different cell signaling pathways [62-65].

In the study by Chang et al. [39], ground brown flaxseed consumption for 6 weeks in post-menopausal women resulted in increased urinary enterolactone excretion, which was higher in European ancestry women. Associations between urinary phytoestrogen enterolactone excretion and 70 functional polymorphisms in 29 steroid hormone metabolizing genes, including genes putatively involved in (poly)phenol excretion and phase II metabolism (BCL2L11, OATP1B1/SLCO1B1, COMT, UGT1A1, SULT1A1), were explored. In European ancestry women, 12% and 17% of the variation in baseline and post-intervention excretion levels was explained by genetic variants. In African ancestry women, this percentage was 16% and 13%, respectively. These associations were more marked among women of African ancestry than among those of European ancestry. Of note, no SNPs were associated in both races. However, these associations were only nominal, failing to achieve the statistical significance threshold after correction for multiple comparisons.

Impact of genetic variations on isoflavone metabolism

Soy is a source particularly rich in isoflavones, which exert pleiotropic actions, by modulating estrogen receptor signaling, cell proliferation and apoptosis, among others, suggesting a potential protective effect against hormone-dependent cancers, osteoporosis, and cardiometabolic diseases [66,67]. Daidzein, genistin and glycitein are the major isoflavones, occurring in *planta* predominantly as β -glucosides. Their absorption involves a preliminar step of hydrolysis catalized by intestinal enzymes, including cytosolic- β -glucosidase (CGG) and lactase-phlorizin hydrolase (LPH). Then they are conjugated by human sulfotransferase (SULTs) and UDP-glucuronosyl-transferases (UGTs), forming phase II metabolites, with glucuronides in excess of sulphates [68]. A common genetic variation consisting in the presence of an additional TA dinucleotide pair in a TA-repeat region ((TA)₇ compared with (TA)₆) of the promoter of the human UGT1A1 gene results in reduced expression of UGT1A1 [69].

Wakeling and Ford [49] recruited a sample of 100 pre-menopausal women. The participants to the “Soy Isoflavone Metabolism Study” consumed a commercial soy supplement as a single bolus dose. The authors measured the urinary levels of isoflavone metabolites (aglycones, 7-

glucuronides, and sulphates of genistein, daidzein, and glycitein) and their relationships with UGT1A1*28 promoter polymorphism and SNPs in other genes involved in absorption and excretion (CBG, LPH, ABCC2/MRP2, and ABCG2/BCRP). ABCG2/BCRP (Breast Cancer Resistance Protein) is an efflux transporter belonging to the ATP-binding cassette family and present in the luminal membrane of the intestine. Urine was collected over 24 h. Large inter-individual differences in isoflavone recovery (mean 39% [range 11-89%], eightfold variation) and metabolites could be observed, with glucuronides representing the major metabolites (72% of total). No statistically significant association between the UGT1A1*28 polymorphism and net recovery in urine of any individual isoflavone metabolite under study could be detected. However, the polymorphism showed other significant associations. More in detail, the UGT1A1*28 minor allele was found to be positively associated with percentage of glycitein excreted in urine as sulphate ($p=0.046$), and, after excluding five participants with both minor alleles of CBG and ABCG2, due to a statistically significant interaction ($p=0.025$), it was positively associated with percentage of glycitein as sulphate in urine ($p=0.014$), negatively with percentage of glycitein as glucuronide ($p=0.028$), positively with combined isoflavones as sulphate ($p=0.035$), and positively with sulphate-to-glucuronide ratio for combined isoflavones ($p=0.036$). Finally, SNPs in CBG, ABCG2/BCRP, and ABCC2/MRP2 were also found to correlate with differences in isoflavone metabolites in urine. In detail, CBG rs358231 was associated with percentage of total isoflavone recovered in urine as glucuronide ($p=0.035$), ABCG2 rs2231142 was negatively associated with percentage of total isoflavone recovered in urine as glucuronide ($p=0.042$), and ABCC2 rs2273697 was associated with total glycitein excretion ($p=0.034$) and total genistein excretion ($p=0.019$).

Equol, or 4',7-isoflavandiol, is a nonsteroidal estrogen derived from the metabolism of isoflavones by the gut microbiota [70]. Equol production occurs in approximately 30–50% of subjects due to variations in their intestinal bacterial composition [71], whereas the contribution of the genetic makeup of the individual is less known. Inter-individual differences in equol production could explain the differential impact of soy intake on health-related outcomes, especially those related to cardiovascular health [72]. Hong et al. [41] attempted to fill in this gap in knowledge, by performing a GWAS of the equol-producing phenotype in a sample of 1,391 Koreans in a context of free diet. In the study population, 70.1% of the participants were equol-producers and exhibited significantly lower blood pressure compared to nonproducers. Five SNPs identified in the HACE1 gene (HECT Domain And Ankyrin Repeat

Containing E3 Ubiquitin Protein Ligase 1) were significantly associated with equol production, the most significant of which was rs6927608: more specifically, individuals with a minor allele of this SNP were less likely to produce equol and displayed more elevated systolic blood pressure values. HACE1 plays a key role in a variety of biological functions and processes, including tumor suppression, by hindering tumor cell proliferation and facilitating the programmed cell death of tumor cells [73]. Moreover, it safeguards the heart, mitigates against oxidative stress, and regulates cellular dynamics [74]. The authors speculated that HACE1 could be involved in host intestinal immune responses and in the maintenance of the pool of equol-producing bacteria, thus mediating the impact of the equol-producing phenotype on heart health [41].

Impact of genetic variations on orange flavanone metabolism

Citrus drinks are a major source of dietary flavanones, including hesperetin, naringenin and their respective glycosides. Flavanones have been associated to a reduction in the risk of cardiovascular diseases and have shown an antihypertensive effect. The modulation of endothelial permeability and vascular reactivity via the regulation of the vascular nitric oxide (NO) synthase pathway may explain in part their protective actions [75]. After ingestion, flavanone glucosides and other glycosides are hydrolysed in the small intestine and in the colon, respectively, and the released aglycones are conjugated by intestinal and hepatic phase II enzymes forming glucuronides, sulfates, sulfo-glucuronides, and diglucuronides [75].

Fraga and coauthors [40] investigated the impact of four SNPs in three genes involved in phase II metabolism or excretion (SULT1A1, SULT1C4, ABCC2/MRP2). Forty-six volunteers ingested a single dose of orange juice. Based on the phenotype of urinary excretion of phase II flavanone metabolites, 25 and 21 subjects were categorized as high- and low-excretors, respectively. A statistically significant relationship between three out of the four of the SNPs under study and the excretion of phase II flavanone metabolites could be found.

Impact of genetic variations on the metabolism of (poly)phenols from free diet

Muhrez et al. [47] explored the impact of genetic variations affecting the gene coding for ABCC2/MRP2 by leveraging a metabolomics-based approach. The authors recruited a sample of 53 individuals, exposed to dietary (poly)phenols ingested in uncontrolled, real-world settings (free diet). A set of 35 metabolites was identified to vary significant in association with

these SNPs. After an iterative series of quality checks and optimization procedures, the final models were able to explain more than 80-85%, 80%, and 81% and predict more than 48-54% of the total variance for three SNPs, respectively. Of note, eight of the urinary metabolites more associated with SNPs in ABCC2/MRP2 were (poly)phenol metabolites.

Another study [46] was carried out in the context of a free diet, without characterizing in detail the dietary habits of the recruited population. This study explored the impact of polymorphisms in the gene PON1 on the performance of an emerging biomarker of flavan-3-ol intake, namely the sum of two gut microbial metabolites specific for this class of flavonoids (here indicated as γ VLM_B): 5-(4'-hydroxyphenyl)- γ -valerolactone-3'-sulfate and 5-(4'-hydroxyphenyl)- γ -valerolactone-3'-glucuronide. The authors hypothesized the role of a family of human serum proteins known as paraoxonases (PONs) in catalyzing the hydrolysis of phenyl- γ -valerolactones (PVLs) into phenylvaleric acids (PVAs), thus affecting the levels of circulating PVLs and so their suitability as nutritional biomarkers. The study consisted of a single acute intake, single arm, non-randomized intervention part, in which 13 healthy male volunteers were recruited and a rapid transformation of PVLs into PVAs could be observed in serum samples collected *ex vivo*, with a half-life of approximately 9.8 ± 0.3 minutes. This conversion was mediated by the PON1 and PON3 isoforms, with PON enzymes being also involved in processing the phase II metabolites of PVLs. In the second part of the study, an ancillary analysis of the EPIC-Norfolk cohort study, five common PON1 genetic variations were investigated to determine their impact on the urinary levels of γ VLM_B, showing a limited influence on the inter-individual variations in γ VLM_B levels and no physiological or clinical effects.

Trial registration, funding/sponsorship

Only one trial [39] was registered. Only two studies [39,48] reported their findings adhering to consolidated checklists (namely, the CONSORT and the TREND checklists, respectively).

Finally, concerning funding/sponsorship, no information was provided for one study [48]. For the remaining studies, funding/sponsorship was provided by national councils/institutes [40-43,49], non-profit/public organizations [47], or companies with significant commercial interests in food products and/or beverages [38,44-46]. In a study [39], one of the authors served as a consultant/advisory board member of the council of a body/institution with significant commercial interests in food products and/or beverages.

Summary of the findings and quality appraisal

The overall quality of the studies here overviewed appears to be low-medium, showing a weak methodological strength, statistical robustness, reproducibility, and generalizability. Only a few studies have applied stringent statistical criteria, and recruited sufficiently large and diverse samples to enable reaching solid and reliable conclusions. Most studies included in the present systematic review show methodological weaknesses and deficiencies, including small samples, biases, confounding, and nominal effect sizes, among others.

4.4.4. Discussion

The present systematic review has synthesized twelve articles that explored the impact of genetic variations on the absorption, distribution, metabolism, and excretion of dietary (poly)phenols. The inter-individual variability in the plasma circulating levels and in the urinary excretion of (poly)phenol metabolites was hypothesized to be influenced, at least partly, by SNPs in the genes encoding for influx and efflux transporters, and phase I and phase II metabolism enzymes. In particular, the association between genetic variation in genes involved in (poly)phenol ADME and changes in plasma and/or urinary levels of (poly)phenol metabolites was explored.

Half of the studies investigated green tea flavan-3-ol ADME, using green tea, green tea extract, or pure EGCG as sources. Other studies explored the bioavailability and metabolism of flavanones, isoflavones and lignans, present in orange juice, soy supplement, and ground brown flaxseed, respectively. Only one study analyzed the impact of free diet on the phenolic metabolome, and two other studies investigated the metabolism of flavan-3-ols and isoflavones deriving from free diet, considering their specific gut microbial metabolites. These findings indicate that the investigation on the association between the variability in (poly)phenol ADME and genetic differences is limited and needs more research, focusing on other classes. Moreover, galocatechins, flavanones, isoflavones, and lignans are not ubiquitous classes but characterize specific foods, being their intake in the daily diet restricted, suggesting that the sources considered in the present review are not representative. Therefore, the study on (poly)phenol ADME and related SNPs should be widened considering other classes, including flavan-3-ols of different origin from green tea, anthocyanins, flavonols, and cinnamic acids, among others.

Considering the genes analyzed in the studies and their role in (poly)phenol ADME, phase I metabolism was the most representative phase, followed by excretion and phase II metabolism. However, taking into account the number of times a gene was studied in all works, the most studied phase resulted to be phase II metabolism, followed by excretion and phase I metabolism. Absorption was the least represented and studied phase, with only a few genes studied in one work. These results are consistent with the crucial role of conjugation in the metabolism of (poly)phenols. Considering the metabolites that were influenced by genetic variability, many of them were phase II conjugates, including methylated forms of gallo catechins (4'-O-methyl-EGC), sulfated and glucuronidated forms of isoflavones (glycitein, genistein, and daidzein), and sulfated, glucuronidated, sulfo-glucuronidated, and diglucuronidated forms of flavanones (naringenin and hesperetin). Other metabolites included alkycones of (gallo)catechins (EGCG, EGC, EC) and isoflavones (glycitein, genistein, and daidzein), phenolic acids (3',4'-dihydroxycinnamic acid, 4'-hydroxyhippuric acid), and gut microbiota metabolites (5-(3',4'-dihydroxyphenyl)- γ -valerolactone, 5-(3',4',5'-trihydroxyphenyl)- γ -valerolactone, 5-(4'-hydroxyphenyl)- γ -valerolactone-3'-sulfate, 5-(4'-hydroxyphenyl)- γ -valerolactone-3'-glucuronide, enterolactone, equol, 3',4'-dihydroxyphenylacetic acid, benzene-1,3-diol).

Regarding phase II metabolism, COMT genetic variants were analyzed mainly concerning gallo catechins, UGT1A1 genetic variants were associated with isoflavone phase II metabolites, and SULT1A1/SULT1C4 were explored together with flavanone phase II metabolites. The most studied genetic variation was rs4680 in COMT gene. However, there was no consensus among studies allowing to associate a particular genetic variant with a reduction/increase of a specific metabolite in urine or plasma. Glucosidases involved in (poly)phenol absorption (CBG/GBA3 and LPH) were analyzed only concerning soy isoflavones, while influx/efflux transporters involved in (poly)phenol excretion were investigated with all sources of (poly)phenols, suggesting their importance in determining (poly)phenol bioavailability.

The findings overviewed are conflicting and can be reconciled only partially by taking into account differences in the methods and protocols implemented. Most studies were conducted in highly controlled situations, in the attempt to avoid or minimize potential biases and confounders. Controlled laboratory research does not necessarily reflect real-life circumstances and real-world settings, also and especially in terms of eating patterns and dietary habits. For instance, the study by Inoue-Choi et al. [42] succeeded in capturing the

incredibly high variety and heterogeneity of green tea drinking habits, in terms of the range in the amounts consumed per day. Also, in this particular study, the timing of urinary sample collection did not reflect the laboratory timing needs and schedule, but was, instead, arranged on the basis of scholarly knowledge and evidence provided by pharmacokinetic/nutrikinetic studies, in such a way that “the levels of urinary tea polyphenols captured in the present study likely represent their near peak levels during a typical day” [42]. On the contrary, the other studies overviewed in the present systematic review may lack ecological validity.

Moreover, some investigations employed small sample sizes and lacked replication populations. Moreover, confounders such as seasonal differences in food intake were generally not adjusted for. Some effects were explored only by means of exploratory post-hoc analyses. Also, given that SNPs vary according to ethnicity, more diverse populations should be explored. A systems genetics-based or a GWAS approach would be ideal and highly promising.

Some studies investigated male- or female-only populations, making it impossible to explore the effect of sex/gender as mediating/moderating the impact of genetic variations on the metabolism of dietary (poly)phenols. There exists a serious lack of data on potential sex/gender differences in the bioavailability and biotransformation of dietary (poly)phenols [54,55,57,76]. As such, future studies should investigate whether and how sex/gender would modify and modulate the complex, non-linear interplay between a given genotype and the consumption of foods and beverages rich in (poly)phenols on the metabolism of dietary (poly)phenols and their beneficial effects. Several factors, including behavioral lifestyles, can impact (poly)phenol ADME. For instance, smoking, drugs, and alcohol can modify the levels of UGTs [77]. Most studies did not report any information concerning these variables, the impact of which and the interactions with the genetic variants should be explored more in depth.

Furthermore, our current understanding of the impact of interindividual genetic variations on the metabolism of dietary (poly)phenols is limited by the number of studies and the knowledge of the functional effects of the SNPs putatively involved in the complex biochemistry and modulation of (poly)phenolic compounds. More specifically, we still do not know to which extent phase II metabolism and other biotransformations occur locally (i.e., within the enterocyte, at the level of the apical and/or basolateral enterocyte membrane) and/or systemically (i.e., mainly in the liver). Taking into account that dietary exposure is a type of particularly complex exposure and reductionist approaches fail to capture underlying heterogeneity and variability, new methodologies – drawing from systems and network

biology and physiology and what has been proposed as “nutritional systems biology” [78] - are warranted. In particular, integrative frameworks, combining *in vivo* and *in vitro* approaches, wet-lab and *in silico*/computational techniques, nutritional epidemiology and mathematical modeling (pharmacokinetics/nutrikinetics), can help address such complex research questions and uncover the impact of a given genotype on the biological activity of ingested (poly)phenols as well as their health-related outcomes, in order to provide personalized dietary recommendations [79,80]. A systems biology approach would be paramount in that most of the investigated polymorphisms showed weak or no significant impact on the metabolism and pharmacokinetic/nutrikinetic parameters of various dietary (poly)phenols. Further explanations of the large variability observed in dietary (poly)phenol plasma concentrations could be interindividual differences in gastrointestinal solubility or, more probably, in the influence of the gut microbiota.

The present systematic review has several strengths, including i) high methodological rigor, ii) transparency and reproducibility, iii) extensive, comprehensive literature search conducted on several databases enhanced by cross-referencing and target journal hand-search, a critical approach, quality appraisal, and iv) complemented by bioinformatics analyses.

On the other hand, it has a few shortcomings, that should be properly acknowledged, including i) the relatively small number of studies included, ii) the high degree of heterogeneity that hinders a meta-analysis, and iii) the low-medium quality of studies here overviewed.

The present systematic review can pave the way for further investigations, that leverage bioinformatics analysis and GWAS-based approaches.

4.4.5. Conclusions

Genetic variations are becoming increasingly recognized as an important modifier of the impact of environmental exposures, including diet, on human physiology and physiopathology. Despite an increasing body of scholarly investigations on (poly)phenols and their dietary intake, still very little is known about the effects of SNPs on (poly)phenol absorption, distribution, metabolism, and excretion in the human body. To the best of our knowledge, this is the first comprehensive investigation of the genetic factors explaining inter-individual variability in the response to dietary (poly)phenol exposure, by conducting a systematic literature review. However, based on the above-mentioned shortcomings, further high-quality research in the field is urgently warranted.

Authors' contributions

PM, DM and DDR contributed to the conception of the review; NT and NLB contributed to literature search, study selection and data extraction; CM and VB contributed to the methodology; NT and NB wrote the manuscript. All the authors performed reviewing and editing of the article and consented to the final article.

Declaration of interest statement

The authors declare no conflict of interest.

Electronic Supplementary Information (ESI)

Supplementary Table 1. Detailed search strategy adopted in the present review.

Search items	Details
Search string	(Polyphenol* OR acetoxypinoresinol* OR alkylmethoxyphenol* OR alkylphenol* OR amentoflavone OR anethole OR angolensin OR anthocyanin* OR apigenin OR arbutin OR arctigenin OR avenanthramide* OR baicalein OR benzoic OR bergapten OR biflavonoid OR biochanin OR bisdemethoxycurcumin OR butein OR butin OR caffeic OR caffeoyl* OR calycosin OR carnos* OR carvacrol OR catech* OR chalcone* OR chicoric OR chlorogenic OR chrysin OR chrysoeriol OR cinnam* OR cirsilineol OR cirsimaritin OR conidendrin OR coumar* OR coumestrol OR curcumin* OR cyanidin OR cyclariciresinol OR daidz* OR dalbergin OR danshensu OR delphinidin OR demethoxycurcumin OR demethyleuropein OR deoxyschisandrin OR dicaffeoyl* OR didymin OR dihydrobiochanin OR dihydrocaffeic OR dihydrochalcone* OR dihydrodaidzein OR dihydroferul* OR dihydroflavonol* OR dihydroformononetin OR dihydrogenistein OR dihydroglycitein OR dihydromyricetin OR dihydro-p-coumaric OR dihydroquercetin OR dihydroresveratrol OR dihydrosinapic OR dimethylmatairesinol OR diosmin OR ellagic OR enterodiol* OR enterolactone* OR epicatechin OR epigallocatechin* OR epirosmannol OR episesamin* OR equol OR eriocitrin OR eriodictyol OR escul* OR estragole OR eugenol OR eupatorin OR ferul* OR fisetin OR flavanol* OR flavanone* OR flavone* OR flavonoid* OR flavonol* OR formononetin OR furanocoumarin* OR galangin OR gallagic OR gallic OR galloyl OR galocatechin* OR gallotannin* OR gardenin OR genist* OR gentisic OR geraldone OR gingerol* OR glycit* OR gomisin* OR guaiacol OR hesper* OR hippuric OR hispidulin OR homoeriodictyol OR homovanillic OR homoveratric OR hydroxybenzaldehyde* OR hydroxybenzoic OR hydroxybenzoketone* OR hydroxycaffeic OR hydroxycinnamaldehyde* OR hydroxycinnamic OR hydroxycoumarin* OR hydroxydanshensu OR hydroxyphenylacetic OR hydroxyphenylpentanoic OR hydroxyphenylpropanoic OR hydroxyphenylpropene* OR hydroxytyrosol OR irilone* OR irisolidone* OR isoferul* OR isoflavonoid* OR isohydroxymatairesinol* OR isolariciresinol* OR isoliquiritigenin* OR isopeonidin OR isopimpinellin OR isorhamnetin OR isorhoifolin OR isosakuranetin OR isotectorigenin OR isoxanthohumol OR jaceidin OR jaceosidin OR juglone OR kaempfer* OR koparin OR lambertianin OR lariciresinol OR lignan* OR ligstroside OR lithospermic OR luteolin OR malvidin* OR matairesinol OR medioresinol OR melanettin OR mellein OR methoxyphen* OR methylgalangin OR morin OR myricetin OR naphthoflavone* OR naphthoquinone* OR naring* OR narirutin OR neodiosmin OR neoeriocitrin OR neohesperidin OR nepetin OR nobiletin OR norathyriol OR nortrachelogenin OR O-Desmethylanholensin OR oleacein OR oleocanthol OR oleoside OR oleuropein OR orobol OR paeoniflorin OR paeonol OR pallidol OR patuletin OR pebrellin OR pelargonidin OR peonidin OR petunidin OR phenacetylglucose OR phenolic OR "phenolic terpene*" OR phenylacetic OR phloretin OR phloridzin OR phlorin OR piceatannol OR pinocembrin OR pinoresinol OR pinosylvic OR pinotin OR poncirin OR procyanidin OR prodelphinidin OR protocathechuic OR provinol OR prunetin OR pseudobaptigenin OR psoralen OR pterostilbene OR puerarin OR punicalagin OR punicalin OR pyrogallol OR quercetin OR resacetophenone OR resveratrol OR rhamnetin OR rhoifolin OR rosmadial OR rosmannol OR rosmarinic OR sakuranetin OR salvianolic OR sanguin OR sativanone OR schisan* OR schottenol OR scopoletin OR scutellarein OR secoisolariciresinol OR sesamin* OR sesamol* OR sesquilignan OR sinap* OR sinensetin OR sitosterol OR spinacetin OR stevenin OR stigmastanol OR stilbene* OR silymarin OR syring* OR tangeretin OR tectoridin OR tectorigenin OR tetramethylscutellarein OR

	theaflavin OR thymol OR tigloylgomicin OR todolactol OR trachelogenin OR tyrosol* OR umbelliferone OR urolithin OR valoneic OR vanilli* OR vanilloyl* OR verbascoside OR vestitone OR viniferin OR violanone OR vitisin OR xanthohumol OR xanthotoxin) AND (human* OR participant* OR patient* OR men OR women OR male* OR female* OR volunteer* OR subject*) AND (diet* OR food* OR nutrition* OR intake) AND (metabol* OR metabotype OR absorption OR distribution OR elimination OR excretion OR bioavailable OR bioavailability OR bioaccessible OR bioaccessibility OR kinetic* OR nutrikinetic*) AND (SNP OR polymorphism* OR “genetic variant*” OR “gene variant*” OR mutation OR genotyp* OR allele* OR homozyg* OR heterozyg* OR “linkage disequilibrium” OR “linkage equilibrium”)
Databases/registries/repositories mined/searched	PubMed/MEDLINE, Scopus, WoS, EMBASE, CAS/ACS, HuGE Navigator Database (PHGKB v8.5)
Inclusion criteria	P (humans) I/E (exposed to dietary (poly)phenols) C (stratified according to their genotype status; age) O (quantification of (poly)phenol-related plasma and/or urinary metabolites by any separation methodology/technique of analytical chemistry) S (any original study, observational/interventional, retrospective/prospective, cross-sectional/longitudinal, randomized/non-randomized, parallel/cross-over, placebo/non-placebo-controlled, pilot, etc.)
Exclusion criteria	P (in vitro studies - cellular -, in silico/computational studies) I/E (not exposed to dietary (poly)phenols) C (not stratified according to their genotype status) O (lack of quantification of (poly)phenol-related plasma and/or urinary metabolites) S (reviews of any type)
Time filter	None applied
Language filter	None applied
Target journals hand-searched	Biochem Pharmacol; Br J Nutr; Cancer Epidemiol Biomarkers Prev; Eur J Pharmacol; Int J Mol Epidemiol Genet; Mol Nutr Food Res; Nutrients; PLoS One

Supplementary Table 2. Overview of the studies excluded with reason.

Study excluded with reason	Reason for exclusion
Dostal et al. (28-30)	Study of the effects of phase II metabolism (COMT)-related SNPs on anthropometric features, obesity-related hormone levels, and glucose homeostasis after exposure to green tea flavan-3-ols
Hursel et al. (31)	Study of the impact of phase II metabolism (COMT)-related genetic variations on basal metabolic rate and substrate oxidation after exposure to green tea flavan-3-ols
McCann et al. (32)	Study of the effects of phase I metabolism (CYP17A1)-related SNPs on breast cancer risk after dietary lignans intake
McCann et al. (33)	Study of the impact of phase I (CYP1B1) and phase II (COMT) metabolism-related genetic variations on the urinary excretion of estrogen metabolites (2-hydroxyestrone and 16 α -hydroxyestrone)
Miller et al. (34)	Study of the effects of phase II metabolism (COMT)-related genetic variations on acute vascular reactivity, blood pressure, and insulin response after exposure to green tea flavan-3-ols
Quian et al. (35)	Study of the effects of SOCS3-related SNPs on the risk of type 2 diabetes after the exposure to fruit and vegetable (polyphenols;
Qin et al. (36)	Study of the impact of phase I (CYP1A1, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2S1, NAT1, and NAT2) and phase II (SULT1E1) metabolism-related genetic variations on the risk of idiopathic male infertility after exposure to dietary lignans and isoflavones
Wu et al. (37)	Study of the impact of phase II metabolism (COMT)-related SNPs on the risk of breast cancer after exposure to green and black tea polyphenols

Supplementary Table 3. Detailed characteristics of the studies included in the present review. Abbreviations: GWAS (genome-wide association study); NA (not available).

Study	Brown et al., 2011 (38)	Chang et al., 2019 (39)	Fraga et al., 2022 (40)	Hong et al., 2012 (41)	Inoue-Choi et al., 2010 (42)	Lorenz et al., 2014 (43)	Miller et al., 2012a (44)	Miller et al., 2012b (45)	Momma et al., 2023 (46)	Muhrez et al., 2017 (47)	Scholl et al., 2018 (48)	Wakeling and Ford, 2012 (49)
Study design	Single-centre, placebo-controlled, double-blinded, cross-over study with age- and BMI-matched groups	Randomized cross-over study	Non-randomized, pre-post design study	Genetic epidemiological study (GWAS), as ancillary analysis of the ongoing population-based cohort KoGES study	Cross-sectional sub-analysis nested within the Shanghai Cohort Study, a population-based, prospective investigation of diet and cancer in 18,244 men	Non-randomized, pre-post design study	Pilot, non-placebo-controlled trial	Randomized, double-blinded, placebo-controlled, crossover trial, with age- and BMI-matched groups	Observational study, as ancillary analysis of the ongoing population-based cohort EPIC-Norfolk study	Metabolomics study	Population-based, open, one-arm nutrkinetic study	Non-randomized, pre-post design study
Country	UK	USA	Brazil	South Korea	China	Germany	UK	UK	UK	France	Germany	UK
Sample size	64 (out of 83); 71 in the ITT dataset, 5 of which excluded due to lack of adherence to the protocol requirements	252 (out of 258); 190 completed all phases	46 (out of 110)	1,391 out of an initial cohort of 10,038 subjects (416 equal nonproducers, 975 equal producers)	660 (out of 774, from an initial list of 1,881)	24	20	47 (out of 50; 3 removed due to lack of adherence to protocol)	16,672	53	84 (out of 89)	100 (out of 212); 97 completed the trial successfully, 3 were excluded after quality checks
Age	40–69y	59.4±6.2y [45–75y] (59.7±6.2 in those who have completed the study)	26.26±4.50y [19–38y]	52.0y (52.9±8.3y in equal nonproducers, 51.6±8.4y in equal producers)	56.7±5.0y	NA	54.6±3.2y [18–70y]	36.2y (37.9±18.2 for the COMT AA group, 34.2±17.2 for the COMT GG group)	NA	23y [20–59y]	24.5±3.9y [19–49y]	33.0±9.3y ([18–50y], median 31.5y)

Sex/gender	M (100.0%)	F (100.0%)	20M (43.5%) and 26F (56.5%)	758M (54.5%), 633F (45.5%); 193M, 223F in equol nonproducers, 565M, 410F in equol producers	M (100.0%)	10M (41.7%) and 14F (58.3%)	14M (70.0%) and 6F (30.0%)	M (100.0%)	9020F (54.1%), 7652M (45.9%)	24M (45.3%) and 29F (54.7%)	30M (35.7%) and 54F (64.3%)	F (100.0%)
BMI	Overweight or obese [28-38]	30.5±7.6 (30.1±7.6 in those who have completed the study); 60 (23.8%) normal; 71 (28.2%) overweight; 121 (48.0%) obese (48 (25.5%) normal; 51 (27.1%) overweight; 89 (47.3%) obese in those who have completed the study)	23.23±2.54 [18.50-29.9]	24.9 (25.1±3.0 in equol nonproducers, 24.8±3.1 in equol producers)	NA	NA	27.4 (27.0±2 in the COMT AA, 27.7±2 in the COMT GG)	24.2 (24.6±2.9 for the COMT AA group, 23.7±2.8 for the COMT GG group)	NA	NA	22.7±2.6	23.4±3.4
Ancestry	Caucasian ancestry	115 African ancestry and 137 European ancestry	NA	Asiatic ancestry	Asiatic ancestry	Caucasian ancestry	Caucasian ancestry	Caucasian ancestry	Caucasian ancestry	Caucasian ancestry	Caucasian ancestry	Caucasian ancestry
Physical activity	Sedentary (30 min of moderate physical activity on most days); strenuous exercise restrained during the intervention period; monitored by means of	NA	Being a high-performance athlete was an exclusion criterion	NA	NA	NA	NA	Intensive exercise restrained 24h before each study visit	NA	NA	No one engaged in "extensive physical activity"	NA

	paper diaries											
Smoking status	Non-smoking as per inclusion/exclusion criteria	50 (19.8%) current smokers, 85 (33.7%) former smokers, 117 (46.4%) never smokers (34 (18.1%) current smokers, 65 (34.6%) former smokers, 89 (47.3%) never smokers)	Non-smoking	730 (52.7%) never smokers, 291 (21.0%) former smokers, 363 (26.2%) current smokers (238 never smokers, 74 former smokers, 100 current smokers in equol nonproducers; 492 never smokers, 217 former smokers, 263 current smokers in equol producers)	60% current smokers	NA	Non-smoking	Non-smoking	NA	NA	56 (66.7%) never smokers, 8 (9.5%) former smokers, 20 (23.8%) current smokers	Non-smoking (as per inclusion/exclusion criteria)
Alcohol intake	0.00±0.00 g/d in the placebo group and 0.01±0.08 g/d in the DGT group; alcohol consumption restrained and screened only during 24h before each timepoint of the intervention	NA	No (alcohol intake was an exclusion criterion)	571 (41.3%) never drinkers, 730 (52.8%) former drinkers, 82 (5.9%) current drinkers; 192 never drinkers, 194 former drinkers, 28 current drinkers in equol nonproducers; 379 never drinkers, 536	49% current consumers of alcoholic beverages on a weekly basis	NA	Alcohol intake restrained 24h prior the study	Alcohol intake restrained 48h before each study visit	NA	NA	75 (89.3%) consumers of alcoholic beverages	NA

	n period (study visit) and during ambulatory blood pressure monitoring; no details about previous alcohol intake			former drinkers, 54 current drinkers in equal producers								
Use of contraceptives	Not applicable	Post-menopausal (without a menstrual cycle in the past 12 months); use of oral antibiotics, hormone replacement therapy, nonprescription hormones or herbal supplements for menopausal symptoms in the 2 months prior to baseline were exclusion criteria	No, hormone therapy was an exclusion criterion	NA	Not applicable	NA	NA	Not applicable	NA	NA	37 (44.0%) ever used contraceptives, 47 (56.0%) never used contraceptives	Pre-menopausal (pregnancy was an exclusion criterion)
Use of drugs	No drugs /medications as per inclusion/exclusion criteria	No use of drugs/medications as per exclusion criteria	No drugs /medications as per inclusion/exclusion criteria	NA	NA	NA	No drugs /medications as per inclusion/exclusion criteria	No drugs /medications as per inclusion/exclusion criteria	NA	NA	No use of drugs as per exclusion criteria	No (use of antibiotics for 3 months prior to the study, and routine use of any medication were exclusion criteria)
Dietary habits	Dark-chocolate intake and consumption of foods	Use of black-cohosh, or flaxseed supplements in the 2 months	No dietary supplement s; use of vitamin and mineral	NA	Daily green tea drinkers	NA	Foods/beverages with flavonoids restrained	Fruit and vegetables of low-medium-high flavonoid content, fruit	NA	NA	10 (11.9%) habitual green tea drinkers	No use of regular soy supplements as per inclusion/exc

	and beverages rich in polyphenols restrained and screened during 24h before each timepoint of the intervention period (study visit) and during ambulatory blood pressure monitoring. Green tea intake prohibited during the entire intervention period	prior to baseline was an exclusion criterion; intake of seeded bakery products and cereals restrained during the 5-month period of the study	supplements, or supplements containing plant foods or extracts were exclusion criteria				24h prior the study	juice, jams and preserves, chocolate and chocolate-containing products, tea, coffee, and soy products intake restrained 48h before each study visit				lusion criteria; isoflavones intake restrained for 3 days prior to the study and on the study day
Dietary assessment	24h food diaries	12 telephone-administered 24-hour dietary recalls assigned randomly throughout the 5-month active intervention period	24h dietary recall	NA	FFQ with 45 food groups	NA	NA	Investigator-led 24 h food recall	NA	NA	NA	NA
Gene(s)	COMT	Genes involved in steroid hormone metabolism (ARPC1A, BAIAP2L1, BCL2L11, BMF, COMT, CYP17A1, CYP19A1, CYP1A1, CYP1B1, CYP3A4, CYP3A5,	SULT1A1, SULT1C4, ABCC2/MRP2	No preferential gene (the whole genome has been explored)	COMT	COMT	COMT	COMT	PON1	ABCC2/MRP2	SLCO1B1/OATP1B1, COMT, ABCC2/MRP2, ABCB1/PGP/MDR1, UGT1A1, SULT1A1	UGT1A1, CBG, LPH, ABCC2/MRP, ABCG2/BCRP

		ESR1, GCKR, JMJD1C, LHCGR, NR2F2, PRMT6, SHBG, SLCO1B1/OATP1 B1, SULT1A1, TSPYL5, UGT1A1, ZBTB10, ZKSCAN5, ZNF652) out of an initial list that included also HHEX, HSD17B, and TDGF3										
SNP(s)	COMT (rs4680)	116 SNPs (including 60 putatively functional SNPs and 56 AIMs) from an initial list of 70 functional SNPs in 29 genes (ARPC1A, BAIAP2L1, BMF, COMT, CYP17A1, CYP19A1, CYP1A1, CYP1B1, CYP3A4, CYP3A5, ESR1, GCKR, HHEX, HSD17B1, JMJD1C, LHCGR, NR2F2, PRMT6, SHBG, SLCO1B1, SULT1A1, TDGF3, TSPYL5, UGT1A1, ZBTB10, ZKSCAN5, ZNF652), and 60 SNPs selected as AIMS	SULT1A1 (rs3760091, rs4788068), SULT1C4 (rs1402467), ABCC2 (rs8187710)	No preferential SNP (all the SNPs have been investigated)	COMT (rs4680)	COMT (rs6269, rs4633, rs4680, rs4818)	COMT (rs4680)	COMT (rs4680)	PON1 (rs662, rs854560, rs705379, rs705381, and rs854572)	ABCC2/MRP2 (rs717620, rs3740066, rs717620, rs8187710), OATP1B1/SLCO1 B1 (rs4149056, rs2306283, rs11045819), Pgp/ABCB1 (rs1045642, rs1128503, rs2032582), COMT (rs4680), SULT1A1 (rs750155), UGT1A1 (rs8175347)	MRP2/ABCC2 (rs2273697, rs3740066, rs717620, rs8187710), OATP1B1/SLCO1 B1 (rs4149056, rs2306283, rs11045819), Pgp/ABCB1 (rs1045642, rs1128503, rs2032582), COMT (rs4680), SULT1A1 (rs750155), UGT1A1 (rs8175347)	UGT1A1 (rs8175347), CBG (rs358231), ABCG2 (rs2231142), and ABCC2 (rs2273697), LPH (rs3754689)
Source, polyphenol (s) and monitored metabolite s	Green tea (Gallo)catechins and gallic acid: EGCG	Ground flaxseed Lignans, mainly secoisolariciresinol-diglucoside	Orange juice Flavanones, mainly hesperetin	Free diet (not better specified) Isoflavones	Green tea in daily free diet (Gallo)catechins	EGCG, pure compound (750 mg) EGCG	Green tea (EGCG 448, EGC 178, EC 96, ECG 66 mg, GC 22,	Green tea (EGCG 460, EGC 116, ECG 94, EC 88, GC 22, GCG 18, C	Free diet (not better specified) Flavan-3-ols (catechins	Free diet (not better specified)	Green tea (Gallo)catechins Plasma (gallo)catechin	Soy Isoflavones, mainly daidzein,

	40.71%, EGC 16.27%, EC 8.74%, ECG 6.02%, GC 2.02%, GCG 1.27%, C 1.16%, GA 0.75%, CG 0.03%) Plasma EGCG and urinary gallo catechin metabolites (EGC and 4'-O-methyl-EGC)	Secoisolariciresinol urinary metabolites: secoisolariciresinol, enterodiol, enterolactone	and naringenin Phase II urinary metabolites (sulfates and glucuronides) of hesperetin and naringenin	Serum equol	Galocatechin urinary metabolite s: EGC, 4'-O-methyl-EGC, EC, 5-(3',4',5'-trihydroxyphenyl)- γ -valerolactone, and 5-(3',4'-dihydroxyphenyl)- γ -valerolactone	Plasma EGCG	GCG 14, C 12, GA 8) (Gallo)catechins and gallic acid Plasma (gallo)catechin metabolites: EGC, ECG, EC, GCG, GC, CG, EGCG, 4'-O-methyl-EGCG, 4''-O-methyl-EGCG, 4',4''-O-dimethyl-EGCG and 3',4',4''-O-trimethyl-EGCG	12, GA 12, CG 2) (Gallo)catechins and gallic acid Plasma (gallo)catechin metabolites (C, EC, EGC, ECG, EGCG, 4'-O-methyl-EGCG, 4''-O-methyl-EGCG, 4',4''-O-dimethyl-EGCG) and urinary (gallo)catechin metabolites (EGC and 4'-O-methyl-EGC)	and procyanidins) Flavan-3-ol gut microbial metabolites in urine: 5-(4'-hydroxyphenyl)- γ -valerolactone-3'-sulfate and 5-(4'-hydroxyphenyl)- γ -valerolactone-3'-glucuronide (sum used as flavan-3-ol intake biomarker)	Several phenolic acids and flavonoids 181 urinary metabolites, including some (poly)phenol metabolites	metabolites: EGCG, EGC, ECG, EC	genistein, and glycitein Phase II urinary metabolites (sulfates and glucuronides) and aglycones of daidzein, genistein and glycitein
Source and mode of consumption	Green tea (530 mg decaffeinated green tea extract with >75% catechins and <1% caffeine per capsule; 2 capsule/d, 800 mg total catechins, 1 h before breakfast and 1 h before the evening meal; about six-to-eight	Ground brown flaxseed (a standardized scoop, equivalent to 10 g/d) or usual diet	Pasteurized orange juice (Citrus sinensis L; a single dose of 500 mL; 25.46 mg per 100 mL of hesperidin and 1.95 mg per 100 mL of narirutin)	Free diet	Green tea	Pure EGCG (750 mg-five capsules of 150 mg each) served with water and a croissant	Decaffeinated green tea extract (1.1 g, containing 836 mg green tea catechins), with a standardized carbohydrate-rich breakfast meal (567 kcal, 107 g carbohydrate, 18.1 g protein, and 7.4 g fat) given after 60 minutes, followed by a	Decaffeinated green tea extract (1.06 g extract, 828 mg green tea catechins, ~8 cups of green tea, EGCG 40.71%, EGC 16.27%, EC 8.74%, ECG 6.02%, GC 2.02%, GCG 1.27%, C 1.16%, GA 0.75%, CG 0.03%, caffeine 0.6%; 550 mg per capsule) or placebo	Free diet	Free diet	Green tea extract (standardized to 225 mg total catechins, 150 mg EGCG, 18 mg EGC, 35 mg ECG, and 10 mg EC plus 1.3 mg caffeine) twice a day (in the morning and in the evening, with a 12-hour interval); a standardized meal and snack were offered 4 h and 10 h after the intake of the capsule.	Commercial soy supplement with water allowed (three capsules) as a single bolus dose; each 750-mg capsule contained 23 mg isoflavone (13.5 mg (32.4 μ mol) daidzin and daidzein, 2.5 mg (5.8 μ mol) genistin and

	cups of moderate-strength green tea daily) or placebo (949 mg lactose per capsule). During the study visit a standardized breakfast meal (100 g Kellogg's Frosties, 300 ml semi-skimmed milk, 250 ml water (energy 2171 kJ (519 kcal); 17 g protein; 99 g carbohydrate; 6 g fat) was offered						standardized low-flavonoid lunch meal (661 kcal, 74.4 g carbohydrate, 15.8 g protein, and 33.3 g fat)	(lactose placebo capsules taken with 250 mL water)				genistein, and 7 mg (15.7 μmol) of glycitin and glycitein), with the aglycone content being 0.03–0.06% of the total isoflavone content; after capsule ingestion, fasted status was continued for 3h
Specimen analyzed	Blood and urine (24 h urine samples)	Blood, and urine (overnight urine samples)	Blood (24 h urine samples) and urine	Blood (serum)	Blood and urine (spot urine samples)	Blood	Blood	Blood and urine (24 h urine samples)	Spot urine	Blood and urine (24 h urine samples)	Blood	Blood and urine (24 h urine samples)
Detection and measurement technique	HPLC coupled with UV/VIS and electrochemical detector for plasma metabolites, and HPLC-MS for	GC-MS	HPLC-QqQ-MS/MS	LC-MS/MS	HPLC coupled with UV/VIS and electrochemical detector	HPLC coupled with UV/VIS and electrochemical detector	UPLC-qTOF-MS and HPLC coupled with UV/VIS and electrochemical detector	UPLC-MS/MS for plasma metabolites, and HPLC-MS for urine metabolites	UPLC-MS	GC-MS	HPLC-MS/MS	HPLC coupled with UV/VIS and electrochemical detector

	urinary metabolites											
Genotyping technique	Direct sequencing	Multiplexing (MassARRAY technology and iPLEX Gold assay)	KASP and agarose gel electrophoresis	Genome-wide human SNP array	PCR-based fluorogenic assay	TaqMan-PCR assay	TaqMan-PCR assay	TaqMan assay (PCR-based, 5'-nuclease allelic discrimination assay)	NA	PCR and direct sequencing	PCR and melting curve analysis	PCR and melt curve analysis
Genotype frequencies and genotypes in HWE	NA	NA	Yes, with one exception (SULT1C4 rs1402467, in both groups of low- and high-excretors)	SNPs with a missing genotype call rate >0.1, minor allele frequency <0.01, and HWE ($p < 1 \times 10^{-6}$) were excluded	Yes	Evidence of linkage disequilibrium	10 GG and 10 AA COMT genotype	25 GG and 25 AA COMT genotype	SNPs have been selected based on their wide distribution in the general population (MAF >20%) and biological activity (they are known to modulate enzyme activity or expression level)	Yes	Yes	Yes (assessed by means of chi-squared tests)
Genetic modeling/test	Four genetic analyses tested (dominant model, recessive model, additive model, and model-free computation)	Additive model	Five genetic models tested (dominant, co-dominant, over-dominant, recessive, log-additive)	Logistic regression model controlling for area, age, and sex/gender and a general linear analysis model with type III sum of square to test the effects of the interactions between genotype	Genotypes were grouped based on the presence of the minor allele	Genotypes were grouped based on the presence of the minor allele	Only GG and AA genotype groups	Only GG and AA genotype groups	Three models (one stratified by sex and adjusted by age, one adjusted by age and biomarker, one adjusted by age and HDL)	Genotypes were grouped based on the presence of the minor allele	Genotypes were grouped based on the presence of the minor allele	Genotypes were grouped based on the presence of the minor allele

				and the equol- producing phenotype								
Statistical analysis	2 df ANOVA, models with age and BMI as covariates	Paired t tests, repeated measures (ANOVA) analysis; linear regression models, repeated measures mixed models, with baseline age and BMI as covariates	Multiple logistic regression models	Chi-squared test, t-test, multiple linear regression, controlling for area, sex/gender, age, smoking, and alcohol consumption	Chi- squared test, ANOVA, multivariable regression models with age, number of cigarettes smoked per day, number of alcoholic drinks consumed per day as covariates	Unpaired Student's t- tests (or their non- parametric al versions, in the scenario of violation of Gaussian distribution)	Pharmacokin etics/nutrikin etics analysis	Three-way interaction analysis (COMT genotype, treatment, and time)	Chi-squared test, Wald test, linear regression models using log- transforme d of metabolite concentrati on values	PCA, OPLS-DA	Pharmacokinetics /nutrikinetics analysis	Multivariate analysis of variance with age and BMI as covariates
Statistical threshold	p<0.05; correction for multiple testing was not conducted	Correction for multiple testing (according to Bonferroni); adjusted p-value threshold of 8.3e-4	p<0.05; correction for multiple testing was not conducted	p-value <1 x 10 ⁻⁵ (instead of the genome- wide significance threshold, to avoid type II errors and screen genetic variants with small effects on the trait of interest)	p<0.05; correction for multiple testing was not conducted	p<0.05; correction for multiple testing was not conducted	p<0.05; correction for multiple testing was not conducted	NA	p<0.05; correction for multiple testing was not conducted	A Bonferro ni correctio n for multiple testing was applied, giving a significan ce threshold 0.0167 (=0.05/3) for associatio n p-values	p<0.05; correction for multiple testing was not conducted	p-value <0.05
Goodness- of-fit and validation	NA	NA	NA	NA	NA	NA	NA	NA	NA	Yes, including R2 and Q	Yes, including goodness-of-fit plots	NA
Interventio n duration	6w	6w	24h	Observationa l	Observatio nal	2h	8h	24h	Observation al	Observati onal	5d	24h

Timepoint(s)	4 timepoints (start of the study, after 6w, after 2w, after 6w)	4 timepoints (start of the study, after 6w, after 2mo, after 6w)	At the start of the study and after 24h	Not applicable	Not applicable	Before and 2 h after the capsule intake	0, 30, 60, 90, 120, 150, 180, 240, 360 and 480 min	At the baseline, at 105, 165, and 225 min (for blood samples), 5.5 h and 18.5 h (for urine samples) for each study visit; a 10d-interval; second study visit	Not applicable	NA	At 0.5, 1, 2, 3, 4, 5, 7, and 9 hours	24h (at 0, 8, and 16h)
Adherence to protocol	Pill count	Uneaten flaxseed was returned to monitor compliance	NA	Not applicable	Not applicable	NA	NA	Monitored (investigator-led 24 h food recall to monitor compliance with low-flavonoid diet)	Not applicable	NA	NA	NA
Metabolite change	↑ plasma EGCG, urinary EGC and urinary 4'-O-methyl EGC by 28-fold and 34-fold	↑ ENL excretion; higher in EA women	NA	Not applicable	Not applicable	↑ plasma EGCG (mostly in the unconjugated (non-sulfated and non-glucuronidated) form)	NA	Lower urinary 4'-O-methyl EGC in the AA genotype group only during the 5.5 h collection period ($p=0.049$)	Not applicable	NA	Large interindividual variability in plasma levels of EGCG, EGC, and ECG	Large interindividual differences with respect to recovery of total isoflavone dose administered (more than eight-fold)
Other findings	No changes in blood pressure, and heart rate; reduction in body weight ($p=0.037$); small effect ($p=0.023$; $p=0.04$)	NA	NA	Five SNPs (rs6927608, rs4946645, rs11759010, rs17065302, rs4245525) in a genetic locus (6q21 region) flanked by HACE1, LIN28B,	NA	Large differences in enzyme activities between different COMT genotypes; increase in COMT activity by 24% after	↑ 50-72% insulin; no changes in blood glucose, vascular reactivity, total nitrite, lipids, plasma total antioxidant capacity or	Significant genotype x treatment interaction in vascular function (digital volume pulse reflection index, $p=0.014$), especially in	No differences in the sum of 5-(4'-hydroxyphenyl)- γ -valerolactone-3'-sulfate and 5-(4'-hydroxyphenyl)- γ -	NA	NA	NA

	after removal of an outlier) on LDL-cholesterol reduction			BVES, and POPDC3, have been associated with equol production. Significant equol x SNP interaction on systolic blood pressure ($p=1.3 \times 10^{-4}$). Nominal effect on diastolic blood pressure ($p=0.046$ controlling for area, age, sex/gender, $p=0.041$, controlling for area, age, sex/gender, alcohol drinking, and smoking).		EGCG supplementation	inflammatory markers; bimodal curves were observed for the non-gallated green tea catechins as opposed to single-peaked curves for the gallated green tea catechins	the AA genotype group exhibiting more beneficial changes from baseline compared to placebo ($p=0.029$); borderline effect for diastolic blood pressure ($p=0.07$) and systolic blood pressure ($p=0.17$)	valerolactone-3'-glucuronide metabolites in men, and only small differences among rs662 and rs705381 alleles in women. No association between SNP (rs662 or rs705381) and systolic blood pressure neither in men nor in women ($p=0.81$ for rs662 and $p=0.19$ for rs705381)			
Study strengths	Power analysis and sample size a priori analysis (however, based on another outcome); compliance/adherence monitored; study design (randomized, placebo-controlled,	Cross-over design; few confounders; adherence/compliance monitored; large number of SNPs; genetic admixture and ancestral analysis	Different genetic models tested; genotype and haplotype analyses	Study design (GWAS), sample size	Sample size; study of dose effect	High doses in vivo	Pharmacokinetic modeling	Power analysis and sample size a priori analysis (however, based on another outcome); study design (randomized, placebo-controlled)	Sample size	Metabolomics approach	Modeling supplement, data stratified by sex/gender, extensive statistical analysis, a priori study protocol	Various SNPs in various genes; a priori sample size and power analysis based on the genetic outcome

	complete crossover trial); extensive genetic analysis and models tested											
Study limitations	Small sample size; treatment order bias (seasonal effects detected); short duration of intervention	Candidate SNP approach; only SNPs with MAF>10% were studied, less common SNPs were not investigated; small sample size; lack of a replication population	Study design	Lack of information concerning the diet of study participants; lack of replication/validation of the findings	Study design (cross-sectional)	Study design (non-randomized, non-placebo controlled), with a lack of a control group	Pilot study, small sample size, study design	Only AA and GG genotype groups; limited number of time points used to measure the levels of circulating catechins	Lack of details concerning the population and the method	No characterization of the cohort	Study design	Short duration of the intervention; study design (non-randomized)
Trial registration (for randomized)	No	Yes (NCT01698294)	Not applicable	Not applicable	No	Not applicable	Not applicable	No	Not applicable	No	Not applicable	Not applicable
Funding/sponsorship	"The authors are all employed by Unilever Research & Development, which is a division of Unilever plc, a company which has a significant commercial interest in tea. Unilever plc provided all funding for the study"	One of the authors is a consultant/advisory board member for U.S. Highbush Blueberry Council. The research was supported by National Cancer Institute (NCI) grants U01CA161809 and P30CA016056	"This work was supported by the Food Research Center (FoRC), CEPID-FAPESP (Research, Innovation and Dissemination Centers, São Paulo Research Foundation), São Paulo, SP, Brazil [grant	"This work was supported by the Korean Genome Analysis Project (4845-301) and the KoGES (4851-302), funded by the Ministry for Health and Welfare, Republic of Korea"	This work was supported by the United States National Institutes of Health (grants R01 CA98497, R01 CA43092, and P01 CA88961).	German Research Council (DFG Exc 257)	Sponsor: Unilever Discover, Colworth, UK	The scholarship of one of the authors has been "sponsored by Unilever Discover, Colworth, UK", authors declare no COIs	Two authors "are employed by Mars Inc., a company engaged in flavan-3-ol research and flavan-3-ol-related commercial activities", while another author "received an unrestricted grant	Sponsors: Ligue Régionale contre le Cancer and the University Hospital of Tours	NA	Funded by the UK Biotechnology and Biological Sciences Research Council (BBSRC; research grant BBE0074571)

			number 2013/07914-8]; São Paulo Research Foundation (FAPESP); and National Council for Scientific and Technological Development (CNPq, Grants 141878/2019-3)"						from Mars, Inc."			
Findings reporting	None	CONSORT	None	None	None	None	None	None	None	None	"Transparent Reporting of Evaluations with Nonrandomized Designs" (TREND) checklist	None

Supplementary Table 4. Detailed outcomes of the studies included in the present review.

Study	Impact of the SNP on polyphenol metabolism	Details
Brown et al., 2011 (38)	Impact of COMT genotype (rs4680) on urinary levels of epigallocatechin (EGC) and 4'-O-methyl-EGC	Mean urinary concentrations of EGC and 4'-O-methyl-EGC lower ($p < 0.01$) in individuals homozygous for the high-activity COMT G-allele. A recessive genetic model for the G allele was found to fit the data well (for EGC, 0.0035 versus 0.013 for the model-free computation, 0.0081 for the additive model, and 0.24 for the dominant model; for 4'-O-methyl EGC 0.0075 versus 0.029 for the model-free computation, 0.021 for the additive model, and 0.37 for the dominant model)
Chang et al., 2019 (39)	Impact of some SNPs in genes involved in xenobiotic phase I and II metabolism, excretion and steroid hormone metabolism on enterolactone urinary excretion, with an effect depending on ethnicity	In European ancestry women, 12% of the variation in baseline enterolactone excretion was explained by rs6738028 in BCL2L11, rs4869739 in ESR1, and rs6259 in SHBG, whilst 17% of the variation in post-intervention enterolactone excretion was explained by rs4869739 in ESR1, rs6259 in SHBG, and rs4149056 in SLCO1B1. In African ancestry women, 16% of the variation in baseline enterolactone excretion was explained by rs3020418 in ESR1, rs740160 in ARPC1A, rs11761528 in ZSCAN5, rs11974702 and rs7809615 in CYP3A5, and rs4633 in COMT, whereas 13% of the variation in post-intervention enterolactone excretion was explained by rs1056827 in CYP1B1, rs3020418 in ESR1, rs740160 in ARPC1A, rs11761528 in ZSCAN5, and rs11974702 and rs7809615 in CYP3A5
Fraga et al., 2022 (40)	Impact of SULT1A1 genotype (rs4788068), SULT1C4 genotype (rs1402467), and ABCC2/MRP2 genotype (rs8187710) on urinary levels of phase II metabolites of orange juice flavanones (hesperetin and naringenin); SULT1A1 genotype (rs3760091) was not statistically associated with orange juice flavanone metabolism and excretion; an association of the GGCC and GCTG haplotypes of ABCC2_rs8187710/SULT1A1_rs3760091/S	SULT1A1 rs4788068 was statistically associated with high excretion of phase II flavanone conjugates, with an OR of 0.2 ([95%CI 0.07–0.61], $p=0.0015$) at the log-additive model, an OR of 0.13 ([95%CI 0.03–0.52], $p=0.0017$) at the dominant model, an OR of 0.15 [95%CI 0.04–0.63] for the T/C genotype and 0.06 [95%CI 0.01–0.72] for the T/T genotype ($p=0.0053$) at the codominant model, an OR of 0.29 ([95%CI 0.09–0.98], $p=0.041$) at the overdominant model, an OR of 0.18 ([95%CI 0.02–1.73], $p=0.095$) at the recessive model; SULT1C4 rs1402467 was statistically associated with high excretion of phase II flavanone conjugates, with an OR of 0.11 ([95%CI 0.03–0.41], $p=5.00 \times 10^{-4}$) at the dominant model, an OR of 0.25 ([95%CI 0.09–0.68], $p=0.0021$) at the log-additive model, an OR of 0.09 [95%CI 0.02–0.46] for the G/C genotype and 0.13 [95%CI 0.02–0.83] for the G/G genotype ($p=0.0022$) at the co-dominant model, an OR of 0.16 ([95%CI 0.04–0.69], $p=0.0084$) at the overdominant model, and an OR of 0.29 ([95%CI 0.05–1.69], $p=0.15$) at the recessive model; ABCC2/MRP2 rs8187710 was statistically associated with high excretion of phase II flavanone conjugates, with an OR of 0.13 ([95%CI 0.01–1.25], $p=0.041$) SULT1A1 rs3760091 was not statistically associated, with an OR of 0.18 [95%CI 0.02–1.73], $p=0.095$) at the recessive model, an OR of 0.55 ([95%CI 0.21–1.45], $p=0.22$) at the log-additive model, an OR of

	ULT1A1_rs4788068/S ULT1C4_rs1402467 with high excretors of flavanone metabolites was observed	0.17 [95%CI 0.01–1.88] for the G/G genotype and 0.91 [95%CI 0.25–3.31] for the G/C genotype ($p=0.25$) at the codominant model, an OR of 0.71 [95%CI 0.20–2.48] ($p=0.59$) at the dominant model, and an OR of 1.36 [95%CI 0.42–4.40] ($p=0.6$) at the overdominant model
Hong et al., 2012 (41)	Significant effects of SNPs clustered in the 6q21 region, flanked by HACE1, LIN28B, BVES, and POPDC3	The most significant SNP was rs6927608 (allele A>C), and individuals with this minor allele were equal nonproducers (with an OR of 0.57 [95%CI 0.45–0.72], $p=2.5 \times 10^{-6}$). Another significant SNP (rs17065302, allele C>G) is found in an activator protein 1 motif, involved in estrogen receptor-mediated regulation of gene expression
Inoue-Choi et al., 2010 (42)	Weak-borderline impact of COMT genotype (rs4680) on urinary levels of green tea catechin metabolites, including EGC, EC, 4'-O-methyl- EGC, 5-(3',4'- dihydroxyphenyl)- γ - valerolactone), 5- (3',4',5'- trihydroxyphenyl)- γ - valerolactone)	↓ urinary levels of five green tea catechin metabolites in carriers of the homozygous low-activity genotype, reduced by 35-45%, compared to those with the wild type high-activity genotype or the heterozygous variant genotype ($p=0.037$ for 5-(3',4',5'-trihydroxyphenyl)- γ -valerolactone, $p=0.047$ for EGC, $p=0.049$ for 4'-O-methyl-EGC, $p=0.057$ for EC, $p=0.099$ for 5-(3',4'-dihydroxyphenyl)- γ -valerolactone, $p=0.007$ for total tea (poly)phenols)
Lorenz et al., 2014 (43)	No impact of COMT genotype (rs4680, rs6269, rs4633, and rs4818) on plasma levels of epigallocatechin-3-O- gallate (EGCG)	While enzymatic COMT activities were affected by the COMT SNPs ($p<0.001$), EGCG plasma levels significantly increased after intervention and were not influenced by any of the COMT SNPs and different enzyme activities. Furthermore, EGCG ingestion did not result in impairment of COMT activity, which significantly increased by 24% after EGCG consumption
Miller et al., 2012a (44)	No impact of COMT genotype (rs4680) on plasma levels of green tea catechins, including EGCG, EGC, ECG, EC and 4'-O- methyl-EGCG	No statistically significant differences based on COMT genotype could be found in terms of maximum concentration, time to reach maximum concentration, area under the time-concentration curve (AUC), EGCG half-life absorption, and EGCG half-life elimination. However, a trend towards a greater maximum concentration and AUC response in the concentration curve for 4'-O-methyl-EGCG in the COMT AA genotype group could be noted. Also, a tendency towards a higher maximum concentration for the AA genotype for EC, EGC and ECG was detected
Miller et al.,	Partial effect of the COMT genotype	A genotypic effect was observed for urinary 4'-O-methyl-EGC during the first 5.5 h, with the COMT GG group exhibiting a greater

2012b (45)	(rs4680) on urinary levels of 4'-O-methyl-EGC after green tea consumption	concentration ($p=0.049$). No differences could be found for the remaining 18.5 h collection or for total EGC during the 5.5 h and 18.5 h collections. Finally, no difference in plasma green tea catechin concentrations could be computed
Momma et al., 2023 (46)	No differences in the sum of 5-(4'-hydroxyphenyl)- γ -valerolactone-3'-sulfate and 5-(4'-hydroxyphenyl)- γ -valerolactone-3'-glucuronide metabolites in men, and only small differences among rs662 and rs705381 alleles in women	Significant effects for the urinary concentration of a specific set of 5-(3',4'-dihydroxyphenyl)-γ-valerolactone metabolites in women only in the EPIC-Norfolk cohort for rs662 ($p=0.002$ adjusting by age or model 1, $p=0.001$ adjusting by age and structurally related (-)-epicatechin metabolites or model 2, $p=0.002$ adjusting by age and HDL or model 3), similar trends for rs705381 ($p=0.035$ for model 1, $p=0.004$ for model 2, and $p=0.029$ for model 3), with slightly marginal effects in men only, in terms of differential concentration of structurally related (-)-epicatechin metabolites ($p=0.056$ adjusting by age, $p=0.060$ adjusting by age and phenyl-γ-valerolactones; $p=0.056$ adjusting by age and HDL). No significant differences in the distribution of rs705381 alleles among the different 5-(4'-hydroxyphenyl)-γ-valerolactone-3'-sulfate and 5-(4'-hydroxyphenyl)-γ-valerolactone-3'-glucuronide metabolite quintiles in men and women ($p=0.4$ for women; $p=0.6$ for men). Significant differences in the distribution of rs662 alleles among different quintiles in women ($p=0.013$), even if the highest difference in the distribution of alleles was only 6 percentage points between the top and bottom quintiles
Muhrez, et al., 2017 (47)	Impact of ABCC2/MRP2 genotype (rs717620, rs3740066, rs2273697) on urinary levels of some phenolic metabolites deriving from (poly)phenols present in free diet	2-(3',4'-Dihydroxyphenyl)acetic acid and 4'-hydroxyhippuric acid were over-excreted in urine of individuals carrying variants of rs717620 and rs3740066, and less excreted in those carrying variant of rs2273697. On the contrary, benzene-1,3-diol (resorcinol) and 3',4'-dihydroxycinnamic acid (caffeic acid) were over-excreted in urine of those carrying variant of rs2273697, and less excreted in urine of individuals carrying variants of rs717620 and rs3740066
Scholl et al., 2018 (48)	Impact of ABCC2/MRP2 genotype (rs717620, rs3740066), OATP1B1/SLCO1B1 genotype (rs4149056, rs2306283), COMT genotype (rs4680), and UGT1A1 genotype (rs8175347 or UGT1A1*28) on the plasma levels of EGCG, EGC and ECG after green tea consumption	Wildtype carriers of ABCC2 rs717620 exhibited 26% less EGCG relative bioavailability than carriers of the variant allele ($p=0.00721$). Additionally, total body clearance of EGCG was lower in carriers of the C allele of rs4149056 in OATP1B1 ($p=0.01212$). Concerning EGC, its pharmacokinetics and/or relative bioavailability were affected by ABCC2 rs3740066 ($p=0.00153$, a higher intercompartmental clearance in wildtype carriers), OATP1B1 rs2306283 ($p=0.00163$, a 35% reduction in total body clearance in wildtype carriers), ABCC2 rs717620 ($p=0.01654$, a reduction in central volume of distribution in carriers of the T allele), UGT1A1*28 ($p=0.01795$, a 26% reduction in total body clearance in wildtype carriers), and COMT rs4680 ($p=0.02350$, a 24% reduction in relative bioavailability in carriers of the low-activity AA genotype)

Wakeling and Ford, 2012 (49)	Impact of UGT1A1 genotype (rs8175347 or UGT1A1*28), CBG genotype (rs358231), ABCG2/BCRP genotype (rs2231142) and ABCC2/MRP2 genotype (rs2273697) on relative and/or net urinary levels of isoflavone metabolites, including aglycones and phase II metabolites of daidzein, glycitein and genistein	No statistically significant association between the UGT1A1*28 polymorphism and net recovery in urine of any individual isoflavone metabolite under study was found. However, the UGT1A1*28 minor allele was found to be positively associated with percentage of glycitein excreted in urine as sulphate ($p=0.046$), and, after excluding five participants with both minor alleles of CBG and ABCG2, due to a statistically significant interaction ($p=0.025$), positively with percentage of glycitein as sulphate in the urine ($p=0.014$), negatively with percentage of glycitein as glucuronide ($p=0.028$), positively with combined isoflavones as sulphate ($p=0.035$), and positively with sulphate-to-glucuronide ratio for combined isoflavones ($p=0.036$). Finally, SNPs in CBG (rs358231, association with percentage of total isoflavone recovered in urine as glucuronide $p=0.035$), ABCG2 (rs2231142, negative association with percentage of total isoflavone recovered in urine as glucuronide $p=0.042$), and ABCC2 (rs2273697, association with total glycitein excretion $p=0.034$, association with total genistein excretion $p=0.019$) were also found to correlate with levels of isoflavone metabolites excreted in urine

Supplementary Table 5. Functional annotation (in terms of biological process) of the genes putatively associated with (poly)phenol bioavailability and metabolism included in the present systematic review.

Gene Ontology term	Cluster frequency	Genome frequency	Corrected <i>p</i> -value	FDR	False Positives	Genes annotated to the term
estrogen metabolic process	7 of 33 genes, 21.2%	37 of 19817 genes, 0.2%	9.91e-11	0.00%	0.00	UGT1A1, CYP1A1, CYP3A4, SULT1A1, CYP1B1, CYP19A1, CYP3A5
steroid metabolic process	11 of 33 genes, 33.3%	311 of 19817 genes, 1.6%	9.41e-10	0.00%	0.00	ESR1, SERPINA6, UGT1A1, PON1, CYP1A1, SULT1A1, CYP1B1, CYP17A1, CYP3A4, CYP19A1, CYP3A5
flavonoid metabolic process	5 of 33 genes, 15.2%	16 of 19817 genes, 0.1%	2.23e-08	0.00%	0.00	UGT1A1, CYP1A1, SULT1C4, SULT1A1, LPH
hormone metabolic process	9 of 33 genes, 27.3%	231 of 19817 genes, 1.2%	5.70e-08	0.00%	0.00	ESR1, UGT1A1, CYP1A1, SULT1A1, CYP1B1, CYP17A1, CYP3A4, CYP19A1, CYP3A5
xenobiotic metabolic process	7 of 33 genes, 21.2%	108 of 19817 genes, 0.5%	2.47e-07	0.00%	0.00	UGT1A1, CYP1A1, CYP3A4, SULT1C4, SULT1A1, CYP1B1, CYP3A5
organic hydroxy compound metabolic process	11 of 33 genes, 33.3%	553 of 19817 genes, 2.8%	4.45e-07	0.00%	0.00	PON1, LHCGR, CYP1A1, SULT1C4, SULT1A1, LPH, CYP1B1, CYP3A4, COMT, CYP19A1, CYP3A5
regulation of hormone levels	10 of 33 genes, 30.3%	501 of 19817 genes, 2.5%	2.97e-06	0.00%	0.00	ESR1, UGT1A1, CYP1A1, SULT1A1, CYP1B1, CYP17A1, CYP3A4, OATP1B1, CYP19A1, CYP3A5
cellular response to xenobiotic stimulus	7 of 33 genes, 21.2%	168 of 19817 genes, 0.8%	5.46e-06	0.00%	0.00	UGT1A1, CYP1A1, CYP3A4, SULT1C4, SULT1A1, CYP1B1, CYP3A5
toxin metabolic process	4 of 33 genes, 12.1%	17 of 19817 genes, 0.1%	8.28e-06	0.00%	0.00	CYP1A1, CYP3A4, CYP1B1, CYP3A5
primary alcohol metabolic process	6 of 33 genes, 18.2%	104 of 19817 genes, 0.5%	9.89e-06	0.00%	0.00	CYP1A1, CYP3A4, SULT1C4, SULT1A1, CYP1B1, CYP3A5

secondary metabolic process	5 of 33 genes, 15.2%	59 of 19817 genes, 0.3%	2.43e-05	0.00%	0.00	CYP1A1, CYP3A4, SULT1C4, CYP1B1, CYP3A5
response to estradiol	6 of 33 genes, 18.2%	124 of 19817 genes, 0.6%	2.84e-05	0.00%	0.00	ESR1, UGT1A1, BCL2L11, NR2F2, CYP1B1, CYP19A1
organic hydroxy compound catabolic process	5 of 33 genes, 15.2%	63 of 19817 genes, 0.3%	3.39e-05	0.00%	0.00	CYP3A4, COMT, SULT1C4, SULT1A1, LPH
xenobiotic catabolic process	4 of 33 genes, 12.1%	25 of 19817 genes, 0.1%	4.36e-05	0.00%	0.00	UGT1A1, CYP1A1, CYP3A4, CYP3A5
xenobiotic transport across blood-brain barrier	3 of 33 genes, 9.1%	6 of 19817 genes, 0.0%	4.65e-05	0.00%	0.00	ABCG2, ABCB1, ABCC2
alcohol metabolic process	8 of 33 genes, 24.2%	360 of 19817 genes, 1.8%	5.68e-05	0.00%	0.00	PON1, LHCGR, CYP1A1, SULT1C4, SULT1A1, CYP1B1, CYP3A4, CYP3A5
lipid hydroxylation	3 of 33 genes, 9.1%	7 of 19817 genes, 0.0%	8.13e-05	0.00%	0.00	CYP1A1, CYP3A4, CYP3A5
lipid metabolic process	13 of 33 genes, 39.4%	1394 of 19817 genes, 7.0%	8.20e-05	0.00%	0.00	ESR1, SERPINA6, UGT1A1, PON1, CYP1A1, SULT1A1, LPH, CYP1B1, CYP17A1, CYP3A4, COMT, CYP19A1, CYP3A5
androgen metabolic process	4 of 33 genes, 12.1%	30 of 19817 genes, 0.2%	9.39e-05	0.00%	0.00	ESR1, CYP3A4, CYP19A1, CYP17A1
olefinic compound metabolic process	6 of 33 genes, 18.2%	158 of 19817 genes, 0.8%	0.00012	0.00%	0.00	CYP1A1, CYP3A4, CYP1B1, CYP19A1, CYP3A5, CYP17A1
response to xenobiotic stimulus	8 of 33 genes, 24.2%	401 of 19817 genes, 2.0%	0.00012	0.00%	0.00	UGT1A1, CYP1A1, SULT1C4, SULT1A1, CYP1B1, ABCB1, CYP3A4, CYP3A5
retinoid metabolic process	5 of 33 genes, 15.2%	84 of 19817 genes, 0.4%	0.00014	0.00%	0.00	UGT1A1, CYP1A1, CYP3A4, CYP1B1, CYP3A5

retinoic acid metabolic process	4 of 33 genes, 12.1%	34 of 19817 genes, 0.2%	0.00015	0.00%	0.00	UGT1A1, CYP1A1, CYP3A4, CYP3A5
diterpenoid metabolic process	5 of 33 genes, 15.2%	87 of 19817 genes, 0.4%	0.00017	0.00%	0.00	UGT1A1, CYP1A1, CYP3A4, CYP1B1, CYP3A5
sex differentiation	7 of 33 genes, 21.2%	282 of 19817 genes, 1.4%	0.00018	0.00%	0.00	ESR1, LHCGR, BCL2L11, NR2F2, CYP1B1, CYP19A1, CYP17A1
terpenoid metabolic process	5 of 33 genes, 15.2%	98 of 19817 genes, 0.5%	0.00031	0.00%	0.00	UGT1A1, CYP1A1, CYP3A4, CYP1B1, CYP3A5
catabolic process	16 of 33 genes, 48.5%	2544 of 19817 genes, 12.8%	0.00038	0.00%	0.00	PON1, CYP1A1, LPH, CYP1B1, OATP1B1, BCL2L11, CYP19A1, ABCC2, CYP3A5, UGT1A1, BMF, SULT1A1, SULT1C4, CYP3A4, COMT, HACE1
porphyrin-containing compound catabolic process	3 of 33 genes, 9.1%	13 of 19817 genes, 0.1%	0.00066	0.00%	0.00	UGT1A1, OATP1B1, ABCC2
tetrapyrrole catabolic process	3 of 33 genes, 9.1%	13 of 19817 genes, 0.1%	0.00066	0.00%	0.00	UGT1A1, OATP1B1, ABCC2
retinol metabolic process	4 of 33 genes, 12.1%	51 of 19817 genes, 0.3%	0.00083	0.00%	0.00	CYP1A1, CYP3A4, CYP1B1, CYP3A5
gonad development	6 of 33 genes, 18.2%	228 of 19817 genes, 1.2%	0.00102	0.00%	0.00	ESR1, LHCGR, BCL2L11, NR2F2, CYP1B1, CYP19A1
porphyrin-containing compound metabolic process	4 of 33 genes, 12.1%	54 of 19817 genes, 0.3%	0.00105	0.00%	0.00	UGT1A1, CYP1A1, OATP1B1, ABCC2
development of primary sexual characteristics	6 of 33 genes, 18.2%	233 of 19817 genes, 1.2%	0.00116	0.00%	0.00	ESR1, LHCGR, BCL2L11, NR2F2, CYP1B1, CYP19A1
isoprenoid metabolic process	5 of 33 genes, 15.2%	131 of 19817 genes, 0.7%	0.00132	0.00%	0.00	UGT1A1, CYP1A1, CYP3A4, CYP1B1, CYP3A5

cellular catabolic process	12 of 33 genes, 36.4%	1486 of 19817 genes, 7.5%	0.00134	0.00%	0.00	UGT1A1, PON1, CYP1A1, BMF, LPH, CYP1B1, OATP1B1, CYP3A4, BCL2L11, COMT, ABCC2, CYP3A5
organic substance catabolic process	14 of 33 genes, 42.4%	2109 of 19817 genes, 10.6%	0.00146	0.00%	0.00	UGT1A1, PON1, CYP1A1, SULT1C4, SULT1A1, LPH, CYP1B1, OATP1B1, CYP3A4, COMT, CYP19A1, HACE1, ABCC2, CYP3A5
regulation of biological quality	16 of 33 genes, 48.5%	2818 of 19817 genes, 14.2%	0.00155	0.05%	0.02	ESR1, CYP1A1, BAIAP2L1, CYP1B1, JMJD1C, OATP1B1, ABCB1, BCL2L11, CYP19A1, CYP3A5, UGT1A1, ARPC1A, SULT1A1, CYP17A1, CYP3A4, NR2F2
small molecule metabolic process	13 of 33 genes, 39.4%	1855 of 19817 genes, 9.4%	0.00214	0.05%	0.02	UGT1A1, PON1, LHCGR, CYP1A1, SULT1C4, SULT1A1, CYP1B1, CYP17A1, ABCG2, CYP3A4, GCKR, CYP19A1, CYP3A5
organic cyclic compound metabolic process	23 of 33 genes, 69.7%	6047 of 19817 genes, 30.5%	0.00223	0.05%	0.02	ESR1, PON1, PRMT6, CYP1A1, ZNF652, CYP1B1, JMJD1C, ABCG2, OATP1B1, GCKR, CYP19A1, CYP3A5, ABCC2, ZKSCAN5, SERPINA6, UGT1A1, SULT1C4, SULT1A1, CYP17A1, CYP3A4, COMT, NR2F2, ZBTB10
tetrapyrrole metabolic process	4 of 33 genes, 12.1%	67 of 19817 genes, 0.3%	0.00251	0.05%	0.02	UGT1A1, CYP1A1, OATP1B1, ABCC2
demethylation	4 of 33 genes, 12.1%	77 of 19817 genes, 0.4%	0.00439	0.05%	0.02	JMJD1C, CYP1A1, CYP3A4, CYP3A5
alkaloid catabolic process	2 of 33 genes, 6.1%	3 of 19817 genes, 0.0%	0.00447	0.05%	0.02	CYP3A4, CYP3A5
pigment metabolic process	4 of 33 genes, 12.1%	80 of 19817 genes, 0.4%	0.00511	0.05%	0.02	UGT1A1, OATP1B1, LPH, ABCC2
reproductive structure development	6 of 33 genes, 18.2%	302 of 19817 genes, 1.5%	0.00518	0.05%	0.02	ESR1, LHCGR, BCL2L11, NR2F2, CYP1B1, CYP19A1
reproductive system development	6 of 33 genes, 18.2%	305 of 19817 genes, 1.5%	0.00548	0.04%	0.02	ESR1, LHCGR, BCL2L11, NR2F2, CYP1B1, CYP19A1

Supplementary Table 6. Functional annotation (in terms of molecular function) of the genes putatively associated with (poly)phenol bioavailability and metabolism included in the present systematic review.

Gene Ontology term	Cluster frequency	Genome frequency	Corrected <i>p</i> -value	FDR	False Positives	Genes annotated to the term
steroid hydroxylase activity	6 of 33 genes, 18.2%	39 of 19817 genes, 0.2%	4.71e-09	0.00%	0.00	CYP1A1, CYP3A4, CYP1B1, CYP19A1, CYP3A5, CYP17A1
estrogen 16- α -hydroxylase activity	4 of 33 genes, 12.1%	8 of 19817 genes, 0.0%	5.05e-08	0.00%	0.00	CYP1A1, CYP3A4, CYP1B1, CYP3A5
aromatase activity	5 of 33 genes, 15.2%	25 of 19817 genes, 0.1%	5.51e-08	0.00%	0.00	CYP1A1, CYP3A4, CYP1B1, CYP19A1, CYP3A5
oxygen binding	5 of 33 genes, 15.2%	37 of 19817 genes, 0.2%	4.46e-07	0.00%	0.00	CYP1A1, CYP3A4, CYP19A1, CYP3A5, CYP17A1
oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, reduced flavin or flavoprotein as one donor, and incorporation of one atom of oxygen	5 of 33 genes, 15.2%	44 of 19817 genes, 0.2%	1.10e-06	0.00%	0.00	CYP1A1, CYP3A4, CYP1B1, CYP19A1, CYP3A5
monooxygenase activity	6 of 33 genes, 18.2%	107 of 19817 genes, 0.5%	2.41e-06	0.00%	0.00	CYP1A1, CYP3A4, CYP1B1, CYP19A1, CYP3A5, CYP17A1
heme binding	6 of 33 genes, 18.2%	140 of 19817 genes, 0.7%	1.20e-05	0.00%	0.00	CYP1A1, CYP3A4, CYP1B1, CYP19A1, CYP3A5, CYP17A1
tetrapyrrole binding	6 of 33 genes, 18.2%	150 of 19817 genes, 0.8%	1.81e-05	0.00%	0.00	CYP1A1, CYP3A4, CYP1B1, CYP19A1, CYP3A5, CYP17A1

iron ion binding	6 of 33 genes, 18.2%	152 of 19817 genes, 0.8%	1.96e-05	0.00%	0.00	CYP1A1, CYP3A4, CYP1B1, CYP19A1, CYP3A5, CYP17A1
oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	6 of 33 genes, 18.2%	185 of 19817 genes, 0.9%	6.24e-05	0.00%	0.00	CYP1A1, CYP3A4, CYP1B1, CYP19A1, CYP3A5, CYP17A1
steroid binding	5 of 33 genes, 15.2%	100 of 19817 genes, 0.5%	7.15e-05	0.00%	0.00	ESR1, SERPINA6, UGT1A1, SHBG, CYP3A4
ABC-type xenobiotic transporter activity	3 of 33 genes, 9.1%	14 of 19817 genes, 0.1%	0.00017	0.00%	0.00	ABCG2, ABCB1, ABCC2
vitamin D 24-hydroxylase activity	2 of 33 genes, 6.1%	3 of 19817 genes, 0.0%	0.00091	0.00%	0.00	CYP1A1, CYP3A4
testosterone 6-beta-hydroxylase activity	2 of 33 genes, 6.1%	4 of 19817 genes, 0.0%	0.00183	0.00%	0.00	CYP3A4, CYP3A5
xenobiotic transmembrane transporter activity	3 of 33 genes, 9.1%	30 of 19817 genes, 0.2%	0.00188	0.00%	0.00	ABCG2, ABCB1, ABCC2
estrogen 2-hydroxylase activity	2 of 33 genes, 6.1%	5 of 19817 genes, 0.0%	0.00305	0.12%	0.02	CYP1A1, CYP3A4
transition metal ion binding	9 of 33 genes, 27.3%	1101 of 19817 genes, 5.6%	0.00638	0.24%	0.04	ZKSCAN5, ESR1, CYP1A1, CYP1B1, CYP17A1, CYP3A4, NR2F2, CYP19A1, CYP3A5
hydroperoxy icosatetraenoate dehydratase activity	2 of 33 genes, 6.1%	7 of 19817 genes, 0.0%	0.00640	0.22%	0.04	CYP1A1, CYP1B1
ABC-type transporter activity	3 of 33 genes, 9.1%	51 of 19817 genes, 0.3%	0.00945	0.21%	0.04	ABCG2, ABCB1, ABCC2

Supplementary Table 7. Functional annotation (in terms of cellular component) of the genes putatively associated with (poly)phenol bioavailability and metabolism included in the present systematic review.

Gene Ontology term	Cluster frequency	Genome frequency	Corrected P-value	FDR	False Positives	Genes annotated to the term
external side of apical plasma membrane	3 of 33 genes, 9.1%	6 of 19817 genes, 0.0%	5.61e-06	0.00%	0.00	ABCG2, ABCB1, LPH

Supplementary Table 8. Bioinformatics analysis of the SNPs studied in the present systematic review.

SNP	Chromosome	Position	Allele	TF BS	Splicing (site)	Splicing (ESE or ESS)	Splicing (abolish domain)	miRNA(miRanda)	miRNA(Sanger)	nsSNP	Stop Codon	Polypeptide	SNPs3D (svm profile)	SNPs3D (svm structure)	RegPotential	Conservation	Nearby Gene	Distance (bp)
rs10012	2	38155894	G/C	--	--	--	--	--	--	Y	--	benign	--	--	0.549557	0.000	CYP1B1	7644 902
rs10046	15	49290278	G/A	--	--	--	--	Y	Y	--	--	--	--	--	0.0	0.000	CYP19A1	1317 127808
rs10242455	7	99078115	A/G	--	--	--	--	--	--	--	--	--	--	--	0.0	0.002	ZNF498 CYP3A5	-10139 -5638
rs10278040	7	98979309	A/G	--	--	--	--	--	--	--	--	--	--	--	NA	0.001	ZKSCAN5 C7orf38	-9928 -2550
rs10454142	2	48499903	C/T	--	--	--	--	--	--	--	--	--	--	--	0.0	0.000	FOXN2 CCDC128	-39965 -21509
rs1045642	7	86976581	G/A	--	--	Y	--	--	--	--	--	possiblydamaging	1	0	0.39625	0.018	ABCB1	5697 203919
rs1056827	2	38155681	C/A	--	--	--	--	--	--	Y	--	benign	--	--	0.616522	0.031	CYP1B1	7431 1115
rs1056836	2	38151707	G/C	--	--	--	--	--	--	Y	--	benign	1	0	0.20363	0.115	CYP1B1	3457 5089
rs10883782	10	1,05E+08	A/G	--	--	--	--	--	--	--	--	--	--	--	0.0	0.017	C10orf26 CYP17A1	-7911 -6356
rs10929302	2	2,34E+08	A/G	Y	--	--	--	--	--	--	--	--	--	--	0.0	0.000	UGT1A8/UGT1A10	139491/120659 16163/16169

rs110 45819	12	2122 1080	A/C	--	--	Y	--	--	--	Y	--	benign	--	--	0.0	0.013	SLCO1B1	45676 62917
rs111 91548	10	1,05 E+08	C/T	--	--	--	--	--	--	--	--	--	--	--	0.0	0.572	CNNM2 NT 5C2	-7937 -1734
rs112 8503	7	8701 7537	G/A	--	--	Y	Y	--	--	--	--	--	--	--	0.1326 81	1.000	ABCB1	46653 162963
rs117 59010	6	1,05 E+08	G/T	--	--	--	--	--	--	--	--	--	--	--	0.0	0.001	HACE1	60334 71492
rs117 61528	7	9895 6737	C/T	--	--	--	--	--	--	--	--	--	--	--	NA	0.000	ZKSCAN5	16528 12644
rs119 74702	7	9900 1887	A/CA/ G/TG	--	--	--	--	--	--	--	--	--	--	--	0.0	0.000	ZNF655	7906 10125
rs121 50660	17	7462 640	G/T	Y	--	--	--	--	--	--	--	--	--	--	NA	0.000	FXR2 SAT2	-3844 -7641
rs124 13409	10	1,05 E+08	A/G	--	--	--	--	--	--	--	--	--	--	--	0.0	0.001	CNNM2	40982 119145
rs140 2467	2	1,08 E+08	C/G	--	--	--	--	--	--	Y	--	benign	--	--	0.0	0.000	SULT1C4	387 9462
rs169 64211	15	4931 7787	A/G	--	--	--	--	--	--	--	--	--	--	--	0.0	0.966	CYP19A1	28826 100299
rs170 65302	6	1,05 E+08	C/G	--	--	--	--	--	--	--	--	--	--	--	0.0	0.000	HACE1	53678 78148
rs174 96332	1	1,07 E+08	A/G	--	--	--	--	--	--	--	--	--	--	--	0.0	0.022	LOC126987 PRMT6	-919953 - 53034
rs180 0440	2	3815 1643	T/C	--	--	--	--	--	--	Y	--	possiblyd amaging	1	0	0.2624 57	0.991	CYP1B1	3393 5153
rs186 4729	8	9835 1365	G/A	--	--	--	--	--	--	--	--	--	--	--	0.0453 21	0.002	PGCP TSPY L5	-126465 -3525
rs203 2582	7	8699 8554	T/C/A	--	--	--	--	--	--	Y	--	benign	1	0	0.1978 85	0.998	ABCB1	27670 181946
rs223 1142	4	8927 1347	T/G	--	--	Y	--	--	--	Y	--	benign	--	--	0.2887 14	1.000	ABCG2	40907 27688

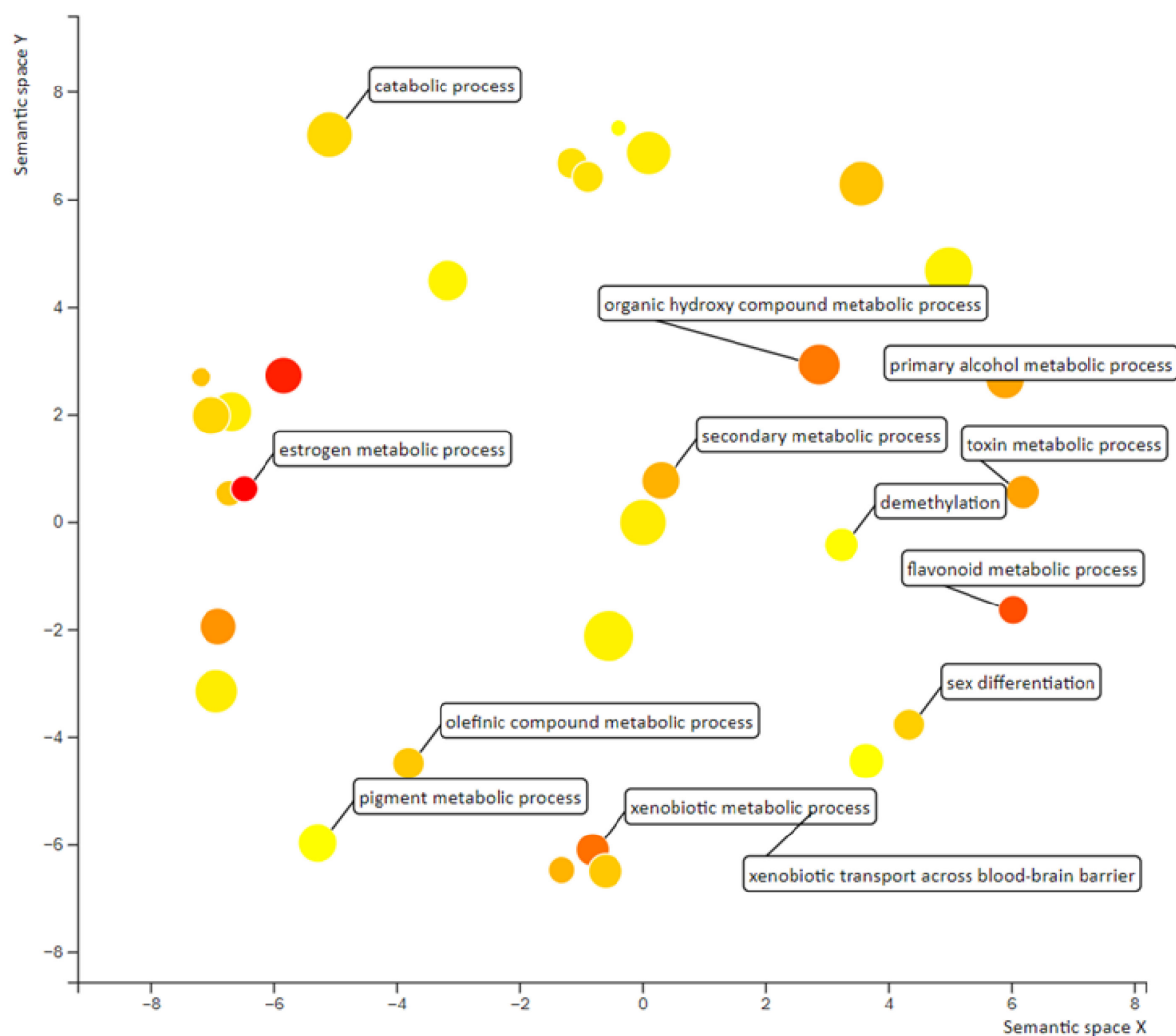
rs227 3697	10	1,02 E+08	A/G	--	--	Y	--	--	--	Y	--	benign	--	--	0.1163 89	0.922	ABCC2	21312 47766
rs230 6283	12	2122 1005	G/A	--	--	Y	--	--	--	Y	--	benign	--	--	0.0	0.000	SLCO1B1	45601 62992
rs241 1984	17	4480 0750	A/G	--	--	--	--	--	--	--	--	--	--	--	NA	0.000	ZNF652 PH B	-5916 -35669
rs241 4095	15	4931 1584	A/G	--	--	--	--	--	--	--	--	--	--	--	0.0	0.000	CYP19A1	22623 106502
rs244 5762	15	4940 5000	C/T	--	--	--	--	--	--	--	--	--	--	--	NA	0.000	CYP19A1	116039 13086
rs247 0893	15	7280 6502	T/C	Y	--	--	--	--	--	--	--	--	--	--	0.0464 9	0.000	CYP1A1 C YP1A2	-1572 -21735
rs247 2297	15	7281 4933	C/T	--	--	--	--	--	--	--	--	--	--	--	0.0	0.001	CYP1A1 C YP1A2	-10003 -13304
rs248 6758	10	1,05 E+08	C/T	Y	--	--	--	--	--	--	--	--	--	--	0.0	0.000	CYP17A1 L OC649704	-190 -294
rs265 28	16	2842 5210	T/C	Y	--	--	--	--	--	--	--	--	--	--	0.1771 73	0.000	IL27	7026 446
rs274 0574	7	9922 0032	T/C	Y	--	--	--	--	--	--	--	--	--	--	0.0866 08	0.000	CYP3A	136279 82077
rs302 0418	6	1,52 E+08	A/G	--	--	--	--	--	--	--	--	--	--	--	0.0	0.000	ESR1	216476 79244
rs358 231	4	2242 9602	T/A	--	--	--	--	--	--	--	Y	--	--	--	0.2645 81	1.000	GBA3/LOC 643751	125914/92356 688/909116
rs374 0066	10	1,02 E+08	T/C	--	--	Y	Y	--	--	--	--	--	--	--	0.1774 3	0.890	ABCC2	61704 7374
rs375 4689	2	1,36 E+08	T/C	--	--	Y	--	--	--	Y	--	benign	--	--	0.2430 31	0.757	LCT	45331 4004
rs375 7318	6	1,52 E+08	A/G	--	--	--	--	--	--	--	--	--	--	--	0.0469 18	0.000	C6orf97	98886 28215
rs376 0091	16	2852 8301	C/G/T	--	--	--	--	--	--	--	--	--	--	--	0.0	0.000	SULT1A1	3887 14066

rs3779195	7	97831298	T/A	--	--	--	--	--	--	--	--	--	--	--	0.0	0.001	BAIAP2L1	70807 37018
rs4124874	2	2,34E+08	T/G	Y	--	--	--	--	--	--	--	--	--	--	0.0	0.001	UGT1A8/UGT1A10	139368/120536 16286/16292
rs4149056	12	21222816	C/T	--	--	Y	--	--	--	Y	--	possiblydamaging	1	0	0.231371	0.740	SLCO1B1	47412 61181
rs4245525	6	1,05E+08	C/T	--	--	--	--	--	--	--	--	--	--	--	0.0	0.000	HACE1	130176 1650
rs440837	8	81624529	A/G	--	--	--	--	--	--	--	--	--	--	--	0.0	0.000	ZBTB10 hCG_1984468	-27364 -9028
rs4409766	10	1,05E+08	C/T	--	--	--	--	--	--	--	--	--	--	--	0.0	0.000	C10orf32	2644 7307
rs4633	22	18330235	C/T	--	--	--	--	--	--	--	--	--	--	--	0.420243	0.002	COMT	20926 6295
rs4646312	22	18328337	C/T	--	--	--	--	--	--	--	--	--	--	--	0.0	0.006	COMT	19028 8193
rs4646450	7	99104254	G/A	--	--	--	--	--	--	--	--	--	--	--	NA	0.000	CYP3A5/CYP3A	20501/20501 11303/197855
rs4680	22	18331271	A/G	--	--	Y	--	--	--	Y	--	benign	1	0	0.288484	0.601	COMT	21962 5259
rs4788068	16	28524224	T/C	--	--	--	--	--	--	--	--	--	--	--	NA	0.999	SULT1A2 SULT1A1	-8332 -190
rs4818	22	18331207	C/G/T	--	--	--	--	--	--	--	--	--	--	--	0.488936	0.668	COMT	21898 5323
rs4869739	6	1,52E+08	A/T	--	--	--	--	--	--	--	--	--	--	--	0.045151	0.005	C6orf97	86575 40526
rs4946645	6	1,05E+08	C/G	--	--	--	--	--	--	--	--	--	--	--	0.0	0.001	HACE1	119567 12259
rs543650	6	1,52E+08	T/G	--	--	--	--	--	--	--	--	--	--	--	0.068447	0.000	C6orf97 ESR1	-168615 -17743
rs6162	10	1,05E+08	A/G	--	--	--	--	--	--	--	--	--	--	--	0.262602	0.000	CYP17A1	6693 309

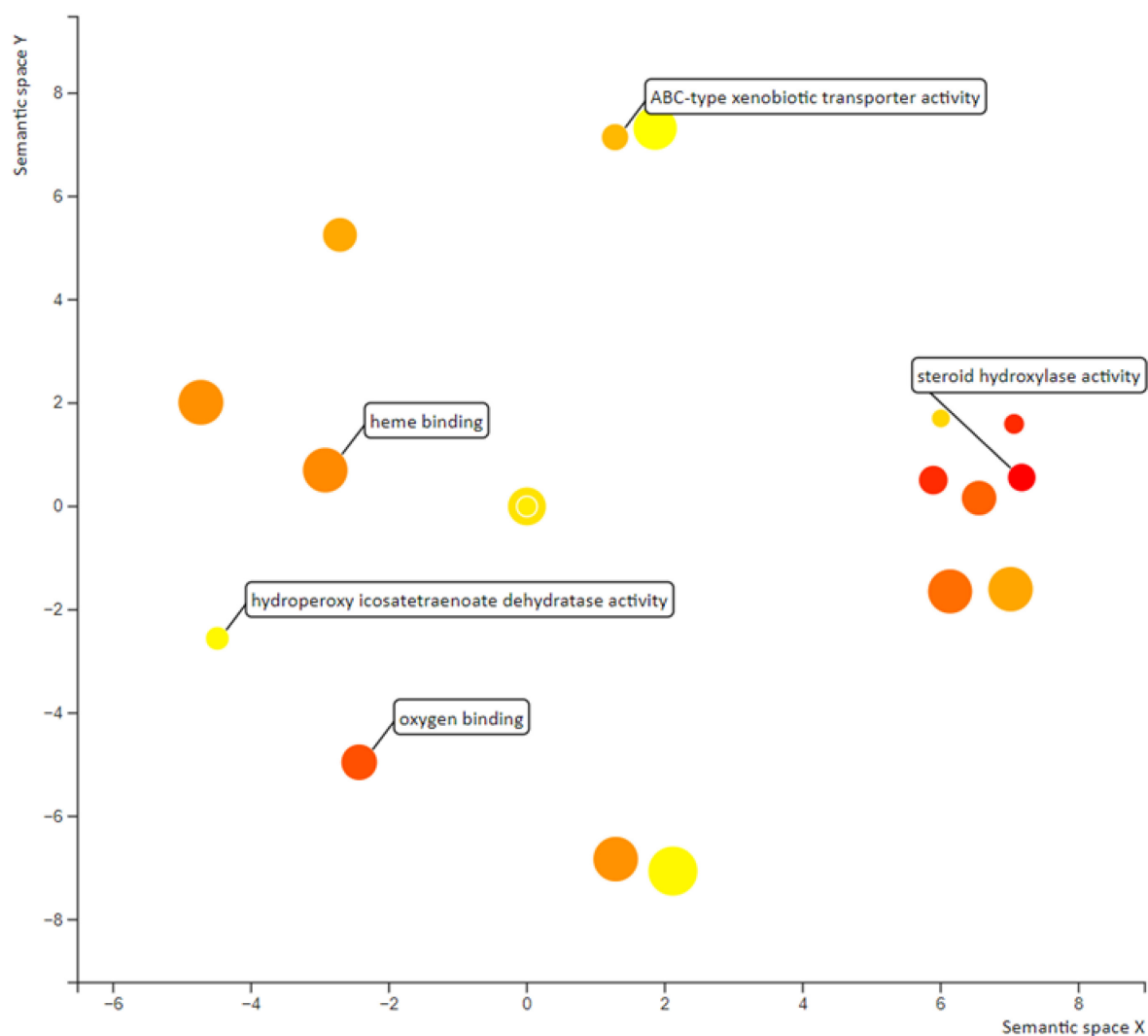
rs6259	17	7477252	A/G	--	--	Y	--	--	--	Y	--	benign	--	--	0.288638	0.478	SHBG	3036 143
rs6269	22	18329952	A/G	--	--	--	--	--	--	--	--	--	--	--	0.0	0.000	COMT	20643 6578
rs662	7	94775382	T/C	--	--	--	--	--	--	Y	--	benign	0	1	0.178979	0.000	PON1	10458 16398
rs6738028	2	1,12E+08	C/G	--	--	--	--	--	--	--	--	--	--	--	0.0	0.000	LOC729312	16137 14761
rs6742078	2	2,34E+08	G/T	--	--	--	--	--	--	--	--	--	--	--	0.0	0.000	UGT1A8/UGT1A10	146348/127516 9306/9312
rs6927608	6	1,05E+08	A/C	--	--	--	--	--	--	--	--	--	--	--	0.0	0.001	HACE1	112157 19669
rs700518	15	49316404	T/C	--	--	Y	--	--	--	--	--	--	--	--	0.415498	1.000	CYP19A1	27443 101682
rs700519	15	49295260	G/A	--	--	Y	--	--	--	Y	--	benign	--	--	0.23781	0.190	CYP19A1	6299 122826
rs705379	7	94791831	A/G	Y	--	--	--	--	--	--	--	--	--	--	0.729891	0.000	PON1 PON3	-51 -35289
rs705381	7	94791885	C/T	Y	--	--	--	--	--	--	--	--	--	--	0.540012	0.000	PON1 PON3	-105 -35235
rs717620	10	1,02E+08	T/C	Y	--	Y	--	--	--	--	--	--	--	--	0.0	0.099	ABCC2	75 69003
rs7181230	15	38148033	A/G	--	--	--	--	--	--	--	--	--	--	--	NA	0.130	LOC100131089 BMF	-2158 -19350
rs727428	17	7478517	T/C	--	--	--	--	--	--	--	--	--	--	--	0.180833	0.001	SHBG ATP1B2	-1122 -16462
rs727479	15	49321839	C/A	--	--	--	--	--	--	--	--	--	--	--	0.045609	0.000	CYP19A1	32878 96247
rs740160	7	98795816	C/T	--	--	--	--	--	--	--	--	--	--	--	0.0	0.000	ARPC1A	34370 6005
rs743572	10	1,05E+08	A/G	Y	--	--	--	--	--	--	--	--	--	--	0.166288	0.001	CYP17A1	6864 138

rs749292	15	49346023	A/G	--	--	--	--	--	--	--	--	--	--	--	0.0	0.001	CYP19A1	57062 72063
rs750155	16	28528073	C/T	--	--	--	--	--	--	--	--	--	--	--	0.170149	0.000	SULT1A1	3659 14294
rs776746	7	99108475	T/C	--	--	--	--	--	--	--	--	--	--	--	0.0	0.000	CYP3A5/CYP3A	24722/24722 7082/193634
rs780093	2	27596107	T/C	--	--	--	--	--	--	--	--	--	--	--	0.0	0.000	GCKR	22897 3948
rs7809615	7	99022714	G/T	--	--	--	--	--	--	--	--	--	--	--	NA	0.000	ZNF655 ZNF498	-10702 -29793
rs7910927	10	64808916	G/T	--	--	--	--	--	--	--	--	--	--	--	0.0	0.003	JMJD1C	211925 86812
rs8023580	15	94509295	C/T	--	--	--	--	--	--	--	--	--	--	--	0.099521	0.000	LOC100132798	37702 162118
rs8049439	16	28745016	C/T	--	--	--	--	--	--	--	--	--	--	--	0.048792	0.014	ATXN2L	3101 11043
rs8175347	2	2,34E+08	(TA)5/6/7/8	--	--	--	--	--	--	--	--	--	--	--	NA	NA	UGT1A8/UGT1A10	142590/123758 13064/13070
rs8187710	10	1,02E+08	A/G	--	--	Y	--	--	--	Y	--	benign	--	--	0.265915	1.000	ABCC2	68791 287
rs854560	7	94784020	A/C/G/T	--	--	--	--	--	--	Y	--	benign	1	1	0.155721	1.000	PON1	19096 7760
rs854572	7	94792632	C/G	Y	--	--	--	--	--	--	--	--	--	--	0.028247	0.004	PON1 PON3	-852 -34488
rs887829	2	2,34E+08	T/C	Y	--	--	--	--	--	--	--	--	--	--	0.0	0.134	UGT1A8/UGT1A10	142279/123447 13375/13381
rs9340835	6	1,52E+08	A/G	--	--	--	--	--	--	--	--	--	--	--	0.0	0.000	ESR1	71245 224475

Supplementary Figure 1. Functional annotation (in terms of biological processes) of the genes putatively associated with (poly)phenol metabolism investigated in the studies included in the present systematic review. Nodes in the diagram are color-coded according to their p-value. Genes in the GO tree are associated with the GO term(s) to which they are directly annotated (biological processes). Only significant hits with a p-value ≤ 0.01 and terms descended from them are included.



Supplementary Figure 2. Functional annotation (in terms of molecular functions) of the genes putatively associated with (poly)phenol metabolism investigated in the studies included in the present systematic review. Nodes in the diagram are color-coded according to their p-value. Genes in the GO tree are associated with the GO term(s) to which they are directly annotated (molecular functions). Only significant hits with a p-value ≤ 0.01 and terms descended from them are included.



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Chapter 5. General discussion

In the present doctoral thesis, four different approaches were applied to investigate the variability in (poly)phenol ADME. Several factors determining the heterogeneity in (poly)phenol bioavailability and metabolism were analyzed, including the native phenolic compound structure, the composition and activity of the gut microbiota, and single nucleotide polymorphisms (SNPs) in genes involved in (poly)phenol ADME. The methodology applied in the first three approaches included the extraction of phenolic metabolites from the biological matrix (fermented slurry, urine, or plasma) and their targeted metabolomics analysis by ultra-high performance liquid chromatography coupled with tandem mass spectrometry (uHPLC-MS/MS). Compounds were monitored in selective reaction monitoring (SRM). Conversely, the fourth approach was based on a critical systematic review of the literature.

The first approach consisted of an *in vitro* study aiming to optimize a protocol of colonic fermentation using flavan-3-ols (Di Pede et al., 2022). The study also explored the influence of the flavan-3-ol structure on the production of phenolic catabolites. Two chemical/structural properties were considered sources of variability: the dimerization and the type of linkage between monomeric units. To reach this objective, three flavan-3-ols, namely (–)-epicatechin (EC) and procyanidin (PC) dimers B2 and A2, were fermented for 48 h using human faecal microbiota, and a total of 40 compounds were simultaneously monitored.

The monitoring of the fermentation conditions through the analysis of pH and faecal microbiota composition confirmed the stability and the colon-like features of the incubation environment, with a pH around 5-6 and a balanced microbial composition in terms of phyla, genera and species, with only slight fluctuations determined by the incubation time and not depending on the fermented substrate. Moreover, the extension of the fermentation time until 48 h and the monitoring of 5 different incubation times resulted in a more comprehensive view of flavan-3-ol kinetics and allowed to quantify additional low molecular weight phenolics, including a catabolite specific to PC_B2 (3',4'-dihydroxyphenylacetic acid) and two downstream catabolites, namely 4-hydroxybenzoic acid and 4-hydroxybenzaldehyde, the latter never reported in a fermentation study before. The results suggest that the flavan-3-ol structure influences microbial catabolism from a quantitative, qualitative and kinetic point of view, as observed in other studies (Appeldoorn et al., 2009; Di Pede et al., 2022; Ou et al., 2014; Stoupi et al., 2010). In detail, flavan-3-ol polymerization and the addition of an ether

linkage in A-type PCs increase the steric hindrance and stiffness of the molecule, with a reduction in bioaccessibility resulting in a delayed and reduced catabolism.

An acute human intervention study further investigated the impact of the flavan-3-ol structure on the production of phenolic metabolites. Three chemical/structural properties were considered sources of variability: polymerization, extent of B-ring hydroxylation, and presence of galloyl moieties. For this purpose, a double-blind, randomized, cross-over, acute intervention trial was performed in 11 healthy subjects consuming 1 mol of total flavan-3-ols from three different sources: a green tea extract (GT), a grape seed extract with high monomeric content (GSM), and a grape seed extract with high procyanidin content (GSP). Baseline spot urine and the urine produced during the 24 or 48 hours after the treatment were collected, and 126 compounds were simultaneously monitored. To analyze more deeply flavan-3-ol metabolism, specific classes of flavan-3-ol metabolites were defined, grouping metabolites according to their aglycon moiety and were referred to as colonic classes. This approach allowed us to focus on colonic catabolism, grouping metabolites originating from the same microbial activities.

Secondary outcomes of the study were methodological analyses that allowed to assess (1) the accuracy and reliability of creatinine-corrected measures of urinary (poly)phenol metabolites and (2) the contribution of endogenous metabolites (mainly small phenolic acids) to the pool of excreted flavan-3-ol metabolites. The evaluation of the impact of non-phytochemical metabolism on the phenolic metabolic profile is essential to avoid biases in the bioavailability estimates: adjusting the 24 h excretion of flavan-3-ol metabolites for the baseline excretion, the bioavailability reduced by 12% for GT, 13% for GSM and 24% for GSP treatment and was mainly driven by HAs, monohydroxylated BAs, dihydroxylated CAs and monohydroxylated PPAs, and to a lesser extent by BZs, BALs, PAAs, monohydroxylated CAs and dihydroxylated PPAs. The background metabolites, including HAs and phenolic acids, were in accordance with those reported by the existing evidence (Clifford & Kuhnert, 2022; Crozier et al., 2010; Goldstein et al., 2003; Kern et al., 2003; Loke et al., 2009).

A total of 75 flavan-3-ol metabolites were quantified in urine samples. PVLs were the most abundant and richest class of metabolites, and specific microbial flavan-3-ol metabolites (PVLs and PVAs) were far higher than SRFMs, in line with existing evidence about the high urinary recovery in colon-derived catabolites after consumption of flavan-3-ol sources (Calani et al.,

2012; Crozier et al., 2010; Del Rio et al., 2010; Rios et al., 2003; Roowi et al., 2010; Ward et al., 2004). Differences in the urinary excretion of phenolic metabolites from a qualitative, quantitative and kinetic point of view were assessed after the intake of different flavan-3-ol sources, indicating that the native flavan-3-ol structure influences the bioavailability and microbial metabolism of this (poly)phenol class, in accordance other studies (Hollands et al., 2020; Ottaviani et al., 2012; Ou et al., 2014; Rios et al., 2003; Stalmach et al., 2009; Ward et al., 2004; Wiese et al., 2015; Zhang et al., 2016). GSM was the most rapidly metabolized extract, while GSP was the most slowly metabolized and showed the highest relative values of metabolites excreted in the second day after the treatment, suggesting that the steric hindrance, the interaction with proteins in the gut lumen and the antimicrobial properties of PCs could slow gut microbiota catabolism, a phenomenon extensively reported in the literature (Santos-Buelga & Scalbert, 2000; Scalbert, 1991). Moreover, flavan-3-ols from GSP had the lowest bioavailability, while flavan-3-ols from GT had the highest values and were associated with the richest metabolic profile from a qualitative and quantitative point of view, in line with existing evidence (Di Pede et al., 2023). The difference in the phenolic metabolic profile between the extracts derives from (1) the peculiar composition of green tea flavan-3-ols, which includes 3',4',5'-trihydroxylated and galloylated forms, leading to the production of 3',5'-dihydroxylated and 3',4',5'-trihydroxylated derivatives, (2) the resistance of PCs to microbial catabolism, leading to a lower production of SRFMs and upstream microbial metabolites (PVLs, PVAs, PPAs), and (3) the additional catabolic pathway of PCs involving the degradation of the upper unit and yielding 3',4'-dihydroxylated PAAs (Mena et al., 2019).

The third approach consisted of a chronic human intervention study, which allowed to explore inter-group (treatment-driven) and inter-individual (mainly gut microbiota-driven) differences in the levels of plasma and urinary phenolic metabolites following a 12-week intake of a cranberry powder in 60 healthy older adults in free-living conditions. Urinary metabotypes related to dietary (poly)phenols were also explored. Up to 13 and 67 metabolites were quantified in plasma and spot urine, respectively. Cranberry consumption led to changes in plasma metabolites, mainly hydroxycinnamates and hippuric acid, in line with other studies reporting acute or chronic consumption of cranberry (Feliciano et al., 2017; Heiss et al., 2022; Rodriguez-Mateos et al., 2016). However, the cranberry treatment did not influence the urinary phenolic profile, suggesting that cranberry (poly)phenols, in particular the A-type PCs

characterizing this fruit, are likely stable in the gut and poorly catabolized, not contributing significantly to the pool of excreted flavan-3-ols or their downstream gut microbiota metabolites, in line with existing evidence (Di Pede et al., 2022; Hollands et al., 2020; Ou et al., 2014; Wiese et al., 2015).

The inter-individual variability in the urinary excretion of (poly)phenol metabolites was assessed using different data sets and a combination of statistical models, using the information collected from previous evidence about flavan-3-ol metabotypes (Mena et al., 2018, 2022). Three phenolic metabotypes were identified: colonic metabolism was the main driver for subject clustering, and 5-(3',4'-dihydroxyphenyl)- γ -valerolactone derivatives were the most relevant metabolite class for metabotyping. This is relevant since the conjugated forms of this metabolite class are the main circulating metabolites of flavan-3-ols (Di Pede et al., 2023; Mena et al., 2019), which are in turn the most important dietary class of (poly)phenols together with CAs (Zamora-Ros et al., 2013; Ziauddeen et al., 2018). Metabotypes were characterized by quali-quantitative differences in the excretion of some gut microbial metabolites and were further confirmed when applying a model only focused on flavan-3-ol colonic metabolites. Metabotype 1 was characterized by 5C-RFMs, including PVLs and 4-OH-PVAs, while metabotype 2 was characterized by 3C-RFMs, namely 3'-hydroxylated CAs and PPAs; metabotype 3 was the most prevalent one and was defined by a low excretion of all metabolites, differing from the other two on a quantitative basis. This outcome confirms the existence of metabotypes in the urinary excretion of flavan-3-ol catabolites proposed for tea and in another cranberry study (Mena et al., 2018, 2022). Moreover, results suggest that (1) flavan-3-ol metabolites may be key in determining (poly)phenol metabotypes, (2) flavan-3-ol metabotypes may be highly predictive of (poly)phenol metabotypes. Interestingly, metabotype allocation was well preserved after a 12-week intervention, indicating their stability over time.

Finally, a systematic literature review was performed to investigate the impact of genetic variations on the bioavailability and metabolism of phenolic compounds. In detail, the relationship between SNPs in genes involved in (poly)phenol ADME and the inter-individual variability in the plasma or urinary levels of (poly)phenol metabolites was explored. Five major databases were questioned and a total of twelve studies were selected (Brown et al., 2011; Chang et al., 2019; Fraga et al., 2022; Hong et al., 2012; Inoue-Choi et al., 2010; Lorenz et al.,

2014; Miller, Jackson, Dadd, Mayes, et al., 2012; Miller, Jackson, Dadd, Nicol, et al., 2012; Momma et al., 2023; Muhrez et al., 2017; Scholl et al., 2018; Wakeling & Ford, 2012). Eight studies were intervention trials, and four were designed as observational. Half of the studies investigated green tea flavan-3-ol ADME, and three single studies explored the bioavailability and metabolism of flavanones, isoflavones and lignans in orange juice, soy and ground brown flaxseed, respectively. Only one study analyzed the impact of an unrestricted diet on the phenolic metabolome, and two other studies investigated the metabolism of flavan-3-ols and isoflavones deriving from a free diet, considering their specific gut microbial metabolites. These findings indicate that the investigation on the association between the variability in (poly)phenol ADME and genetic differences is limited and should be widened, focusing on other, less food-specific classes, including flavan-3-ols of different origin from green tea, anthocyanins, flavonols, and cinnamic acids, among others.

In total, 88 SNPs in 33 genes were studied, more than half of which were related to drug/xenobiotic metabolism. Phase I metabolism was the most representative phase, followed by excretion and phase II metabolism. However, considering the number of times a gene was studied in all works, phase II metabolism was the most investigated, followed by excretion and phase I metabolism, absorption being the least represented and studied phase. These results are consistent with the crucial role of conjugation in the metabolism of (poly)phenols. Considering the metabolites that were influenced by genetic variability, many were phase II conjugates, including methylated forms of galliccatechins, and sulfated and glucuronidated forms of isoflavones and flavanones. Other metabolites included aglycones of (gallo)catechins, isoflavones, phenolic acids and benzene derivatives, and gut microbiota metabolites both in an aglyconic and a conjugated form, including several PVLs, enterolactone and equol. Regarding phase II metabolism, COMT genetic variants were analyzed mainly concerning galliccatechins, UGT1A1 genetic variants were associated with isoflavones, and SULT1A1/SULT1C4 were explored together with flavanones. Glucosidases involved in (poly)phenol absorption (CBG/GBA3 and LPH) were analyzed only concerning soy isoflavones, while influx/efflux transporters involved in (poly)phenol excretion were investigated with all (poly)phenol sources, suggesting their importance in determining (poly)phenol bioavailability. However, there was no consensus among studies allowing to associate a particular genetic variant with a change in the levels of a specific metabolite in urine or plasma, suggesting the need for more high-quality research in the field.

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Chapter 6. Conclusions

This Doctoral Thesis investigated the variability in (poly)phenol bioavailability and metabolism, also analyzing some putative determining factors in order to gain new and further insights in the field of (poly)phenol research. Based on four different approaches applied in the present research, it can be concluded that:

1. New protocols for *in vitro* human fermentation studies are needed to explore the catabolism of flavan-3-ols and the variation in the biotransformation of different native structures, such as monomeric and oligo/polymeric flavan-3-ols. These studies should monitor the incubation conditions (pH, gut microbiota composition) to maintain colon-like environment features. Moreover, the extension of the fermentation time until 48 h and the addition of intermediate incubation times can lead to a more detailed and comprehensive view of flavan-3-ol kinetics, allowing the detection of transient and downstream catabolites;
2. Acute human intervention studies aiming to investigate flavan-3-ol ADME and the influence of the native structure on flavan-3-ol bioavailability and metabolism should consider the contribution of endogenous metabolites to the pool of circulating flavan-3-ol metabolites. To achieve this objective, a placebo-controlled, cross-over study should be designed, with strict control of lifestyle (diet, physical activity, smoking) during the intervention days and, if possible, the adoption of a standard diet. These precautions would avoid biases in the bioavailability estimates and allow the adjustment of the contribution of each phenolic class to the total excreted amount of phenolic metabolites, obtaining a more realistic picture of the phenolic metabolic profile.
3. Further *in vitro* and intervention trials exploring flavan-3-ol biotransformation and the influence of the native structure on flavan-3-ol metabolism are needed to (1) identify valuable biomarkers of flavan-3-ol intake to be used in interventional and epidemiological studies, (2) investigate flavan-3-ol mechanism of action in *in vitro* studies, using flavan-3-ol metabolites in their physiological circulating concentrations, (3) identify putatively distinct urinary phenolic metabotypes associated with the consumption of different flavan-3-ol sources and resulting in varying health effects, and (4) assess the inter-individual variability in flavan-3-ol metabolism and the associated metabotypes and biological response.
4. Chronic human intervention studies designed to investigate the health effects associated with (poly)phenol consumption should take into account (1) the bioaccessibility,

bioavailability and metabolism of the administered (poly)phenols, ensuring that a relevant dose could reach the target cells, tissues and organs, (2) the inter-individual variability in the quali-quantitative production of phenolic metabolites, and (3) the factors influencing the heterogeneity in (poly)phenol ADME and biological response, including personal characteristics (age, sex), lifestyle, medication, pathophysiological status, gut microbiota activity and composition, and genetic background. In this context, the identification of metabolotypes in the urinary excretion of (poly)phenol metabolites and their determining factors could help unveil the health-promoting activity of phenolic metabolites associated with the consumption of (poly)phenol-rich foods or supplements. To explore the inter-individual variation in the excretion of phenolic compounds specifically derived from the administered poly)phenols, some precautions should be taken: (1) the control of the volunteers' diet using dietary records/recalls during the sampling period and the day(s) before and, if possible, giving specific recommendations such as avoiding the intake of (poly)phenol-rich foods in the same period; (2) the optimization of the time and type of sampling, preferring the collection of 24 h urine or at least providing harmonized recommendations on food consumption timing when spot urine samples are collected.

5. New methodologies such as a systems genetics-based or a genome-wide association study (GWAS) approach are warranted to explore the impact of genetic variations, in particular genetic polymorphisms, on the inter-individual variability in (poly)phenol ADME. Integrative frameworks, combining *in vivo* and *in vitro* approaches, *in silico*/computational techniques, intervention trials, nutritional epidemiology and mathematical modeling (pharmacokinetics/nutrikinetics), can help uncover the impact of a given genotype on the biological activity of the ingested (poly)phenols and their health-related outcomes. Integrating with metabotyping techniques, this strategy may be a critical success factor in personalized approaches when considering (poly)phenols, providing tailored dietary advice to decrease disease risks.

In conclusion, the findings of this Doctoral Thesis represent a starting point for improving the available knowledge on the variability in (poly)phenol bioavailability and metabolism, helping in the development of new concepts and methodologies and the building of a new scenario based on preventive, evidence-based, personalized nutrition strategies with these major dietary plant bioactives.

Acknowledgements

I would like to acknowledge my PhD supervisor, Professor Daniele Del Rio, who gave me the opportunity to do a PhD in Food Science, always trusted and supported me, and gave me valuable advice. Also, I thank him for the human and scientific values he has transmitted to me. I thank my co-tuturs, Prof. Pedro Mena and Letizia Bresciani, for guiding me on my PhD path. Their teachings and expertise were priceless, and our scientific debates were stimulating and enriched me as a person and researcher. Also, I must express my gratitude to Dr. Luca Calani, for all the scientific teaching and technical support he gave me.

Then, I would like to thank all the persons (past and present) of the Human Nutrition Unit of the University of Parma, because the contribution of everyone was important for my education and scientific growth: Claudia, Giuseppe, Cristiana, Marta, Costanza, Laila, Cristiano, Sole, Elisabetta, Nicola, Elena, Cinzia and Beatrice. A special thank you goes to my Spanish teammates, Vicente, Lorena, Vanessa and Silvia, whose collaboration made lighter the laboratory work and whose company helped me overcome some difficult moments.

Thanks to all the researchers I had the possibility to work with, especially Doctor David Vauzour, and thanks to Professor Chiara Dall'Asta for her great availability and professionalism.

Infine, ringrazio i miei genitori per l'immenso affetto che mi hanno mostrato durante tutto il mio percorso scolastico e universitario, per il costante supporto, per la fiducia che mi hanno sempre mostrato, per i valori che mi hanno trasmesso: la cultura del lavoro, la tenacia, la generosità. Ringrazio mio fratello, perché nella nostra diversità caratteriale è sempre riuscito a smussare i lati più testardi e rigidi del mio carattere, spesso alleggerendomi l'animo e confortandomi con un semplice gesto. Ringrazio tutta la mia famiglia, perché ognuno di loro mi ha fatto crescere e mi ha aiutato a raggiungere questo traguardo: la zia Mari, che rimarrà sempre la mia tata, gli altri miei zii, i miei cugini che sono stati compagni di gioco e risate, le mie nonne Maria e Nora, che con la loro rispettiva dolcezza e intraprendenza hanno formato lati del mio carattere, i miei nonni Francesco e Angiolino, che pur nella loro assenza in questi ultimi anni sono sempre stati tra i primi sostenitori delle mie passioni.

Ringrazio le mie amiche per il sostegno morale, l'incoraggiamento, i momenti di spensieratezza che mi hanno donato: Bea, Gio, Bi, Sivi, Ele, Nene e Beatrice. Ringrazio i miei amici per il confronto e i tanti momenti leggeri condivisi: Paglia, Cats, Marco, Miki, Ste, Dario, Fabri.

Infine, ma non per importanza, ringrazio le tante persone della grande comunità di *Juventibus*, perché l'ingresso in questa calorosa famiglia mi ha dato conforto e strappato risate nel difficile periodo del lockdown, creando un legame più forte di quello che potessi mai immaginare. In particolare, un sentito grazie va a Max, che mi ha introdotto in questo mondo (ero, sono e rimarrò sempre una zampiniana di ferro), a Luca e alla sua coinvolgente estrosità, e ai miei fantastici compagni di *Juventiboh* (Matt, Jacopo, mister Zenzola, Leo, Gio, Tamita), iniziato quasi per gioco e diventato il nostro spazio virtuale ed emotivo fatto di sorrisi, confronto, supporto, compagnia. Ringrazio anche tutte le persone incontrate in questo meraviglioso percorso bianconero, ognuna delle quali mi ha lasciato qualcosa. Un ultimo, sincero e profondo ringraziamento va alla Vecchia Signora, che, specialmente in questi anni talvolta duri di dottorato, mi ha fatto sentire sempre viva: perché, come diceva qualcuno, la Juve è *"motivo di gioia e orgoglio, ma anche di delusione e frustrazione, comunque emozioni forti, come può dare una vera e infinita storia d'amore"*.

Curriculum vitae



Address Via Emilia 1536, 29010 Alseno (Italy) **Date of birth** 07/05/1995

Personal description I have a strong interest in the fields of nutrigenomics and nutrimetabolomics, the epigenetics of ageing and longevity, and the relationship between health, diet and gut microbiota. Moreover, I have a passion for journalism, philosophy, politics and sport, especially skating and football. I love poetry, music, cinema and the mountains, and I have a penchant for teaching and scientific dissemination activity. I am fond of my independence and I have a strong collaboration spirit and a great constancy, determination and willingness to learn. Finally, I have confidence in human relations and I believe in personal initiative.

WORK EXPERIENCE

Figure skating coach (December 2014 - December 2018)

Amateur sports association “Felix” in Fiorenzuola D’Arda (Italy).

Description: teaching children the basics of skating and organizing their first competition.

Background in the field: this work experience was preceded by a coaching course attended in Bologna and organized by the UISP Committee of Emilia-Romagna. Moreover, I practised agonistic skating for 6 years, attending many competitions at provincial, regional and national level.

EDUCATION

PhD in Food Science (November 2020-present)

Department of Food and Drug, University of Parma (Italy).

Laboratory of Phytochemicals in Physiology, Human Nutrition Unit.

Topic: Multiple approaches to investigate the variability in (poly)phenol bioavailability and metabolism

Objectives: Investigating the variability in (poly)phenol bioavailability and metabolism and its determining factors applying multiple approaches, ranging from *in vitro* human fermentation studies, acute and chronic human intervention trials and systematic reviews of the literature. Moreover, the inter-individual variability in (poly)phenol ADME is explored in order to identify putative metabotypes in the urinary excretion of phenolic metabolites.

Material and methods: Analysis of the (poly)phenol composition of plant extracts and powders by ultra-high performance liquid chromatography coupled with mass spectrometry (uHPLC-MSⁿ); extraction of (poly)phenol metabolites from faecal slurries and plasma and urine samples using liquid-liquid extraction and microelution solid phase extraction (μSPE) methods; targeted metabolomics analysis by ultra-high performance liquid chromatography coupled with tandem mass spectrometry (uHPLC-MS/MS); metabotyping using multivariate statistical analysis techniques including Principal Component Analysis (PCA) and Partial Least Squares-Discriminant Analysis (PLS-DA).

Supervisor: Professor Daniele Del Rio.

Co-tutors: Professor Pedro Mena and Professor Letizia Bresciani

Qualification to the profession of Biologist - Section A (July 2020)

University of Parma (Italy).

Mark of state examination: 28/30.

Master of Science in Genomic, Molecular and Industrial Biotechnology (September 2017-April 2020)

Department of Chemistry, Life Sciences and Environmental Sustainability, University of Parma (Italy).

Final mark: 110/110 with honors.

Thesis title: Assessment of the inter-individual variability in the metabolism of inulin and flavan-3-ols: a partially randomized cross-over intervention study.

Thesis Supervisor: Professor Daniele Del Rio.

Internship: period of 8 months in the Laboratory of Phytochemicals in Physiology (Human Nutrition Unit, Department of Food and Drug, University of Parma).

Technical skills gained during internship: organization and handling of a partially randomized cross-over intervention study; running of a challenge breath test with recruited volunteers and analysis of breath samples with hydrogen/methane analyzer; extraction of (poly)phenols and phenolic metabolites from plant extracts (green tea, grape seed) and biological samples (urine); qualitative and quantitative analysis of monomeric, oligomeric and polymeric flavan-3-ols with ultra-high performance liquid chromatography coupled with mass spectrometry (uHPLC-MSⁿ);

Bachelor of Science in Biotechnology (September 2014-December 2017)

Department of Chemistry, Life Sciences and Environmental Sustainability, University of Parma (Italy).

Final mark: 110/110 with honors.

Thesis title: Specificity and putative molecular targets of a polyphenolic metabolite with anti-amyloidogenic activity.

Thesis Supervisor: Professor Simone Ottonello.

Internship: period of 3 months in the Laboratory of Molecular Biology (Biomolecular, Genomic and Bioinformatic Sciences Unit, Department of Chemistry, Life Sciences and Environmental Sustainability, University of Parma).

Technical skills gained during internship: culture of yeast models of neurodegenerative diseases (Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, Huntington's disease) and growth assays (spot assay, drug assay); RNA preparation and quantification, RNA analytical electrophoresis, real-time qPCR.

Diploma in Scientific High School (June 2014)

Institution of higher education "Paciolo-D'Annunzio" in Fidenza (Italy).

Final mark: 100/100 with honors.

TRAINING

Summer School "Innovative approaches to substantiate the link between dietary polyphenols and health - From clinical to mechanistic research" (12-15 September 2022)

2nd PhenolAcTwin Summer School organized by INRAE in Paris (France).

Main topics: meta-analysis on the health effects of polyphenols; microbiome analysis to investigate the relationship between polyphenols and gut microbiota; use of nutrigenomics to elucidate the cellular and molecular mechanisms of polyphenols; in-silico docking to identify new molecular targets of polyphenol metabolites.

AIRC-UniPR School "Evidence-based approach to nutrition in cancer prevention and management" (26-28 November 2021)

Virtual school organized by the University of Parma and the Italian Association for Cancer Research.

Main topics: molecular and genetic basis of the relationship between cancer and nutrition; genes and nutrition in cancer prevention; studies used to demonstrate the effects of diet; nutrition and lifestyle in primary prevention; secondary prevention and nutritional management of the cancer patient; communication on cancer and nutrition.

International course in Healthy Food Design (7-10 June 2021)

Online course held by the University of Wageningen and organized by the Graduate School VLAG.

Aim: providing the essential skills for designing healthy foods for specific categories of consumers in the European legislative framework of nutritional and health claims

Main topics: definitions and legislative European framework of nutritional and health claims; a step by step approach on how to design healthy foods for specific categories of consumers; main bioactive ingredients for healthy food design; the consumer perception of healthy claimed foods; preparation of an EFSA dossier for asking for a health claim; failure cases of healthy food design in the food industry.

Summer School in Applied Human Nutrition (September 2020)

Virtual school organized by the NNEdPro (Need for Nutrition Education/Innovation Programme) Global Centre for Nutrition and Health in Cambridge and by the School of Advanced Studies on Food and Nutrition of the University of Parma.

Main topics: basic concepts in human nutrition; nutrition research methods and topics; nutrition in disease prevention and in healthcare; global and public health and nutrition policy.

PERSONAL SKILLS

Languages

Mother tongue: Italian.

Other language(s): English – B2.

Communication skills

Good verbal and written communication skills gained during my educational path; ability to clearly and accurately disclose the acquired knowledge.

Organisational skills

Good organisational and time management skills gained during my master's degree internship and my experience as a skating coach.

Other skills

Ability to work in group and in stressful conditions (presence of a public or a deadline).

Driving licence

B

CONGRESSES, CONFERENCES AND SEMINARS

IV International Conference on Food Bioactives and Health

Conference organized in Prague (Italy), 18-21 September 2023.

Workshop on the developments in the Italian PhD Research on Food Science Technology and Biotechnology

Workshop organized by the University of Turin in Asti (Italy), 19-21 September 2022.

“Phenolic metabolites after acute and chronic consumption of different flavan-3-ol sources”

III International Conference on Food Bioactives and Health

Conference organized in Parma (Italy), 21-24 June 2022.

Title of the poster presentation: *Influence of the flavan-3-ol structure on the production of phenolic metabolites after consumption of different flavan-3-ol sources by healthy subjects*

PhD International Seminar on Functional Foods, Bioactives and Human Health

International seminar organized by RCC (Real Colegio Complutense) Harvard Study Group on Functional Foods, Bioactives and Human Health, 20 May 2022

Title of the oral presentation: *Phenolic metabolites and their inter-individual variability after chronic cranberry consumption*

X International Conference on Polyphenols and Health 2022

Conference organized in London (United Kingdom), 20-23 April 2022.

Title of the poster presentation: *Influence of the flavan-3-ol structure on the production of phenolic metabolites after consumption of different flavan-3-ol sources by healthy subjects*

XLII National Congress of the Italian Society of Human Nutrition 2022

Congress organized by the Italian Society of Human Nutrition in Napoli (Italy), 4-6 April 2022.

Title of the poster presentation: *Influence of the flavan-3-ol structure on the production of phenolic metabolites after consumption of different flavan-3-ol sources by healthy subjects*

Seminar “Nutrition and cancer”

Seminar organized by the Italian Society of Human Nutrition (Emilia-Romagna and Marche branch), 22 October 2021.

Symposium “Personalized Nutrition- Science or Fiction?”

Virtual symposium organized by The Royal Swedish Academy of Sciences National Committee in Nutrition and Food Sciences, 19 October 2021.

ILSI Europe Annual Symposium 2021 – Nurturing the next generation

Virtual symposium organized by the International Life Sciences Institute (ILSI), 5 October 2021.

Seminar “Interaction between diet and gut microbiota: news from the research and focus on the gut-brain axis”

Virtual seminar organized by the IRCCS Institute in Bologna, 21 September 2021.

International Conference on Polyphenols 2020

Virtual conference organized by the University of Turku (Finland), 13-15 July 2021.

Nutrimi 2021 - The Digital Forum for Nutrition

Virtual conference, 30 April 2021.

Nutrimi 2020 - The Digital Forum for Nutrition

Virtual conference, 6 and 7 July 2020.

Conference “The new nutrition era: from molecular mechanisms to human health”

Conference in the IFOM-IEO Campus in Milan (Italy), 12 April 2018.

Symposium “Keep an eye on the cancer”

Symposium organized by the University of Parma (Italy), 13 November 2017.

Seminar “Living in someone’s gut: a Bifidobacterial perspective”

Seminar organized by the University of Parma (Italy), 21 November 2017.

PUBLICATIONS

Unravelling phenolic metabotypes in the frame of the COMBAT study, a randomized, controlled trial with cranberry supplementation

Food Research International, 2023 June 28, Volume 172, Page 113187
<https://doi.org/10.1016/j.foodres.2023.113187>

Chronic Consumption of Cranberries (*Vaccinium macrocarpon*) for 12 Weeks Improves Episodic Memory and Regional Brain Perfusion in Healthy Older Adults: A Randomised, Placebo-Controlled, Parallel-Groups Feasibility Study

Frontiers in Nutrition, 2022 May 19, Volume 9, Page 849902
<https://doi.org/10.3389/fnut.2022.849902>

(Poly)phenolic compounds and gut microbiome: new opportunities for personalized nutrition

Microbiome Research Reports, 2022 January 1, Volume 1
<https://doi.org/10.20517/mrr.2022.06>

Development of an Oral (Poly)Phenol Challenge Test (OPCT) to Identify Aggregate Metabotypes for Dietary (Poly)Phenols and Their Drivers: A Study Protocol

Current Developments in Nutrition, 2022 June 14, Volume 6, Issue Supplement_1, Page 1148
<https://doi.org/10.1093/cdn/nzac072.020>